Midkine expression is correlated with an adverse prognosis and is down-regulated by p53 in oral squamous cell carcinoma

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Abstract. Midkine (MK) expression has been documented to be inversely correlated with the prognosis of patients with various tumors, but the mechanism of this relationship has not been well characterized. Recent studies have also correlated p53 expression with prognosis of patients with oral squamous cell carcinoma (OSCC). We evaluated the relationship between MK expression and clinicopathological features of patients with OSCC to clarify the influence of p53 status on MK expression in OSCC cells. Our results showed that patients with MK over-expression in OSCC cells had a significantly lower 5-year survival rate compared with patients with low MK expression. Immunohistochemical analyses demonstrated overexpression of MK protein in OSCC samples with mutant p53. Cell culture experiments with human lingual squamous cell carcinoma revealed that the MK gene was regulated by the wild-type p53 gene. Thus, MK expression may affect prognosis via the p53 status and mutation of the p53 gene, and MK may be an attractive target for therapeutic intervention in patients with cancer cells with mutant p53.

Introduction

Oral cancer is one of the most common types of human cancer worldwide (1). Oral squamous cell carcinoma (OSCC) comprises at least 90% of all oral malignancies (2). The prognosis for patients with OSCC is still not good despite better diagnostic techniques and treatment innovations. Improved survival of patients with OSCC requires better methods for prediction of prognosis and novel therapies that focus on molecular targets.

Several oncogenes and tumor suppressor genes were previously implicated in the pathogenesis of various cancers (3-7). Midkine (MK) is a novel heparin-binding growth factor found as a product of a retinoic acid-responsive gene (8). MK has been reported to be intensely expressed in various human tumors including lung (9), breast (10), gastric (11), colorectal (12), esophageal (13), liver (14), thyroid (15), prostate (16) and pancreatic (17). In addition, MK expression has been correlated with poor prognosis in patients with neuroblastomas (18), astrocytomas (19), pancreatic cancers (17) and gastrointestinal stromal tumors (20). In a previous study, MK expression was increased in blood samples from OSCC patients and was associated with prognosis (as assessed by 5-year survival) but not with other conventional prognostic factors such as clinical stage, tumor size and cervical lymph-node metastasis (21). That report suggested that the mechanism of the association of MK with prognosis was different from that for tumor proliferation and metastasis. MK expression has been demonstrated to have a significant relationship with drug resistance, which may be involved in influencing prognosis in OSCC. Kato *et al* (22) recently reported that the intensity of p53 immunoreactivity was strongly correlated with prognosis and that no significant relationship between p53 expression and other clinicopathological parameters was found in OSCC. In addition, the p53 tumor suppressor gene has been documented to contribute to drug resistance (23). We thus hypothesized that a pathway of communication between MK and p53 may exist. In this study, we analyzed MK expression in OSCC tissues and examined its relationship to clinicopathological data. Furthermore, we investigated whether MK expression could be regulated by p53 in OSCC cells.

Materials and methods

Patients and samples. A total of 113 primary OSCC specimens were collected from patients who underwent radical

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treatment at the Department of Oral and Maxillofacial Surgery, Kumamoto University Hospital. We excluded patients who presented with distant metastases. We also collected four normal oral mucosal specimens, which we excised surgically during tooth extractions. Biopsy and normal specimens were either flash frozen in liquid nitrogen and stored at -80˚C or fixed in 10% formaldehyde solution and embedded in paraffin. We obtained informed consent from all patients. All OSCC patients were staged according to the 1997 UICC TNM Classification of Malignant Tumours (24). Clinicopathological data, including age, sex, tumor size, cervical lymph node metastasis, degree of differentiation of OSCC and prognosis, were obtained from patient files.

Western blot analysis. To assess the expression of MK in OSCC and oral mucosal samples, Western blot analysis was performed. The samples were homogenized in lysis buffer (M-PER mammalian protein extraction reagent; Pierce, Rockford, IL, USA) containing protease inhibitors (Protease Inhibitor Cocktail; Nacalai Tesque Inc., Kyoto, Japan). Samples that contained 400 μg of protein were diluted with phosphate-buffered saline (PBS) to 1 ml and were incubated on ice for 1 h with 50 μl of 50% heparin-Sepharose gel (Heparin Sepharose CL-6B; GE Healthcare, Buckinghamshire, England). The gels were washed 3 times with PBS, and heparin-Sepharose-bound molecules were eluted with 25 μl of electrophoresis sample buffer at 95˚C for 5 min. Samples were loaded onto a 15% SDS-polyacrylamide gel and were transferred to pure nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% skim-milk in PBS containing 0.1% Tween-20. The rabbit monoclonal anti-human MK antibody diluted 1:2000 and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody diluted 1:2000 were used as primary and secondary antibodies, respectively. For an internal control, an antibody against ß-actin (AC-15; Sigma-Aldrich, St. Louis, MO, USA), diluted 1:2000, was used. The immunocomplex was visualized by means of the ECL+ Western blotting detection system (GE Healthcare) according to the manufacturer's instructions. For quantification of MK expression, we calculated the density and dimension of the bands by using a computer graphic analytical software (LAS-4000 mini EPUV; Fujifilm, Tokyo, Japan).

Immunohistochemistry (IHC). Paraffin-embedded 4-μm-thick sections were prepared, blocked with 0.3% hydrogen peroxide in methanol, and microwaved for 15 min. After being incubated with Protein Block serum-free (Dako, Glostrup, Denmark), sections were incubated overnight at 4˚C with mouse monoclonal anti-human MK antibody (IP14), which was provided by Cell Signals Inc. (Yokohama, Japan), and rabbit polyclonal anti-human p53 antibody (RSP53; Nichirei Corp., Tokyo, Japan) (MK dilution 1:100, p53 dilution 1:1000). After a 30-min incubation with anti-mouse labeled polymer (EnVision+ System HPR; Dako), 3,3'-diaminobenzidine was used as the chromogen. Sections were counterstained with hematoxylin to enhance nuclear detection.

MK immunoreactivity was assessed independently, in a blinded fashion, by two observers, with consensus achieved for all 93 tumors. MK expression was categorized into four

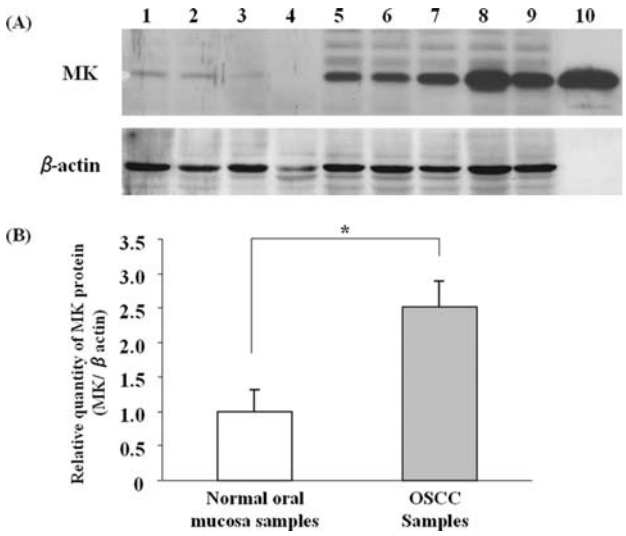

Figure 1. (A) Western blot analysis for MK expression in OSCC samples. Lanes 1-3, normal oral mucosa, and lanes 5-9, OSCC samples. (B) Quantitative evaluation of normal oral mucosa and OSCC samples by Western blot analysis using the analysis software. The statistical significance was evaluated using Student's t-test. *p<0.005.

stages on the basis of the percentage of positive cells, with scores as follows: negative (score points 0): <5% of cells positive; weak (score points 1): 6-30% of cells positive; moderate (score points 2): 31-60% of cells positive; and strong (score points 3): >61% of cells positive. OSCC samples were recorded as overexpressing MK when >30% of the tumor cells (MK scores of 3 and 4) exhibited immunoreactivity. For p53 expression, cases with >5% of positively stained tumor cells were defined as positive.

Cell culture and treatments. Human lingual SCC cells (SAS and HSC-4) were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). These cells were maintained in RPMI-1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), at 37˚C in 5% CO_2. On the day before the experiments were performed, cells were seeded at a density of $5x10^5$ cells per 35-mm tissue culture dish. Either an empty vector (as the control) or the wild-type p53 expression plasmid (pCDM8-p53), which was cloned into the pCDM8 expression vector (25), was transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Lipofectamine 2000 diluted in Opti-MEM (Gibco) was mixed with total DNA at a ratio of 1:4 (DNA-Lipofectamine 2000) and was applied to subconfluent cells in RPMI-1640 medium with 10% FBS. As the small interfering RNA (siRNA) for p53, a Silencer Validated siRNA targeting human p53 (Ambion/Applied Biosystems, Foster City, CA, USA) and a Silencer Negative Control no. 1 siRNA (Ambion/Applied Biosystems) for the control siRNA were used. siRNA for p53 was transfected into SAS cells by using Lipofectamine 2000 according to the manufacturer's instructions. p53 siRNA (25 pmol, diluted in Opti-MEM) and control siRNA (25 pmol, diluted in Opti-MEM) in 500 μl of medium were transfected into 50% confluent cells to knock-down p53

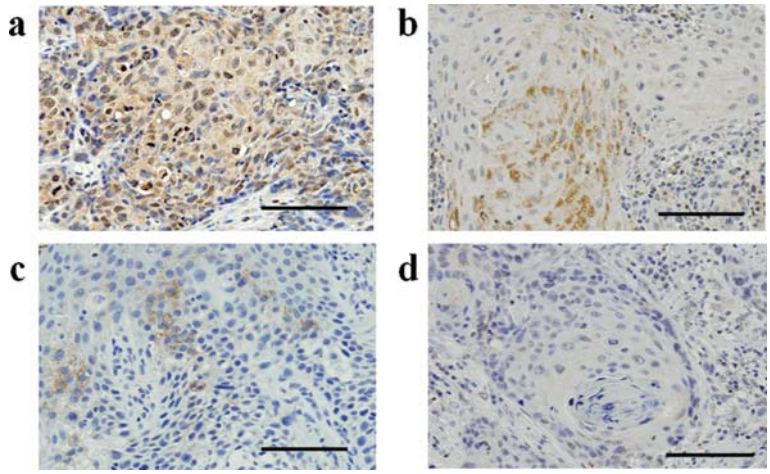

Figure 2. MK immunohistochemistry in OSCC samples. (A) Strong (score 3): 100-61% for MK expression, (B) moderate (score 2): 60-31% for MK expression, (C) weak (score 1): 30-5% for MK expression, and (D) negative (score 0): lower 5% for MK expression (A) to (D): original magnifications, x200, bars = 200 μm.

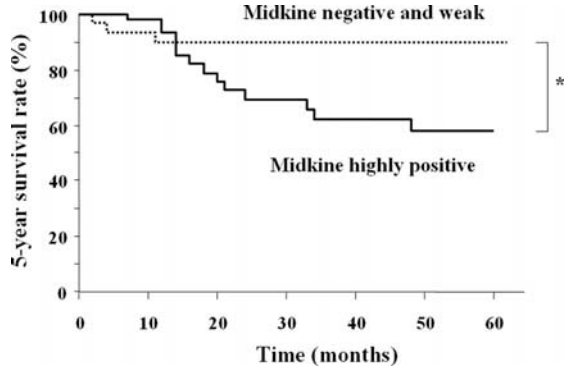

Figure 3. Survival rate of OSCC patients with high or low MK immunoreactivity. Dotted line: five-year survival rate of patients with MK immunoreactivity of 0 and 1 MK score (n=31), and black line: that of patients with MK immunoreactivity of 3 and 4 MK score (n=62). The statistical significance was evaluated using log-rank test. *p<0.05.

gene expression. Cells were moved to serum-free RPMI-1640 medium at 5 h after transfection and were harvested at 36 h after transfection.

RNA isolation and real-time PCR. Total RNA was extracted from cells by using TRIzol (Invitrogen) and was reverse transcribed to cDNA by using the ExScript RT reagent (Takara Bio Inc., Otsu, Japan). All PCR reactions were performed via the LightCycler 480 System (Roche Diagnostics, Basel, Switzerland) with a LightCycler 480 SYBR-Green I Master kit (Roche Diagnostics). The following conditions were used for each reaction: initialization for 10 sec at 95˚C and then 45 cycles of amplification, with 5 sec at 95˚C for denaturation, and 20 sec at 60˚C for annealing and elongation. After amplification, the temperature was slowly raised to above the melting temperature of the PCR product to measure fluorescence and thereby to determine the melting curve. The primers used for real-time PCR were as follows: p53 forward: 5'-ACT AAGCGAGCACTGCCCAAC-3', p53 reverse: 5'-CCTCAT TCAGCTCTCGGAACATC-3'; MK forward: 5'-AAGGCGC GCTACAATGCTC-3', MK reverse: 5'-CATCCAGGCTTG GCGTCTA-3'; ß-actin forward: 5'- TGGCACCCAGCACAA TGAA-3', and ß-actin reverse: 5'-CTAAGTCATAGTCCGC CTAGAAGCA-3'. The threshold cycle values for each gene amplification cycle were normalized by subtracting the threshold cycle value calculated for the ß-actin gene. Normalized gene expression values were expressed as the relative quantity of gene-specific mRNA. All standards and samples were analyzed in triplicate.

Statistical analyses. The Shapiro-Wilk test was used for normality of data, after which data were evaluated via Student's t-test and analysis of variance (for normal distributed data) or via the Kruskall-Wallis test (for non-normal data). Survival curves were plotted by using the Kaplan-Meier method and were analyzed with the log-rank test for univariate analysis. All analyses were performed with the JMP IN Version 5.1 (SAS Institute Japan, Tokyo, Japan). $p<0.05$ was considered statistically significant.

Results

MK expression and its correlation with prognosis in OSCC. Fig. 1 shows the results of Western blot analysis for MK expression in normal oral mucosa and OSCC samples. Quantitative evaluation with the computer graphic software revealed higher intensity bands corresponding to MK protein in five OSCC samples but not in normal oral mucosa samples.

According to IHC, MK expression was localized in the cytoplasm and some nuclei in OSCC cells (Fig. 2). The immunoreactive intensity of MK protein in 93 OSCC samples was semiquantitatively evaluated: 32 showed strong reactions; 30, moderate reactions; and 19, weak reactions. Twelve had no reaction (negative). Statistical analysis of the MK points scored according to IHC results indicated no significant correlations with the clinicopathological factors of tumor size, cervical lymph node metastasis and pathological differentiation (Table I). However, OSCC patients with low MK scores (negative and weak) had a significantly greater 5-year survival rate than did those with high MK scores (strong and moderate) (90.0 vs. 57.7%; $p<0.05$) (Fig. 3).

Table I. Relationship between clinical parameters and MK scores in patients with OSCC.

Clinical parameters	No. of patients	MK protein (MK score points ± SD)	p-values
Sex			
Male	54	1.85±1.09	0.610
Female	39	1.91±1.00	
Age (years)			
<60	24	1.83±1.09	0.604
≥60	69	1.90±1.02	
Clinical stage			0.498
I-II	34	1.88±0.95	
III-IV	59	1.88±1.08	
Tumor size (mm)			
<40	54	1.91±0.99	0.380
≥40	39	1.84±1.10	
Cervical lymph node metastasis (N factor)			
N(-)	49	1.78±0.98	0.852
N(+)	44	2.00±1.08	
OSCC differentiation			
Well	59	1.76±1.04	0.329
Moderately	25	2.12±0.97	
Poorly	9	2.00±1.12	

Relationship between MK and p53 immunoreactivity in OSCC tissues. IHC demonstrated positive p53 immunoreactivity in 58 of 93 cases (62.4%). Positive p53 staining in OSCC samples occurred not only in cancer cells but also in part of the basal mucosal layers in epithelial dysplasia. Mutant p53 proteins have longer half-lives than wild-type protein and tend to be detected by IHC, so we also exa-mined samples for p53 gene mutations (26,27). Fifty-eight of 93 samples showed such mutations. When we compared the immunoreactive association of p53 with MK in OSCC, when MK immunoreactivity was positive, the localization patterns of those immunoreactive proteins were similar (Fig. 4). MK scores in p53-positive samples and those in p53-negative samples were 2.21±0.89 and 1.39±1.02, respectively. A statistically significant correlation was thus observed between MK points and p53-positive and p53-negative samples ($p<0.001$) (Fig. 4B).

Effect of p53 gene transfection and inhibition on MK expression in OSCC cell lines. Because MK overexpression was strongly related to p53 mutation, we used pCDM8-p53 and p53 siRNA to assess the effect of p53 gene transfection and inhibition, respectively, on MK expression in OSCC cell lines. Although both pCDM8-p53-transfected SAS cells and HSC-4 cells evidenced increased p53 mRNA levels, the p53 gene transduction efficiency in HSC-4 cells with a p53 dominant-negative mutation (codon 248) was much lower than that in SAS cells. In p53-transfected cells, MK mRNA significantly decreased in SAS cells compared with empty vector-transfected cells, whereas that in HSC-4 cells did not change significantly (Fig. 5A and B).

We also analyzed the MK mRNA level by knocking down p53 by using siRNA in SAS cells. After transfection, p53 mRNA levels significantly decreased, and MK mRNA levels significantly increased compared with levels of control cells (Fig. 5C).

Discussion

In this report, we demonstrated that MK overexpression in OSCC tissues led to a worse prognosis and that this MK overexpression occurred in p53 mutant cancer cells. We also confirmed that MK expression was regulated by expression of p53 in OSCC cell cultures. MK has been well documented to play important roles in the survival (28-30), growth (31,32), and migration (33-35) of many cells, which may contribute to oncogenesis and tumor progression. Therefore, it was not surprising to find MK overexpression in various tumors and that it was related to prognosis. Our analyses revealed significantly higher MK expression in OSCC samples than in normal mucosa samples. However, our results also indicated that expression of MK protein was not related to clinical stage, tumor size, or cervical lymph node metastasis in OSCC patients. Nevertheless, patients with high MK scores as evaluated by IHC had a significantly lower 5-year survival rate compared with patients with low MK scores ($p<0.05$). These results support findings from our previous study, indicating that S-MK concentrations may be a useful marker

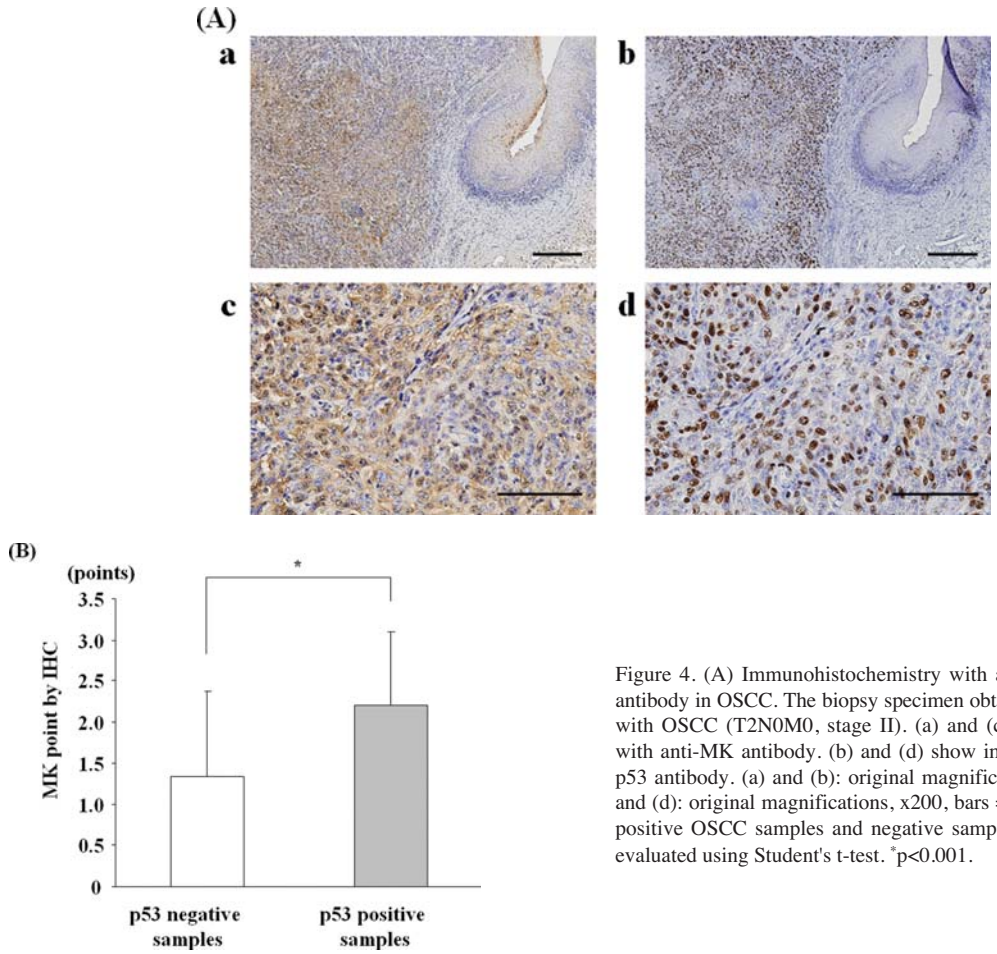

Figure 4. (A) Immunohistochemistry with anti-MK antibody and anti-p53 antibody in OSCC. The biopsy specimen obtained from a 70-year-old female with OSCC (T2N0M0, stage II). (a) and (c) show immunohistochemistry with anti-MK antibody. (b) and (d) show immunohistochemistry with anti-p53 antibody. (a) and (b): original magnifications, x40, bars = 500 μm, (c) and (d): original magnifications, x200, bars = 200 μm. (B) MK point in p53 positive OSCC samples and negative samples. Statistical significance was evaluated using Student's t-test. $^{*}p<0.001$.

for predicting prognosis of OSCC patients (21). Ruan *et al* (36) also reported that high MK expression in OSCC samples confirmed by IHC was significantly correlated with poorer clinical outcome, and the level of MK expression was correlated with tumor size and stage. However, in gastrointestinal stromal tumors (20) and pancreatic head carcinoma (17), MK protein expression determined by IHC was correlated with a poor prognosis but had no relationship to tumor size and lymph node metastasis. With regard to analysis of serum MK concentrations, similar results were obtained for esophageal squamous cell carcinoma (37) and endometrial carcinoma (38). These findings lead us to theorize that MK expression may serve a dominant function in prognosis, with the mechanisms for tumor proliferation and metastasis being different.

Recent studies revealed that MK contributes to in anticancer drug resistance. Qi *et al* (30) reported that MK had cytoprotective activity, in that cisplatin (CDDP) induced apoptotic cell death through enhancement of Bcl-2 expression in both murine kidney and cultured Wilms' tumor cells (G401 cells). Mirkin *et al* (39) reported that MK indirectly mediated intercellular cytoprotective signals that originated from cells with acquired drug resistance to protect neighboring drug-sensitive cells and contribute to development of resistance to chemotherapy. In addition, Kang *et al* (40) used microarray analysis to perform global gene expression analysis of gastric cancer cell lines with acquired drug resistance to 5-fluorouracil, doxorubicin and CDDP. They reported that MK was an important factor related to drug resistance in all drug-resistant cell lines. They described a strong contribution of MK to multidrug resistance in gastric cancer cells. In that study, all patients were treated with S-1 (TS-1; Taiho Pharmaceutical, Tokyo, Japan) adjuvant chemotherapy after curative surgery and radiotherapy. Thus, we also believed that MK could be associated with prognosis, presumably through a drug resistance mechanism. Further studies are needed, however, to clarify the relationship between drug sensitivity of OSCC and MK expression.

Many studies have instead focused on p53, a major tumor suppressor gene, which has an important role in apoptosis induction and contributes strongly to chemotherapy and radiotherapy resistance. With regard to prognosis, numerous studies have used the meta-analytical approach. Statistically significant differences were observed between p53 alterations and poor prognosis in various cancers (41-44). Moreover, Kato *et al* (22) reported that p53-positive immunoreactivity was manifested at the invasive front of OSCC, and concluded that p53 was an indicator of prognosis, even though the correlation between expression of p53 protein and clinicopathological findings was not significant. Those findings suggested that p53 gene alteration and MK gene expression may have a similar function in apoptosis and drug resistance. We thus suspected that the interaction between these genes may be important and hypothesized that one MK expression pathway is regulated by p53 gene. This hypothesis was supported by our findings: OSCC samples lacking p53 function, caused by a p53 gene mutation, had increased MK protein expression as confirmed by IHC. *In vitro* studies revealed that p53 gene overexpression, as resulting from

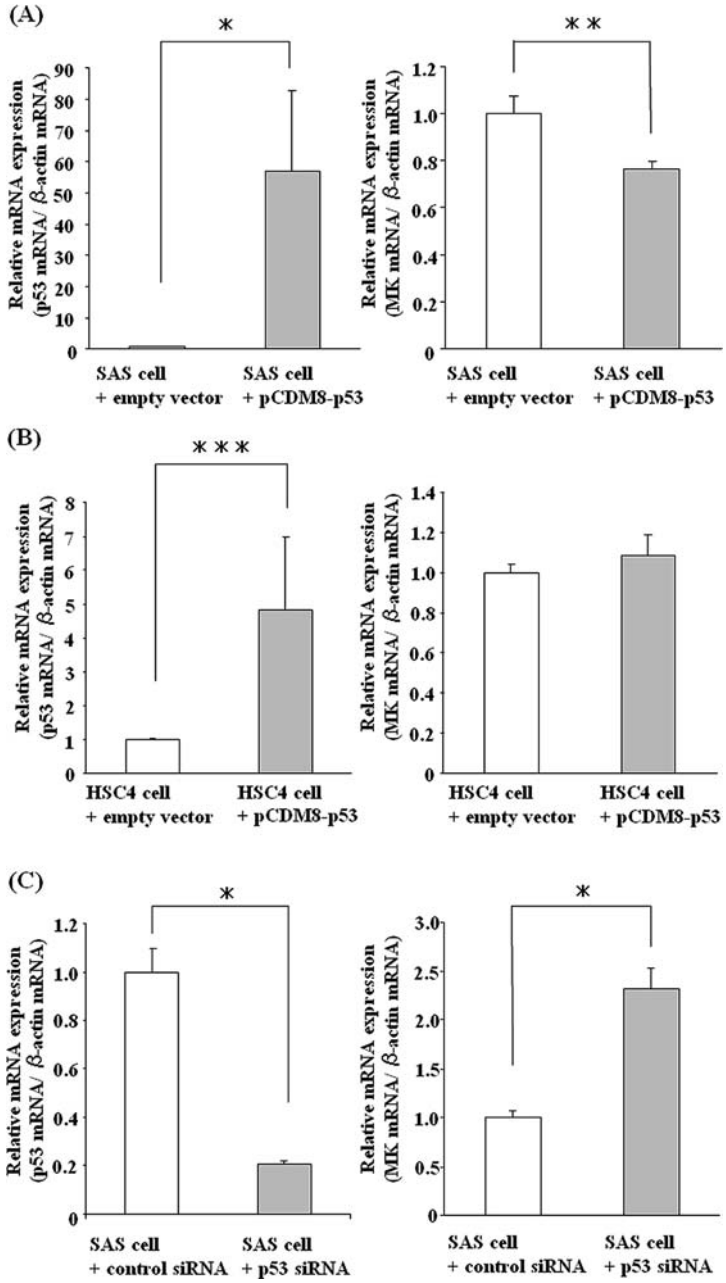

Figure 5. (A) Effect of p53 gene transfection on MK expression in SAS cells. (B) Effect of p53 gene transfection on MK expression in HSC-4 cells. (C) Effect of p53 gene inhibition using siRNA on MK expression in SAS cells. The statistical significance was evaluated using Student's t-test. $^{*}p<0.001$, $^{**}p<0.01$, $^{***}p<0.05$

gene transfection, facilitated reduced MK gene expression in SAS cells, which had the wild-type p53 gene. HSC-4 cells, with a mutant p53 gene in DNA-binding domains and a dominant-negative effect, did not show significant effects on MK gene expression (Fig. 5A and B). As Fig. 5C indicates, p53 gene inhibition by siRNA increased MK gene expression, which clearly supported our hypothesis.

Previous studies of MK were mainly concerned with various physiological and pathological mechanisms, but the signals and mechanisms regulating MK expression were not well understood. Expression of MK was, however, found to be activated by the *wt1* Wilms' tumor gene (45) and nuclear factor-κB (NF-κB) (46,47). Reynolds *et al* reported that hypoxia-inducible factor-1α (HIF-1α) (through hypoxia) (48) and thyroid transcription factor-1 (TTF-1) (49) up-regulated MK expression. Because p53 is well known to contribute to expression of NF-κB and HIF-1α, p53 may also control MK expression indirectly via these transcription factors. Yu *et al* (50) also used the MK promoter-mediated luciferase assay and discovered that transcriptional activity of the MK promoter was regulated by the p53-dependent pathway. Thus, MK could be regulated by p53, either directly or indirectly.

With regard to human drug-resistant cancer (51-54) and advanced cancer (55,56), adenovirus-mediated wild-type p53 gene transfer treatment was performed and produced a good response. However, the efficacy of this treatment depends on the p53 gene status of the target tumor cells. In p53 mutant cells, which have a dominant-negative function,

this treatment has no benefit. Therefore, MK may become a new target gene only if MK strongly contributes to drug resistance downstream of the p53 pathway.

In conclusion, MK proteins were highly expressed and were designated as an independent prognostic factor in patients with OSCC. MK expression possessed a strong relationship with p53 gene status, and MK gene expression was regulated by the p53 gene. These results may lead to trials to inhibit MK expression by using an MK antibody and siRNA against drug-resistant cancer. Future studies of MK may elucidate anticancer drug resistance and contribute to improvement in prognosis of various cancers.

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