Deletion of 1p32-p36 Is the Most Frequent Genetic Change and Poor Prognostic Marker in Adenoid Cystic Carcinoma of the Salivary Glands

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Abstract

Purpose: Adenoid cystic carcinoma (ACC) is a relatively uncommon salivary gland malignancy known for its protean phenotypic features and pernicious clinical behavior. Currently, no effective therapy is available for patients with advanced nonresectable, recurrent, and/or metastatic disease. The purpose of this study is to identify prognostic factors other than tumor stage that can be used to predict the outcome of the patients with ACC.

Experimental Design: We used comparative genomic hybridization (CGH) to identify copy number aberrations in 53 primary ACCs. Array CGH and fluorescence *in situ* hybridization analysis was used to validate CGH results on selected cases. We correlated these copy number aberrations with clinicopathologic factors using Pearson's χ^2 or by the two-tailed Fisher exact test. The disease-specific survival and disease-free intervals were generated by the Kaplan-Meier product limit method.

Results: Chromosomal losses (n = 134) were more frequent than gains (n = 74). The most frequent genetic change was the loss of 1p32-p36 in 44% of the cases followed by 6q23-q27, and 12q12-q14. The most frequently gained chromosomal regions were 8 and 18. Of the chromosomal aberrations, loss of 1p32-p36 was the only abnormality significantly associated with patient's outcome.

Conclusions: This study, for the first time, identifies loss of 1p32-p36 as a significant aberration in ACC. Molecular characterization of 1p32-36 region using the available genomic technologies may lead to the identification of new genes critical to the development of novel therapeutic targets for this disease copy number aberration.

Adenoid cystic carcinoma (ACC) is an uncommon salivary gland malignancy characterized by heterogeneous phenotypic features and persistently progressive biological behavior (1–6). Histopathologically, ACC manifests three histologic patterns, including the solid, cribriform, and tubular forms, in variable combination and dominance (7). The solid pattern is typically associated with aggressive clinical course. ACCs are known for their proclivity to perineural invasion, which significantly contribute to the intractable nature of this disease (1, 8, 9). Surgical resection with and without postoperative radiotherapy remains the primary treatment modality for the management of this disease. However, patients with advanced local presentation, recurrence, and/or distant metastasis have limited therapeutic options (8, 9). Therefore, elucidation of molecular events that predict the malignant risk is critical in understanding the biology, clinical behavior, and management of ACC.

Previous conventional cytogenetic studies of ACC, although limited in numbers and scope, have revealed translocations involving chromosome 6q with multiple chromosomal partners (10, 11). Subsequent analysis, using comparative genomic hybridization (CGH) of these tumors have reported recurrent chromosomal gains of 16p, 17q, 19, and 22q13 and losses at 6q23-qter, 12q12-q13, 13q21-q22, and 19 (12, 13). Recently, an array CGH study of 18 paraffin-embedded primary ACCs have reported frequent gains at 9q33.3-q34.3, 11q13.3, 11q23.3, 19q13.3-p13.11, 19q12-q13.43, 21q22.3, and 22q13.33 (14). Several molecular genetic studies also have been attempted to define the genetic alterations and identified frequent loss of heterozygosity at 6q, 12q12-q13, and 17p chromosomal regions, suggesting the presence of putative tumor suppressor genes on these chromosomes (15–19). Gene expression studies of ACC have identified a gene signature that is associated with early development, cell cycle regulation, apoptosis, myoepithelial differentiation, and extracellular matrix (20, 21). In addition, overexpression of transcriptional

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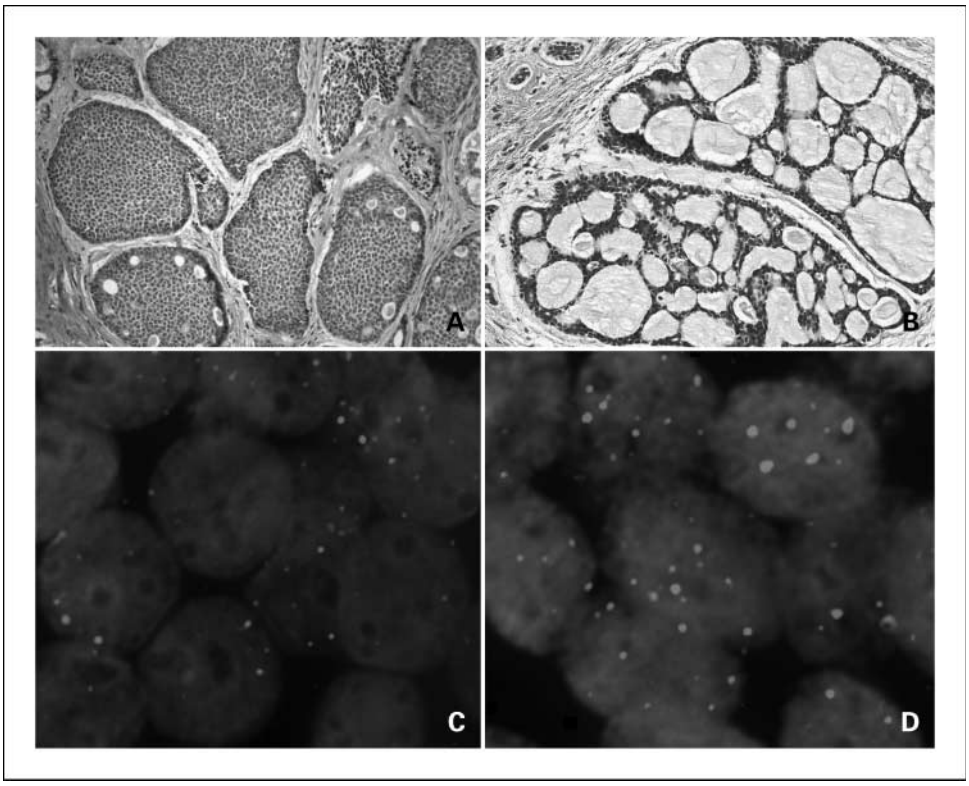

Fig. 1. H&E-stained images of solid (*A*) and cribriform ACC (*B*). FISH analysis on ACC sections using KIT containing BAC clone-RP11-586A2 (*C-D*). Increased copy number for KIT was noted in 502A5 (4-6 copies) and 386G1 (>6 copies).

factors *SOX4* and *AP-2γ*, and members of Wnt/β-catenin pathway have been reported in ACC (21).

In this study, we conducted comprehensive genetic characterization of 53 primary ACCs, the largest cohort to date, by chromosomal CGH and correlated the results with clinicopathologic factors and patients survival. We report frequent losses at 1p32-p36, 6q23-q27, and 12q12-q14. Of these, loss of 1p32-p36 was significantly associated with poor prognosis in patients with ACC.

Materials and Methods

Tumor specimens. Fifty-three ACCs with fresh-frozen tissue from equal number of patients accessioned at the head and neck tissue repository of the M. D. Anderson Cancer Center from 1991 to 2004 formed the materials for this study. All tumors were harvested by one head and neck pathologist and quality controlled to ensure the nature and the neoplastic cell composition in each specimen, each tumor specimen contained at least 85% tumor cells. All tissues in this study were obtained after Institutional Review Board–approved informed consents were signed.

The patients population composed of 28 males and 25 females, and age ranged between 27 and 85 y with a mean of 52 y. Tumors were located in the parotid gland (13), submandibular gland (5), maxillary sinus and nasal cavity (18), oral cavity (12), lacrimal gland (3), and upper trachea (2). Tumor size ranged from 2 to 15 cm with mean size of 4.1 cm. Although, no pure morphologic type was noted, there were 2 predominantly tubular, 1 mixed, 31 cribriform, and 19 solid phenotypes. H&E-stained images of solid and cribriform were shown in Fig. 1A and B. Margins of resection were free in 42 and 11 undetermined in the sinonasal region.

CGH. High-molecular weight DNA was extracted from tumor tissue and normal placenta by standard methods and subjected to CGH according to the previously published method with some modifications

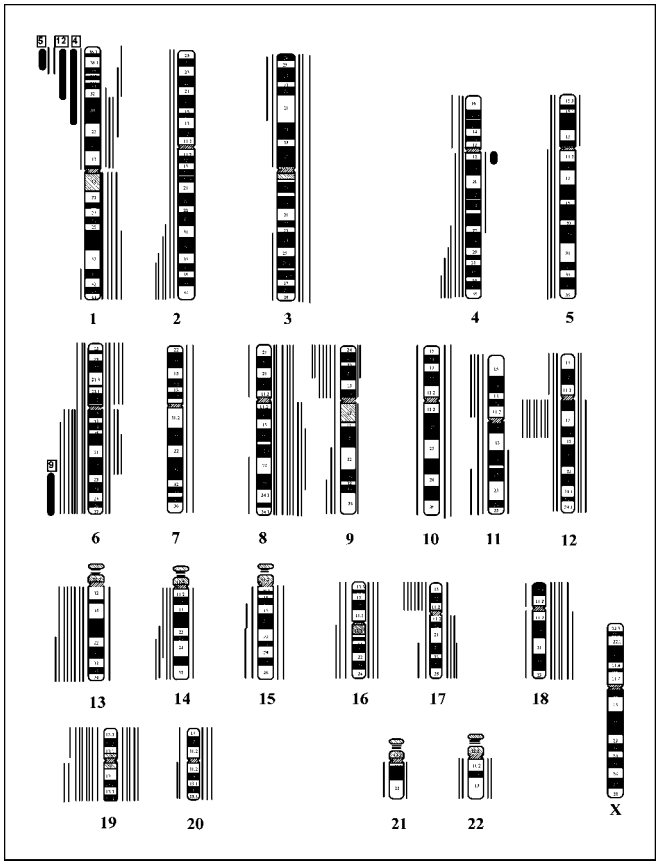

Fig. 2. Ideogram showing DNA copy number changes identified by CGH in 53 ACC tumors. *Thin vertical lines on either side of the ideogram,* losses (*left*) and gains (*right*) of the chromosomal region. *Thick bar,* high-level amplification (*right*).

(22). The metaphase preparations were captured and processed using the QUIPS (Applied Imaging).

Array CGH. The array used in this study consists of 2,500 human BACs, which were spaced ~1 megabase across the whole genome. These arrays were obtained from Spectral Genomics. The array CGH was done on DNA extracted from case 179F6 439A6 and 386G1. The experiments were done according to the manufacturer's protocol. Arrays were prehybridized with human Cot-I DNA (Life Technologies Invitrogen) and salmon testes DNA to block the repetitive sequences on BACs. One microgram of normal DNA (reference) and tumor DNA (test) was labeled with cy5-dUTP and cy3-dUTP, respectively, by random priming. To avoid dye bias, we did dye swap experiments for each sample. The probe mixture is dissolved in hybridization mixture, denatured, cooled, and mounted with 22 × 60 mm coverslip. Hybridizations were done in sealed chambers for 16 to 20 h at 37°C. After posthybridization washes, arrays were rinsed, dried with compressed air, and scanned into two 16-bit TIFF image files using Gene Pix 4000A two-color fluorescent scanner (Axon Instruments, Inc.) and quantitated using GenePix software (Axon Instruments).

Fluorescence* in situ *hybridization. Fluorescence *in situ* hybridization (FISH) was done on paraffin sections from cases 98E2, 104H4, 123B6, 131A8, 179F6, 185G8, 386G1, 459G5, and 461G1 to validate losses from 1p35-36 using bacterial artificial chromosome (BAC) clones RP11-219C24 and RP11-163M9 and control clone, RP11-201K10 (1q21). FISH was also done on paraffin sections from 386G1 and 502A5 to validate amplification/gain of 4q12 using *KIT* containing BAC clone RP11-586A2. We confirmed the map positions of all clones on normal human metaphase chromosomes by FISH. The clones from 1p35-p36 and 1q21/4q12 were labeled with Spectrum Green and Spectrum Orange, respectively (Vysis), by nick translation. Hybridization and FISH analysis was done as described previously (22). To determine the deletion status, 200 individual interphase nuclei were analyzed for each case.

Statistical methods. Correlations between copy number aberration (CNA) and clinicopathologic factors were assessed for statistical significance by Pearson's χ^2 or, where there are fewer than 10 subjects in any cell of a 2 × 2 grid, by the two-tailed Fisher exact test. Estimations of type II error (power) were calculated using a two-tailed two-proportion Z test model for unequal N. Curves describing overall and disease-specific survival and disease-free intervals were generated by the Kaplan-Meier product limit method. The statistical significance of differences between the actuarial curves was tested by the log-rank test. Follow-up time was the time from first appointment at the University of Texas M. D. Anderson Cancer Center for the primary tumor of concern until the date of last contact or death for survival measurements. These statistical tests and frequency enumerations were done with the assistance of the *Statistica* statistical software application (StatSoft, Inc.).

Results

Overview of CNAs in ACC. Figure 2 summarizes the DNA copy number changes identified in the present study. The CGH analysis showed that the chromosomal losses (n = 134) were more frequent than gains (n = 74). Under representation of chromosomes 1p (44%), 6q (32%), 12q (18%), 19 (17%), 9p (13%), 13q (17%), and 17p (13%) were observed in descending order. The most notable and novel finding in the present study was the frequent and consistent loss of 1p (44%) with the commonly deleted site at 1p32-p36 region (Fig. 2). The other common deletions were located at 6q at 6q23-q27,

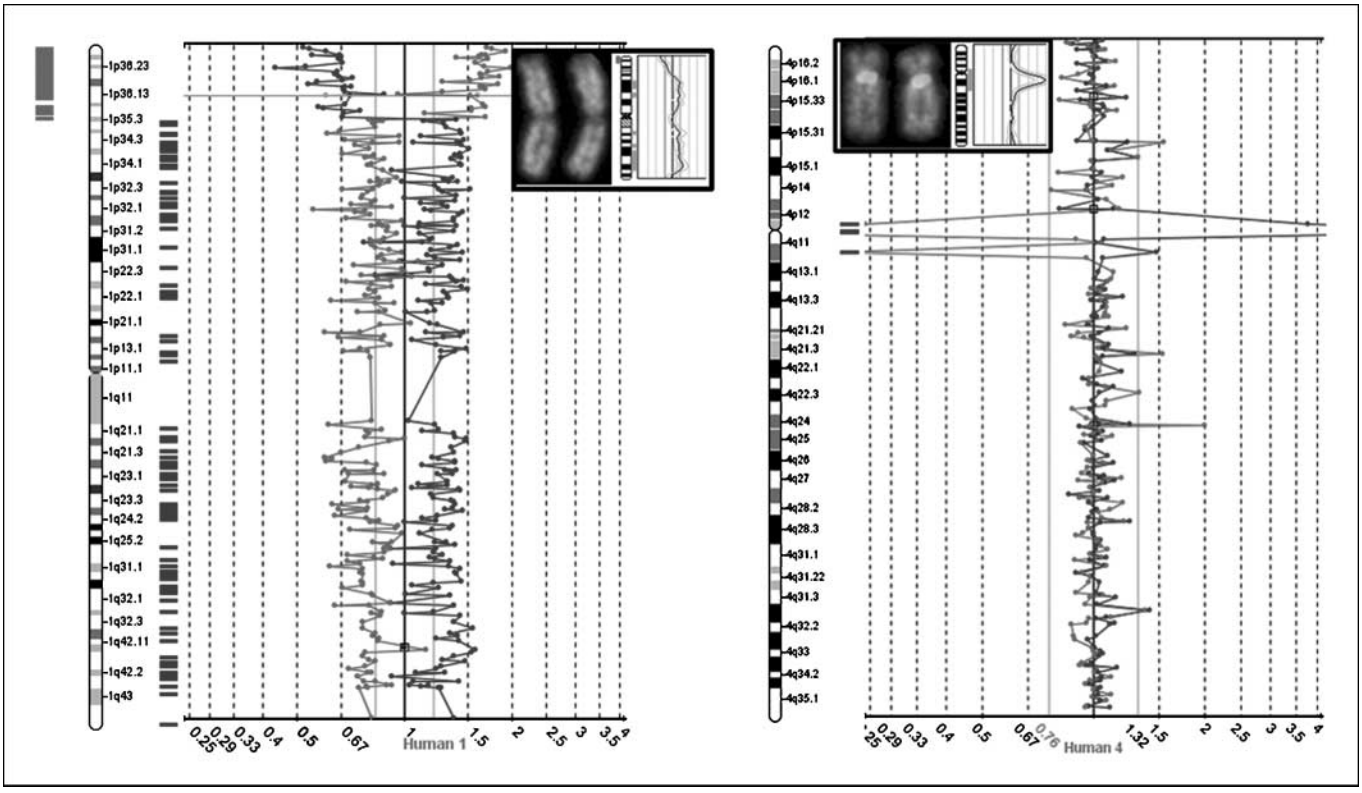

Fig. 3. The array CGH profiles from 179F6 and 386G1 showing loss of 1p32-36 (*left*) and amplification of 4q12 (*right*). In array CGH experiment, the tumor and normal DNA were labeled with Cy5 and Cy3, respectively. The dye reversal experiment was also done to avoid dye bias and hybridization artifacts. The mean ratios were plotted along the length of the chromosome. *Insets,* partial chromosomal CGH karyotypes for chromosome 1 and 4 and corresponding ratio profiles. *Vertical bars on the right of the ideogram,* threshold values of 0.80 and 1.20 for loss and gain, respectively.

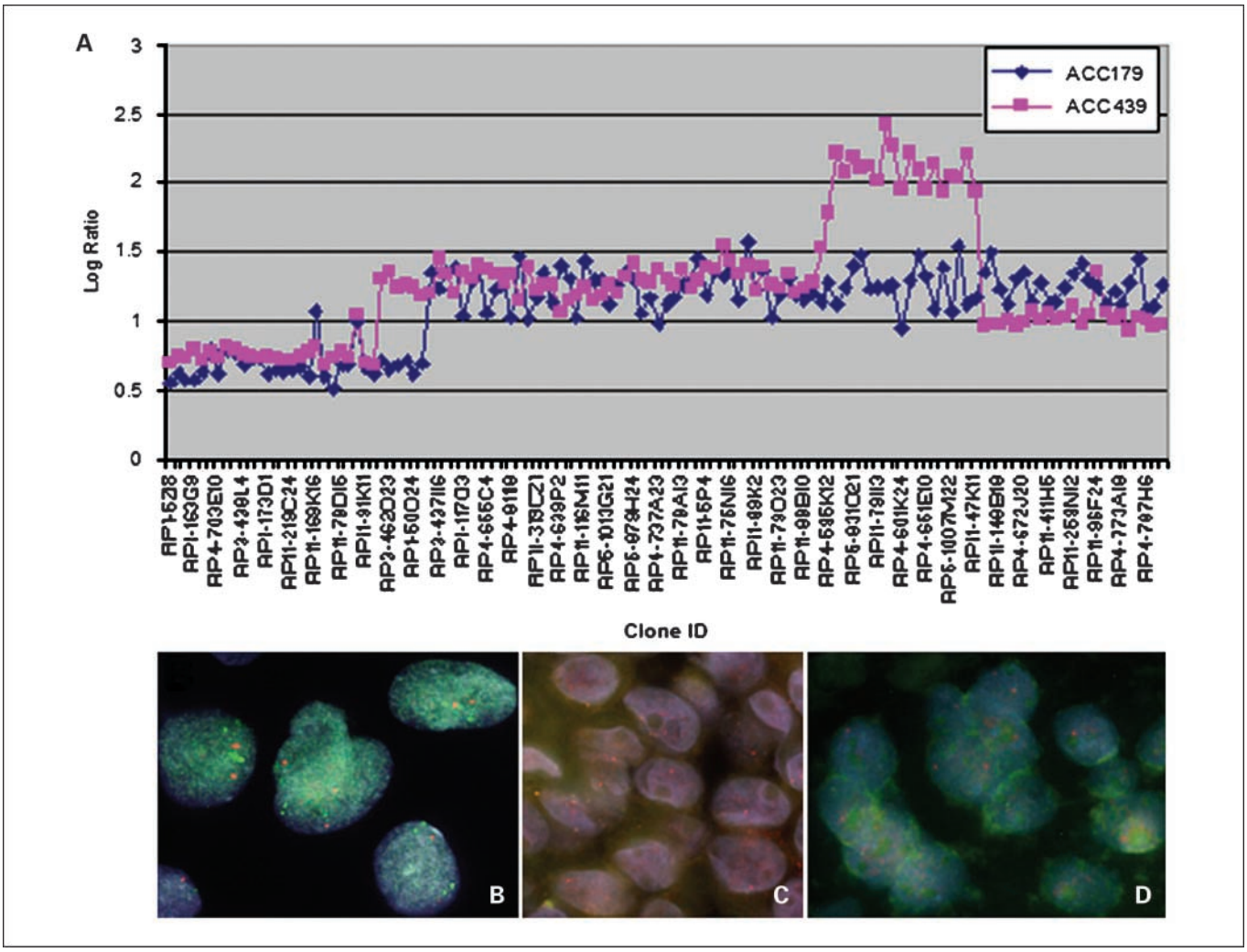

Fig. 4. *A,* array CGH profiles for chromosome 1 for cases 179F6 and 439A6 showing the loss of distal 1p. The clones on chromosome 1 are arranged (pter to qter) based on University of California Santa Cruz mapping positions (*top*). FISH validation for 1p36 losses identified in ACC (*B-D*). The control probe (RP11201K10-1q21) labeled with spectrum orange and target probe (RP1-163G9 and RP1-163M99-1p36) labeled with spectrum green (*bottom*). The cocktail of this probe hybridized to interphase nuclei form cases control (*B*), 98E1 with loss of two copies (*C*), and 123B6 loss of 1 copy of target probe (*D*).

and 12q12-q14 regions. The most frequently gained regions were localized to chromosomes 8 and 18 in 38%, and 11% of the cases, respectively. High-level of amplification was detected at chromosome 4q12 region (Fig. 3).

Loss of 1p32-p36 is the most common genetic change in ACC. The most novel and consistent finding in this study is the loss of chromosome 1p32-p36 regions in 44% of the ACCs analyzed (Fig. 2). Because of the variable hybridization pattern on chromosome 1p terminal region by cCGH, we further validated this finding by array CGH and FISH. We subjected two cases (179F6 and 439A6) to array CGH to confirm and refine the deletions in 1p region identified by cCGH (Fig. 4A). In both cases, array CGH corroborated the findings of cCGH. In case 179F6, the deletion was ~27.5-Mb region spanning from chromosome 1p34 to 1p36 between the RP1-62I8 and RP11-261P19 clones. The deletion in case 439A6 was ~19.3 Mb and spanning between RP1-62I8 and RP11-91K11 clones. Based on our array CGH analysis, we identified two commonly deleted BAC clones (RP11-219C24 and RP11-163M9) from chromosome 1p36.

FISH analysis using the combination of these two BAC clones on interphase nuclei and metaphase spreads of normal lymphocytes from healthy donors yielded two signals in both interphase nuclei and metaphase chromosomes along with control clone, RP11-201K10 (1q21; Fig. 4B). To validate the use of this probe in paraffin-embedded tissues, FISH analysis was done on cases 98E1, 104H4, 123B6, 131A8, 179F6, 185G8, 386G1, 459G5, and 461G1. This analysis consistently yielded two copies for the control probe RP11-201K10 (1q21) and loss of two copies and one copy of the 1p36 target probe (RP11-219C24, RP11-163M9) in 2 and 7 cases, respectively (Fig. 4C and D).

Chromosomal amplifications are infrequent in ACC. Our results revealed chromosomal amplification at 4q12 in one case (386G1). Subsequent array CGH analysis refined two distinct amplicon peaks at this region amplicon 1 and amplicon 2 (Fig. 3). The amplicon 1 covered 3.5-Mb regions between RP11-65IC2 and RP11-80L11 with peak amplification for clone RP11-571I18. Another amplicon (amplicon 2) was noted at 5.9 Mb distal to amplicon 1 and confined to a single clone, RP11-91C3. To assess whether the 4q12 amplicon contributes

to an increased copy number of *KIT* gene from 4q12, FISH was done on tissue sections from the case 386G1 and 502A5 (gain of 4q12-q27). Consistent with CGH and array CGH data, we found high-level amplification of *KIT* in 386G1 (>6 copies) and 502A5 (4-6 copies; Fig. 1C and D).

Correlations of CNAs with clinical variables. To evaluate the clinical significance of common CNAs, we stratified the 53 ACC patients based on the presence or absence of specific cytogenetic lesions in their tumors. Frequent CNAs were correlated with age, gender, site (submandibular, maxilla, parotid, oral cavity, and orbit), histologic subtype (solid and tubular and cribriform), recurrence, metastasis, perineural invasion, and overall survival (Table 1). Age, gender, tumor size, and metastasis did not show any significant correlation with CNAs. Chromosomal loss at 1p32-p36 ($P = 0.002$), 2q33-q37 ($P = 0.02$), and 6q22-q27 ($P = 0.004$), and gain of chromosome 8 ($P = 0.003$) were significantly correlated with solid tumor phenotype. Among the sites, oropharynx was significantly correlated with losses at 12q12-q13.3 ($P = 0.036$) and loss of 1p32-p36 ($P = 0.035$), chromosomes 4 ($P = 0.001$), and 5 ($P = 0.006$) with orbit. We classified perineural invasion into low and high based on the extent of invasion the nerves involved, and correlated with frequent CNAs. Patients with high perineural invasion were significantly correlated with loss of chromosome 19 ($P = 0.005$).

The most common chromosomal loss of 1p32-p36 was significantly associated with shorter overall survival time compared with the patients without this aberration (Fig. 5). We also correlated each chromosomal band from 1p32-p36 (1p32, 1p33, 1p34, 1p35, and 1p36) region individually with overall survival. Each chromosomal band from 1p32-p36 region was significantly correlated with poor overall survival for 3- and 5-year period. Our data substantiate the significant correlation between the loss of 1p32-p36 chromosomal region and overall survival at 3 years ($P = 0.0001$) and 5 years ($P = 0.003$; Fig. 5). The prognostic significance of this genetic aberration has not been previously reported, and subsequent identification of gene(s) from this region may aid in developing therapeutic targets for ACC.

Discussion

Our study, the largest to date, identify a novel deletions at the distal region of chromosome 1p in ACC. The loss of chromosome 1p occurred in 44% of the cases and common

Table 1. Correlation between CNAs and clinical variables in 53 ACCs.

Clinical variable	Genetic aberration	Percentages in clinical variable with alteration*	*P**
Gender	No significant correlations		
Age group			
<45 y	No significant correlations		
45-65 y	Gains at 1q11-q25	20.0%	0.028
>65 y	No significant correlations		
Size of tumor	No significant correlations		
Site of tumor			
Maxilla	No significant correlations		
Parotid gland	No significant correlations		
Oropharynx	Losses at 12q12-q13.3	41.7%	0.036
Submandibular gland	No significant correlations		
Orbit	Losses at 1p32-p35	100%	0.029-0.035
	Losses at 4p16-q35	66.7-100%	<0.001-0.013
	Losses at 5p15-q35	66.7%	0.002-0.006
	Gains at 6p25-q22	66.7%	0.013-0.021
	Gain of 8	100%	<0.001-0.004
Histologic type			
Solid	Losses at 1p32-p36	57.9-68.4%	0.002-0.02
	Losses at 2q34-q37	26.3%	0.024
	Losses at 4q11-q27	15.8-21.1%	0.017
	Losses at 4q31-q35	31.6	0.009-0.024
	Losses at 5q11-q35	15.8%	0.049
	Losses at 6q23-q27	52.6-57.9%	0.004
	Losses at 9p13-q22	21.1%	0.017
	Gains at 6p25-q22	21.1-26.3%	0.005-0.017
	Gain at 8	26.3-36.8%	0.003-0.024
Cribiform	No significant correlations		
Perineural invasion			
Low	No significant correlations		
High	Loss of 19	40%	0.005
Distant metastasis	No significant correlations		
3-y overall survival	Losses at 1p32-p36	80-100%	<0.0001-0.003
	Gain of 8	40-50%	0.014-0.019
5-y overall survival	Losses at 1p32-p36	69.2-84.6%	0.003-0.018
	Losses at 2q33-q37	30.8%	0.041
	Gains at 1p31-p22	30.8%	0.041
	Gains at 1q31-q44	30.8%	0.041
	Gains at 8p11-p23	30.8%	0.041

*Range of values found over the correlated chromosomal region.

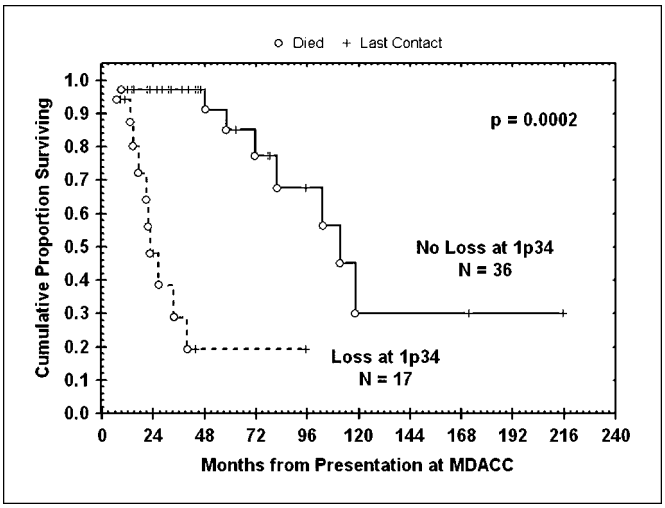

Fig. 5. Overall survival plot for chromosome 1p34 in patients with ACC.

region was mapped to 1p32-p36. This finding strongly suggests that this region harbor tumor suppressor gene(s) associated with the development of at least a subset of ACCs. In addition, loss of chromosome 1p32-p36 region was correlated significantly with poor outcome in patients with ACC. Interestingly, previous studies of different malignancies have also reported an association between loss of the distal region of chromosome 1p and aggressive tumor behavior and poor outcome (23). Several genes (*p73* and *CHD5*) were identified from 1p36 region and shown to function as tumor suppressor gene. p73 is one of the tumor suppressor genes located at 1p36.3 and shown to be frequently deleted in neuroblastoma and other human cancers (24). The functional studies suggested that transcriptional silencing of this gene by hypermethylation of a CpG island in the 5′ untranslated region may contribute to development and/or progression of lymphoid malignancies (25). In addition, the data showed that when p73 overproduced, activate the transcription of p53-responsive genes, and inhibit cell growth in a p53-like manner by inducing apoptosis (26). Recently, the *CHD5*, a tumor suppressor gene, has been identified using chromosome engineering to generate mice with either a gain or loss of a region corresponding to human 1p36 (27). Functional studies revealed that *CHD5* play a fundamental role in the control proliferation, apoptosis, and cell senescence through p19(ARF)/p53 pathway. *CHD5* is a member of the chromodomain gene family and is preferentially expressed in the nervous system (28). The role of these two genes in ACC tumorigenesis remains to be determined.

Other frequently deleted chromosomal regions in our cohort were 6q, 9p, 12q12-q14, 19, and 13q. Consistent with previous studies by CGH and loss of heterozygosity, we identified 32% of the cases with losses on 6q with common region of deletion at 6q23-q27 (12, 13, 16). In the present study, the frequent losses on 6q have a significant propensity to solid histologic type. Previous loss of heterozygosity studies identified thrombospondin-2 as a candidate tumor suppressor gene in ACC (16). The *thrombospondin-2* gene is a potent inhibitor of tumor growth and angiogenesis, but no functional significance for this gene in ACC has been reported. Loss of 12q12-q13 has also been reported in 33% of ACCs by CGH (12). The combined genetic and genomic efforts fail to identify putative tumor suppressor gene(s) from 12q12-q13. However, down-regulation of several genes from this region has been reported (17). In contrast to chromosomal losses, gains were infrequent. The most frequent gains were mapped to chromosome 8 and 18, and gain of chromosome 8 was significantly associated with solid type in the present study. Previous studies by CGH and array CGH, reported gain of 22q13 in a significant number of ACCs, but we observed in low frequency in our cohort of tumors (13, 14).

In conclusion, our comprehensive genetic study using CGH, FISH, and array CGH identified a novel site of deletion at 1p32-p36 that has not been reported previously in ACC. Further, genomic studies using high-resolution single nucleotide polymorphism and expression arrays may eventually aid in the identification of tumor suppressor genes from this region in ACC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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