

Analysis of Microsatellite Instability and Frameshift Mutation of TGF β Type II Receptor Gene in Oral Squamous Cell Carcinomas

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(Received for publication, March 3, 1999)

ABSTRACT

In the present study, replication errors (RERs) at microsatellite loci were determined in oral squamous cell carcinomas (OSCCs). Moreover frequencies of microsatellite instability (MI) in 34 single primary cancers (SCs) were compared with those in 11 oral cancers of multicentric or multiple primary cancers (MCs). In 3 (27.3%) of 11 MCs and 2 (5.9%) of 34 SCs, RER phenotype was observed at two microsatellite loci on chromosomes 1q and/or 2p. All the RER(+) cases could be detected using BAT40 marker (1q). In addition, none of OSCCs showed mutation of TGF β receptor type II (TGF β RII). These results probably indicate that genetic instability may play a little role in carcinogenesis of OSCCs and development of oral cancers of MCs, but RERs occurred in cancers of sibling patients with multicentric cancer, and in one of them allele loss at *hMLH1* was observed. The cancers of these patients may be related to genetic defects of the mismatch repair system.

INTRODUCTION

Microsatellites are short-repeated nucleotide sequences interspersed throughout the human genome. The repeating unit comprising a microsatellite can be as short as one or two nucleotides. Alterations of microsatellites consisting of extra or missing copies occur at relatively high frequencies in hereditary non-polyposis colorectal cancer (HNPCC)¹⁻³. A tumor phenotype displaying frequent replication errors (RERs) has been associated with HNPCC^{4,5}. The RER phenotype is

believed to be associated with a DNA mismatch repair defect in the tumor cells as a consequence of inadequate function of either *hMLH1*, *hMSH2*, *hPMS1*, *hPMS2* or *GTBP* involved in mismatch repair^{1,3,6-10}. The RER(+) has been reported not only in tumors associated with HNPCC but also in sporadic form of tumors that developed in colon, stomach and others^{3,4,11,12}. On the other hand, Horii *et al.* previously reported that the RER(+) was observed high-frequent in MCs than in SC¹³. The results suggest that genetic instability may play a important role in development of MCs. The detection of RER in tumors may constitute a useful marker for screening of development of MCs¹³. On the other hand, Simada *et al.* described that frequency of RER(+) in esophageal cancers of MCs was very low, and defects of DNA mismatch repair system were not demanded in the development of these multiple tumors¹⁴.

TGF β inhibits growth of various epithelial cells and TGF β RII is the primary ligand for this¹⁵. Recently, it was reported that TGF β RII frequently mutated in colon and other cancers of RER(+) ^{16,17}.

Mutation of *p53* is the most common genetic alteration associated with human cancer¹⁸. A major function of *p53* is believed to be as a cell cycle check point gene and to induce by DNA damage with a cell cycle arrest at G1-S interface¹⁹⁻²¹. Tumor cells lacking wild-type *p53* do not display this DNA damage-induced cell cycle arrest^{22,23}. In addition, it is known that wild-type *p53* protein stimulates DNA repair through *Gadd 45* and *ERCC 3*^{24,25}. Thus, it is especially interesting to reveal incidence of genetic changes of *p53* gene in the RER(+) and it is necessary to make clear an association between genetic changes, such as mutations in *p53* genes and genetic instability caused by abnormalities of DNA mismatch repair system.

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MATERIALS AND METHODS

Tumor specimens and DNA preparation

Forty-five OSCCs including 11 oral cancers of MCs were collected from surgical specimens. These materials were immediately snap-frozen in liquid nitrogen or fixed with formalin and embedded in paraffin. In addition, corresponding normal tissues or lithium heparinized blood were collected as a control source of normal leukocyte DNA. Genomic DNA was isolated by proteinase K digestion and phenol-chloroform extraction, as described previously^{26,27}.

MI and LOH of TP53, hMLH1 and hMSH2

Microsatellite primer sets for the four loci examined in the present study were previously described as follows: D2S123²⁸, BAT25¹⁶, BAT26¹⁶ and BAT40¹⁶. Polymerase chain reaction (PCR) was performed in 15.0 μ l reaction volumes with 20–50 ng of genomic DNA, 4.5 mM MgCl₂, 200 μ M each deoxynucleotide triphosphate, 2.0 pmol of labeled with [γ -³²P]ATP and unlabeled primer, reaction buffer (X10) and 0.5 units of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). Conditions were standardized for all reactions and consisted of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C for 40 cycles. A final extension cycle of 3.5 min at 72°C was also included¹². PCR products were electrophoresed in 6% polyacrylamide-8M urea gels and autoradiographed for 8–24 hours on Fuji RX film. MI was scored when new alleles appeared in the tumor tissue, compared with the normal tissue.

TP53, hMLH1 and hMSH2 CA dinucleotide repeat primer sets were previously described as follows: TP53²⁹, D3S1611 and CA21¹. Allele loss was scored blind by two individuals independently. The signal intensity of the normal and tumor alleles were also analyzed by scanning densitometry. Allele loss was inferred when

the ratio of two alleles in the tumor tissues was below 50% of that in the matching normal tissues.

TGF β RII assay

A 73bp fragment carrying TGF β RII gene adenine-mononucleotide repeat was amplified, as previously described using primer sets BAT RII^{16,17}.

RESULTS

The detail of microsatellite markers and TGF β RII adenine-mononucleotide repeat marker were shown in Table 1. Of 11 oral cancers of MCs, MI was observed in 3 cases (27.3%) (Fig. 1a, 2, Table 2). In Cases M27 and MF29, RER phenotypes were observed using both D2S123 and BAT40 markers (Fig. 1a, Table 3). Of 34 sporadic OSCCs, MI was found in 2 cases (5.9%) (Fig. 1a, 2, Table 2). In Cases S22 and S44, RER phenotype was observed using only BAT40 markers (Table 3). All the RER phenotypes could be detected with BAT40 marker.

On the other hand, we examined mutation of TGF β RII and LOH at *p53*, *hMLH1* and *hMSH2* loci with these intragenetic or proximal microsatellite markers (TP53, D3S1611 and CA21), and compared with RER status (Table 3). In all tumors, the 10bp poly A tract of TGF β RII was remained as both wild type alleles (Fig. 1b). LOH at TP53 was 7 tumors (36.8%) of 19 informative cases. Of RER(+) cases, Cases MF21 and S22 showed allele loss. Cases M27, MF29 and S44 showed homozygous alleles as uninformative cases (Table 3). Frequency of LOH at D3S1611 was 8.0% (2/25). Allele loss at D3S1611 was observed in Case MF21 and S44 of RER(+) cases (Table 3, Fig. 2). Frequency of LOH at CA21 was 8.3% (2/24). Allele loss at CA21 was observed in Case S44 of RER phenotype cases (Fig. 1a).

Cases MF21 and MF29 having multicentric oral cancers were in sibling relation. This family consisted of

Table 1 Microsatellite markers and TGF β type II receptor adenine-mononucleotide repeat marker

Marker	Locus	Position in locus
D2S123 (CA) ₂₈	2p16	linked to <i>hMSH2</i> gene
BAT25 (A) ₂₅	4q12	within an intron of <i>c-kit</i> oncogene
BAT26 (A) ₂₆	2p21–22	within intron 5 of <i>hMSH2</i> gene
BAT40 (A) ₄₀	1q13.1	within an intron of 3- β -hydroxysteroid dehydrogenase gene
BATRII (A) ₁₀	3p22	TGF β type II receptor codons 125–128

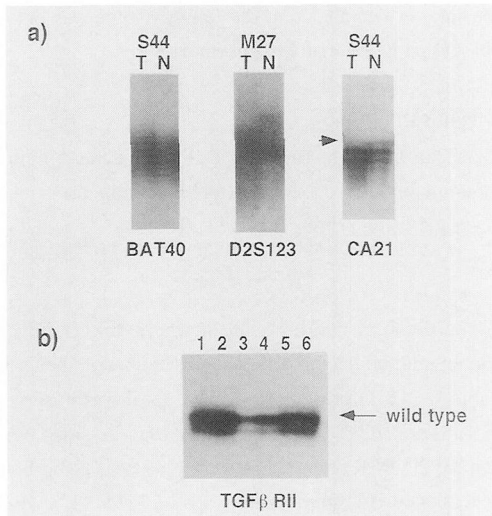


Figure 1 a) RER phenotypes and allele loss of hMSH2 gene with proximal marker CA21 in OSCCs. T; tumor, N, normal control. Arrowhead; allele loss in tumor. b) A 73bp fragment carrying *TGFβ* RII gene adenine mononucleotide repeat shown in Lanes 1 (MF29), 2 (MF21), 3 (S22), 4 (MF27), 5 (S44) and 6 (S28). All the cases showed wild type alleles.

Table 2 Frequency of RER+ cases in oral squamous cell carcinomas

Single oral cancer	2/34 (5.9%)
Oral cancer of multicentric or multiple primary cancers	3/11 (27.3%)
$\chi^2 = 3.21 < 3.84$	$P = 0.0853 > 0.05$

Table 3 RER phenotype cases of oral squamous cell carcinomas

case No.	age and sex ^a	location	TNM class. and stage ^b	other primary cancer	microsatellite markers ^c	RII mutation ^d	p53 LOH ^e	<i>hMLH1</i> LOH ^f	<i>hMSH2</i> LOH ^g
M27	71 F	gingiva	T4N2aM0, IV	esophagus	D2S123 and BAT40	wt	/	MI	/
MF29	51 M	palate	T2N1M0, III	tongue	D2S123 and BAT40	wt	/	/	—
MF21	55 F	gingiva	T4N0M0, IV	palate	BAT40	wt	LOH	LOH	—
S22	46 M	tongue	T1N0M0, I	—	BAT40	wt	LOH	nd	nd
S44	68 F	gingiva	T3N0M0, III	—	BAT40	wt	/	LOH	LOH

a. F; female, M, male

b. TNM class.; TMN classification and clinical staging of the oral cancers were according to criteria of the Japan Society for Head and Neck.

c. RER phenotype: Microsatellite instability observed in these microsatellite markers.

d. Mutation of *TGFβ*-RII at a 10bp poly A tract: wt, wild type alleles.

e, f, g. LOH of TP53, *hMLH1* (D3S1611) and *hMSH2* (CA21) CA repeats regions: —, heterozygous, /; homozygous, nd; not determined, MI; microsatellite instability.

four brothers and four sisters. Of them, 3 siblings were suffered from oral cancer (Fig. 2). They had no habits of drinking and smoking, and no specific backgrounds. Of oral cancers of MCs without these cases, frequency of MI was 11.1% (1/9).

DISCUSSION

According to a previous study³⁰, MI occurred in 7% of tumor samples of principally OSCCs. It is suggested that MI seems to occur during the clonal expansion stage in oral tumors and there is no evidence linking genomic instability for presence of *p53* mutations. In this study, frequency of MI in sporadic OSCCs was 5.9% and RER was observed also at early stage of cancer (Case S22). MI did not depend on sex, age and *p53* LOH of cancers. On the other hand, Simada *et al.* described that the frequency of RER(+) in esophageal cancers of multiple primary cancers was very low, and genetic defects in DNA mismatch repair pathway did not play important roles in the development of these multiple tumors¹⁴. We determined MI in oral cancers of MCs. The frequency of RER(+) was very low (27.3%) (11.1%, if the familial cases excluded). These results indicate that genetic instability may play a little role in carcinogenesis of OSCCs and development of oral cancer of MCs. To detect MI in OSCCs, BAT40 was a good marker. Using only this marker, it must be better to know whether or not a oral cancer is caused by defecting the DNA mismatch repair system. Interestingly, we experienced multiple oral cancers family. Familial or hereditary OSCC was very rare. We analyzed genetic instability for oral cancers as Cases MF29

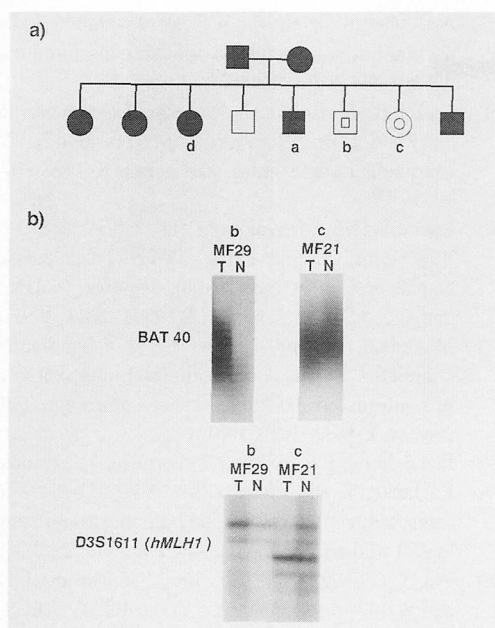


Figure 2 a) Pedigree chart of a family with multiple oral cancers. a; OSCC, primary site: buccal (age: 50 years-old) and tongue (52 years-old), b; OSCC, Case MF29, c; OSCC, Case MF21, d; s/o colon cancer (29 years-old). b) MI and allele loss at hMLH1 gene in Cases MF29 and MF21. Allele loss at hMLH1 gene was observed in Case MF21. Case MF29 showed homozygous alleles as uninformative case. T; tumor, N, normal control.

and SF21. The cancers of these patients may be caused by genetic instability. And in germine of these patients, there was a possibility of genetic defect of the mismatch repair system.

TGF β RII has a 10 bp poly A tract in its open reading frame and its mutations in cancers were concentrated at this region as small insertion or deletion. In colon and gastric cancers of RER(+), mutations were frequently observed at this region of TGF β RII¹⁶. Mutation of this gene provides a common route for RER(+) carcinogenesis in both of upper and lower gastrointestinal tract. In this study, we found no mutations at this gene in RER(+) OSCCs. Whereas RER(+) oral cancers may proceed by a different mechanism and perhaps oral cancers have not commonly required TGF β resistance.

In conclusion, infrequent MI and no mutation of TGF β RII were shown in OSCCs including oral cancers of MCs. In the cancers of sibling patients, RER pheno-

type and allele loss at hMLH1 were observed. Further work is needed to examine MI in dysplasias of oral mucosa, and to detect mutation of hMLH1 gene in germline.

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