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Loss of 6q or 8p23 is associated with the total number of DNA copy number aberrations in adenoid cystic carcinoma

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Abstract. We analyzed 10 adenoid cystic carcinomas (ACCs) of the salivary glands by array-based comparative genomic hybridization (a-CGH) using DNA chips spotted with 4,030 bacterial artificial chromosome clones. After the data smoothing procedure was applied, a total of 88 DNA copy number aberrations (DCNAs) were detected. The frequent (≥30%) DCNAs were loss of 6q23-27 and 8p23, and gains of 6p, 6q23, 8p23 and 22q13. High-level gains were detected on 12q15, including *MDM2* in two cases. These two cases showed an immunohistochemically high-level (>50%) expression of MDM2 and a low-level expression of p53 (<20%). Furthermore, the total number of DCNAs was significantly greater in ACCs with loss of 6q compared to other ACCs, and in ACCs without the loss of 8p23 compared to other ACCs, respectively. Although limitations exist, a-CGH detected several candidate chromosomal imbalances associated with accumulation of DCNAs in ACCs.

Introduction

Adenoid cystic carcinoma (ACC) is a rare type malignant epithelial tumor that mostly occurs in the major and minor salivary glands. However, it has also been reported in other sites, e.g., the breast, lung and trachea, and accounts for 2-4% of all malignant head and neck tumors (1,2). Microscopically, the nucleus of ACC cells often shows monotonous and inconspicuous atypia. Furthermore, this tumor is usually slow to metastasize, but often severely invades along the nerves. The growth of these tumors is often persistent and unpredictable (3). Genomic aberrations present in the tumor play an important role in the carcinogenesis and/or progression of the carcinoma. In tumors that occur in the same organ, there are those with a high number of DNA copy number aberrations (DCNAs) and those with a low number of DCNAs. We have reported that abnormalities tend to be seen with a lot of chromosomes when a certain DNA copy number aberration exists, i.e., gains of 5p15, 7p, 17q11-22 and 18p and losses of 3p14-21, 4p and 9p in oral squamous cell carcinoma (4) and loss of 5q14-21 in intestinal type gastric cancer (5).

Karyotyping analyses have demonstrated frequent translocations of 6q21-24, 9p13-23 and 17p12-13 in ACCs (6-8). According to research on the loss of heterozygosity (LOH), LOH rates have been highest at 6q23-25 (9). Chromosome-based comparative genomic hybridization (c-CGH) (10) revealed losses of 12q12-13, 6q23-qter, 13q21-22 and gains of 9q, 11q, 16p, 17q, 19, 21q and 22q as recurrent aberrations (11-13). Recently, an expression analysis of ACCs using array-based CGH (a-CGH) (14) has detected candidate key proteins, i.e., SOX4 and AP-2 γ, in 15 cases of ACCs. Compared to the analyzed number of cases, information from a-CGH is generally too abundant. Thus, it is useful to refine the chromosomal regions detected by a-CGH. Frierson *et al* (14) used a cluster analysis (15) for narrowing the data. We have reported a useful method (16) to refine and define DCNAs using the adaptive weights smoothing (AWS) procedure with the gain and loss analysis of DNA (GLAD) algorithm (17,18). After data smoothing by this method (16), the graph of the log ratio obtained by a-CGH looks like the graph obtained by c-CGH. However, the information of the breakpoint, gain and loss is more correct because it is based on a-CGH having a higher resolution than c-CGH (19).

The purpose of the present study using a-CGH with data refining was to confirm the trends of DCNAs in ACCs and to obtain information associated with accumulations of genomic aberrations in ACCs.

Materials and methods

Patients. We examined 10 surgically resected ACCs in this study (Table I). The patients consisted of 5 males and 5 females with an average age of 64.5 (range, 51-78) years. None of the patients had any history of chemotherapy or radiotherapy

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Table I. Clinical and histopathological data of the 10 ACC patients.

No.	Age/Gender	Type[a]	Tumor site	TNM	Stage
1	61/F	Cri	Mouth floor	T1N0M0	I
2	65/M	Sol	Upper lip	T1N0M0	I
3	66/M	Sol	Mouth floor	T1N0M0	I
4	78/F	Cri	Mouth floor	T2N0M0	II
5	77/M	Cri	Tongue	T2N0M0	III
6	69/M	Sol	Lower lip	T4N0M0	IV
7	58/M	Cri	Submandibular gland	T3N0M0	III
8	51/F	Cri	Palate	N/A	N/A
9	57/F	Cri	Submandibular gland	T1N0M0	I
10	63/F	Cri	Mouth floor	T2N0M0	II

[a]Histological subtype: Cri, cribriform; Sol, solid. M, male; F, female; N/A, not available.

before surgery. The pathological staging of these tumors was made according to the tumor-node-metastasis (TNM) classification of the International Union Against Cancer (19). The tumor tissue specimens were stored frozen at -80˚C until use. The study protocol including informed consent for this study was approved by the Institutional Review Board for Human Use, Yamaguchi University School of Medicine.

DNA extraction. High-molecular weight DNA was extracted from each tumor specimen using a DNA extraction kit (DNeasy Tissue Kit, Qiagen Co., Hilden, Germany) according to the manufacturer's instructions after the microdissection (20). Control DNA was obtained from Promega (Madison, WI, USA) and was used as the reference.

Array-based comparative genomic hybridization (a-CGH). A commercial array (MAC array Karyo 4K®, Macrogen, Inc., Seoul, Korea) was used in this study. This array consists of 4,030 BAC clones spotted in duplicated at an average spacing about 1 Mb over the whole genome. Information for each BAC clone is available at http://www.macrogen.co.kr. The BAC clones in this platform were established by Macrogen, Inc. The experiments were performed as previously described (21-23).

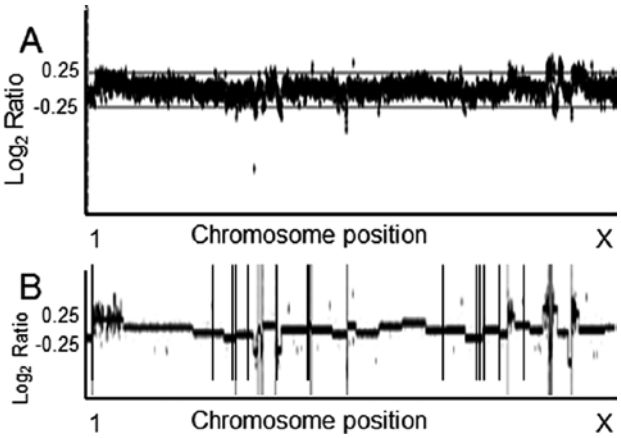

Figure 1. The fluorescence intensity ratios (log base 2) of the tumor vs. the reference DNA for each bacterial artificial chromosome (BAC) clone. The vertical axis shows the log ratio and the horizontal axis shows the position of the chromosome (from 1p to 22q, Xp and Xq). (A) An example (case 7) of an array-based comparative genomic hybridization profile (original data with normalization). The fluorescence intensity ratios (log base 2) of the tumor vs. the reference DNA were evaluated for each BAC clone. (B) The simple profile pattern was obtained by processing original data in an adaptive weights smoothing (AWS) mode. The vertical break lines on the graph show break points expected. In total, DCNAs were detected at 18 chromosomal segments (regions) in this example.

Analysis of a-CGH. After hybridization, the slides were scanned on a GenePix 4000A scanner (Axon Instruments, Union City, CA, USA) with GenePix Pro 5.0 software (Axon Instruments). The 16-bit fluorescence intensity images were quantified and analyzed using a software program (MacViewer version 1.7.7, Macrogen, Inc.) optimized for analysis of this array. The fluorescence spots were defined using the automatic grid feature and were adjusted manually. Thereafter the ratio of the red:green channel of each clone was calculated, and all CGH ratios were converted to log base 2 (log2) ratios. The clones with log2 ratios that exceeded ±0.25 were considered gain or loss of the copy number. We defined a log2 ratio >1.0 as an amplification. Inadequate spots were flagged by manual inspection. In order to define chromosomal regions with DSCNAs, the method using the AWS procedure with the GLAD algorithm (16) was applied to the ratio data. Because three consecutive spot points were needed to estimate each data point with AWS, several clones were omitted from evaluation as noise using the data smoothing algorithm. We also defined the frequent loss or gain when the aberration was detected at the frequency of 30% or more.

Statistical analysis. The differences of the mean value of the DCNAs between the two groups were examined by the two-tailed paired Student's t-test. A difference was considered to be significant at $p<0.05$.

Immunohistochemical analysis. We prepared 4-μm thick sections from formalin-fixed and paraffin-embedded specimens. Immunohistochemical staining using MDM2 (Mdm2

Table II. DNA copy number aberrations detected by a-CGH in 10 ACCs.

Case no.	DNA copy number aberrations
1	-1p36.1-36.3, -5q13, -6q23-27, +10q26, **+12q15**, +18p, +18q, -22q11, +22q12-13, +22q13.
2	+8p23, -9p11-21, -9q11-34, -10q24-26, -12q12-15, **+12q15**, -12q15-24, -14q32, -17p, +19p, +19q, +22q.
3	+6p, 6q11-25, -6q25, +6q25-27, -8p23, -15q21, +16p, +16q
4	-5q11, -8p23
5	+6p, +6q11-23, -6q23-27, +9p23-24, -10p12-15, -10q24-25
6	-1p34-36, -4p15-16, +6p, +6q23, -6q24-27, +8p, +8q, +9p23-24, -9p11-13, -9q11-12, +9q21, -11p12-14, -11q11, -11q14-22, -15q11, +16p13, +18p, +18q
7	+1p11-34.3, +1q, -5q13, -6q22-25, -6p12-21.2, -6q24-27, +8p23, -9q22, +17q21-25, +20p, -20q11, +20q11-12, -20q12-13.1, +20q13.1-13.3, -22q11.1, +22q11.1-13
8	-8p23, -9q21-23
9	-8p23, -8q21.3
10	+2p15, -3q27, -5p14-15.1, -6q22-24, -8p23, -11p, -11q, -17p, -17q11-22, -18q22, -20p, -Xq21-27

a-CGH, array-based comparative genomic hybridization; ACC, adenoid cystic carcinoma. Gains are marked with '+' and losses with '-'. Amplifications are shown in bold.

p53 binding protein homolog), p53 or p21 antibody were performed. Briefly, deparaffinized sections were heated in sodium citrate buffer (0.01 M, pH 9.0 for MDM2 and pH 6.0 for the others) for 30 min in a microwave oven (650 W), and were treated with 3% hydrogen peroxide in absolute methanol. Then the sections were incubated for 3 h at room temperature with primary antibodies diluted in Dako Real antibody Diluent S2022 (Dako Cytomation, Glostrup, Denmark) against p53 (DO7, 1:100, Novocastra Laboratories, Newcastle, UK), MDM2 antibody (M4308, 1:200, Sigma-Aldrich, St. Louis, MO) and p21 antibody (556430, 1:100, BD Biosciences Pharmingen, San Diego, CA). For subsequent reactions a Histofine® SAB-PO (M) immunohistochemical staining kit (Nichirei, Tokyo, Japan) was used. Immunoreactivity was visualized with 3,3'-diaminobenzidine. The nuclei were counterstained with Mayer's hematoxylin. Negative controls were run by omitting the primary antibodies.

Nearly the same areas of the tumor in serial sections were evaluated for MDM2, p53 or p21. From over 500 tumor cells, cells with nuclear staining were recorded as p53 or p21 expression cells and cells with nuclear and cytoplasmic staining were recorded as MDM2 expression cells. The expression status of MDM2, p53 and p21 were classified into three categories as follows: low-level, 0-19%; middle-level, 20-50%; and high-level, >50%, in tumor cells. Epithelial cells in normal part that was adjacent to the tumor were evaluated by a similar method for the control in 4 samples.

Results

DCNAs detected by a-CGH. After data smoothing, a total of 88 DCNAs were detected in 10 ACCs by a-CGH (Fig. 1). The total number of DCNAs ranged from 2 to 18, and the average number per tumor was 8.8, with a standard deviation of 5.83 (Table II). Seventeen recurrent DCNA (gain or loss) regions were detected (Table II). The most frequent loss regions were 6q25 (6/10), 8p23 (5/10), 6q24 (5/10), 6q27 (4/10), 6q23 (3/10) and 6q26 (3/10). On the other hand, the most frequent gain regions were 6p (3/10), 6q 23 (3/10), 8p23 (3/10) and 22q12-13 (3/10). Amplification was detected only two times, and both of these were observed at the narrow segment of 12q15 including *MDM2* (Fig. 2). The averages of the total number of DCNAs in ACCs were associated with several specific DCNAs (Table III). The average number was greater in tumors with 6q loss (6 cases) than in tumors without 6q loss (4 cases) (mean ± SD, 11.7±4.6 vs. 4.5±5.0, p=0.048). In addition, the number was greater in tumors without 8p23 loss (5 cases) than in tumors with 8p23 loss (5 cases) (5.2±4.6 vs. 12.4±4.8, p=0.017). Moreover, the number was greater in tumors with 8p23 gain (3 cases) than in tumors with 8p23 loss (5 cases) (5.2±4.6 vs. 15.3±3.1, p=0.007). The raw data files of a-CGH with a format including BAC start, BAC end, STS and gene information were registered in the Gene Expression Ominibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) to serve as a public archive (GSE22328).

Many candidate genes were in the frequent DCNAs (gains) detected by a-CGH. The amplification of 12q15 was composed of three neighboring BAC clone spots on the chromosome, including *MDM2*, *CPM* (carboxypeptidase M), *SLC35E3* (solute carrier family 35, member E3), *RAP1B* (RAP1B, member of RAS oncogene family) and *NUP107* (nucleoporin 107 kDa) on the chip. A gain in region 22q12-13 included *SYN3* (synapsin III) and *TIMP3* (TIMP metallopeptidase inhibitor 3). For the frequent loss regions in 6q, the candidate genes were *ESR1* (estrogen receptor 1) at 6q25.1, *MAS1* (a tyrosine kinase protooncogene) at 6q25.3, *RPS6KA2* (ribosomal protein S6 kinase), *MLLT4* (myeloid/lymphoid or mixed-lineage leukemia), *C6orf54* (an uncharacterized protein) and *THBS2* (thrombospondin-2) at 6q27.

MDM2, p53 and p21 expression. The immunohistochemical results are summarized in Table IV. All 10 cases of ACC showed middle- or high-level positive status for MDM2 (high-level 8 and middle-level 2). Middle-or high-level of p53

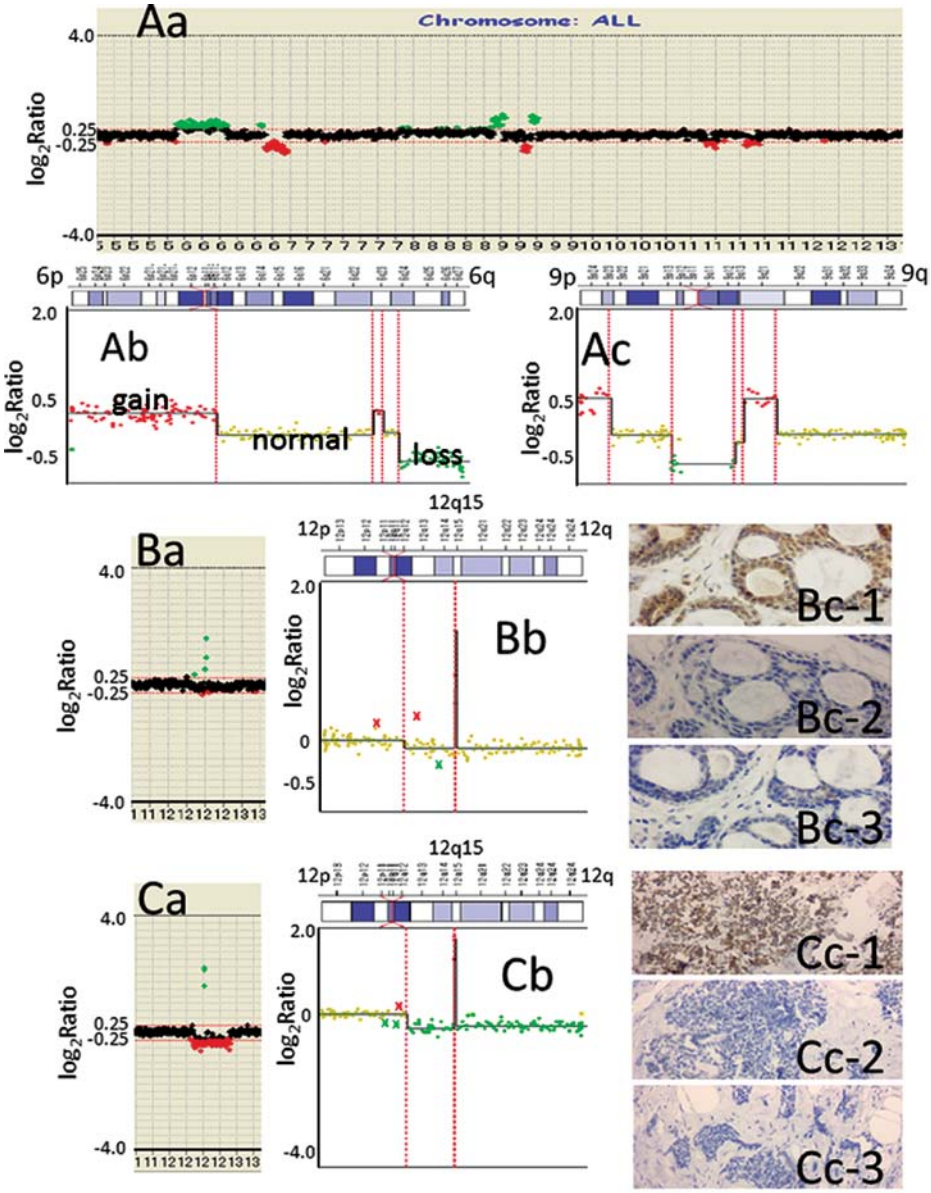

Figure 2. (A) The fluorescence intensity ratios (log base 2) of the tumor vs. the reference DNA for each bacterial artificial chromosome (BAC) clone. (Aa) An example (case 6) of an array-based comparative genomic hybridization (a-CGH) profile (original data with normalization). The 16-bit fluorescence intensity images were quantified and analyzed using a software program (MacViewer version 1.7.7, Macrogen, Inc.) optimized for analysis of the array. The dot plot DNA copy number gain indicated by the green color and DNA copy number loss indicated by the red color. (Ab and Ac) These simple profiles are obtained by processing original data in an application adaptive weights smoothing model. DNA copy number aberrations (DCNAs) are detected on the chromosomal segments; (Ab) 6p gain (red dots), 6q23 gain and 6q24-27 loss (green dots), (Ac) 9p23-24 gain, 9p11-13 loss, 9q11-12 loss and 9q21 gain. A red line shows a breakpoint. (B and C) Two examples (adenoid cystic carcinoma, case 1 and 2) of a-CGH profile, (Ba and Ca) original data with normalization and (Bb and Cb) data smoothing, and (Bc-1 and Cc-1) the MDM2, (Bc-2 and Cc-2) p53 and (Bc-3 and Cc-3) p21 expressions in these samples. Note that a narrow amplification (with a fluorescence intensity ratio >1.0) segment of 12q15 including *MDM2* is seen. Several clones are omitted from evaluation as noise 'x' using the data smoothing algorithm. (Bc and Cc) The histological subtype of these tumors are the cribriform type (B) and solid type (C). The tumors show high-level expression of MDM2 (Bc-1 and Cc-1), low-level expression of p53 (Bc-2 and Cc-2) and p21 (Bc-3 and Cc-3). Original magnification x200.

expression was detected in 7 cases and all cases were classified as being middle-level positive. Middle- or high-level of p21 expression was detected in 5 cases (middle-level 4 and high-level 1). ACC cases with 12q15 genomic amplification showed high-level expression of MDM2, low-level expression of p21 and low-level expression of p53 (Fig. 2). In this study, normal epithelial cells in the salivary gland were used as control; all four samples were classified into the low-level (nearly no) expression of MDM2 or p53, and the three of the four samples were classified into the positive (middle-level) of p21.

Discussion

As previously shown, the accuracy obtained by cross-validation was low, even when 74 colorectal cancer samples were analyzed by using the same BAC-array chip used in this study (24). Therefore, data refining to obtain the reliable DNA aberrations is important. Frierson *et al* (14) used a cluster analysis (15) for narrowing the data from 15 cases of ACCs by a-CGH with cDNA. In order to define chromosomal regions with DCNAs, a smoothing method using the AWS procedure with GLAD

Table III. Relationship between the average of the total number of DCNAs in ACCs and particular DCNAs (10 cases).

Type	DCNAs[a]	p-value
6q loss		
Yes (n=6)	11.7±4.6	0.048
No (n=4)	4.5±5.0	
8p23 loss		
Yes (n=5)	5.2±4.6	0.017
No (n=5)	12.4±4.8	
8p23		
8p23 loss (n=5)	5.2±4.6	0.007
8p23 gain (n=3)	15.3±3.1	

[a]The average of the total number of DNA copy number aberrations (DCNAs); ACC, adenoid cystic carcinoma.

Table IV. Immunohistochemical expression of MDM2, p53, and p21 in ACCs.

Case no.	MDM2	p53	p21
1[a]	H	L	L
2[a]	H	L	L
3	H	L	L
4	H	M	M
5	H	M	M
6	M	M	L
7	M	M	H
8	H	M	M
9	H	M	M
10	H	M	L
A	L	L	M
B	L	L	M
C	L	L	M
D	L	L	M

ACC, adenoid cystic carcinoma. [a]12q15 (including *MDM2*) amplification case. Low-level expression (L), 0-19%; middle-level expression (M), 20-49%; high-level staining (H), 51-100%. A-D, Reference from normal salivary gland.

algorithm (16) was applied to the ratio data in this study. Using this method, we previously reported that a copy number gain of the 6p arm is linked with advanced hepatocellular carcinoma (19). After data smoothing by this method (16), the graph of the log ratio obtained by a-CGH looks like the graph obtained by c-CGH (Fig. 2). However, the information of the breakpoint, gain and loss is more correct because it is based on a-CGH which has a higher resolution than c-CGH (19). After data smoothing, a total of 88 DCNAs were detected in 10 ACCs by a-CGH. The average number per tumor was 8.8, and the standard deviation was 5.83. Only 17 of recurrent DCNAs (gain or loss) were detected. The mathematical method adapted was helpful to remove noise and limit the number of candidate chromosomal segments from the original data (Fig. 1). The representative candidate chromosomal segments were as follows: the loss regions were, 6q25, 8p23, 6q24, 6q23 and 6q26, and the gain regions were 6p, 6q 23, 8p23 and 22q12-13. The past and present research results (6-14) reveal that the 6q loss, especially 6q23-26, is a representative DCNA of ACC. In the array used, BAC clones of *ESR1*, *MAS1*, *RPS6KA2*, *MLLT4*, *C6orf54* and *THBS2* were indicated to be affected by this loss. Besides 6q, another candidate chromosomal segment is 22q12-13 (12,25), which includes *SYN3* and *TIMP3*. The former gene is a member of synapsins and is associated with synaptic vesicles. Its expression appears to be neuron-specific in normal tissue (26). The latter gene is associated with suppression of invasion or metastasis (27). These genes at 22q12-13 may thus be associated with ACC nerve invasion.

The only amplicon that we observed in our results was 12q15, which included *MDM2* and two consecutive spots on the array (Fig. 2). Furthermore, the 12q15 amplification (log2 ratio >1.0) was detected two times. Bernheim *et al* reported that there was a 12q15 gain in 17 ACC samples using an oligo-44K-CGH chip (25). On the other hand, Sequeiros-Santiago *et al* reported *ERBB1*, *CCND1* and *PIK3CA* gene amplifications to be frequently detected in ACC of the salivary glands using a PCR-based semi-quantitative approach. However, no MDM2 gene amplification was detected in 24 samples of ACC (28).

Furthermore, the middle-or high-level expression of MDM2 was observed in all ACC samples, and was not observed in the normal salivary gland tissues used for comparison in the present results. Interestingly, the cases with *MDM2* amplification showed low-level expression of p21 and p53. MDM2 regulates the activity of p53 (29) and p21 is a transcriptional target of p53. In the present study, a remarkable 12q15 amplification pattern was detected; the amplification was detected in the loss region of 12q12-24 (Fig. 2Cc-2). It seems that the MDM2 gene amplification is responsible, at least in part, for the overexpression of MDM2 protein in ACC. Furthermore, we have reported mucinous adenocarcinoma with *MDM2* amplification arising from a minor salivary gland (30).

In the present study, the total number of DCNAs was significantly greater in ACC samples with loss of 6q than in other ACC samples, and in ACC without loss of 8p23 than in other ACC samples. The chromosomal region affected by such DCNAs varies, according to the type of tumor. For instance, we reported loss of 5q14-21 in intestinal type gastric cancer (5) and loss of 11q23-24 in endometrial cancer (31). In our present results, we demonstrated that 8p23 was a unique segment which showed recurrent loss or gain.

The sample size in this study was small, so further large-scale studies are necessary. However, we have shown that a-CGH with data refining is useful to confirm the trends of DCNAs in ACCs and to obtain information associated with accumulations of genomic aberrations in ACCs.

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