Increased expression of CENP-H gene in human oral squamous cell carcinomas harboring high-proliferative activity

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Abstract. We examined the expression of Centromere protein H (CENP-H) mRNA in 38 oral squamous cell carcinomas (SCCs), 2 epithelial dysplasias and 5 normal gingivae using the real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). The mean expression level of *CENP-H* mRNA was higher in oral SCCs (0.11±0.08) than epithelial dysplasias (0.03±0.01) and normal gingivae (0.027±0.01). The expression level of *CENP-H* mRNA was significantly higher in oral SCCs than normal gingivae (Mann-Whitney U test, P=0.005). We also found a significant association between the level of *CENP-H* mRNA expression and clinical stage in oral SCCs (Mann-Whitney U test, P=0.04). We next studied the expression of CENP-H in 17 oral SCCs immunohistochemically. A significant correlation between the expression levels of CENP-H protein and the Ki-67 labeling index was found (Mann-Whitney U test, P=0.005). These results indicate that human CENP-H is closely linked to the increased or abnormal cell proliferation in malignant conditions.

Introduction

The kinetochore is a large complex protein structure that assembles in the centromeric region of each sister chromatid and is responsible for establishing and maintaining the connection with the mitotic spindle (1,2). Kinetochore proteins can be divided into two groups: constitutive kinetocore proteins and faculative kinetochore proteins (3). Constitutive kinetocore proteins are found at centromeric foci throughout the cell cycle, comprising CENP-A, CENP-B and CENP-C (4-6). CENP-A is a central element of the human kinetochore protein concentrated in the inner kinetochore plate (4). CENP-C is also constitutively present in the inner kinetochore plate, which is closely associated with the centromeric heterochromatin (6). Faculative kinetochore proteins are localized in kinetochores tansiently during mitosis, comprising CENP-E and CENP-F (7,8). CENP-F plays several important roles in mitotic events, including centromere/kinetochore maturation, chromosome alignment and segragation, and anaphase spindle stabilization (9,10).

Human centromere protein-H (CENP-H) has been identified and discoverd as a constitutive component of the centromere (11). Confocal microscopic analysis of HeLa cells with anti-human CENP-H-specific antibody demonstrated that CENP-H is present at the inner kinetochore plate and colocalizes with CENP-A and CENP-C in both interphase and metaphase (12). The observation of a conditional loss of function mutant of CENP-H in the vertebrate cells revealed that CENP-H is a fundamental conponent of the active centromere complex (13). These identifications suggest that CENP-H might play an important role in kinetochore organization and function throught out the cell cycle.

Recently, overexpression of the CENP-H have been reported in human colorectal cancer tissues as well as cancer cell lines with chromosomal instability (14). However, no report exists on the expression of the CENP-H gene in oral squamous cell carcinomas. In this study, we examined the expression of human *CENP-H* mRNA and protein in oral squamous cell carcinomas to clarify the correlation between CENP-H expression and clinicopathological factors.

Materials and methods

Tissue samples. We examined 38 oral squamous cell carcinomas (SCCs), two epithelial dysplasias and five normal gingivas. Tumor tissues and specimens of normal gingivae were obtained with informed consent and approval from the institutional review board at Hiroshima University Dental Hospital (Japan) between 1995 and 2005. The mean age of the patients was 65.5 years. The oral SCC samples were derived from the tongue, upper gingiva, lower gingiva, buccal mucosa and floor of the mouth. The clinical staging was determined according to the International Union Against Cancer TNM classification (15). The primary tumors were classified histopathologically as well or moderately differentiated, based on the classification of the World Health Organization (16).

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For molecular analyses, tissue samples obtained at the time of surgery were frozen immediately in liquid nitrogen and stored at -80˚C. We confirmed microscopically that the tumor specimens consisted mainly of carcinoma tissue and that the specimens of normal gingiva did not exhibit any tumor cell invasion or show significant inflammatory involvement.

RNA extraction and quantitative RT-PCR analysis. RNA was extracted with an RNAeasy Mini Kit (Qiagen, Hilden, Germany). One microgram of total-RNA was subjected to a reverse-transcriptase reaction using the First Strand cDNA Synthesis kit (Amersham Biosciences, Uppsala, Sweden). The quantitation of mRNA levels was carried out using a real-time fluorescence detection method according to the method of Eads *et al* (17). The fluorescence was detected by the laser detector of the Line Gene Fluorecent Quantitative Detection System (Bio Flux, Tokyo, Japan) and the detection was carried out by measuring the binding of a fluorescence dye, SYBR Green I, to double-stranded DNA. The PCR was run in microtubes in a volume of 20 μl. The reaction mixture contained 1.0 μg of cDNA, 10 μl of SYBR Green PCR Master Mix (TOYOBO, Osaka, Japan), and 10 pmol of each pair of oligonucleotide primers. The primer sequences were: CENP-H; 5'-TGCAAGAAAAGCAAATCGAA-3' (sense), 5'-ATCCCAAGATTCCTGCTGTG-3' (antisense), and G3PDH; 5'-ACCACAGTCCATGCCATCAC-3' (sense), 5'-TC CACCACCCTGTGGCTGTA-3' (antisense). The PCR program was as follows: initial melting at 95˚C for 30 sec followed by 40 cycles at 95˚C for 15 sec, 55˚C for 10 sec and 72˚C for 15 sec. The quantitation of CENP-H mRNA relative to an internal control, G3PDH, was performed by the ΔCt method (18).

Immunohistochemistry. Avidin-biotin-peroxidase complex immunostaining was performed as described previously (19). CENP-H staining using an anti-CENP-H polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (diluted 1:100) was graded as high (at least 10% of tumor cells showed moderate to intense immunoreactivity) or low (<10% of tumor cells showed weak or no immunoreactivity). For the Ki-67 labeling index, an immunohistochemical analysis was performed using an anti-Ki-67 monoclonal antibody (Dako, Copenhagen, Denmark). The proportion of tumor cell nuclei stained by Ki-67 was calculated for each tumor in x200 microscopic fields. All tumor cell nuclei stained brown above the background level, regardless of intensity, were considered positively stained. We presented the Ki-67 positive cell ratio as the number of tumor cells immunostained by Ki-67 per more than 1000 carcinoma cells in each case (Ki-67 labeling index).

Statistical methods. The results of quantitative RT-PCR analysis were compared with the patients clinicopathological information using the Mann-Whitney U test and Spearmans correlation coefficient by rank test. P-values of <0.05 were regarded as statistically significant.

Results

Expression of CENP-H mRNA in oral SCCs. We examined the expression of *CENP-H* mRNA in 38 oral SCCs and 2 epithelial dysplasias and 5 normal gingivas by real-time RT-PCR. Results of a quantitative RT-PCR analysis of one oral SCC sample are shown in Fig. 1. The mean expression level of *CENP-H* mRNA was higher in oral SCCs (0.11±0.08) than normal gingivae (0.027±0.01) and epithelial dysplasias (0.03±0.01) as shown in Fig. 2. The expression level of *CENP-H* mRNA was significantly higher in oral SCCs than normal gingivae (Mann-Whitney U test, P=0.005). In addition, oral SCCs showed high levels of *CENP-H* mRNA expression compared to epithelial dysplasias, although the difference was not significant (Mann-Whitney U test, P=0.082). The expression of *CENP-H* mRNA was not correlated to clinicopathological factors such as age, gender, tumor type and tumor location. Data on *CENP-H* mRNA expression, tumor size, clinical stage and lymph node metastasis are summarized in Table I. The expression level of *CENP-H* mRNA was higher in stage III/IV oral SCCs than in stage I/II oral SCCs. A significant association was found between the level of CENP-H mRNA expression and clinical stage (Mann-Whitney U test, P=0.04). The patients with larger tumors showed higher levels

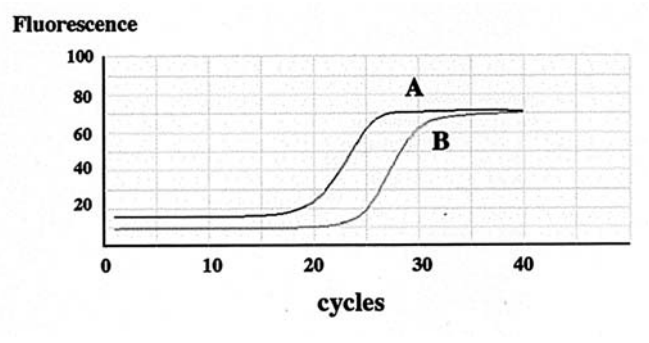

Figure 1. Amplification curves of real-time quantitative PCR analysis in an oral SCC sample: (A), G3PDH; (B), CENP-H. Fluorescence, fluorescence of SYBR Green I dye.

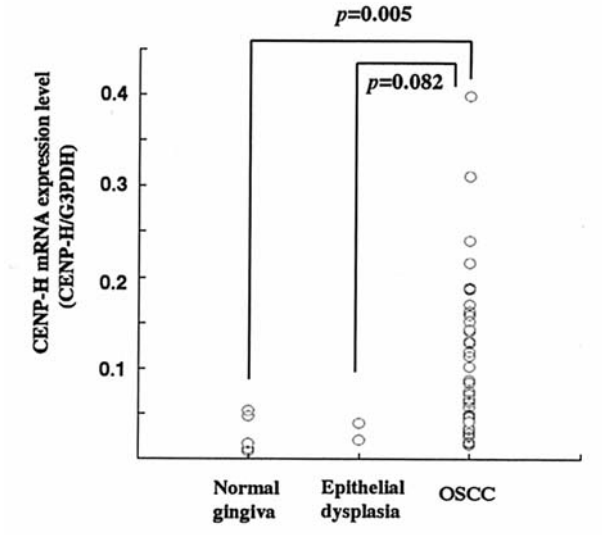

Figure 2. Levels of *CENP-H* mRNA expression in normal gingivas, epithelial dysplasias and oral SCCs: each point represents the *CENP-H* mRNA expression level. *CENP-H* mRNA expression levels were significantly higher in oral SCCs than normal gingivae (Mann-Whitney U test, P=0.005).

Table I. Expression of *CENP-H* mRNA in OSCCs and its correlation with clinicopathological parameters.

	Case no.	Expression level of CENP-H	
		Mean ± SD	P-value[a]
Sex			
Male	21	0.12±0.02	0.96
Female	17	0.10±0.01	
Site			
Tongue	15	0.091±0.014	0.39
Upper gingiva	5	0.17±0.047	
Lower gingiva	14	0.11±0.018	
Buccal mucosa	1	0.024	
Oral floor	3	0.168±0.11	
Histology[b]			
Well	13	0.102±0.015	0.30
Moderate	25	0.118±0.028	
Tumor size[b]			
T1	4	0.043±0.01	0.12
T2	17	0.095±0.014	
T3	6	0.157±0.045	
T4	11	0.140±0.031	
Clinical stage[b]			
I+II	17	0.077±0.01	0.04
III+IV	21	0.14±0.02	
Lymph node metastasis			
Positive	12	0.11±0.018	0.90
Negative	26	0.12±0.018	

[a]P-value, the correlation was analyzed using the Mann-Whitney U test or Kruskal-Wallis test and p-values are shown. P-values <0.05 were regarded as statistically significant. [b]According to the American Joint Committee on Cancer Staging Manual, 5th edition.

of *CENP-H* mRNA expression, although a significant association was not found.

Immunohistochemistry for CENP-H in oral SCCs. We next studied the expression of CENP-H in 17 oral SCCs immunohistochemically. The results of the immunohistochemical analysis reflected the levels of *CENP-H* mRNA expression obtained by quantitative RT-PCR. Normal gingivae showed weak or no immunoreactivity for CENP-H (Fig. 3A). Epithelial dysplasias showed weak CENP-H staining (data not shown). Seven (41%) out of the 17 oral SCCs showed CENP-H positive staining, and the remaining 10 (59%) cases were negative for CENP-H. Most of the oral SCCs with higher levels of CENP-H mRNA showed CENP-H positive staining (Fig. 3B).

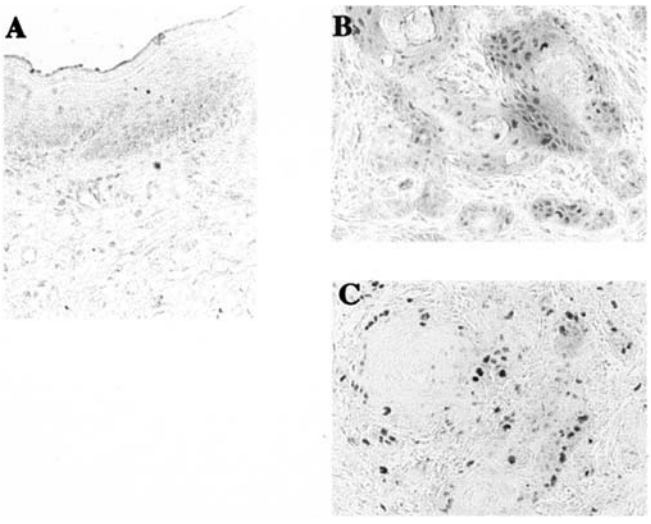

Figure 3. Immunohistochemistry for CENP-H and Ki-67 in oral SCC: (A), Expression of CENP-H in normal gingiva. (B), Expression of CENP-H in oral SCC. (C), Expression of Ki-67 in oral SCC.

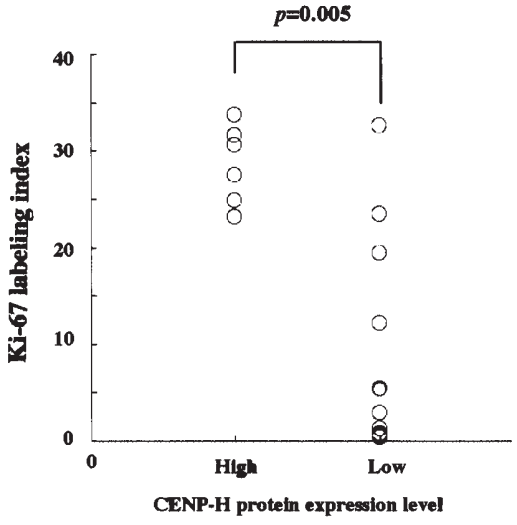

Figure 4. Correlation between CENP-H protein expression levels and Ki-67 labeling index and in oral SCCs. CENP-H expression levels were graded as high (at least 10% of tumor cells showed moderate to intense immunoreactivity) or low (<10% of tumor cells showed weak or no immunoreactivity). A significant correlation was found between the CENP-H protein expression levels and Ki-67 labeling index (Mann-Whitney U test, P=0.005).

Immunohistochemistry for Ki-67 in oral SCCs. We further examined the expression of Ki-67 in oral SCCs immunohistochemically to investigate the correlation between CENP-H expression and proliferating activity. All the normal gingivae and epithelial dysplasias had a low Ki-67 labeling index. The index was compared with the expression levels of CENP-H protein in the tumors. The average Ki-67 labeling index in the cases with CENP-H high expression and with low expression was 28.6±4.1 and 8.76±10.8, respectively, indicating a significant correlation between the Ki-67 labeling index and CENP-H expression in oral SCCs (Mann-Whitney U test, P=0.005) (Fig. 4).

Discussion

Chromosome segregation in mitosis depends on kinetochores, complex protein structures that assemble at the centromeres of chromosomes (10). Kinetochores play a significant role in the check-point mechanism that delays the onset of anaphase until all the chromosomes achieve bipolar attachments (20). Recently, a number of kinetochore components have been identified. CENP-H is a protein of the fundamental components of the centromere-kinetochore complex throughout the cell cycle, localizing outside centromeric heterochromatin. CENP-A and CENP-C, constitutive kinetochore proteins, localize to the inner kinetochore plate (4,6). CENP-H colocalizes with CENP-A and CENP-C in both interphase and metaphase (11,12). CENP-H is also capable of interacting with mitotic centromere associated kinesin (MCAK), which is one of the kinetochore proteins localize to the centromeric heterochromatin, as well as to the outer face of the kinetochore (12,21). Although these observations suggest that CENP-H plays an important role in kinetochore assembly and interactions between inner kinetochore plate and outer plate, the functions of CENP-H are not well understood.

CENP-F is a faculative kinetochore protein that gradually accumulates during the G2 and M phases, localizing in the kinetochore region of the centromeres and in the spindle midzone (2,22). CENP-F is able to interact with CENP-E and the spindle check-point component Bub1 (23). CENP-F plays a significant role in kinetochore assembly and the spindle checkpoint. Esguerra *et al* reported a correlation between the expression of CENP-F and the Ki-67 labeling index in oral SCCs (24). We have also reported that *CENP-F* mRNA levels were higher in patients with oral SCCs harboring lymph node metastasis than in cases without metastasis (25). Furthermore, oral SCC patients with lower *CENP-F* mRNA levels had a better survival rate than those with higher *CENP-F* mRNA levels (25). We also reported a significant correlation between *CENP-F*/*Bub1* expression and the Ki-67 labeling index in salivary gland tumors (26,27). These results suggest the usefulness of the *CENP-F* gene as an additional diagnostic tool for oral SCCs and salivary gland tumors.

In this study, we first analyzed the expression of the *CENP-H* gene in human oral SCC patients. We found that levels of *CENP-H* mRNA were higher in oral SCCs than in epithelial dysplasias and normal gingivae. We also found an association between *CENP-H* mRNA expression and clinical stage in oral SCCs. Furthermore, we demonstrated a significant association between the expression level of CENP-H protein and the Ki-67 labeling index which has been used as a proliferation marker of oral squamous cell carcinomas (28,29). These results indicate that expression of the human *CENP-H* gene is associated with tumor-proliferating activity in human oral SCCs. Furthermore, we investigated an association between expression levels of *CENP-H* mRNA and oral SCC patients survival rates. Oral SCC patients with lower *CENP-H* mRNA levels had a better survival rate than those with higher *CENP-H* mRNA levels (Shigeishi *et al*, unpublished data).

Autoantibodies to centromere protein have been observed in many patients with malignant tumors or rheumatic disorders (30,31). Recently, anti-CENP-H antibodies were identified in the sera of patients with Sjogren's syndrome (32). The observation suggest that anti-CENP-H antibody is a useful marker for Sjogren's syndrome. However, the clinical significance of anti-CENP-H antibody has not been investigated in human cancers. The identification of anti-CENP-H antibody in oral SCC patients is needed to confirm the increased expression of CENP-H in oral SCCs.

The identification of a specific factor for predicting clinical outcome in cases of oral SCCs would be helpful for selecting effective treatments. The present study suggests the usefulness of the *CENP-H* gene as an additional diagnostic tool for oral SCCs.

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