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Genome-Wide Profiling of Acquired Uniparental Disomy Reveals Prognostic Factors in Head and Neck Squamous Cell Carcinoma

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Abstract

Acquired uniparental disomy (aUPD) leads to homozygosity facilitating identification of monoallelically expressed genes. We analyzed singlenucleotide polymorphism arraybased genotyping data of 448 head and neck squamous cell carcinoma (HNSCC) samples from The Cancer Genome Atlas to determine the frequency and distribution of aUPD regions and their association with survival, as well as to gain a better understanding of their influence on the tumor genome. We used expression data from the same dataset to identify differentially expressed genes between groups with and without aUPD. Univariate and multivariable Cox proportional hazards models were performed for survival analysis. We found that 82.14% of HNSCC samples carried aUPD; the most common regions were in chromosome 17p (31.25%), 9p (30.13%), and 9q (27.46%). In univariate analysis, five independent aUPD regions at chromosome 9p, two regions at chromosome 9q, and the *CDKN2A* region were associated with poor overall survival in all groups, including training and test sets and human papillomavirus (HPV)negative samples. Fortythree genes in areas of aUPD including PDL1 and CDKN2A were differentially expressed in samples with aUPD compared to samples without aUPD. In multivariable analysis, aUPD at the *CDKN2A* region was a significant predictor of overall survival in the whole cohort and in patients with HPVnegative HNSCC. aUPD at specific regions in the genome influences clinical outcomes of HNSCC and may be beneficial for selection of personalized therapy to prolong survival in patients with this disease.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the seventh most common cancer worldwide; more than half a million new patients are diagnosed each year [1]. Incidence has increased, especially among young patients, because of increasing prevalence of human papillomavirus (HPV) [2,3]. The 5year overall survival (OS) rate is better in patients with HPVassociated HNSCC than in those whose tumors are not associated with HPV [4].

Loss of heterozygosity (LOH) results from loss of one of two parental alleles present in each genome. In most cases LOH results in cells having a single copy of one parental allele and loss of the other allele. Acquired uniparental disomy (aUPD) also called copyneutral LOH) is a subset of LOH wherein a chromosomal region or whole chromosome is lost and reduplicated. aUPD is not associated with changes in copy number. Thus each cell harbors two copies of a single parental allele rather than one copy each of two parental alleles. Both regulatory and open reading frames are monoallelic and any alterations in promoter, enhancer or regions either as the result of germline SNPs or methylation that are included in the aUPD could alter the expression or stability of mRNAs or the stability of function of their protein products. aUPD thus has the potential to expose effects of homozygosity for existing germline and somatic aberrations including mutations, deletions, methylation (hypo or hyper), complex structural alterations, and imprinted genes [5–10]. aUPD can be a consequence of mitotic recombination that usually results in segmental aUPD where only a portion of the chromosome arises from a single parent. Whole chromosome aUPD is usually the consequence of anaphase lagging of one chromosome, and with duplication of the whole chromosome [11–15]. Moreover, loss of chromosomal segments or wholechromosome, and frequently duplication of the retained allele in subsequent replication can be consequence of breakagefusionbridge (BFB) events in cancer [16]. Thus, a UPD can be a result of BFB and thereafter replication of the retained allele [16]. Genomic copy number alterations, gene expression, miRNA expression, and protein expression are well studied in HNSCC. Accumulating data have shown that genomic events including loss of heterozygosity (LOH) and epigenetic changes pre-

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sent in tumors can be used as prognostic biomarkers for cancer [17–19]. However, genomewide profiling of aUPD in HNSCC is very limited with most studies having small sample sizes [20–22]. Of note, aUPD profiling and association between aUPD regions and survival have been reported in a variety of malignancies including MDS, MDS/MPD, and secondary AML [23]. However, allelebased level changes in the genome and their association with clinical outcome and survival are poorly characterized, and acquired uniparental disomy (aUPD) has not been studied as a potential prognostic factor in HNSCC.

Previously, we have shown that smallest overlapping regions of aUPD were associated with etiologic factors such as alcohol intake, smoking, HPV and *TP53* mutation status of HNSCCs [24]. In the current study, we profiled genomewide aUPD to determine the frequency and distribution of aUPD in HNSCC and to determine whether any smallest overlapping regions (SORs) of aUPD were associated with survival and clinical characteristics of disease in a large data set. Importantly, we assessed expression of mRNA for genes located in areas of aUPD to determine effects of monoalellic gene expression due to aUPD on gene expression. This represents the first large scale comprehensive study of aUPD regions and their association with survival in HNSCC.

Materials and Methods

Patient Samples

Clinical and patient demographic data were retrieved from TCGA (http://portal.gdc.cancer.gov/). We noted HPV status as reported by TCGA and by Nulton et al. [25,26]. Patient characteristics are summarized in Table S1a, and all samples are listed in Table S1B. Overall survival (OS) was calculated from the date of diagnosis of head and neck cancer to the date of death or last followup. Recurrencefree survival (RFS) was calculated from the date of diagnosis of HNSCC to the date of recurrence or last followup. Sample and clinical data were based on a November 2015 freeze from TCGA data portal.

Genomic Data and aUPD Analysis

Genomic data (CEL files) were retrieved from TCGA data portal. Genotyping console software (Affymetrix) was used to perform quality control, and then to generate CHP files. Affymetrix contrast QC threshold was used for both tumor and matching samples. Data that did not pass the quality control check were removed from further analysis; 448 samples (448 tumor and 448 matching normal) passed the quality control check and were used in the study. aUPD analysis and detection of SORs of aUPD were performed using Copy Number Analyzer for GeneChip (CNAG v4.0) software (http://www.genome.umin.jp) by using tumor and matching normal data as described previously [10,27]. The SORs of UPD are described based on the 3 and 5 endpoints of aUPD regions. The Dec 2013 human genome browser (NCBI Build 38/hg38; http://genome.ucsc.edu) was used for identification of gene localization. Telomeric aUPD was defined as aUPD occurring in the telomeric region with one breakpoint. When at least two breakpoints appeared, it was defined as centromeric aUPD (also called interstitial). Segmental aUPD was defined as all centromeric and telomeric regions. When aUPD occurred in wholep arm or wholeq arm of chromosome, it was defined as wholep arm or wholeq arm aUPD. If aUPD occurred in the whole chromosome, it was considered wholechromosome aUPD (Figure S1*A*). Total aUPD represented both segmental and wholechromosome aUPD. We recruited normalized HiSeq gene expression data from Cancer Genome Browser (https://xena.ucsc.edu/) to determine differentially expressed genes between samples with and without aUPD.

Statistical Analysis

The nonparametric KruskalWallis test was used to analyze the correlation between aUPD (total, telomeric, centromeric, segmental, wholechromosome, wholep arm, and wholeq arm) and clinical characteristics (stage, grade, HPV status, and gender). Univariate Cox proportional hazards models were used to determine the effects of SORs of aUPD, gender, age, stage, and grade on OS and RFS. KaplanMeier analysis and logrank p values were calculated to identify survival differences between groups. A multivariable Cox proportional hazards model was used to select prognostic markers. This study complied with REMARK criteria [28]. A twotailed Student t test was used to compare expression of genes between samples with aUPD and those without aUPD for identified SORs. To correct for multiple comparisons, we adjusted the p values by obtaining the BenjaminiHochberg false discovery rate [29]. A finding was considered significant when the twosided P value was less than .05. Statistical analyses were performed in STATA v10 (STATA Corp., College Station, TX).

Results

Profiling SORs of aUPD

We analyzed singlenucleotide polymorphismbased arrays generated by The Cancer Genome Atlas (TCGA) from 448 HNSCCs to identify the frequency and distribution of segmental and wholechromosome aUPD. A total of 1591 aUPD regions were found across all of the chromosomes (per sample range 0 to 27, mean 3.56, median 3.0), including 1407 segmental and 184 wholechromosome events in the entire data set. The frequency of HNSCC samples harboring at least one aUPD was 82.14% (368 of the 448 samples). The most common instances of aUPD were found in chromosomes 17p (31.25%), 9p (30.13%), 9q (27.46%), and 13q (18.53%) (Figure S1*B*).

Next we tested for differences in the frequency of total, telomeric, centromeric, segmental region, wholechromosome, wholep arm, and wholeq arm aUPD among patient samples by disease stage, grade, and HPV status. In KruskalWallis tests, the frequency of total, telomeric, centromeric, segmental aUPD was significantly higher in patients with stage III and IV disease than in those with stage I and II disease (total: $P = .005$, telomeric: $P = .011$, centromeric: $P = .021$, segmental: $P = .002$), but no differences were found by disease stage in wholep arm ($P = .278$), wholeq arm ($P = .375$), and wholechromosome ($P = .4821$; Figure 1). In contrast to disease stage, the frequency of aUPD was not correlated with grade (total: $P = .733$, telomeric: $P = .793$, centromeric: $P = .133$, segmental: $P = .826$, wholechromosome: $P = .819$, wholep arm: $P = .675$, wholeq arm: $P = .352$; Figure S2). The frequencies of total ($P = .036$) aUPD were significantly higher in HPVnegative patients than in HPVpositive patients. However, no differences were found according to HPV status for telomeric ($P = .472$), centromeric ($P = .962$), segmental ($P = .102$), wholechromosome ($P = .188$), or wholep arm ($P = .810$), and wholeq arm ($P = .389$) aUPD (Figure S3).

Association of Recurrent SORs of aUPD With Survival

We identified 23 SORs of aUPD, including *CDKN2A*, across all chromosomes. Independent SORs were identified at chromosomes 2q (two regions), 6p (one region), 9p (10 regions), 9q (four regions), 11q (two regions), 13q (one region), and 17p (two regions), as well as *CDKN2A* (at chromosome 9p) (see Table 1 and Supplemental Table S2). Next, we tested whether any of these SORs were associated with OS or recurrencefree survival (RFS). First we randomly divided samples into a training and a test sets (Table S1) and assessed whether the SORs were associated with survival. In univariate analysis, SORs of aUPD at chromo-

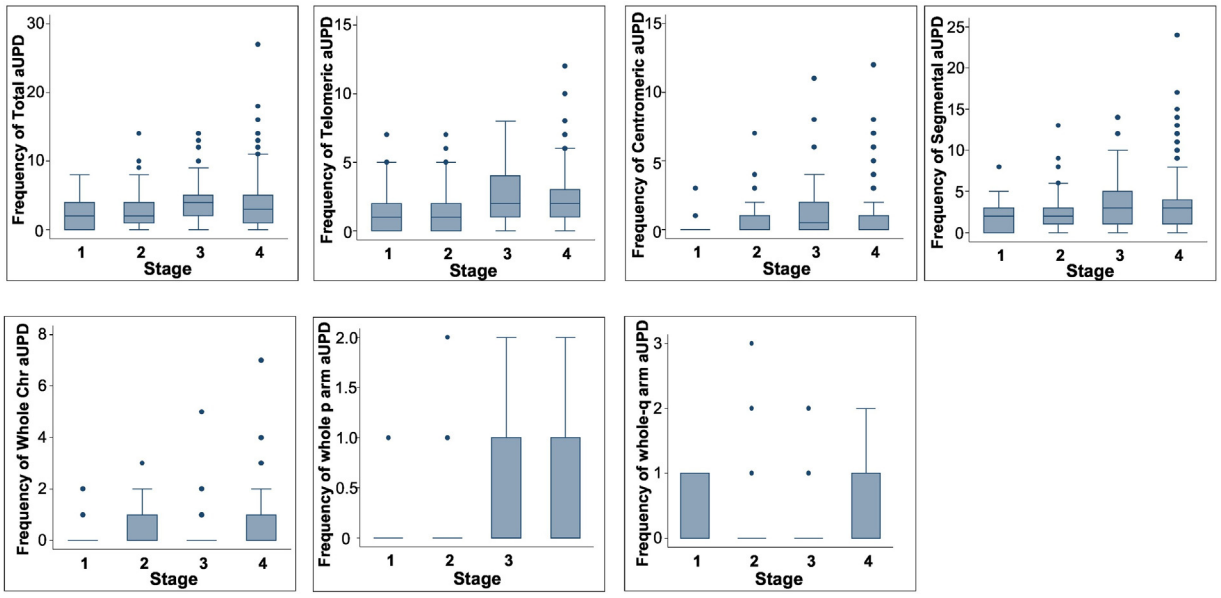

Figure 1. Frequency of total, telomeric, centromeric, segmental, whole-chromosome, whole-p arm, and whole-q arm acquired uniparental disomy (aUPD) in patients with head and neck squamous cell carcinoma, stratified by disease stage. The frequency of total, telomeric, centromeric, and segmental aUPD was significantly higher in patients with stage III and IV disease than in those with stage I and II disease, but no differences were found by disease stage in whole-p arm, whole-q arm, and whole-chromosome.

some 9p (9p24.3; $P = .044$, 9p24.1; $P = .020$, 9p23p22.3; $P = .009$, 9p22.3p22.2; $P = .014$, 9p21.3_1; $P = .017$, 9p21.3_2; $P = .024$, 9p21.3p21.2; $P = .044$, 9p21.2; $P = .008$, 9p21.1; $P = .044$, and 9p13.3; $P = .029$, respectively; Table 1, Figs. 2 and 3, Table S2a, and Figure S4) and *CDKN2A* ($P = .045$; Table 1, Figure 3), as well as gender ($P = .022$; Table 1, Figure S4), were associated with shorter OS in the training set. In the test set, one SOR of aUPD at chromosome 6p12.3 ($P = .018$), seven SORs at chromosome 9p (9p24.3; $P = .008$, 9p24.1; $P = .007$, 9p23p22.3; $P = .029$, 9p22.3p22.2; $P = .011$, 9p21.3_1; $P = .010$, 9p21.3_2; $P = .018$, and 9p21.3p21.2; $P = .033$, respectively), two SORs at 9q (9q33.2; $P = .025$; and 9q34.13; $P = .026$), and one SOR at the *CDKN2A* (9p21.3; $P = .008$) were associated with reduced OS (Table 1, Figures 2 and 3, Table S2B, Figures S4 and S5). In the test set, only two SORs of aUPD at 11q (11q22.3; $P = .008$; and 11q25;

Table 1. Univariate analysis of clinical variables and the SORs of aUPD serving as covariates of survival in patients with head and neck squamous cell carcinoma

	Training set		Test set		All samples		HPV-negative	
Covariate	HR (95% CI)	P[a]	HR (95% CI)	P[a]	HR (95% CI)	P[a]	HR (95% CI)	P[a]
OS								
Age >50 vs. 50	1.15 (0.57-2.31)	.700	0.80 (0.48-1.31)	.370	0.88 (0.59-1.31)	.530	0.87 (0.57-1.33)	.523
Stage I&II vs. III&IV	1.30 (0.75-2.27)	.350	1.07 (0.67-1.71)	.779	1.19 (0.83-1.70)	.354	1.13 (0.77-1.67)	.529
Grade 1 vs. 2&3&4	1.96 (0.84-4.56)	.120	1.24 (0.69-2.23)	.464	1.42 (0.89-2.29)	.145	1.32 (0.78-2.24)	.297
Gender	1.71 (1.08-2.72)	**.022**	1.43 (0.92-2.22)	.115	1.55 (1.13-2.13)	**.007**	1.58 (1.12-2.22)	**.009**
aUPD 6p12.3	0.43 (0.11-1.77)	.242	1.99 (1.13-3.54)	**.018**	1.41 (0.84-2.36)	.196	1.32 (0.76-2.29)	.332
aUPD 9p24.3	1.65 (1.01-2.69)	**.044**	1.76 (1.16-2.68)	**.008**	1.62 (1.21-2.17)	**.001**	1.75 (1.25-2.45)	**.001**
aUPD 9p24.1	1.77 (1.09-2.87)	**.020**	1.79 (1.17-2.72)	**.007**	1.81 (1.32-2.74)	**<.0001**	1.82 (1.30-2.55)	**<.0001**
aUPD 9p23-p22.3	1.89 (1.17-3.05)	**.009**	1.61 (1.05-2.47)	**.029**	1.74 (1.27-2.39)	**.001**	1.79 (1.27-2.51)	**.001**
aUPD 9p22.3-p22.2	1.80 (1.13-2.86)	**.014**	1.73 (1.13-2.65)	**.011**	1.66 (1.24-2.23)	**.001**	1.85 (1.32-2.60)	**<.0001**
aUPD at CDKN2A	1.71 (1.01-2.88)	**.045**	2.15 (1.23-3.78)	**.008**	1.96 (1.34-2.85)	**<.0001**	1.98 (1.31-2.97)	**.001**
aUPD 9p21.3_1	1.77 (1.11-2.84)	**.017**	1.75 (1.14-2.67)	**.010**	1.80 (1.31-2.46)	**<.0001**	1.82 (1.29-2.55)	**.001**
aUPD 9p21.3_2	1.72 (1.07-2.76)	**.024**	1.68 (1.09-2.57)	**.018**	1.73 (1.26-2.37)	**.001**	1.76 (1.25-2.47)	**.001**
aUPD 9p21.3-p21.2	1.63 (1.01-2.63)	**.044**	1.60 (1.04-2.46)	**.033**	1.65 (1.20-2.26)	**.002**	1.71 (1.22-2.41)	**.002**
aUPD 9p21.2	1.91 (1.19-3.08)	**.008**	1.43 (0.92-2.20)	.108	1.64 (1.19-2.25)	**.002**	1.79 (1.27-2.52)	**.001**
aUPD 9p21.1	1.67 (1.01-2.73)	**.044**	1.29 (0.82-2.01)	.269	1.45 (1.04-2.02)	**.027**	1.51 (1.06-2.15)	**.024**
aUPD 9p13.3	1.74 (1.06-2.86)	**.029**	1.56 (0.99-2.47)	.057	1.64 (1.17-2.29)	**.004**	1.62 (1.13-2.33)	**.009**
aUPD 9q22.33	1.56 (0.94-2.59)	.082	1.38 (0.87-2.19)	.171	1.47 (1.05-2.07)	**.025**	1.58 (1.10-2.27)	**.014**
aUPD at 9q31.3	1.62 (0.99-2.65)	.054	1.52 (0.97-2.38)	.067	1.58 (1.13-2.19)	**.007**	1.70 (1.20-2.43)	**.003**
aUPD at 9q33.2	1.48 (0.90-2.44)	.120	1.67 (1.07-2.60)	**.025**	1.59 (1.14-2.21)	**.006**	1.66 (1.16-2.37)	**.005**
aUPD at 9q34.13	1.34 (0.81-2.20)	.259	1.65 (1.06-2.57)	**.026**	1.52 (1.10-2.12)	**.012**	1.62 (1.14-2.30)	**.007**
DEL at CDKN2A	1.24 (0.71-2.18)	.454	1.54 (0.88-2.70)	.132	1.38 (0.94-2.02)	.097	1.28 (0.85-1.93)	.235
RFS								
aUPD 11q22.3[b]	1.25E-14 (0-0)	1.000	3.62 (1.41-9.33)	**.008**	3.60 (1.44-9.00)	**.006**	3.66 (1.45-9.23)	**.006**
aUPD 11q25[b]	1.25E-14 (0-0)	1.00	4.07 (1.68-9.75)	**.002**	4.04 (1.74-9.43)	**.001**	3.66 (1.45-9.23)	**.006**

Abbreviations: SOR, smallest overlapping region; aUPD, acquired uniparental disomy; HR, hazard ratio; CI, confidence interval; HPV, human papillomavirus; OS, overall survival; RFS, recurrence-free survival; DEL, deletion.

[a] $P < .05$ was used to select features; boldface indicates statistically significant variables.

[b] aUPD-positive sample size was small in these two variables.

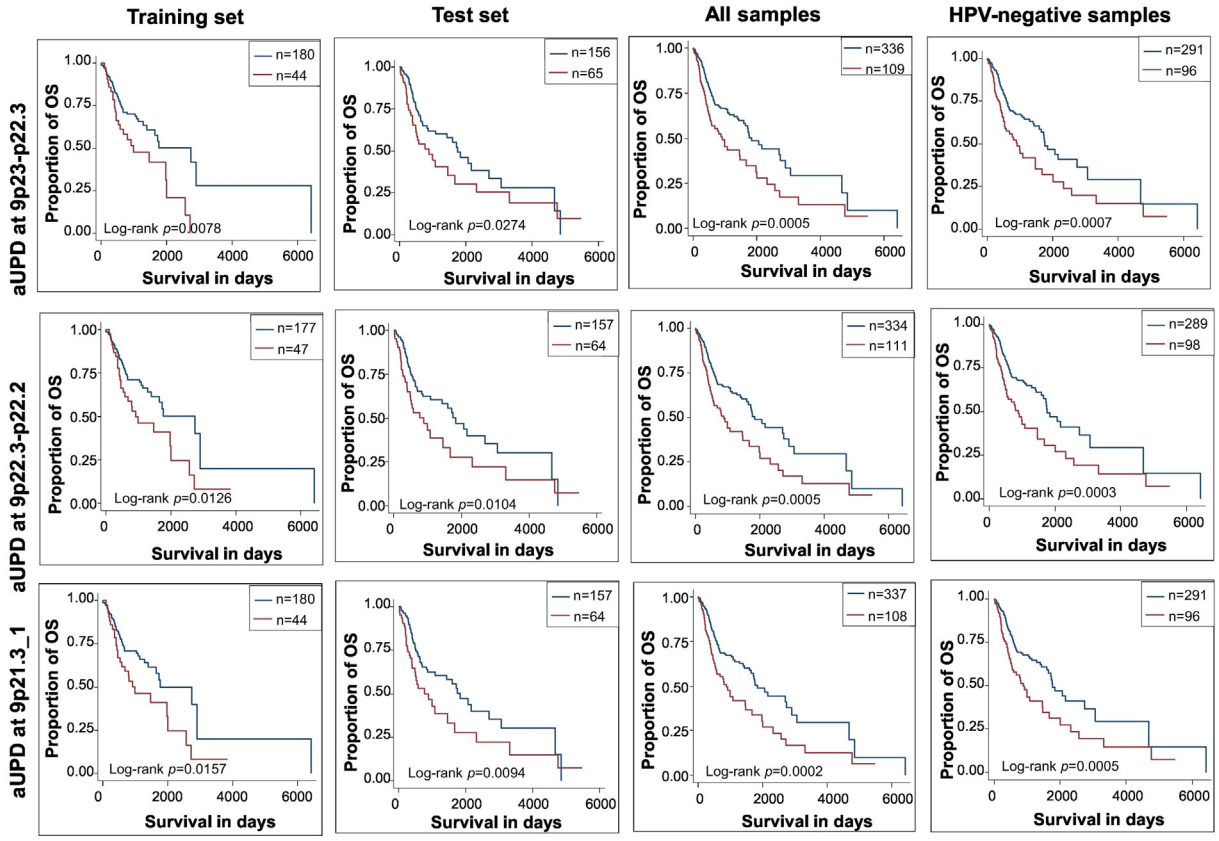

Figure 2. Kaplan-Meier plots of overall survival (OS) for acquired uniparental disomy (aUPD) at chromosomes 9p23-p22.3, 9p22.3-p22.2, and 9p21.3_1 have shown worse OS than the samples without aUPD in the training set and test set from patients with head and neck squamous cell carcinoma, as well as in all samples and human papillomavirus (HPV)-negative patients only.

$P = .002$) were associated with reduced RFS (Table S2B). In the training set, none of the SORs of aUPD were associated with RFS (Table S2a). In multivariable analysis, 9p21.2 ($P < .0001$) in training set, and 9p24.1 ($P < .0001$) and 9p23p22.3 ($P < .0001$) in test set were associated with worst OS, while none of SOR of aUPD was a significant predictor of RFS in training and test sets (Table 2).

Then we tested all samples to determine whether the SORs were associated with survival. In univariate analysis, aUPD in 14 independent SORs was associated with reduced OS in all samples; 10 regions at chromosomes 9p (9p24.3; $P = .001$, 9p24.1; $P < .0001$, 9p23p22.3; $P = .001$, 9p22.3p22.2; $P = .001$, 9p21.3_1; $P < .0001$, 9p21.3_2; $P = .001$, 9p21.3p21.2; $P = .002$, 9p21.2; $P = .002$, 9p21.1; $P = .027$, and 9p13.3; $P = .004$) and four at chromosome 9q (9q22.33; $P = .025$, 9q31.3; $P = .007$, 9q33.2; $P = .006$, and 9q34.13; $P = .012$; Table 1, Table S2C, Figures 2 and 3, Figures S4 and S5). Conversely, only two SORs of aUPD at chromosome 11q (11q22.3; $P = .006$, and 11q25; $P = .001$) were associated with reduced RFS in all HNSCC samples (Table 1, Figure S4). Next, we analyzed associations between aUPD or deletion at the *CDKN2A* region and survival. We found that aUPD at the *CDKN2A* region ($P < .0001$) was associated with poor OS (Figure 3, Table 1), but deletion in the same region was not associated with OS ($P = .097$) in all samples. Moreover, we tested whether age, gender, stage, and grade were associated with survival and found that only gender was associated with reduced OS ($P = .007$; Figure S4); but age ($P = .530$), grade ($P = .145$), and stage ($P = .354$) were not associated with OS. In multivariable analysis, four aUPD regions at chromosome 9p (9p21.3_1; $P = .027$, 9p21.2; $P < .0001$, and 9p21.1; $P = .017$, and *CDKN2A*; $P = .034$) were significant predictors of OS, and one region at chromosome 11q (11q25, $P = .034$) was a significant predictor of RFS (Table 2).

When we tested only HPVnegative samples, we found that the same 14 SORs of aUPD were associated with shorter OS: chromosome 9p (9p24.3; $P = .001$, 9p24.1; $P < .0001$, 9p23p22.3; $P = .001$, 9p22.3p22.2; $P < .0001$, 9p21.3_1; $P = .001$, 9p21.3_2; $P = .001$, 9p21.3p21.2; $P = .002$, 9p21.2; $P = .001$, 9p21.1; $P = .024$, and 9p13.3; $P = .009$) and chromosome 9q (9q22.33; $P = .014$, 9q31.3; $P = .003$, 9q33.2; $P = .005$, and 9q34.13; $P = .007$; Table 1, Figures 2 and 3, Table S2D, Figure S4). Similar to all samples, only two SORs at chromosome 11q (11q22.3; $P = .006$, and 11q25; $P = .006$) were associated with shorter RFS. In addition, the SOR of aUPD at *CDKN2A* ($P = .001$; Figure 3) was associated with shorter OS, but deletion at the same region was not associated with OS ($P = .235$). Gender ($P = .009$) was also associated with reduced OS. In multivariate analysis, SORs of aUPD at chromosome 9p21.3p21.2 ($P < .0001$), 9p21.1 ($P = .029$) and *CDKN2A* (9p21.3; $P = .041$) were significant predictors of OS, and only SOR of aUPD at chromosome 11q22.3 ($P = .006$) was a significant predictor of RFS (Table 2). In contrast to all samples and HPVnegative samples, none of the SORs were associated with OS or RFS in HPVpositive samples.

Association of Differentially Expressed Genes in the SORs of aUPD with Survival

Seventeen of 22 SORs in all samples, including the *CDKN2A*, and additional one region in the test set, were associated with survival. The 18 SORs contained 135 genes (Table S3A). The number of genes in these

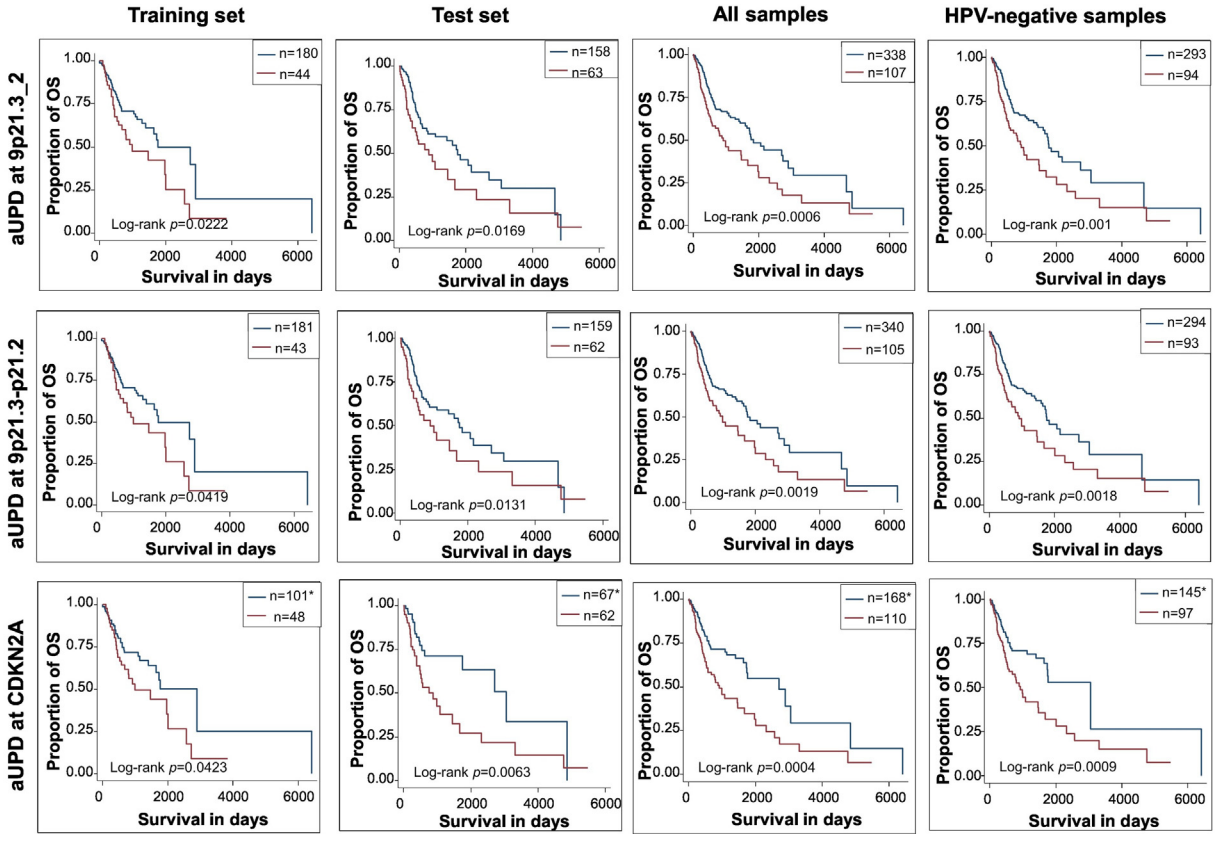

Figure 3. Kaplan-Meier plots of overall survival for acquired uniparental disomy (aUPD) at chromosomes 9p21.3_2 and 9p21.3-p21.2 and *CDKN2A* have shown shorter OS than the samples without aUPD in the defining regions in the training set, test set, all samples from patients with head and neck squamous cell carcinoma, as well as human papillomavirus (HPV)-negative patients only. *Samples with aUPD at *CDKN2A* region was compared with samples without aUPD and deletion for the same region; aUPD-Neg; aUPD-Pos.

Table 2. Multivariable analysis of clinical and genetic covariates for OS and RFS in patients with head and neck squamous cell carcinoma

Variable	HR (95% CI)	P^{a}	q
OS			
Training set			
9p21.2	2.95E+09 (8.67+ 07-1.01+ 11)	<.0001	<.0001
Test set			
9p24.1	4.54E+07 (5.88E+06-3.50E+08)	<.0001	<.0001
9p23-p22.3	5.06-08 (1.95E-09-1.32E-06)	<.0001	<.0001
All samples			
9p21.3_1	61.34 (1.59-2369.73)	.027	.037
9p21.2	7.73E+08 (1.05E+08-5.67E+09)	<.0001	<.0001
9p21.1	0.26 (0.09-0.79)	.017	.029
CDKN2A	0.13 (0.02-0.85)	.034	.037
HPV-negative samples			
9p21.3-p21.2	7.85E-10 (1.05E-10-5.88E-09)	<.0001	<.0001
9p21.1	0.29 (0.09-0.88)	.029	.037
CDKN2A	0.13 (0.02-0.92)	.041	.041
RFS			
All samples			
11q25	8.63 (1.18-63.18)	.034	.037
HPV-negative samples			
11q22.3	3.66 (1.45-9.23)	.006	.012

Abbreviations: OS, overall survival; RFS, recurrence free survival; HR, hazard ratio; CI, confidence interval; q, Benjamini-Hochberg false discovery rate.

Only variables that were significant in univariate analysis were included in multivariate analysis.

[a] $P < .05$ was used to select features.

18 SORs varied. Two SORs at chromosome 9p (9p21.3_2 and 9p21.1) did not contain any open reading frames (ORF), however both contain long noncoding RNA and pseudogenes, and 9p21.3_2 also harbors regulatory elements (Supplementary Table S3B). One of independent SOR at 6p12.3, four at chromosome 9p (9p24.3, 9p23p22.3, 9p21.3_2, and 9p21.3p21.2, one at 9q31.3, and one at 11q25 contained only one proteincoding gene; the remaining SORs consisted of multiple genes (e.g., 9p13.3 had 51 genes; Table S3A). Moreover, miRNAs or and noncoding RNAs as well as pseudogenes and regulatory elements (promotor or enhancers) are mapped in the 18 regions explored in this manuscript and may contribute to the correlations with outcomes (Supplementary Table S3B).

Next, we tested whether the expression of these 135 genes differed between samples with and without aUPD. Fiftysix genes were significantly differentially expressed between samples with and without aUPD. With BenjaminiHochberg false discovery rate correction, only 43 genes were significantly differentially expressed. Fortyone of these 43 genes had significantly higher expression in SORs of aUPDpositive samples than in aUPDnegative samples. Significantly overexpressed genes included *CD274* (also known as *PDL1*; $q = 8.99E06$) in SOR at chromosome 9p24.1, and *DCTN3* ($q = 2.86E07$) and *VCP* ($q = 2.86E07$) at chromosome 9p13.3. We found that only two of the 43 genes had significantly lower expression in aUPDpositive samples than in aUPDnegative samples: *CDKN2A* at chromosome 9p21.3 ($q = 7.58E04$) and *GGTA1* at chromosome 9q33.2 ($P = 3.78E04$; Table S3).

Discussion

Our results indicate that aUPD is widespread in HNSCC, and specific SORs of aUPD in the genome influence survival. We also found that

genes within these SORs were differentially expressed between those with and without aUPD.

The mechanisms influencing cancer development may vary among the telomeric, centromeric, whole arm and whole chromosomes aUPD. Thus, in the current study, we analyzed the data for the wholep and wholeq arm, whole chromosome, telomeric and centromeric aUPD. We found that the frequency of total aUPD, as well as telomeric and centromeric was associated with stage. However, the frequency of aUPD was not associated with grade. These findings contrast with those of our previous report in highgrade serous epithelial ovarian cancers, in which the frequency of aUPD was associated with grade but not with stage [10]. This suggests that aUPD can mediate different functions across cancer lineage.

We identified a total of 18 SORs of aUPD that were associated with shorter OS or RFS. Recently aUPD has been shown to occur at areas encompassing imprinted genes in tumors [8]. The 18 SORs encompass 135 genes and the expression of 43 of the 135 genes within the defined aUPD regions was significantly different compared with samples without aUPD in the same regions. Thus the monoallelic expression of the 43 differentially expressed genes may alter expression. Differentially expression of the 43 genes identified to be located in aUPD SOR in the current study may confer an advantage for the tumor, and may contribute to tumor aggressiveness. Fortyone of the 43 differentially expressed genes had significantly higher expression and only two had lower expression in samples with aUPD compared with those without aUPD. If these genes are in areas encompassing imprinting [8], only the nonimprinted gene was selected. This finding indicates that many of these genes may exhibit gain of function activity. PDL1 hypomethylation has been observed in HNSCC in TCGA and other data sets. PDL1 hypomethylation is inversely correlated with mRNA and protein expression [25,30]. If a hypomethylated PDL1 allele undergoes reduction to homozygosity through aUPD, this could result in increased PDL1 mRNA expression as is observed in samples with aUPD compared to those without aUPD.

Six SORs of aUPD at chromosome 9p (9p23p22.3, 9p22.3p22.2, 9p21.3_1, 9p21.3_2, 9p21.3p21.2, and the *CDKN2A*) were associated with shorter OS in all groups, including the training and test sets, all samples, and HPVnegative samples. Moreover, seven SORs at chromosome 9p (9p24.3, 9p24.1, 9p23p22.3, 9p22.3p22.2, 9p21.3_1, 9p21.3_2, 9p21.3p21.2) and two SORs at chromosome 9q (9q33.2 and 9q34.13) were associated with reduced OS in the test set, all samples, and HPVnegative samples. In contrast, only two SORs of aUPD at chromosome 11q (11q22.3 and 11q25) were associated with RFS in the test set, all samples, and HPVnegative samples. This may be partly because data for RFS in some samples were missing decreasing the power to detect associations. Although only a few samples had SORs of aUPD at chromosome 11q (11q22.3 and 11q25), the overall concordance of the data among the training set, test set, all samples, and HPVnegative samples was notable.

Previously aUPD (in 3 out16 samples) and deletion (in 13 out16 samples) at chromosome 9p were reported in HNSCC [22], and at chromosome 3p and 17 [20]. However these regions were not refined and association between aUPD regions and survival was not assessed due to the small sample sets. LOH at 8p21.2 and 9p21.2 has been reported to be associated with shorter survival in HNSCC samples [17]. LOH at 9p was also found to be predictive for local relapse in HNSCC [19]. However, in both LOH studies microsatellite markers were employed to identify LOH in limited regions in HNSCC samples and the LOH was not further segregated into copy number loss and aUPD [17,19]. Genomewide LOH analysis was performed in the TCGA HNSCC paper based on deletion, but LOH or aUPD were not analyzed for association with gene expression or survival in the TCGA HNSCC paper [25].

CD274 (also known as *PDL1*), *RCL1*, *PDCD1LG2 (also known as PDL2), KIAA1432*, *JAK2* at chromosome 9p24.1, and *VCP*, *DCTN3*, *STOML2*, *C9orf23*, and *GALT* at chromosome 9p13.3, and *CDKN2A* at chromosome 9p21.3 and *TTF1* at chromosome 9q34.13 provide examples of significantly differentially expressed genes between samples with and without aUPD that were associated with OS in all samples, HPVnegative samples, and the test set. *CD274* (also known as *PDL1*) encodes programmed cell death protein1 ligand 1, which is an immune inhibitory receptor ligand, and interaction of this ligand with its receptor, PD1 (programmed cell death protein1), inhibits Tcell activation and cytokine production and enables immune escape. PDL1 is primarily expressed in cancer cells, parenchymal cells, and myeloid cells, whereas PD1 is primarily expressed in tumorinfiltrating lymphocytes. Activation of the PD1/PDL1 axis occurs in tumors either through innate immune resistance or adaptive immune resistance [31]. Previously, overexpression of PDL1 has been shown in a variety of cancers, including HNSCC [32], gastric cancer [33], cervical cancer [34], and squamous carcinoma of the cervix and vulva [35], and PDL1 overexpression confers resistance to radiation in HNSCC [36]. Notably, targeting PDL1 with antiPDL1 monoclonal antibody decreased PDL1 expression in a variety of tumors, including HNSCC [32,36,37]. Our results indicate that PDL1 and PDL2 are significantly overexpressed in samples with aUPD compared with those without aUPD. These findings provide potential insight into the mechanisms of PDL1 and PDL2 overexpression in HNSCC and support previous reports. Our results also suggest that identification of aUPD in defined regions may help to select patients for individualized therapy. *TTF1*, which encodes transcription termination factor 1 was also overexpressed in samples with aUPD in defined regions. *TTF1* expression and blood vessel invasion were shown to correlate with PDL1 expression in sarcomatoid lung carcinoma [38]. Moreover, overexpression of *TTF1* has been shown to be associated with poor prognosis in colorectal cancers [39].

Of note, we found that aUPD at the *CDKN2A* region was associated with OS in all samples and in HPVnegative samples, but not in HPVpositive samples. Indeed, expression of CDKN2A in samples with aUPD was significantly lower than in samples without aUPD. In contrast, deletion at the same region was not associated with OS or RFS in any of the sample groups. Other genes with significantly higher expression in samples with aUPD in defined regions compared with those without aUPD included mitochondrial genes (*HINT2*, *ACAT1*), genes involved in galactose (*GALT*, galactose1phosphate uridyltransferase) and carbohydrate metabolism (*GBA2*, glucosidase, beta [bile acid] 2), genes involved in nucleotide exchange (*KIAA1432* and *C9ORF100*), genes involved in oxidative stress defense (*ERMP1*, endoplasmic reticulum metalloprotease 1), ion channel and DNA excision repair genes (*XPA*, xeroderma pigmentosum, complementation group A), and nonreceptor tyrosine kinase gene (*JAK2*). In other studies, downregulation of *ERMP1* and *C9orf100* significantly reduced cell proliferation and migration [40–42]. *CAT1* encodes a mitochondrially localized enzyme that catalyzes the reversible formation of acetoacetylCoA from two molecules of acetylCoA and regulates pyruvate dehydrogenase complex [43]. Inhibition of *ACAT1* decreases cell proliferation and tumor growth [43].

In addition, *VCP* (valosincontaining protein), a member of the AAAATPase protein family, encodes a protein that interacts with other proteins to regulate endoplasmic reticulum–associated protein degradation. This involves multiple cellular functions during mitosis, including regulation of the spindle pole body, vesicular trafficking, and membrane fusion [44]. Expression of *VCP*, *DCTN3*, and *STOML2* independently plays a role in increasing cell growth and anchorageindependent growth during the development of invasive oral carcinoma [45]. *VCP* also promotes growth, invasion, and metastasis in colorectal cancer through activation of STAT3 signaling [46]. Inhibition of *VCP* expression suppresses West Nile virus infection [47], as well as suppressing the cell cycle, inducing endoplasmic reticulum stress, and inducing caspasemediated cell death in ovarian cancer cells [48]. *ANP32B* encodes

histone chaperone acidic nuclear phosphoprotein 32B (ANP32B) and plays an antiapoptotic role. Overexpression of ANP32B has been shown to lead to accumulation of henipavirus matrix (Hendra virus; HeV M) and nuclear proteins (Nipah virus; NiV M) [49]. Downregulation of ANP32B induces apoptosis in myeloid leukemia cells [50]. Collectively, with recent evidence support roles for the microbiome and a number of viruses in human cancer, it is possible that HeV M and NiV M may be involved in HNSCC development. Further, inhibition of ANP32B expression may enhance apoptosis in HNSCC cells. Our findings indicate that genes involved in galactose, carbohydrate and mitochondrial metabolism, nucleotide exchange, oxidative stress defense, and ion channels may be also involved in the pathogenesis of HNSCC, and inhibition of expression of these genes may reduce cell growth or invasion.

In summary, we demonstrated associations with patient outcome and identified genes in specific SORs of aUPD that are differentially expressed in patients with aUPD, compared with patients without aUPD. These differentially expressed genes may be an indicator of tumor aggressiveness, and in turn affect survival. Therefore, aUPD analysis may be a useful tool to select targeted therapy for this heterogeneous disease.

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Conflicts of Interest

The authors declare no potential conflicts of interest.

Authors' contributions

MT conceived and coordinated the study. MT and GBM designed the study. MT developed the methodology. MT, WL, CIA, GBM analyzed and interpreted the data; MT draft the manuscript; MT, WL, CIA and GBM critically discussed the data and revised the manuscript. All authors read and approved final manuscript.

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