Loss of heterozygosity of the PTH/PTHrP type 1 receptor in oral squamous cell carcinoma

HITOSHI MIYASHITA[1], SHIRO MORI[1], YUTAKA FUKUMOTO[3], ATUSHI SATO[4],
MANABU FUKUMOTO[2] and HIROSHI KAWAMURA[1]

[1]Division of Maxillofacial Surgery, Department of Oral Medicine and Surgery, Graduate
School of Dentistry, and [2]Department of Pathology, Institute of Development, Aging and Cancer,
Tohoku University, Sendai 980-8575; [3]Department of Oral Surgery, Tokyo Metropolitan Futyu Hospital,
Tokyo 183-8524; [4]Department of Oral Surgery, Sendai Medical Center, Sendai 983-8520, Japan

Abstract. Parathyroid hormone-related protein (PTHrP) is produced by various types of carcinomas, and is an important factor in the development of bone metastasis. The coexpression of PTHrP and parathyroid hormone/parathyroid hormone-related protein type 1 receptor (PTHR1) in cancer predicts poor patient survival. While genetic transformations of thyroid hormone receptor ß (THRß) have been reported as being associated with reduced survival in patients with oral squamous cell carcinoma (OSCC), the details of transformations in PTHR1 have not been extensively analyzed. The aim of this study was to examine loss of heterozygosity (LOH) and microsatellite instability (MSI) in PTHR1 in OSCC. Analysis of genetic transformations using microdissected clinical tissues revealed that the proportions of LOH and MSI in PTHR1 were 30.0 (3/10) and 20.0% (2/10), respectively. Furthermore, the proportion of carcinomas which developed with LOH on the chromosome of PTHR1 and without LOH for tumor suppressor genes such as *p53*, *FHIT*, *APC*, *BRCA1*, *BRCA2* and *DCC* was 20.0% (2/10). These observations suggest that transformations in PTHR1 may be involved in carcinogenesis in human OSCC.

Introduction

Parathyroid hormone-related protein (PTHrP) is in part responsible for the clinical syndrome termed humoral hypercalcemia of malignancy (HHM), and has been implicated as an important factor in the development of bone metastasis (1). PTHrP is produced by various types of carcinomas, including oral squamous cell carcinoma (OSCC) cells (2). HHM in patients with OSCC attributed to PTHrP appears to be an ominous prognostic sign (3). It has recently been reported that the coexpression of PTHrP and its type 1 receptor (PTHR1) in early breast cancer predicts poor patient survival (1). Furthermore, a mutant PTHR1 is a candidate gene for enchondroma, common benign cartilage tumors of the bone which have the potential for malignant transformation to chondrosarcoma (4).

PTHR1 is located on 3p22-p21.1. Chromosome 3p, which includes PTHR1 and thyroid hormone receptor ß (THRß) located on 3p24.1-p22, has one of the highest incidences of loss of heterozygosity (LOH) at loci for OSCC (5,6). LOH in the THRß gene has already been reported, and has been associated with reduced survival (7). However, the details of transformations including both LOH and microsatellite instability (MSI) in PTHR1 have not been extensively analyzed.

LOH and MSI are important events in the carcinogenesis of various types of carcinoma, including OSCC. LOH implies the loss of microsatellite loci, suggesting genetic loss, whereas MSI indicates replication error. Human carcinomas develop through a multistep process involving the activation of oncogenes and the inactivation of tumor suppressor genes (TSGs). Chromosomal regions with LOH indicate genomic regions which may harbor TSGs. Recently, the role of TSGs such as *p53*, *APC*, *BRCA1*, *BRCA2*, *DCC* and *FHIT* was analyzed in numerous human solid tumors (8-12).

The purpose of the present study was to elucidate the involvement of PTHR1 in the carcinogenesis of human sporadic OSCC.

Materials and methods

Patients and tumor samples. Seventeen OSCCs and their adjacent non-neoplastic tissues were obtained from surgical procedures performed at the Tohoku University Dental Hospital and the Sendai Medical Center, Japan. Diagnostic verification, tumor subtyping and grading were performed by pathologists. Grading and pathological criteria of oral carcinoma were determined using the TNM classification.

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Correspondence to: Dr Hitoshi Miyashita, Division of Maxillofacial Surgery, Department of Oral Medicine and Surgery, Graduate School of Dentistry, Tohoku University, 4-1 Seiryo-mach, Aoba-ku, Sendai 980-8575, Japan
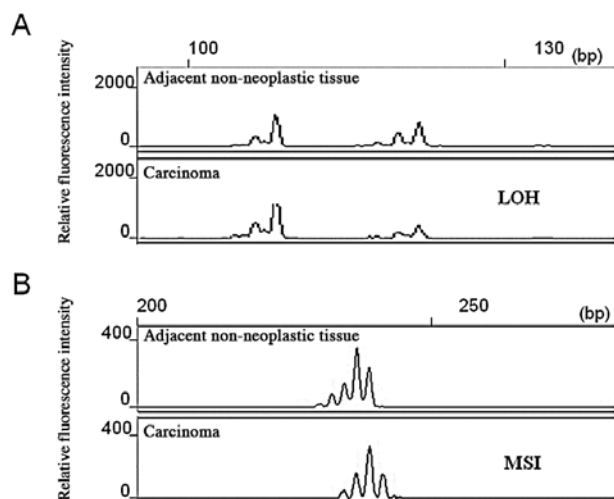E-mail: miyashita@mail.tains.tohoku.ac.jp

Figure 1. Representative result of (A) loss of heterozygosity (LOH) and (B) microsatellite instability (MSI) analysis in carcinoma tissue compared with adjacent non-neoplastic tissue.

The clinical and pathological characteristics of the cases are summarized in Table I.

DNA extraction. Before the tumor samples were processed for DNA extraction, sections from all the samples stained with hematoxylin and eosin underwent careful histopathological examination, which revealed the presence of a large amount of contaminating stroma. After deparaffinization of 10-μm sections stained with hematoxylin, neoplastic components were collected by a laser captured microdissection system (LMS, ver. 3.50, Carl Zeiss, Germany) to minimize the contamination of non-neoplastic stromal cells. Non-neoplastic tissues were used as normal controls. The dissected tissues were collected in an Eppendorf tube and incubated overnight at 58˚C in digestion mixture (0.01 M NaCl, 0.5 M Tris-HCl, pH 8.0, 20 mM EDTA, 0.05% Tween-20R and 0.1 mg/ml proteinase K). The samples were then heated to 95˚C for 10 min to inactivate proteinase K activity. After digestion, DNA was extracted with phenol/chloroform treatment and ethanol precipitation.

Analysis of LOH and MSI. The microsatellite markers used, all of which were purchased from Research Genetics (Huntsville, AL), are listed in Table I. Details regarding all markers can be found at the Genome Database (http://www.gdb.org). PCR reaction for LOH and MSI analysis was performed in a total volume of 10 μl containing 50 ng of DNA, dNTP at a final concentration of 20 μM, 0.4 μM of each primer and 0.25 U of Ex-Taq DNA polymerase (Takara Shuzo, Shiga, Japan). After the mixture was heated for 10 min at 94˚C, PCR was performed for 45 cycles at 94˚C at the appropriate annealing temperature and at 72˚C for 1 min, each followed by 72˚C for 10 min. After denaturation of the PCR products at 94˚C for 2 min, samples were subjected to electrophoresis using a Performance Optimized Polymer 4 in a 310 Genetic Analyzer (Applied Biosystems, Foster, CA).

LOH analysis was performed at loci which included two endocrine hormone receptor genes and six tumor suppressor genes, and also at nine loci with frequencies of LOH reported in OSCC (Table I). All markers used were dye-labeled. LOH
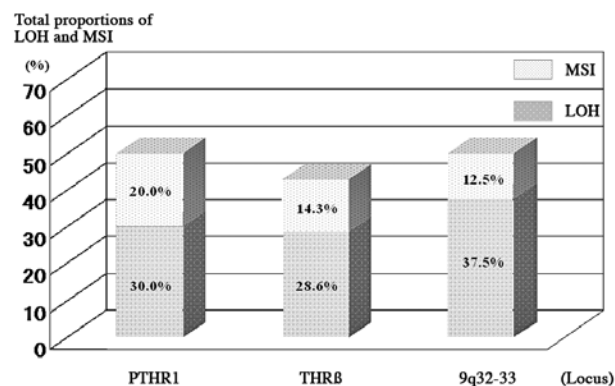

Figure 2. The proportions of loss of heterozygosity (LOH) and microsatellite instability (MSI) on loci including 3p21.2-14.2 (PTHR1), 3p24.1-21.3 (THRß) and 9q32-33.

was defined as a reduction of ≥50% in the band intensity of one of the tumor sample alleles when compared by Gene Scan version 2.1 to a homozygous normal tissue control (Fig. 1A). Cases with a homozygous normal tissue pattern were not informative for LOH analysis. For MSI analysis, eight microsatellite markers recommended by the National Cancer Institute for the detection of MSI were used (Table I). A locus with shifted bands or extra bands as compared to normal controls was defined as MSI-positive (Fig. 1B).

Results

LOH at loci on chromosomes including endocrine hormone receptors. We examined LOH at two loci on chromosomes including PTHR1 and THRß. The results of LOH analysis are shown in Tables I and II. The proportions of allele losses on 3p22.-21.1 (candidate gene, PTHR1) and 3p24.1-22 (THRß) were 30.0% (3/10, LOH cases/informative cases) and 28.6% (2/7) in the human microdissected OSCC tissues, respectively. The frequencies of LOH in THRß were similar to those of previous studies. As a result, the proportion of cases with at least one LOH at these loci was 23.5% (4/17).

MSI in OSCC. Only two cases reached MSI at one locus of the TSGs, on 18q21 (DCC) and 3p21.1-14.2 (FHIT). However, the proportions of MSI on 8p21.1-11.2, 3p24.1-26.5 and 9p22 (IFNα) were 33.3 (3/10, MSI cases/ informative cases), 30.7 (4/13) and 27.3% (3/11), respectively. Furthermore, the proportions of MSI at loci on 3p22.-21.1 (PTHR1) and 3p24.1-22 (THRß) were 20.0 (2/10) and 14.3% (1/7), respectively. In contrast, the proportion of MSI with eight microsatellite markers was 0% (Tables I and II).

High frequency of LOH and MSI at loci in OSCC. Total proportions of LOH and MSI at loci on 3p21.2-14.2 (PTHR1), 3p24.1-21.3 (THRß) and 9q32-33 reached 50.0 (5/10, LOH and MSI cases/informative cases), 42.9 (3/7) and 50.0% (4/8), respectively (Fig. 2). Moreover, at loci on 3p24.1-26.5 and 8p21.1-11.2, proportions were 46.1 (6/13) and 40.0% (4/10), respectively.

LOH of the PTHR1-mediated carcinogenic pathway independent of TSGs. We examined LOH on chromosomes where novel

Table I. Loss of heterozygosity and microsatellite instability analysis in OSCC.

Sample no.			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Stage			I	III	II	II	III	III	II	IV	IV	IV	III	I	II	II	II	IV	I
Histology			W	W	W	W	M	M	W	M	W	P	W	W	W	W	M	W	W
Gene	Position	Locus																	
Endocrine hormone receptor																			
PTHR1	3p21.1	D3S1606	▲	○	○	▲	—	○	—	—	○	●	—	●	●	△	○	△	△
THRß	3p24.1	THRß	△	△	●	△	○	—	—	▲	○	△	○	○	●	△	△	—	—
Tumor suppressor gene																			
p53	17q13.3	TP53	○	○	●	●	○	○	○	○	○	○	△	△	△	—	△	●	○
APC	5q21-22	D5S346	○	△	○	●	○	●	○	○	○	●	—	○	○	○	○	○	△
BRCA1	17q21	D17S579	○	○	—	●	○	○	○	○	○	○	—	—	—	—	—	—	●
BRCA2	13q12	D13S267	—	○	—	—	●	○	○	○	△	△	○	○	○	○	△	○	●
DCC	18q21	D18S59	—	○	○	○	○	○	○	—	○	▲	—	○	▲	○	○	—	—
FHIT	3p21.1-14.2	D3S1300	○	○	○	○	○	—	○	○	○	○	—	○	○	△	○	△	●
The frequency of LOH reported at loci for OSCC																			
	3p14.1-12	D3S1562	—	○	—	—	○	○	○	○	○	○	△	○	△	●	○	△	○
	3p14.1-13	D3S1296	○	○	○	—	○	○	●	○	—	●	△	○	△	—	△	△	—
	3p24.1-26.5	D3S192	○	○	▲	—	○	●	▲	○	—	●	○	○	▲	▲	○	—	—
	8p21.1-11.2	D8S298	▲	○	—	—	○	○	—	●	▲	●	△	△	△	▲	○	△	○
	9p21	D9S171	—	○	—	▲	○	○	△	—	○	●	△	△	△	△	△	—	—
	9p22	IFNα	○	▲	○	○	○	○	○	●	○	○	△	△	△	△	○	○	△
	9p23-22	D9S162	○	—	—	—	—	○	△	—	○	○	△	○	△	△	△	—	●
	9p24	D9S286	○	●	—	—	●	○	○	○	○	○	○	○	—	▲	○	—	○
	9q32-33	D9S177	—	△	—	○	—	△	△	●	○	●	○	○	—	▲	●	—	—
Microsatellite instability																			
	2p16	BAT25	○	○	○	○	○	○	○	○	○	○	△	○	△	○	○	○	○
	2p16	D2S123	○	○	○	○	○	○	○	○	○	●	○	○	○	○	○	○	○
	8p12	D8S87	○	○	○	○	○	○	○	○	—	—	○	○	○	○	△	—	—
	13q11	D13S175	○	○	○	○	○	○	○	○	—	○	○	—	△	○	○	○	●
	13q14.1-14.3	D13S153	○	○	○	●	○	○	○	○	—	○	○	○	△	○	○	○	○
	17q11.2-12	D17S250	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	—	○
	18q22.1	D18S55	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	—
	20p	D20S100	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	—	○

●, LOH; ○, normal; △, non-infomative; —, reaction failure; ▲, MSI. W, well-differentiated; M, moderately differentiated; P, poorly differentiated.

Table II. Proportion of loss of heterozygosity and microsatellite instability in OSCC.

Gene	Locus	MSI/inf (%)		LOH/inf (%)	
Endocrine hormone receptor					
PTHR1	D3S1606	2/10	(20.0)	3/10	(33.3)
THRß	THRß	1/7	(14.3)	2/7	(28.6)
Tumor suppressor gene					
p53	TP53	0/12	(0.0)	4/12	(33.3)
APC	D5S346	0/15	(0.0)	2/15	(13.3)
BRCA1	D17S579	0/10	(0.0)	1/10	(10.0)
BRCA2	D13S267	0/12	(0.0)	1/12	(8.3)
DCC	D18S59	1/13	(7.7)	1/13	(7.7)
FHIT	D3S1300	1/13	(7.7)	1/13	(7.7)
Frequency of LOH reported at loci for OSCC					
	D3S1562	0/11	(0.0)	1/11	(9.1)
	D3S1296	0/9	(0.0)	1/9	(11.1)
	D3S192	4/13	(30.7)	2/13	(15.4)
	D8S298	3/10	(30.0)	1/10	(10.0)
	D9S171	0/7	(0.0)	2/7	(28.6)
	IFNα	3/11	(27.3)	0/11	(0.0)
	D9S162	0/10	(0.0)	3/10	(30.0)
	D9S286	0/10	(0.0)	2/10	(20.0)
	D9S177	1/8	(12.5)	3/8	(37.5)
Microsatellite instability					
	BAT25	1/15	(6.7)	0/15	(0.0)
	D2S123	0/17	(0.0)	1/17	(5.9)
	D8S87	0/14	(0.0)	0/14	(0.0)
	D13S175	0/15	(0.0)	1/15	(6.7)
	D13S153	0/14	(0.0)	1/14	(7.1)
	D17S250	0/16	(0.0)	0/16	(0.0)
	D18S55	0/15	(0.0)	0/15	(0.0)
	D20S100	0/16	(0.0)	0/16	(0.0)

MSI/inf, number of microsatellite instability/informative cases; LOH/inf, number of loss of heterozygosity/informative cases.

TSGs such as *p53*, *APC*, *DCC*, *BRCA1*, *BRCA2* and *FHIT* were located, in order to evaluate the independence of LOH on the chromosome of PTHR1. As shown in Tables I and II, the proportions of LOH on 17q13.3 (candidate gene, p53), 5q21-22 (APC), 17q21 (BRCA1), 13q12 (BRCA2), 18q21 (DCC) and 3p21.2-14.2 (FHIT) were 33.3, 13.3, 10.0, 8.3, 7.7 and 7.7%, respectively. The proportion of carcinomas with LOH on the chromosome of PTHR1 but without LOH of novel TSGs was 20.0% (2/10).

Discussion

LOH analysis for PTHR1 was performed with the proportion of LOH on PTHR1 reaching 30.0% (3/10 cases) in human sporadic OSCC. In total, 4 of 15 cases (26.7%) revealed LOH in endocrine hormone receptors including PTHR1 and THRß. Although the proportion of PTHR1 was not high, it suggests that an anomaly of PTHR1 could be one of the crucial steps in carcinogenesis in human OSCC.

Microsatellites are widely-distributed repetitive DNA sequences composed of short tandemly-repeated nucleotide motifs. In some neoplasms, these sequences exhibit a form of genetic instability characterized by the gain or loss of repeat units at multiple independent loci. Such transformations have been observed to accumulate in cells defective for DNA repair activities. MSI has also been observed in a variety of sporadic malignancies (13-16), and plays an important role in the carcinogenesis of human carcinoma (13). Although the proportion of MSI with eight microsatellite markers was 0%, MSI on PTHR1 was revealed to be 20% (2/10 cases).

Our results suggest that the carcinogenesis of 30.0% of OSCCs might be due to abnormalities in PTHR1. Two cases revealed LOH in PTHR1, but no LOH was found in tumor suppressor genes (TSGs) such as *p53*, *FHIT*, *APC*, *BRCA1*, *BRCA2* and *DCC*. This indicates that LOH of the PTHR1-mediated carcinogenic pathway is independent of that of TSGs in human OSCC.

According to recent studies, coexpression of PTHrP and PTHR1 predicts poor patient survival in breast carcinoma (1). Coexpression of PTHrP and PTHR1 was also prevalent in paired primary prostate cancer and bone metastases (17). This coexpression suggests that autocrine PTHrP-mediated

stimulation may be a mechanism of escape from normal growth regulatory pathways. More importantly, positive PTHR1 expression in early breast cancer is linked with reduced patient survival (1). PTHR1 is also expressed in breast cancer bone metastasis and promotes autocrine proliferation in breast carcinoma cells (18). The presence of PTHR1 in the primary tumor, as opposed to PTHR1 itself, plays a dominant role in determining clinical outcome. The increased frequency and level of PTHR1 expression in bone metastases compared to primary tumors suggest that the receptor may play a role in the metastatic process. Therefore, as has been reported, treatment designed to inhibit PTHR1 function, such as monoclonal antibodies or synthetic antagonists (19,20), may offer improved clinical outcome in patients with carcinomas expressing PTHR1 (1).

In human OSCC, it has been reported that tumor-derived PTHrP may act locally to influence tumor growth as well as the differentiation and resorption of bone (21). Moreover, the clinical syndrome of humoral hypercalcemia of malignancy (HHM), attributed to PTHrP, has been implicated as an important factor in the development of bone metastasis and appears to be a prognostic sign (3). However, PTHR1 expression and its role in human OSCC has not yet been investigated and remains unclear. Our results indicate that, although no correlation was found between LOH in PTHR1 and clinical factors including tumor stage and histology, it is possibile that the transformation of PTHR1 may be involved in carcinogenesis in human OSCC.

Because LOH analysis is suggestive of TSG candidates, further evidence is required to verify that abnormality of PTHR1 contributes to carcinogenesis. We plan to further investigate the expression of PTHR1 or the coexpression of PTHrP and PTHR1 using immunohistochemical analysis, and to examine the relationship between their expression and clinical implications including tumor development, metastasis and patient survival in human sporadic OSCC.

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