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Frequent microsatellite alterations of chromosome locus 4q13.1 in oral squamous cell carcinomas

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BACKGROUND: Studies have revealed that losses of chromosome 4q24–25 regions are frequent in cancers including head and neck squamous cell carcinoma. Our previous comparative genomic hybridization analysis showed extensive losses of chromosome arm 4q in oral squamous cell carcinoma (OSCC).

METHODS: To be more precise in mapping the potential regions of allelic losses and to understand the microsatellite instability (MSI) on 4q involving in oral pathogenesis, we performed allelotypings using eight polymorphic markers. Microsatellite analyses were first performed on 100 randomly selected controls to confirm the high informative rates of markers. Twenty OSCC tissues were microdissected from surgical specimens for microsatellite alterations (MA) analysis.

RESULTS: MA was observed in 95% OSCC cases. The most eminently altered locus was 4q13.1 (75%), followed by 4q22.2 and 4q32.1 (55%). Allelic losses also occurred most frequently on these loci. Thirty-five percent cases had MA spanning 4q13.1 to 4q21.1. MSI occurred in 35% OSCC, at a lesser extent compared with allelic losses. The most common locus for MSI was 4q21.2 (20%). In addition, 4q MSI was significantly associated with the lymph node metastasis of OSCC ($P = 0.01$). So far, most tumor suppressor genes on 4q have not been specified.

CONCLUSION: Our results were additive to previous findings and proposed novel scenario of suppressor loci located at 4q13.1–21.1 whose inactivation could be important for progression of OSCC.

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Oral squamous cell carcinoma (OSCC) is the third most frequent neoplasm in developing nations and fourth leading neoplasm in male population in Taiwan (1). It is known that tumorigenesis is a multistep process resulting from the accumulation of detrimental genetic alterations (2). Inactivation of tumor suppressor gene (TSG) plays important roles in tumorigenesis of OSCC. Allelic losses as homozygous deletion (HD) and loss of heterozygosity (LOH) at polymorphic loci are key events of TSG. For TSG with LOH, the remained allele could be inactivated by mutation or promoter hypermethylation (2). Researches have indicated that OSCC showed a plethora of genetic aberrations, including losses of 3p, 4q, 8p, 9p, 13q and 18q (1, 3–13). Allelic loss of 4q or entire chromosome 4 was common in neoplasms, including bladder, cervical, colorectal, hepatocellular, breast, skin and esophageal carcinomas, as well as head and neck squamous cell carcinomas (HNSCCs), which include OSCC (1, 7–9, 11–18). Furthermore, 4q losses were important for genesis and progression of OSCC (1, 19). Phenotypic tumor suppression has been observed with the introduction of chromosome 4 into human cancer cells (20). Thereby, chromosome 4 is likely to contain TSGs that are frequently inactivated in neoplasms. Researches have disclosed that loci around 4q24–25 were frequently lost in HNSCC (7–10). Another region frequently lost in HNSCC was 4q28–29 (9). However, none of the genes on these regions was shown to be a TSG.

Genetic instability is a common event in cancers; this instability might cause the accumulation of mutations that confer growth advantages for tumor cells. Impaired DNA repair capacity, also called mutator phenotype, is usually responsible for genomic instability. Microsatellite instability (MSI) is a pattern of genomic instability with high rates of frameshift mutations observed in short tandem repeat sequences. It is represented by insertions or deletions of nucleotides. The genomic instability in HNSCC seems to be rather widespread (2, 21). Although it was generally believed that allelic loss was more prominent than MSI in HNSCC (22, 23), MSI of 3p, 9p and 17p have become important markers for the

development and progress of HNSCC (22). A recent study addressed that the promoter hypermethylation could be the possible underlying mechanism for the inactivation of DNA repair genes in HNSCC that conferred the susceptibility for genetic instability (24).

Previously, we have identified 4q loss in OSCC using comparative genomic hybridization (CGH) (1). Most of the 4q losses were rather extensive. CGH cannot detect small deletions, which might underscore the chromosomal losses in neoplasms. As microsatellite alterations (MA) have been recognized as important events in HNSCC, and being suggested involved in both suppressor and mutator pathways, the present allelotypings were undertaken to determine the MA involving chromosome 4q in OSCC.

Materials and methods

Subjects

Surgical specimens were obtained from 20 OSCC. The resected tumor tissues were snap frozen immediately until use. All patients were male. The ages of the patients ranged from 34 to 73 years with a mean of 54 years. The most common primary site was buccal mucosa (50%, 10 cases), for 80% patients were areca chewers. In histopathological grading, 30% (six cases), 60% (12 cases) and 10% (two cases) of OSCC showed well, moderate and poor differentiation, respectively. Sixty percent (12 cases) of the patients presented with lymph node metastasis (LNM) and 75% (15 cases) of the patients were at stage IV. One hundred normal individuals were randomly selected from people who came for routine physical checkup. This study was approved by an ethics reviewing committee. After an informed consent was obtained, blood was drawn from subjects. A leukocyte cell pellet was obtained from the buffy coat by centrifugating the whole blood. Representative thin tumor sections were stained with hematoxyline and eosin (H&E) first using frozen section. Microdissection was performed to remove non-cancerous tissue under microscopy to achieve at least an 80% purity of the neoplastic cell population. DNA was isolated by Qiagen Mini Kit (Qiagen, Valencia, CA, USA).

Microsatellite analysis

Multiple informative loci markers D4S3029 (4q13.1), D4S1517 (4q21.1), D4S414 (4q22.2), D4S407 (4q25), D4S430 (4q27), D4S424 (4q31.22), D4S413 (4q32.1) and D4S1529 (4q34.1), with an average of approximately 15 cM between markers, were used for 4q allelotyping (Table 1). Primer pairs were used to identify specific dinucleotide and tetranucleotide repeats polymorphisms. The fluorescent dyes used for primer labels and types of polymorphism in marker segments were described in Table 1. The sequences used to amplify the marker segments were obtained from Genome Data Base (GDB) and Marshfield database. A 10 ng genomic DNA was used as template. The amplicons were detected by laser fluorescence on ABI377 autosequencer (Applied Biosystems, Foster City, CA, USA). Data were analyzed by genescan analysis and genotyper software (Applied Biosystems).

Intensity calculations and comparisons were performed on the amplicons of patient's leukocyte DNA and OSCC DNA to define MA, including HD, LOH and MSI. A reproducible detection of the absence of

Table 1 Primers used for analysis

Marker (locus)	*Sense primer (5′–3′)*	*Antisense primer (5′–3′)*	*Dye*	*Type*
D4S3029 (4q13.1)	[a]gccagggtcctaactgat	ggctcaatgtcctcaga	FAM	DN
D4S1517 (4q21.1)	[a]agctaactactctccacccatac	ccatgctaagtttatgatgtctg	FAM	DN
D4S414 (4q22.2)	ttgcacaaagcatcagcc	[a]tcaggaacctcagcccat	HEX	DN
D4S407 (4q25)	[a]ataatatcctttgatcctttcgcta	aaatttggttatttttaagcaaact	TAMRA	DN
D4S430 (4q27)	[a]taaccctgtatatgttaatgtgc	ggacccagtcttgctatg	FAM	DN
D4S424 (4q31.22)	[a]gcgctcttggtatatggtacag	tgtgggcaacgtcactc	HEX	DN
D4S413 (4q32.1)	[a]tctgaatatagtgctccagaaa	caatcagtgggtttttgaa	HEX	DN
D4S1529 (4q34.1)	gcaaaagagtgaaattctatc	[a]ttctgatatatagtgagggc	TAMRA	TN

DN, dinucleotide repeats marker; TN, tetranucleotide repeats marker.
[a]Labeling site.

Table 2 Informative rate of 4q markers of normal individuals

	Informative rate (%)					
Marker (locus)	*GDB*	*Present study*	*Range of amplicon size (bp)*	*Most frequent alleles (bp)*		
D4S3029 (4q13.1)	85	79	155–185	157	173	177
D4S1517 (4q21.1)	80	89	200–240	212	218	222
D4S414 (4q22.2)	89	70	225–250	237	239	241
D4S407 (4q25)	87	88	105–140	116	124	126
D4S430 (4q27)	81	79	115–145	118	120	134
D4S424 (31.22)	84	65	170–200	174	178	180
D4S413 (4q32.1)	85	48	120–166	126	138	154
D4S1529 (4q34.1)	80	85	180–220	197	201	205

amplicon for a marker from OSCC DNA indicated a HD. LOH was determined by calculating the band intensity of normal and OSCC alleles following the formula: (intensity of normal allele 2/intensity of normal allele 1)/(intensity of OSCC allele 2/intensity of OSCC allele 1) to obtain a ratio. The ratio <0.5 or >2.0 indicated LOH (25). MSI was defined by the occurrence of at least one extra band in OSCC DNA. A case showing MA in less than 50% (i.e. four) evaluated markers was defined as partial MA. More than two independent experiments were performed to confirm the reproducibility of each analysis.

Statistical analysis

The variants were analyzed using Fisher's exact test. Differences between the values were considered significant when $P < 0.05$.

Results

Microsatellite analysis was initially applied for detecting the informative rate of various markers in normal population, as race difference may cause great discrepancies in polymorphisms. The data revealed by genotyper in Table 2 showed a slight disparity in the informative rate between our population and the GDB. It indicated that nearly all markers selected exhibited high informative rate in our study. Even the worst marker, D4S413, displayed an informative rate of 48%. The ranges of amplicon size and the most frequent alleles detected in our population were organized in Table 2.

Microsatellite analysis was then used to determine the frequency of MA from 4q in OSCC. Representative patterns of LOH and MSI were illustrated in Fig. 1. The MA of all cases was plotted in Fig. 2, with 95% (19 cases) of OSCC showing MA in one or more informative markers. Partial MA was observed in 60% (12 cases) of OSCC. In 35% (seven cases) of OSCC, the MA was extensive, spanning all or >4 markers per case (Cases 1–7 in Fig. 2). There were 76 MA in total being identified. The distribution of these MA in 4q did not show discrepancy, with 42 (55%) MA distributed in more centromeric markers and the other 34 (45%) MA distributed in more telomeric markers (Fig. 2). Although informative rate of D4S413 (4q32.1) in OSCC cases was only 50%, it was noted that nearly all-informative cases in D4S413 had MA.

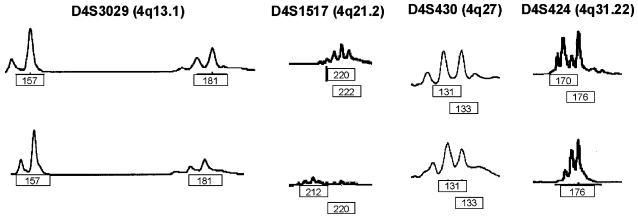

Figure 1 Microsatellite alleotyping. Markers D4S3029, D4S1517, D4S430 and D4S424 were amplified. The analysis of amplicons was carried out on an ABI377 autosequencer. The allele sizes are indicated in the boxes below the peaks. *Y*-axis indicates the intensity of fluorescence. The upper row is leukocyte DNA. The lower row is OSCC DNA. Note the LOH in D4S3029, D4S430 and D4S424, and both LOH and MSI in D4S1517. The allele 170 in D4S424 was completely deleted in OSCC counterpart. The D4S1517 peaks were adjusted to minimal level for better illustrating peak 212. Note the loss of allele 220 and the occurrence of allele 212 in OSCC.

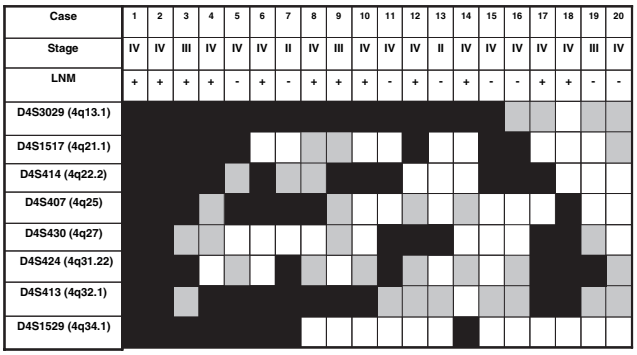

Figure 2 Distribution of microsatellite alterations. Filled boxes, loci with MA; shadow boxes, loci not informative; open boxes, loci without MA. Note the extensive MA in cases 1–7, partial MA in cases 8–19, and the absence of MA in case 20. Loci 4q13.1 to 4q21.1 were regions with highest density for MA.

The detailed frequencies of various MA were summarized in Table 3. MA was observed in all markers, with D4S3029 (4q13.1) as the most eminently altered one (75%), followed by 55% in D4S414 (4q22.2) and D4S413 (4q32.1). Thirty-five percent cases had MA at both 4q13.1 and 4q21.1. Allelic losses, characterized by HD and LOH, were also prominent in these discrete markers. Only 35% (seven cases) of OSCC had MSI.

Table 3 4q microsatellite alterations (MA) in oral squamous cell carcinoma (OSCC)

Marker (Locus)	*HD*	*LOH*	*MSI*	*LOH and MSI*	*MA*
D4S3029 (4q13.1)	6/20 (30)	9/13 (69)	0	0	15/20 (75)
D4S1517 (4q21.1)	0	4/17 (24)	4/20 (20)	0	8/20 (40)
D4S414 (4q22.2)	4/20 (20)	6/16 (38)	1/20 (5)	0	11/20 (55)
D4S407 (4q25)	4/20 (20)	3/16 (19)	0	1/20 (10)	8/20 (40)
D4S430 (4q27)	2/20 (10)	4/14 (29)	1/20 (5)	0	7/20 (35)
D4S424 (31.22)	2/20 (10)	5/13 (38)	1/20 (5)	0	8/20 (40)
D4S413 (4q32.1)	5/20 (25)	3/5 (60)	2/20 (10)	1/20 (10)	11/20 (55)
D4S1529 (4q34.1)	2/20 (10)	5/20 (25)	1/20 (5)	0	8/20 (40)
Total[a] (%)	25	39	10	2	76

HD, homozygous deletion; LOH, loss of heterozygosity; MSI, microsatellite instability.
[a]Total number of alterations in all cases.

Table 4 4q microsatellite instability (MSI) and clinical parameters [Fisher's exact test (%)]

	MSI		
	Presence (n = 7) [n (%)]	*Absence (n = 13) [n (%)]*	*P-value*
Lymph node metastasis			
Absence ($n = 8$)	0	8 (62)	0.01
Presence ($n = 12$)	7 (100)	5 (38)	
Stage			
I–III ($n = 5$)	0	5 (38)	0.11
IV ($n = 15$)	7 (100)	8 (62)	

Four cases were MSI-low, having only one MSI; and three cases were MSI-high, having two or more MSI. D4S1517 (4q21.1) was the region exhibiting highest ratio of MSI (20%). Among 76 MA identified, 33% was HD, 51% was LOH, 13% was MSI, and 3% exhibited both LOH and MSI (Table 3). Allelic losses were much prevalent than MSI. No significant association between patients' ages, differentiation grades, LNM as well as clinical stage and the status of MA or allelic loss was observed. However, all OSCC carrying 4q MSI had regional LNM (Table 4). A statistically significant association lied between the regional LNM and the 4q MSI status in OSCC ($P = 0.01$).

Discussion

The patterns of genetic or chromosomal alterations in a tumor might indicate certain phenotypic disruption or neoplastic features. Alterations on 4q, mostly losses, were detected in > 50% human cancers, which suggested the presence of TSGs on this chromosomal arm (1, 15, 18). Previous reports indicated that 4q was lost in approximately 50% OSCC or HNSCC (1, 7–9, 11–13). Hence, 4q has been implicated to play an important role in the genesis of OSCC. We performed allelotyping to identify MA of 4q and found that the overall frequencies of MA at any site of 4q were high in OSCC. Whereas, allelic losses were common in nearly all (95%) cases, MSI were only detected in 35% cases. In this study, 35% OSCC were found to have extensive MA which agreed our previous CGH observations (1). Moreover, there were additional 60% OSCC exhibiting partial or scattered MA, which suggested the better sensitivity of allelotyping in detecting small alterations. One-third of MA was identified as HD, which brought us the concern of possible false results from the incompetence of PCR reaction. However, the input DNA was purified from microdissected frozen sections, which should maintain their high integrity. The reproducibility of the experiments and the absence of preferential loss of chromosomal substances had substantiated the existence of 4q HD in OSCC.

With microdissected samples, 4q allelic loss identified in our study was higher than previous report. It suggested that the 4q alterations might be more prevalent than studies shown before. Discrete regions of 4q24–26 and 4q33–34 have been mapped in multiple neoplasms for losses (7, 8, 16–18). Approximately 40% alterations on 4q24 and 4q34 identified in OSCC agreed with previous findings. Studies have shown that in up to 75% HNSCC had the region 4q24–25, or near this site, deleted (7–10). However, our data identified more regions, including 4q13.1, 4q22.2 and 4q32.1, were frequently altered in OSCC. The alterations of 4q13.1 were particularly eminent (75%). Moreover, 4q13.1–21.1 was the region altered most frequently in OSCC, centromeric to hotspot regions identified previously. These specific regions, plus the regions frequently lost in cancers as previously reported, suggested that there may be multiple TSGs in 4q. Their inactivation may be important in the carcinogenesis. We are currently performing finer mapping of 4q hotspot regions, particularly 4q13.1, by high-density microsatellite markers to identify minimal overlapping region. These processes could aid elucidating the putative TSG in these regions. In this study, no relevance between clinical parameters and MA or allelic losses was found. It might due to most likely the high prevalence of alterations.

We found that 4q allelic losses were more prominent than 4q MSI, which supported the dominant role of the suppressor pathway in comparison with the mutator pathway in oral carcinogenesis. Interestingly, a recent paper showed the relationship between genetic instability of OSCC and areca consumption of the patients (26). Such linkage was not observed in our study, as the vast majority of patients were areca chewers. However, we found that all patients with MSI had LNM, which was drastically contrasting to the result that only 38% patients without MSI had LNM (Table 4). The findings implicated that gains of more genetic instability, as reflected by increase in 4q MSI, and the accompanied accumulation of mutations, could induce the acquirement of metastatic phenotypes. Understanding of genetic events frequently occurred in OSCC may be important for detection or prediction of tumor progression. This is the first study demonstrating that 4q MSI could be extrapolated for evaluating the metastatic status of OSCC. As MSI, in 3p, 9p and 17q, have been markers for early carcinogenic process (22), the cooperative use of MSI states in multiple chromosomal arms might add strength in discerning the neoplastic progress of OSCC.

This systematic allelotyping demonstrated the frequent MA in distinctive 4q region. To understand the roles of 4q alterations in multistage tumorigenesis, particularly that of 4q13.1, it is necessary to analyze further on lesions of preinvasive stage or at early stage of oral carcinogenesis (19). As TSGs in 4q have not been well characterized, they worth intensive studies.

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