Background and Aims. Bone morphogenetic proteins (BMPs) have recently been shown to be involved in the genesis and progression of a wide variety of carcinomas. The present study was undertaken to estimate the effect of BMP-4 on squamous cell carcinoma of the head and neck (SCCHN) in tissue and cell levels.

Methods. In this study, immunohistochemistry, Western blotting and RT-PCR were utilized to detect the expression of BMP-4, Smad1 and phosphorylated Smad1 in SCCHN tissues or SCCHN cell lines. Those three proteins in tissues were further correlated with prognosis of SCCHN by Kaplan-Meier analysis. The epithelial-mesenchymal transition (EMT)-associated changes in SCCHN cells were detected after stimulation by human BMP-4 recombinant protein and knockdown of Smad1 gene. Meanwhile, the effect on invasiveness and migration was evaluated by invasion and scratch assays, respectively.

Results. BMP-4 and p-Smad1 protein were overexpressed in SCCHN tissues with cervical lymph node metastasis, which was significantly higher than those without metastasis. The expression of BMP-4 and p-Smad1 protein was negatively correlated with the prognosis of SCCHN. BMP-4 promoted the invasiveness and migration through EMT, which was demonstrated by morphological alterations, loss of E-cadherin, increase of vimentin and activation of the Smad1 signal pathway. Knockdown of Smad1 expression suppressed BMP-4 induced EMT in both cell lines and weakened the invasiveness and migration of Tu686 and Tu212 in vitro.

Conclusions. Our results demonstrate that BMP-4 protein may contribute to the malignant metastasis of SCCHN, which presents as a novel prognostic marker and a potential therapeutic target for patients with SCCHN.

Key Words: Bone morphogenetic protein-4, Squamous cell carcinoma of the head and neck, Epithelial-mesenchymal transition, Invasiveness.

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) is one of the most frequent cancers that lead to death and represents 6% of all cancers worldwide (1,2). Overall 5-year survival for patients with this type of cancer is among the lowest of the major cancer types (3). The current treatments for SCCHN mainly are surgery, radiotherapy and adjuvant chemotherapy. Despite the fact that therapies are continuously updated and improved, the 5-year survival rate of patients with SCCHN has not significantly improved, which is mainly due to its early tumor metastasis (4). Therefore, identification of biomarkers associated with metastasis and prognosis could predict tumor behavior and guide treatment of SCCHN. Effective measures can be
taken to control the malignant progression of SCCHN by clarifying the molecular mechanisms of metastasis.

Epithelial to mesenchymal transition (EMT) is a developmental process characterized by loss of epithelial markers, gain of mesenchymal markers and changes in cellular morphology and phenotype with increased ability to migrate and invade (5–7). It is reported that transforming growth factor β1 (TGF-β1) plays an important role in the process of EMT in a variety of cancer cells such as stomach, breast, skin, colon and oral cancers (8–13). BMPs are members of the TGF-β superfamily. More than 20 different bone morphogenetic protein (BMP) isoforms have been identified in mammals and Drosophila. They are initially identified by their capacity to induce endochondral bone formation and development in invertebrates, BMPs have recently been shown to modulate the transcription of target genes (7).

In addition to regulating bone formation, cell differentiation and development in vertebrates, BMPs have recently been shown to be involved in the genesis and progression in a wide variety of carcinomas. It is reported that progression of many epithelial cancer cells is associated with BMP-mediated EMT such as ovarian cancer (17,18), colorectal cancer (19), gastric cancer (20), gliomas (21) and breast cancer (22). However, there are scarce reports on the relationship between BMPs and squamous cell carcinoma. Thus, this study aims to evaluate the correlation of BMP-4, Smad1 and p-Smad1 expression in different SCCHN tissues with the prognosis, to explore the BMP-4-signaling pathway and to determine the functional consequences of BMP-4 signaling in SCCHN cells.

Materials and Methods

Patient Materials

A total of 89 SCCHN primary tumor tissue specimens (including laryngeal and hypopharyngeal cancers) were obtained from January 2002–September 2009 in the Department of Otorhinolaryngology, Xiangya Hospital, Central South University, Changsha, China. Patients were undergoing hemilaryngectomy, total laryngectomy or hypopharyngeal lumpsurgery. None of the subjects had a previous history of malignancy, radiation or chemotherapy. Recurrence and metastasis were diagnosed through clinical examination, radiological data, and surgical and pathological examination. Tissues were eventually validated by pathologists. The study was approved by the Research Ethics Committee of Central South University, Changsha, China. Informed consent was obtained from all patients. All specimens were handled anonymously according to ethical and legal standards.

Immunohistochemistry

Immunohistochemical staining was performed on paraffin-embedded tissues. Paraffin-embedded sections were dewaxed in xylene and rehydrated in a graded series of ethanol. Antigen retrieval was carried out in 10 mM citrate buffer (pH 6.0) for 10 min at 100°C in a microwave oven. Slides were incubated with 3% H2O2 and blocked with 5% serum/2% BSA in PBS. After incubation in normal serum, tissue slides were incubated with primary antibodies (BMP-4 antibody, sc-12721, 1:50 dilution; Smad1 antibody, sc-7965, 1:50 dilution; p-Smad1 antibody, sc-12353, 1:50 dilution) obtained from Santa Cruz Biotechnology (Santa Cruz, CA) overnight at 4°C. Slides were then stained with biotinylated secondary antibody (1:500 dilution) from DAKO (Carpinteria, CA) for 1 h. Immunoreactive proteins were visualized with 3’,3’-diaminobenzidine and counterstained with Mayer’s hematoxylin. Negative control slides were probed with normal goat serum under the same experimental conditions. Slides were visualized under microscopy. The degree of immunostaining of formalin-fixed, paraffin-embedded sections was reviewed and scored by two independent pathologists in a blinded fashion. The proportion of stained cells and the extent of staining were used as evaluation criteria. For each case, at least 1,000 tumor cells were analyzed and the percentage of positively stained nuclear tumor cells was recorded. One score was given according to the percent of positive cells as follows: 0: no staining; 1: ≤5% of the cells; 2: 6–35% of the cells; 3: 36–70% of the cells; 4: ≥71% of the cells. A score was then calculated according to the intensity of staining as negative staining: 1 point; weak staining (light yellow): 2 points; moderate staining (yellowish brown): 3 points; and strong staining (brown): 4 points. A final score was then calculated by multiplying the above two scores. If the final score was equal or higher than four, the tumor was considered high expression; otherwise, the tumor was considered low expression (23,24).

Follow-up

Complete clinical and pathological data was obtained from all patients; 7/89 patients were lost to follow-up because of telephone number changes or relocating. Therefore, the follow-up rate was 92.1%. Recurrence and metastasis were diagnosed through clinical examination, radiological data, and surgical and pathological examination. Overall survival (OS) and disease-free survival (DFS) were calculated from the date of surgery to the date of death or that of tumor relapse, respectively. Deaths from other causes were treated as censored cases. Follow-up time was 5 years for each patient (range: 2–60 months, mean = 43.24 months, SD = 19.796).
Cell Culture and BMP-4 Stimulation Test

SCCHN cell lines Tu686 and Tu212 were established from a primary tumor at the base of the tongue and from a primary human hypopharynx tumor, respectively. The above two SCCHN cell lines were kindly provided by Dr. Zhuo (Georgia) Chen (Emory University Winship Cancer Institute, Atlanta, Georgia). The two cell lines were maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM/F12 medium (1:1) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 IU/mL streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

Both Tu686 and Tu212 cells were plated at 11 × 10⁴ cells per dish in culture dishes. After 24 h, the medium was changed into serum-free medium for another 12 h, and the cells were then treated with the indicated concentrations of Recombinant Human BMP-4 (PeproTech, name city, England) for 72 h. As for time intervention, after fasting for 12 h for both Tu686 and Tu212 cells, the cells were treated with 100 ng/mL BMP-4 for the indicated hours. The cells were then assayed for EMT by inverted microscopy. Western blotting and RT-PCR were performed on BMP-4 signal molecules and EMT markers.

Western Blotting Analysis

Whole cell lysates were prepared. Fifty μg of protein from each sample was mixed with gel loading buffer (2×: 125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.1% bromophenol blue and 2.5% β-mercaptoethanol), boiled for 5 min, separated by 8% SDS-polyacrylamide gel and then transferred onto polyvinylidene difluoride membranes. The blotted membranes were incubated with anti-Smad1 (sc-59780, dilution 1:100), anti-Smad4 (sc-56479, dilution 1:300), anti-E-cadherin (sc-73259, dilution 1:500) (antibodies were all from Santa Cruz Biotechnology) or fluorescent control siRNA (sc-36869, Santa Cruz Biotechnology,) or fluorescent control siRNA (sc-36869, Santa Cruz Biotechnology) according to the manufacturer’s protocol. Cells were then treated with BMP-4 for another 72 h. Expression of proteins or RNA and the invasiveness and migration of two cells were assayed according to protocols as described above.

Knockdown of Smad1 Gene

Tu686 and Tu212 cells were plated at 8 × 10⁴ cells per well and infected with siRNA Smad1 (sc-29483, Santa Cruz Biotechnology,) or fluorescent control siRNA (sc-36869, Santa Cruz Biotechnology) according to the manufacturer’s protocol. Cells were then treated with BMP-4 for another 72 h. Expression of proteins or RNA and the invasiveness and migration of two cells were assayed according to protocols as described above.

Statistical Analysis

SPSS v.11.0 for Windows statistical analysis software package was used for data analysis. Continuous variables were
expressed as mean ± SD. Student two-sample t-test was used for comparison of continuous variables. Single factor analysis of variance (one-way ANOVA) was applied to compare means of multiple samples. Spearman’s correlation coefficient and Kaplan-Meier analysis were applied for comparison of proteins and survival analysis, respectively. Curves for survival analysis were compared using the log-rank test; \( p \) value <0.05 was considered to be statistically significant.

**Results**

Expression of BMP-4, Smad1 and p-Smad1 in Different SCCHN Specimens and Survival Analysis

BMP-4, Smad1 and p-Smad1 signals were all brown granules, mainly located in the cytoplasm (Figure 1A). Higher expression of BMP-4 and p-Smad1 was observed in SCCHN primary tissue specimens with cervical lymph node metastasis. There was no significant difference for Smad1 between specimens. Meanwhile, it was detected that BMP-4 was positively correlated with p-Smad1 (\( r_s = 0.548 \)) but not Smad1 (\( r_s = 0.013 \)).

Until the last follow-up of September 30, 2009, 7/89 cases of SCCHN were lost to follow-up. There were a total of 40 deaths in 5 years. Overall 5-year survival rate was ~51.22%. According to immunohistochemistry, 82 patients with SCCHN were divided into low and high BMP-4 groups, low and high Smad1 groups, and low and high p-Smad1 groups, respectively. Kaplan-Meier analysis and the log-rank test demonstrated that the levels of BMP-4 and p-Smad1 were negatively correlated with patient survival time (\( \chi^2 = 5.85, p = 0.01562 \) and \( \chi^2 = 4.14, p = 0.0418 \)), respectively. Higher levels of BMP-4 or

![Figure 1. Immunostaining of BMP-4, Smad1 and p-Smad1 in SCCHN tissues and survival analysis. (A) Two kinds of specimens were stained with BMP-4, Smad1 and p-Smad1, respectively. Images were taken under inverted microscopy at ×200. a,c,e: Primary tissues without cervical lymph node metastasis were stained with BMP-4, Smad1 and p-Smad1, respectively. b,d,f: Primary tissues with cervical lymph node metastasis were stained with BMP-4, Smad1 and p-Smad1, respectively. (B) All tissue slides were divided into low-expression groups and high-expression groups according to the expression of BMP-4, Smad1 and p-Smad1. Kaplan-Meier analysis was applied to compare the prognosis of SCCHN and the expression of three proteins. a,b,c: Survival curves for the comparison of prognosis of SCCHN with BMP-4, Smad1 and p-Smad1, respectively.](image-url)
p-Smad1 correlated with shorter survival time. Expression level of Smad1 was not correlated with patient survival time ($\chi^2 = 0.20, p = 0.6587$) as shown in Figure 1B.

**BMP-4 Induced Morphological Changes in SCCHN Cells**

Tu686 and Tu212 cells underwent morphological changes after 72 h of stimulation with 100 ng/mL BMP-4 (Figure 2). Cells treated with BMP-4 turned into more narrow, long strips or fusiform shapes, stretched out tentacles, and the contacts among them were loosened.

**Expression Changes of EMT-related Genes After BMP-4 Stimulation**

With the increased concentration of BMP-4 (0–300 ng/mL), the expression of p-Smad1 and vimentin in both Tu686 and Tu212 cells was increased dose-dependently. The expression of E-cadherin was decreased in the same manner, and the expression of Smad4 was not significantly changed (Figure 3A). We then detected the proteins at 15 min to 6 h after treatment and found no considerable change for the p-Smad1 and other EMT-associated proteins. We finally chose 0, 12, 24 and 72 h for the time contrast. The expression of p-Smad1 and vimentin in both Tu686 and Tu212 cells was increased with time, whereas E-cadherin decreased with time (Figures 3B–3D).

**BMP-4 Stimulation Promoted Invasiveness and Migration in SCCHN Cell Lines**

We measured the effect of BMP-4 on cell invasion in both Tu686 and Tu212. Cells that penetrated through the filters to the other side of inserts in BMP-4-treated groups were much more than those of untreated groups (Figure 4A). We further evaluated the changes of capability for migration after treatment of BMP-4 in those two cell lines. Cells treated with BMP-4 showed much higher motility and achieved more wound closure compared with their controls at both 24 and 48 h (Figure 5).

**Effect of Smad1 Gene on EMT, Invasiveness and Migration in SCCHN Cells**

Smad1 was successfully silenced after being transfected with siRNA Smad1 in both Tu686 and Tu212 cells. With the treatment of 100 ng/mL BMP-4 for 72 h, E-cadherin was significantly upregulated, whereas p-Smad1 and vimentin were significantly downregulated (Figure 6A).

Predictable changes could be seen in cell invasiveness and migration. With the treatment of 100 ng/mL BMP-4 for 72 h in both Tu686 and Tu212, the cells that penetrated through the filters to the other side of the inserts in Smad1 knockdown groups were much less than those of control groups (Figure 4B). It was important to note that by knockdown of Smad1 and with the treatment of 100 ng/mL BMP-4 for 72 h, cells showed poorer motility and achieved less wound closure compared with their controls whose Smad1 was not silenced (Figure 6B).

**Discussion**

Early metastasis of SCCHN seriously affects patient prognosis. Efforts have been made to improve diagnosis,
BMP-4 Induced EMT and Invasiveness in SCCHN

We demonstrated that BMP-4 and p-Smad1 in primary SCCHN tissues with cervical lymph node metastasis were higher than those without metastasis, whereas there was no difference for Smad1 between the two tissues. It is generally considered that the signal transduction pathway of BMPs is mediated by Smads 1, 5, and 8, and their activated forms are the phosphorylated products (29). The positive correlation between BMP-4 and p-Smad1 can further verify this conclusion. We further demonstrated the clinical significance of BMP-4 in the prognosis of SCCHN. It was found that high expression of BMP-4 and p-Smad1 was associated with poor prognosis of SCCHN. Increased expression of BMP-4 or p-Smad1 in metastatic primary tumor may be able to change the microenvironment of tumor progression that led to its poor prognosis. The results of the present study have implications for our understanding of BMP-4-induced EMT and its clinical values.

Figure 3. Expression of EMT-associated proteins or genes in SCCHN cells with treatment of BMP-4. (A) Smad4 expression in Tu686 and Tu212 after treatment by indicated concentration of BMP-4 (0, 10, 100 and 300 ng/mL) for 72 h. (B,C) Expression of epithelial marker-E-cadherin, mesenchymal marker-vimentin, total and phosphorylated Smad1 after treatment by indicated concentration of BMP-4 in Tu686 and Tu212 cells, respectively. (D) Expression of EMT-associated proteins or genes in SCCHN cells with treatment of 100 ng/mL BMP-4 for 0, 12, 24, 48 and 72 h.
were consistent with the reports on colon adenocarcinomas (30) and breast cancer (31).

EMT has been considered to be an event following malignant transformation to endow cancer cells invasive and metastatic competence (32). It is not easily observed in histological examinations of cancer tissue sections, even by experienced pathologists, which may be transient during cancer progression (33,34). Evidence of EMT includes loss or delocalization of junctional E-cadherin and unexpected expression of mesenchymal markers such as the intermediate filament protein vimentin (35). Therefore, we designed an in vitro EMT model to prove BMP-4-induced EMT. As early as 12 h after BMP 4 treatment, Tu686 cells began to exhibit a spindle-shaped, narrower morphology, stretched out tentacles, and the contacts between them were loosened, whereas nontreated cells were more rounded and closed. Such changes could also be observed in Tu212 cells. In addition to phenotype, the changes of E-cadherin and vimentin further verified the BMP-4-induced EMT, which was consistent with the study by Gordon et al. in which BMP-4 activated Smad1 signal pathway and induced EMT in pancreatic cancer cells (36). It was reported that by forming complexes with Co-Smad such as Smad4, phosphorylated Smad1 could regulate transcription of target genes in nucleus in BMP-mediated signal pathway (7,37). Results showed that the changes of Smad4 after the incentive of BMP-4 were not significant, suggesting that BMP-4 did not act through the regulation of Smad4. BMP-4 may induce EMT through phosphorylation of Smad1. Changes of migration and invasive-ness after BMP-4 incentive suggested that BMP-4-induced EMT promoted the motility of cancer cells. Increased p-Smad1 expression demonstrated that it played a role in defining migration and invasion of cells that had undergone EMT in response to BMP-4.

In order to demonstrate the effect of Smad1 in BMP-4-induced EMT, we further silenced Smad1 gene and then detected the change of invasiveness and migration in SCCHN cells under the stimulation of BMP-4. It was observed that by knockdown of Smad1, E-cadherin
was upregulated, vimentin was suppressed and the capability of both invasion and migration was inhibited. siRNA Smad1 may offset the effect of BMP-4, which further verified the effect of p-Smad1 on the BMP-4-induced EMT and thus promoted invasiveness and migration in SCCHN cells. However, we did not observe morphological changes after siRNA Smad1 transfection due to the toxic effect of the transfected reagent. Our results further demonstrated that BMP-4 may induce EMT through the phosphorylation of Smad1 in SCCHN and elevated invasiveness and migration abilities. BMP-4 mediated signal pathway played a role in the invasiveness of SCCHN and has potential value for the treatment of SCCHN metastasis by blocking Smad1.

Together the results of the present investigation revealed that BMP-4 and p-Smad1 overexpression in metastatic primary cancer tissues may contribute to their aggressive behavior, and detection of BMP-4 may have clinical values in the diagnosis of early metastasis of SCCHN. Down-regulation of Smad1 protein may represent a promising therapeutic strategy for the prevention and therapy of metastasis. Although we have demonstrated the effect of Smad1-mediated signal pathway in BMP-4-induced EMT in SCCHN, the signal cascade is still unknown. There may be other signal pathways or molecules involved in EMT, which needs further study. In addition, how BMP-4 works in vivo should be further verified by animal experiments.

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Figure 5. BMP-4-induced migration in Tu686 and Tu212 cells. (A,B) Images were taken for wound closure of cells at 0, 24 and 48 h after treatment with or without 100 ng/mL BMP-4. Graphs represent the average number of migrating cells from five independent fields. Error bars were the standard error of the mean. Difference was considered significant when \( p < 0.05 \) (*).
References


Figure 6. The effect of Smad1 gene on BMP-4-induced EMT in SCCHN cell lines. (A) Expression for E-cadherin, mesenchymal marker-vimentin, total and phosphorylated Smad1 after different treatments. (B) Images were taken for wound closure of cells 24 h after different treatments. Graphs represent the average number of migrated cells from five independent fields. Error bars were the standard error of the mean. Difference was considered significant when \( p < 0.05 \) (*).