Abstract. Receptor for hyaluronan-mediated motility (RHAMM) has previously been characterized as a cell surface receptor for hyaluronan and a microtubule-associated intracellular hyaluronan binding protein. We examined the expression of RHAMM mRNA in 43 oral squamous cell carcinomas (SCCs) and 7 normal gingivae by real-time RT-PCR. The expression level of RHAMM mRNA was significantly higher in oral SCCs than normal gingivae (P=0.0047). Forty out of 43 oral SCCs showed expression of RHAMM splice variant (48 bp deletion). We immuno-histochemically confirmed the protein expression of RHAMM in oral SCCs with higher levels of RHAMM mRNA. Patients with oral SCC who had high RHAMM expression had shorter survival rates than patients with low expression. However, it was not statistically significant. It has been reported that RHAMM interacts with spindle assembly factors such as microtubule-associated protein (TPX2). To investigate the expression of microtubule-associated protein in oral SCCs, mRNA expression of TPX2 was also examined by real-time RT-PCR. The expression level of TPX2 mRNA was significantly higher in oral SCCs than normal gingivae (P=0.046). Furthermore, a significant correlation between the mRNA expression levels of TPX2 and RHAMM was recognized (P=0.011). The results indicate that there is a strong correlation between the mRNA expression levels of TPX2 and RHAMM in oral SCCs. Our observations suggest that the up-regulations of human RHAMM and TPX2 gene correlate with the malignant condition and might be linked to the increased or abnormal cell proliferation in human oral SCCs.

Introduction

The receptor for hyaluronan-mediated motility (RHAMM) has been identified as a cell surface receptor for hyaluronan and microtubule-associated protein which interacts with the actin cytoskeleton (1,2). RHAMM localizes to the centrosome maintaining the spindle integrity through direct interaction with microtubules (3). The amino terminus of RHAMM directly interacts with microtubules, while the carboxy-terminus is essential for centromeric localization (2-4). RHAMM interacts with spindle assembly factors such as microtubule-associated protein (TPX2) (4). TPX2 is a microtubule-associated protein and is required for microtubule formation at kinetochores in mammalian cells (5). TPX2 initiates spindle assembly by bundling the microtubules and by activating Aurora A kinase in a microtubule-dependent manner (6). Aurora A kinase accumulates at centrosomes from S phase to the end of mitosis and has been implicated in centrosome maturation and spindle assembly (7). Expression of Aurora A is associated with genetic instability and poor prognosis in human cancers (8). RHAMM is also involved in regulating extracellular-regulated kinase (ERK) (9). RHAMM can bind ERK kinase and control the expression of ERK. ERK is activated by upstream kinase Raf and MEK and play a role in cell defferentiation and proliferation (10).

RHAMM colocalizes with TPX2 during mitosis in myeloma cells (4). The carboxy-terminal leucine zipper of RHAMM is required for its spindle pole localization and this domain mediates the interaction of TPX2 (11). Furthermore, overexpression of RHAMM has been associated with centrosomal abnormalities in myeloma cells (4). These observations suggest that RHAMM and TPX2 are structural components of the spindle and contribute to maintaining spindle integrity during mitosis.

Previously, overexpression of RHAMM has been reported in different tumor types including multiple myeloma, breast cancer, endometrial cancer and colorectal cancer (12-16). Expression of RHAMM induces tumor proliferative activities and correlates with poor prognosis in multiple myeloma (17). These observations suggest that expression of RHAMM correlates with tumor progression in several types of human cancers. Human RHAMM cDNA was cloned and contained 725 amino acids that encoded an 85 kDa protein (18). Three
distinct RHAMM gene products, full-length RHAMM, splice variant of 48 bp deletion (RHAMM-48) and variant of 147 bp deletion (RHAMM-147), were cloned from multiple myeloma cells (12). All of the detected RHAMM splice variants contain exon 4, which is alternatively spliced in murine RHAMM (12). Deletion of RHAMM exon 4 disrupts interaction with microtubules at the amino-terminus (4). RHAMM exon 4 plays a significant role in microtubule interaction during mitosis. Deletion of RHAMM-48 and RHAMM-147 lie outside of hyaluronan-binding domains (4). Although the importance of these deletion variants is not apparent, the deletion variants might encode conformationally altered RHAMM and different localization from that of full-length RHAMM.

In human carcinomas, RHAMM participates in tumor progression and metastasis. In the present study, we examined the expression of the human RHAMM gene in oral squamous cell carcinomas to clarify the correlation between RHAMM expression and clinicopathological factors. Expression of the human RHAMM gene in oral squamous cell carcinomas from 1995 to 2007. The oral SCC samples were derived from the tongue, upper gingiva, lower gingiva and buccal mucosa. The clinical staging was determined according to the International Union Against Cancer TNM classification (19). The primary tumors were classified histopathologically as well- or moderately-differentiated in the World Health Organization (WHO) classification (20). For molecular analyses, tissue samples obtained at the time of surgery were frozen immediately in liquid nitrogen and stored at -80°C.

**Materials and methods**

**Tissue samples.** We examined 43 oral squamous cell carcinomas (SCCs) and seven normal gingivae. 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 2007. The oral SCC samples were derived from the tongue, upper gingiva, lower gingiva and buccal mucosa. The clinical staging was determined according to the International Union Against Cancer TNM classification (19). The primary tumors were classified histopathologically as well- or moderately-differentiated in the World Health Organization (WHO) classification (20). For molecular analyses, tissue samples obtained at the time of surgery were frozen immediately in liquid nitrogen and stored at -80°C.

**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). mRNA levels were quantified using a real-time fluorescence detection method (21). Fluorescence was detected using the laser detector of the fluorescent quantitative detection system (LineGene FQD-33A, Bio Flux, Tokyo, Japan) and was analyzed by electrophoresis using 2% agarose gels. For the sequencing reaction, the BigDye Terminator v1.1 cycle sequencing kit (Perkin-Elmer ABI, Foster City, CA) was used. Amplified DNA fragments were sequenced by ABI PRISM 310 genetic analyzer (Perkin-Elmer ABI).

**Immunohistochemistry.** Immunohistochemical staining was performed by the immunoperoxidase technique following antigen retrieval with microwave treatment (500 W, 5 min) in citrate buffer pH 6.0. After peroxidase block by 3% H2O2-methanol for 10 min, specimens were blocked with PBS containing 5% normal horse serum (Vector Laboratories, Inc., Burlingame, CA). For the staining of RHAMM, anti-RHAMM monoclonal antibody (Sanbio B.V., The Netherlands) (diluted 1:100) was used. After 6 h incubation at room temperature with primary antibody, specimens were rinsed briefly with

**Detection of splice variants of RHAMM and sequencing analysis.** One microgram of total RNA was subjected to a reverse-transcriptase reaction using the first-strand cDNA synthesis kit (Amersham Biosciences). To detect two splice variants, RHAMM-48 (48 bp deletion) and RHAMM-147 (147 bp deletion), we used the primer sets as described previously (12). We first examined the expression of two splice variants in several oral cancer cell lines. We could detect RHAMM-48 splice variant in HSC3 cell lines. HSC3 cell lines were used as a positive control for the detection of RHAMM-48. We could not detect RHAMM-48 splice variant in HO-1-u-1 cell lines. HO-1-u-1 cell lines were used as a negative control. The primer sequences for RHAMM-48 were: 5'-GGCCGTCAACATGTCTTTTCTA-3' (sense) and 5'-TTGGGCTATTTTCCCTTGAGACTC-3' (antisense); RHAMM-147, 5'-AGGAGGAACACAGCTGAAAGG-3' (sense) and 5'-TCTCCACACCTTGAGCTGA-3' (antisense). The target sequence was amplified in a 50 μl reaction volume containing 1 μg cDNA, 0.2 mM dNTPs, 1.5 mM MgCl2, 0.2 μM each primer and 1.0 U Platinum Taq (Invitrogen, San Diego, CA). PCR amplification consisted of 35 cycles (94°C for 1 min, 57°C for 30 sec and 72°C for 2 min) after the initial Taq Gold activation step (94°C for 7 min). After the PCR was completed, 10 μl of PCR product were analyzed by electrophoresis using 2% agarose gels. For the sequencing reaction, the BigDye Terminator v1.1 cycle sequencing kit (Perkin-Elmer ABI, Foster City, CA) was used. Amplified DNA fragments were sequenced by ABI PRISM 310 genetic analyzer (Perkin-Elmer ABI).
PBS and incubated with secondary antibody (anti-mouse IgG antibody, Medical and Biological Laboratories, Tokyo, Japan) (diluted 1:100) for 1 h at room temperature. Specimens were rinsed with PBS and incubated with DAB (Dako, Tokyo, Japan). RHAMM staining was graded as positive (at least 10% of tumor cells showed moderate to intense immunoreactivity) or negative (<10% of tumor cells showed weak or no immunoreactivity). According to this grading protocol, we examined all sections of the immunostained specimens randomly to make the grading as objective as possible (one slide was examined by at least two people). We counted at least 1000 tumor cells or normal gingival epithelial cells per one slide.

Statistical methods. The results of quantitative RT-PCR analysis were compared with the patient’s clinicopathological information using the Mann-Whitney U test, Kruskal-Wallis test and Spearman’s correlation coefficient by rank test. The overall survival rates were calculated by the Kaplan-Meier method and analyzed by means of the log-rank test. P-values <0.05 were regarded as statistically significant.

Results

Expression of RHAMM mRNA in oral SCCs and normal gingivae. We examined the expression of RHAMM mRNA in 43 oral SCCs and 7 normal gingivae by real-time RT-PCR. The mean expression level of RHAMM mRNA was higher in oral SCCs (0.20±0.16) than normal gingivae (0.039±0.035) as shown in Fig. 1A and B. The expression level of RHAMM mRNA was significantly higher in oral SCCs than normal gingivae (Mann-Whitney U test, P=0.0047). Data on RHAMM mRNA expression, tumor size, clinical stage and lymph node metastasis are summarized in Table I. The expression of RHAMM mRNA was not correlated to clinicopathological factors such as histology, tumor size and lymph node metastasis.

Correlation of mRNA expression levels between TPX2 and RHAMM. We also examined the expression of TPX2 mRNA in 43 oral SCCs and 7 normal gingivae by real-time RT-PCR. As shown in Fig. 1B, mRNA expression levels of TPX2 were significantly higher in oral SCCs (0.33±0.32) than normal gingivae (0.12±0.063) (Mann-Whitney U test, P=0.046). Data on TPX2 mRNA expression, tumor size, clinical stage and lymph node metastasis are summarized in Table I. TPX2 mRNA was significantly higher in stage III/IV oral SCCs than in stage I/II oral SCCs (Mann-Whitney U test, P=0.010). Next, the co-expression of TPX2 and RHAMM were examined. A significant correlation was found between the mRNA expression levels of RHAMM and TPX2 (Spearman’s correlation coefficient by rank test, P=0.011) (Fig. 2).

Expression of RHAMM splice variants in oral SCCs. Expression of two splice variants, RHAMM-48 and RHAMM-147, were examined in 43 oral SCCs and 7 normal gingivae. RHAMM-48 was identified in oral SCC cell lines HO-1-u-1 using PCR analysis, which amplifies two products (Fig. 3B). RHAMM-48 could not be identified in oral SCC cell lines HSC3 using PCR analysis. HO-1-u-1 and HSC3 cell lines were used as a control for the detection of RHAMM splice variants. We confirmed PCR products of 613 bp corresponding to RHAMM and 565 bp corresponding to 48 bp deletion of RHAMM by sequencing analysis. Forty-eight bp deletion of RHAMM was detected by sequencing of 565 bp PCR products (Fig. 3C).
Forty out of 43 oral SCCs showed expression of RHAMM-48 splice variants (Fig. 4A). We could not detect expression of RHAMM-48 in the other 3 oral SCCs. Two (N4 and N6) out of 7 normal cases showed expression of RHAMM-48 (Fig. 4A). We could not detect expression of RHAMM-48 in the other 5 normal cases (Fig. 4A). On the other hand, expression of RHAMM-147 could not be detected in any of the 43 oral SCCs and 7 normal gingivae (Fig. 4B).

Immunohistochemistry for RHAMM in oral SCCs. We next studied the expression of RHAMM protein immunohistochemically in 13 oral SCCs with higher levels of RHAMM mRNA (expression level >0.1). Normal gingiva showed weak or no immunostaining (Fig. 5A). Twelve out of 13 oral SCCs with higher levels of RHAMM mRNA showed positive staining. RHAMM expression was observed in the cytoplasm of the cancer cells (Fig. 5B).

Correlation between mRNA expression levels of RHAMM and survival rates of oral SCC patient. A total of 35 oral SCC cases were investigated for survival rates. The 14 cases whose tumors expressed increased levels of RHAMM mRNA (expression level >0.1) showed a worse survival rate than the 21 cases expressing RHAMM mRNA at lower levels (expression level <0.1). However, a significant difference was not found (log-rank test, P>0.05) (Fig. 6).

Discussion

RHAMM has previously been characterized as a cell surface receptor for hyaluronan and a microtubule-associated intracellular hyaluronan binding protein (1,2). RHAMM mRNA expression has been identified in normal human tissues including colon, stomach, ovary and testis (24). Over-expression of RHAMM has also been reported in several human cancers (13-16). In the present study, we investigated the expression of RHAMM mRNA in oral SCCs. The expression of RHAMM mRNA was significantly higher in
Figure 3. Expression of splice variants of RHAMM in oral SCC cell lines. (A) Location of RHAMM splice variants (RHAMM-48 and RHAMM-147) and PCR primers are shown. (B) Expression of RHAMM-48 was identified in HO-1-u-1 oral SCC cell lines using PCR analysis. H3, HSC3 cell; HO, HO-1-u-1 cell. (C) Sequencing analysis of 565 bp PCR products corresponding to 48 bp deletion using RHAMM-48 antisense primer.

Figure 4. Expression of splice variants of RHAMM in oral SCCs and normal gingiva. (A) Expression of RHAMM-48 was identified in oral SCCs and normal gingiva using PCR analysis. H3, HSC3 cell; HO, HO-1-u-1 cell. G3PDH was used as a positive control. (B) Expression of RHAMM-147 was not identified in oral SCCs and normal gingiva using PCR analysis.
oral SCCs than normal gingivae. RHAMM protein expression was observed in oral SCCs with higher levels of RHAMM mRNA. On the other hand, normal gingiva showed weak or no immunostaining. We examined the correlation between the expression of RHAMM mRNA and different clinical characteristics, including tumor size, clinical stage and lymph node metastasis. Previous studies showed a correlation between RHAMM expression and clinicopathological factors in human cancers (14-17). RHAMM expression was associated with tumor progression in epithelial tumors such as breast and endometrial cancers (13,14). Although we could not find a significant correlation between expression of RHAMM expression levels and clinical characteristics, patients with tumors that have low expression of RHAMM mRNA have longer survival rate than patients with high expression.

Two splice variants of RHAMM have been described in malignant cells in multiple myeloma patients (12). RHAMM-48 and RHAMM-147 splice variants were overexpressed in multiple myeloma cells and all of the RHAMM variants contain exon 4, which is alternatively spliced in murine RHAMM (12). High expression of RHAMM-48 splice variant was correlated with poor survival of myeloma patients (17). We detected expression of RHAMM-48 splice variant in 40 out of 43 oral SCCs. In contrast, 2 of the 7 normal gingivae showed expression of RHAMM-48. Our results suggested that increased expression of RHAMM and high incidence of RHAMM splicing might contribute to the biological response such as tumor proliferation in oral SCC.

Previously, Maxwell et al reported that RHAMM interacted with the spindle assembly factors such as dynein and TPX2 (4). The spindle assembly factors ensure fidelity of chromosome segregation by delaying the cell cycle until all chromosomes are correctly attached to the spindle. TPX2 is a cell cycle-associated protein and plays a significant role in the microtubule formation at kinetochores (5), although its overexpression induces monopolar spindle structures in Xenopus egg extracts (25). Overexpression of TPX2 has been reported in several human cancers (22). In the present study, we found that the expression level of TPX2 mRNA was significantly increased in oral SCCs compared with normal gingivae. TPX2 mRNA was significantly higher in stage III/IV oral SCCs than in stage I/II oral SCCs. Patients with tumors that have high expression of TPX2 mRNA have a lower survival rate than patients with low expression (data not shown). Furthermore, a significant relationship was found between the mRNA expression levels of RHAMM and TPX2. These results indicate a strong correlation between TPX2 and RHAMM in oral SCCs. Our observations suggest that the up-regulation of human RHAMM and TPX2 correlate with the malignant condition. In conclusion, increased expression of RHAMM and TPX2 might play an important role in malignant transformation of the oral epithelium and proliferation of oral SCC.

Acknowledgements

This study was supported by a Grant-in-Aid from the Japanese Ministry of Education, Culture, Sports and Technology.
References


