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Combinatorial use of bone morphogenetic protein 6, noggin and SOST significantly predicts cancer progression

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Emerging evidence has indicated a role of the bone morphogenetic proteins (BMP) in the pathogenesis of certain cancers. The signaling of BMP family members is tightly regulated by their antagonists, including noggin and SOST, which are, in turn, positively regulated by BMP, thereby forming a negative feedback loop. Consequently, the expression of these antagonists should be taken into account in studies on the prognostic significance of BMP. In the present paper, we correlated protein and mRNA expression levels of BMP6, noggin and SOST, alone or in combination, with patient survival in various types of cancer. We found that BMP6 alone was not significantly correlated with esophageal squamous cell carcinoma patient survival. Instead, a high level of inhibitor of differentiation 1, a downstream factor of BMP6, was associated with shorter survival in patients whose tumors stained strongly for BMP6. Knockdown of noggin in esophageal cancer cell line EC109, which expresses BMP6 strongly and SOST weakly, enhanced the non-adherent growth of the cells. Noggin and SOST expression levels, when analyzed alone, were not significantly correlated with patient survival. However, high BMP6 activity, defined by strong BMP6 expression coupled with weak noggin or SOST expression, was significantly associated with shorter survival in esophageal squamous cell carcinoma patients. We further confirmed that BMP6 activity could be used as a prognostic indicator in prostate, bladder and colorectal cancers, using publicly available data on BMP6, noggin and SOST mRNA expression and patient survival. Our results strongly suggest that BMP6, noggin and SOST could be used in combination as a prognostic indicator in cancer progression. (*Cancer Sci* 2012; 103: 1145–1154)

Despite the fact that the 5-year survival rate for esophageal cancer improved from 5% to 19% in the USA between 1975 and 2005, survival is still poor relative to common cancers originating from other sites.[1] The predominant subtype of esophageal cancer in Asian countries is esophageal squamous cell carcinoma (SCC),[2] which has a poor prognosis.[3] Attempts are being made to identify environmental and genetic susceptibility factors of esophageal SCC.[4–6] In addition, the molecular basis of esophageal SCC progression is still largely unknown.

The bone morphogenetic proteins (BMP) are family members of the TGF-β superfamily of cytokines, whose first identified function was the promotion of bone formation. The activity of BMP is marshaled by their intrinsic antagonists, which include noggin and SOST.[7] BMP signaling plays an important role in the normal development of the esophagus; dysregulation of BMP and their native antagonists can result in improper development of the esophagus in the embryonic stage.[8–10] Altered expression of BMP also results in an inflammatory response in the esophagus,[11,12] as well as onset of Barrett's esophagus, a premalignant lesion of esophageal adenocarcinoma.[13] The significance of BMP6 in esophageal SCC was revealed when a high level of BMP6 expression was correlated with shorter survival.[14] Moreover, BMP6 is upregulated in prostate cancer,[15,16] clear cell renal carcinoma[17] and osteosarcoma.[18]

Bone morphogenetic proteins regulate the expression of inhibitor of differentiation (Id) proteins in various cellular contexts.[19–21] We have previously shown that Id-1 is overexpressed in esophageal SCC, and its expression is an independent predictor of shorter survival.[22] BMP activate Smad1/5/8 phosphorylation, which leads to translocation of Smad4 into the nucleus, thereby activating the transcription of Id proteins in a Smad-dependent manner.[23–26] However, the relationship between Id proteins and BMP in esophageal SCC is not known.

In the present study, we investigate the role of BMP6 in esophageal SCC development and progression, and examine whether BMP6 and its antagonists, noggin and SOST, or downstream effectors, such as Id-1 and pSmad1/5/8, are associated with patient survival.

Materials and Methods

Patients and specimens. Patient data and specimens were collected and described previously,[22] and are summarized in Table 1.

Immunohistochemical staining. Immunohistochemical staining for Id-1 in the same cohort was performed previously.[22] The staining of BMP6, noggin and SOST was performed as previously described.[15] Staining of phospho-Smad1/5/8 was performed as previously described using pSmad1/5/8 antibody at a concentration of 1:100 (Cell Signaling, Danvers, MA, USA; Cat. no. 9511).

Evaluation of immunohistochemical staining results. The stained sections were reviewed by two independent observers (KWC and YPC), who had no prior knowledge of the clinicpathological data of the patients. All three cores of the same specimens were scored independently and the highest score among the three cores was used for later statistical analysis. In the survival analyses, strong expression of BMP6 was stratified

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Table 1. Patient clinical and pathologic features

	Number of cases	%	Median (range)
Age	81		67 (41–87) years
Sex			
Male	62	77	
Female	19	24	
T-stage			
T1	2	3	
T2	13	16	
T3	49	61	
T4	17	21	
N-stage			
N0	30	37	
N1	51	63	
M-stage			
M0	68	84	
M1	13	16	
pTNM staging			
Stage II	32	40	
Stage III	36	44	
Stage IV	13	16	
Histological grade			
Well-differentiated	13	16	
Moderately-differentiated	48	59	
Poorly-differentiated	20	25	

as high level staining, while weak to moderate expression of BMP6 was stratified as low level staining. The staining of noggin, SOST and pSmad1/5/8 was evaluated as for BMP6. Specimens were further stratified into two groups based on their expression levels of all of BMP6, noggin and SOST. Those stained with a high level of BMP6, and low levels of both noggin and SOST were classified as having high BMP6 activity; patterns other than that were classified as having low BMP6 activity, as previously described.[15] The staining of Id proteins was evaluated previously.[22]

Statistical analysis. Statistical analysis was performed using IBM SPSS 19.0 (Armonk, NY, USA). The significance of the differences in the protein expression levels between normal epithelium and esophageal SCC specimens was analyzed by chi-square-test. Correlations between expression levels of different proteins and stages of the disease were analyzed by Spearman's rank correlation test. Kaplan–Meier analysis was performed using either log-rank or Wilcoxon–Gehan tests. Cox regression was performed with forward conditional stepwise selection with an inclusion value of $P < 0.05$. The differences between groups were compared using Student's t-test. $P < 0.05$ was considered statistically significant in all tests.

Cell lines. Immortalized esophageal epithelial cell line NE1 was maintained in keratinocyte serum-free medium (KSFM) (Invitrogen, Paisley, UK). Esophageal SCC cell lines HKESC-1, HKESC-2 and HKESC-3 were maintained in alpha-MEM supplemented with 10% FBS. Esophageal SCC cell lines SLMT-1, EC1, EC109 and EC18 were maintained in RPMI1640 supplemented with 10% FBS. Viral packaging cell line 293T was maintained in DMEM supplemented with 10% FBS. Stable EC109 shScr, shNOG-1 and shNOG-2 cells were generated by lentiviral infection of pLKO.1 plasmid expressing scramble shRNA (Addgene ID: 17920, a gift from Professor SA Stewart), noggin shRNA1 (obtained from Sigma [St. Louis, MA, USA], ID NM_005450.2-452s1c1) and noggin shRNA2 (Sigma ID: NM_005450.2-676s1c1). Stably infected cells, selected using puromycin and having undergone fewer than 10 passages, were used for all assays performed in the present study.

Reverse transcription quantitative PCR. RNA was extracted using Trizol (Invitrogen) and was reverse transcribed using the Superscript III first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed using Taqman gene expression assays (Invitrogen) for BMP6 (ID: Hs01099594_m1), noggin (ID: Hs00271352_s1) and SOST (ID: Hs00228830_m1) according to the manufacturer's instructions.

Western blot analysis. Western blotting was performed as previously described. BMP6 (R&D system, Minneapolis, MN, USA) and noggin (Abcam, Cambridge, UK) antibodies were used at concentrations of 0.5 and 2.5 μg/mL, respectively. Phospho-Smad1/5/8 antibody (Cell Signaling Technology) and actin antibody (Sigma) were used at dilutions of 1:1000 and 1:10 000, respectively.

Colony formation assay. A total of 500 EC109 cells were seeded in a six-well plate and allowed to grow for 2 weeks. The cells were fixed with 70% ethanol and the colonies were stained with 0.1% crystal violet. The plates were photographed and colonies formed were enumerated.

Soft agar assay. EC109 cell suspension in culture medium containing 0.35% (w/v) low melting point agarose (Invitrogen) were overlaid on a base layer of solidified culture medium that contained 0.7% (w/v) low melting point agarose. The mixture was allowed to solidify and was incubated at 37°C for 3 weeks. A few drops of medium were added every 2 days. The plates were photographed and the colonies formed were enumerated.

Analysis of cancer patient microarray data. A prostate cancer patient dataset (GSE 21035),[27] a bladder cancer dataset (GSE 13507)[28] and three colorectal cancer datasets (GSE 14333,[29] GSE 17536 and GSE 17537)[30] (the only available carcinoma patient datasets in the Gene Expression Omnibus database of the Prognoscan platform[31] with microarray data for BMP6, noggin and SOST, as well as survival data) were investigated. The datasets were pre-processed as previously described.[32]

Results

Bone morphogenetic protein 6 expression in esophageal squamous cell carcinoma. Here, by reverse transcription quantitative PCR, we found that BMP6 was significantly overexpressed in six out of seven esophageal SCC cell lines compared to the immortalized non-transformed esophageal epithelial cell line NE1 (Fig. 1A). Similarly, we also observed an increase in BMP6 protein level by western blot in esophageal SCC cell lines compared to NE1 cells (Fig. 1B). Immunohistochemical staining of 84 human esophageal SCC and 33 non-tumor esophageal epithelium specimens revealed that BMP6 was either moderately or strongly expressed in 76 out of 80 esophageal SCC specimens (Fig. 1C), which is in line with the previous report showing that BMP6 was expressed in the majority of the esophageal SCC specimens.[14] The expression level of BMP6 was significantly lower in non-tumor esophageal epithelium compared to the esophageal SCC specimens (χ^2-test, $P < 0.001$), with only 7 out of 32 non-tumor specimens having moderate or strong staining of BMP6 (Fig. 1D). However, the association between BMP6 protein expression and survival was not statistically significant (log-rank test, $P = 0.288$).

Significance of inhibitor of differentiation 1 expression in bone morphogenetic protein 6-high tumors. Bone morphogenetic protein 6 and Id-1 expression levels were found to be significantly positively correlated in the esophageal SCC specimens in our current cohort (Spearman's rank test, $r = 0.227$, $P = 0.046$; Fig. 2A). BMP6 expression was also significantly correlated with Id-3 expression (Spearman's rank test, $r = 0.324$, $P = 0.005$; Fig. 2C). Conversely, BMP6 expression did not correlate with that of Id-2 (Spearman's rank test, $r = 0.165$, $P = 0.147$; Fig. 2B). More importantly, we found that a low

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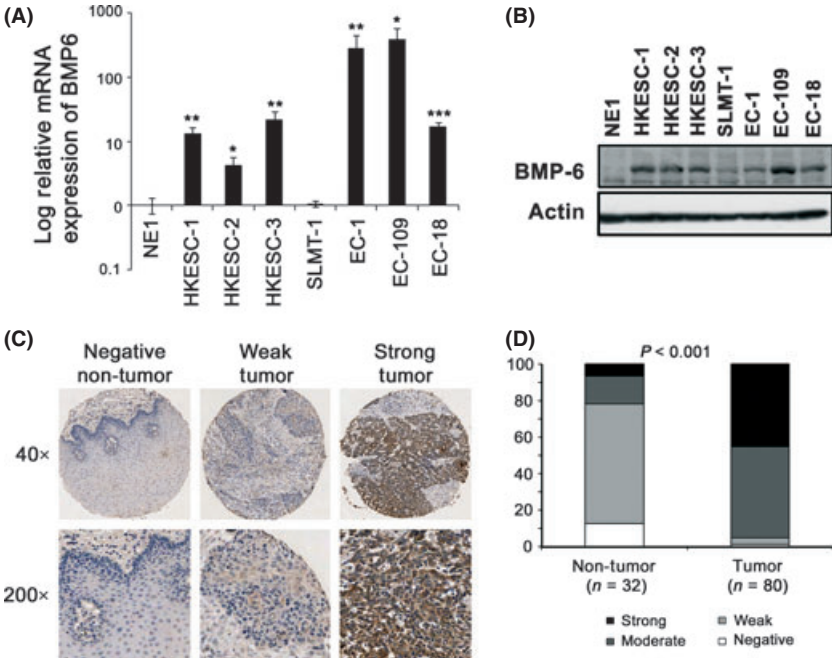

Fig. 1. Expression of bone morphogenetic protein 6 (BMP6) in esophageal squamous cell carcinoma (SCC) cell lines and human specimens. (A) mRNA expression of BMP6 in normal esophageal cell line NE1 and seven oesophageal SCC cell lines. (B) Corresponding protein expression of BMP6 in esophageal cell lines. (C) Representative images of immunohistochemical staining of BMP6 in human esophageal SCC patients. (D) Histogram showing the percentage of cases with various levels of BMP6 immunohistochemical staining.

level of Id-1 expression was associated with longer survival in patients whose tumors stained strongly for BMP6 (Wilcoxon–Gehan test, $P = 0.042$; Fig. 2D). As Id-1 expression is controlled by BMP6-activated Smad,[20,24] this result provides indirect evidence that tumors with higher levels of BMP6 can be divided into two distinct groups that differ in their degrees of aggression based on Id-1 expression. Tumors that translate a high level of BMP6 to transactivate its downstream target Id-1 are more aggressive than those whose BMP6 signaling is interrupted and cannot affect Id-1 activation.

Knockdown of noggin in EC109 cells reduces their non-adherent growth. BMP6 has upregulates expression of noggin and SOST to form a negative feedback loop,[33,34] in which noggin and SOST inhibit BMP6.[35,36] Noggin mRNA was overexpressed in six out of seven esophageal SCC cell lines (Fig. 3A), while SOST mRNA was overexpressed in four out of seven esophageal SCC cell lines (Fig. 3B) compared to NE1 cells. All seven cell lines overexpressed at least one BMP6 antagonist. We also found that noggin mRNA levels were positively correlated with BMP6 mRNA levels in the eight esophageal cell lines (Spearman's rank test, $r = 0.81$, $P = 0.015$), suggesting that BMP6 and noggin are co-expressed in esophageal cell lines. We investigated the biological implications of uncoupling this co-expression in EC109 cells, which express a high level of BMP6 and noggin, and a low level of SOST. Knockdown of noggin in EC109 cells, by shNog-1 shRNA, resulted in a reduced expression of noggin at the protein level and a concomitant increase in phospho-Smad1/5/8 (pSmad, Fig. 3C), and this occurred independent of the presence or absence of serum (Fig. 3D). The consequential increase in BMP signaling activity in turn resulted in a significant increase in non-adherent growth of the cells (Fig. 3E). This increase in non-adherent growth cannot be explained by an increase in proliferation rate as noggin knockdown did not significantly affect the 2-D colony formation ability of the cells (Fig. 3E). These biological implications of noggin interference were mirrored in parallel experiments using a second shRNA sequence targeting noggin (Fig. 3F,G).

Significance of noggin and SOST expression in esophageal squamous cell carcinoma expressing high levels of BMP6. Noggin (χ^2-test, $P < 0.001$; Fig. 4A,B) and SOST (χ^2-test, $P < 0.001$, Fig. 4C,D) were overexpressed in esophageal SCC specimens compared to non-tumor esophageal epithelium. This is in accordance with the esophageal cell line studies, when both proteins were overexpressed in transformed cell lines compared to an immortalized cell line. In the entire esophageal SCC patient cohort, neither noggin nor SOST expression levels were significantly correlated with patient survival (Wilcoxon–Gehan test: $P = 0.665$ and $P = 0.634$ for noggin and SOST, respectively).

Low level expression of SOST was associated with shorter survival in patients with high BMP6, but not in patients with low BMP6 (Wilcoxon–Gehan test, $P = 0.024$ and $P = 0.195$, respectively; Fig. 4E,F). The expression level of noggin was not significantly associated with patient survival either in the high or low BMP6 patient group. When both BMP antagonists were considered in tandem, we found that the expression of both noggin and SOST at low levels was associated with significantly shorter patient survival in the high BMP6 sub-group of patients, but, once again, not in the low BMP6 sub-group (Wilcoxon–Gehan test: $P = 0.021$ and $P = 0.355$, respectively; Fig. 4G,H). These results suggest that the expression of noggin and SOST is an important prognostic indicator in patients whose tumors strongly express BMP6, and further supports the hypothesis that unchecked BMP6 activity worsens the prognosis of esophageal SCC patients.

Prognostic significance of bone morphogenetic protein 6 activity in esophageal squamous cell carcinoma. Based on BMP6, noggin and SOST expression, patients can be assigned as having high or low BMP6 activity. Patients with high BMP6 activity had a mean survival time of 8.4 months (95% confidence interval [CI] = 2.7–14.1), whereas patients with low

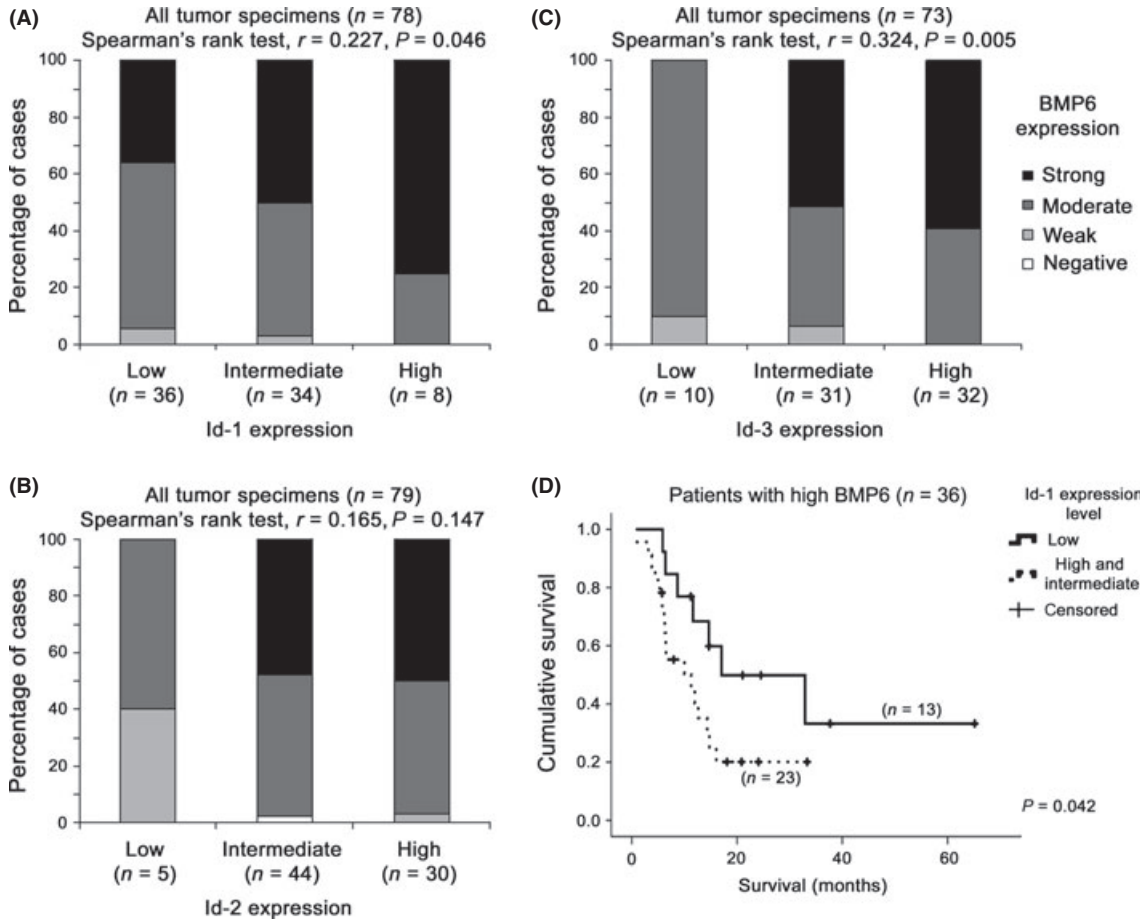

Fig. 2. The correlation between bone morphogenetic protein 6 (BMP6) and Id protein expression and patient survival. (A) The association between protein expression levels of BMP6 and inhibitor of differentiation (Id)-1 in esophageal squamous cell carcinoma (SCC) patients. (B) The association between protein expression levels of BMP6 and Id-2 in esophageal SCC patients. (C) The association between protein expression levels of BMP6 and Id-3 in esophageal SCC patients. (D) Kaplan–Meier analysis for Id-1 expression levels in BMP6-high esophageal SCC patients.

BMP6 activity had a significantly longer (Wilcoxon–Gehan test, $P = 0.002$) mean survival time of 22.4 months (95% CI = 17.6–27.2) (Fig. 4I). By Cox-regression analysis, we further found that high BMP6 activity ($P = 0.014$) and higher T-stage ($P = 0.002$) were independent predictors of shorter survival in our patient cohort (Table 2).

Prognostic significance of pSmad1/5/8 expression in esophageal squamous cell carcinoma. We also investigated whether pSmad1/5/8 expression level is correlated with BMP6 activity or prognosis of our patient cohort. Indeed, in the esophageal SCC specimens, pSmad1/5/8 expression level did not significantly correlate with BMP6 activity ($P > 0.05$). This result suggests that pSmad1/5/8 might not directly reflect the activity of the BMP6 pathway, as phosphorylation of Smad1/5/8 is also controlled by other BMP and TGF-β. This indicates that in esophageal SCC specimens, pSmad1/5/8 expression alone cannot be used as a surrogate of BMP6 activity. Nonetheless, high level expression of pSmad1/5/8 is significantly correlated with a shorter survival time of esophageal SCC patients ($P = 0.001$; Fig. 5A,B). In contrast to Id-1 and BMP6 antagonists, pSmad1/5/8 expression level correlated with survival of esophageal SCC patients, regardless of BMP6 expression status ($P < 0.05$; Fig. 5C,D).

Bone morphogenetic protein 6, noggin and SOST mRNA can also be used in combination for predicting prognosis in patients with prostate, colorectal and bladder cancers. We further examined DNA microarray datasets that comprised 198 prostate cancer patients, 458 colorectal cancer patients and 165 bladder cancer patients. mRNA expression levels of BMP6, noggin and SOST were analyzed along with survival status, and correlations between expression levels and patient survival were investigated. As shown in Figure 6, none of BMP6 (Fig. 6A), noggin (Fig. 6B) or SOST (Fig. 6C) mRNA expression levels alone were significantly associated with patient survival. Noggin mRNA expression was not significantly associated with patient survival in either BMP6-low (Fig. 6D) or BMP6-high (Fig. 6E) patients. In contrast, SOST mRNA expression, although not significantly associated with survival in patients expressing low levels of BMP6 (Fig. 6F), was significantly associated with a shorter survival time in BMP6-high patients ($P = 0.046$; Fig. 6G). Expression levels of noggin and SOST were not associated with patient survival in BMP6-low patients (Fig. 6H), while low level expression of both noggin and SOST were significantly associated with a shorter survival time in BMP6-high patients ($P = 0.003$; Fig. 6I). As with the esophageal SCC cohort, we attempted to delineate the influence of BMP6 activity on the survival of our prostate cancer cohort. As before, prostate cancer patients with high BMP6 activity had a significantly shorter survival time than those with low BMP6 activity ($P = 0.009$; Fig. 6J).

Similar results were obtained using datasets from colorectal and bladder cancers. Colorectal cancer patients whose primary tumors express high BMP6 activity had a significantly shorter survival time compared to those whose primary tumors express

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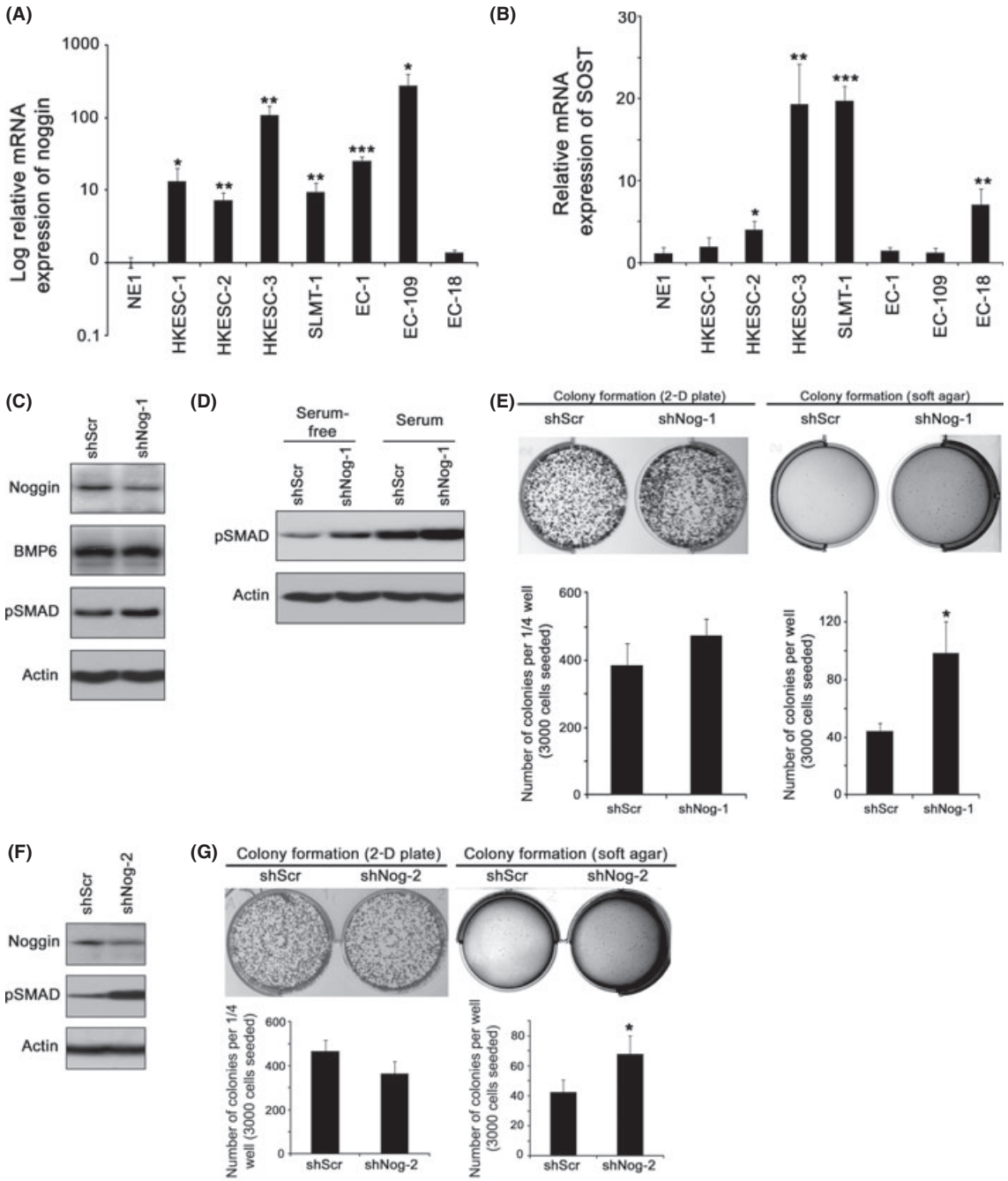

Fig. 3. Role of noggin expression in esophageal squamous cell carcinoma (SCC) cell lines. (A) Expression of noggin mRNA in esophageal SCC cell lines. (B) Expression of SOST mRNA in esophageal SCC cell line. (C) Protein expression of noggin, BMP6, pSMAD and actin in EC109 shScr and EC109 shNoggin-1 cells. (D) Expression of pSMAD in EC109 shScr and EC109 shNoggin-1 cells in the absence or presence of serum. (E) The ability of EC109 shScr and EC109 shNoggin-1 cells to form colonies in 2-D culture and 3-D soft agar. (F) Protein expression of noggin, pSMAD and actin in EC109 shScr and EC109 shNoggin-2 cells. (G) The ability of EC109 shScr and EC109 shNoggin-2 cells to form colonies in 2-D culture and 3-D soft agar.

low BMP6 activity ($P = 0.033$; Fig. 7A). Bladder cancer patients who express high levels of BMP6 activity had a significantly shorter overall survival ($P = 0.031$; Fig. 7B) and disease-specific survival ($P = 0.046$; Fig. 7C) compared to patients who express low levels of BMP6 activity.

Discussion

The BMP protein family consists of more than 20 members. They can play positive and negative roles in cancer progression, depending on the family member and the site of disease.[37–39] BMP6 has been shown to be overexpressed in esophageal SCC, and high level expression of BMP6 has been correlated with shorter patient survival.[14] However, the expressions of noggin and SOST, BMP6-inducible BMP6 antagonists, were not investigated in those studies, so the activity of BMP6 was not understood. In the present study, we have demonstrated that expression levels of noggin and SOST were correlated with survival in patients with BMP6-high tumors. In addition, BMP6 activity, which is determined by the expression levels of BMP6

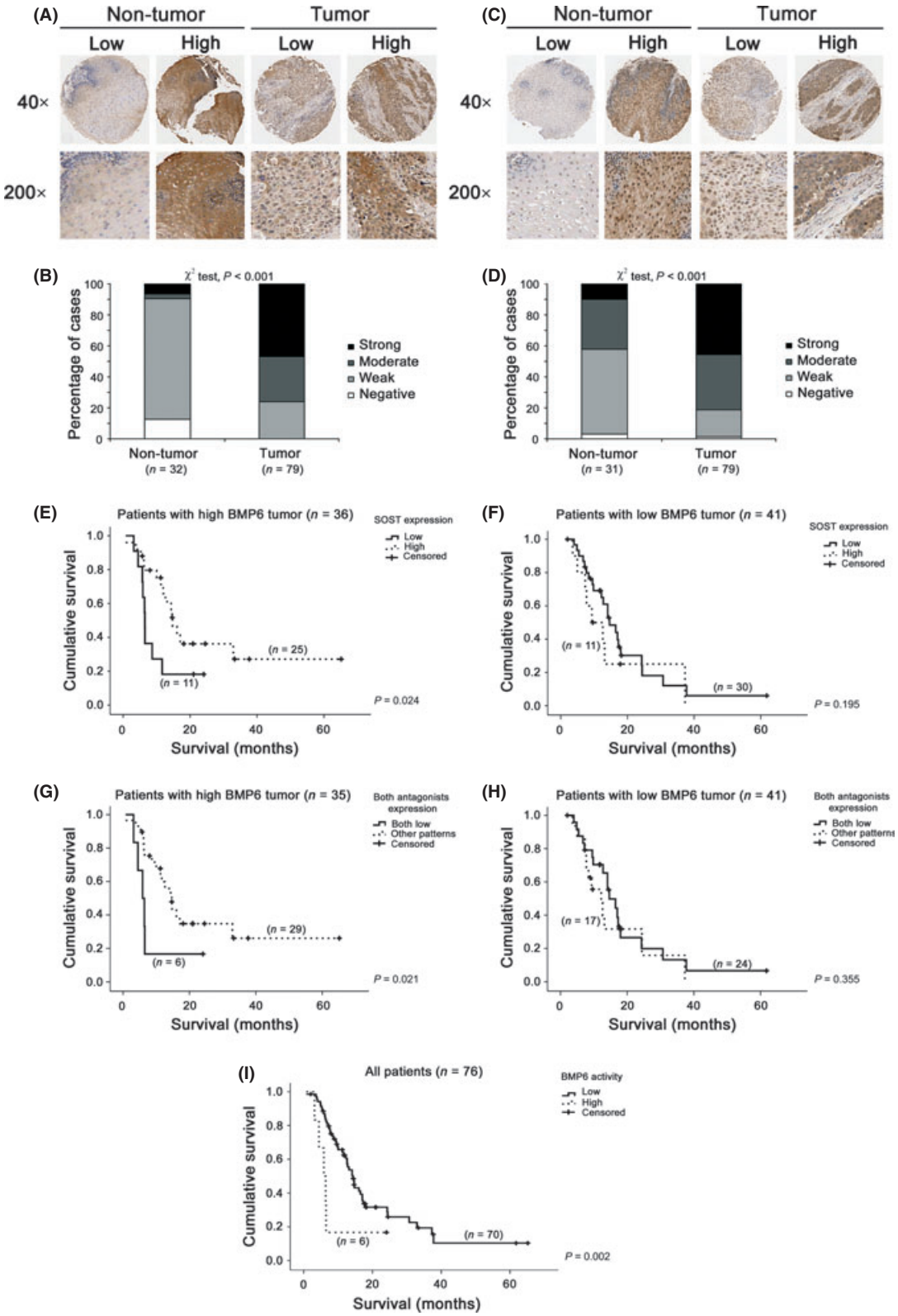

Fig. 4. Immunohistochemical staining of noggin and SOST in esophageal squamous cell carcinoma (SCC) patient specimens and Kaplan–Meier analysis of the esophageal SCC patient cohort. (A) Representative images of immunohistochemical staining of noggin. (B) Percentage of cases with various immunohistochemical staining levels of noggin. (C) Representative images of immunohistochemical staining of SOST. (D) Percentage of cases with various immunohistochemical staining levels of SOST. (E) Low levels of expression of SOST were significantly associated with a shorter survival time of esophageal SCC patients expressing high levels of bone morphogenetic protein 6 (BMP6) (F) but not in patients expressing low levels of BMP6. (G) Low level expressions of both noggin and SOST were significantly associated with a shorter survival time of esophageal SCC patients expressing high levels of BMP6 (H), but not in patients expressing low levels of BMP6. (I) High BMP6 activity was significantly associated with a shorter survival time in the whole esophageal SCC patient cohort.

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Table 2. Cox regression analyses of overall survival of esophageal squamous cell carcinoma patients

Clinical-pathological parameters	Univariate analysis		Multivariate analysis	
	RR (95% CI)	*P*	RR (95% CI)	*P*
M-stage	2.077 (1.089–3.960)	0.026		
T-stage	1.882 (1.232–2.875)	0.003	2.018 (1.281–3.179)	0.002
High BMP6, low sclerostin	2.303 (1.110–4.780)	0.025		
High BMP6, low noggin and sclerostin	2.824 (1.107–7.209)	0.030	3.271 (1.273–8.408)	0.014

BMP6, bone morphogenetic protein 6; CI, confidence interval; RR, relative risk.

itself, as well as its intrinsic antagonists, including noggin and SOST, was an independent predictor of survival in our esophageal SCC patient cohort. As BMP6 activates its two antagonists, thereby generating another level of regulation in its signal transduction, we speculate that both the overexpression of BMP6 and the uncoupling of the regulation of its antagonists are required for esophageal SCC progression in a BMP6-dependent fashion. Indeed, by using public data on prostate, colorectal and bladder cancer, we found that BMP6 activity could be a prognostic indicator for patient survival in multiple malignancies.

Previous studies have indicated that BMP6 is upregulated in clear cell renal carcinoma[17] and osteosarcoma,[18] but in neither case was it correlated with progression of the tumors. The results we present here indicate that BMP6 antagonists should be included in expression profiling to determine the intrinsic biological activity of BMP6 in patients, rather than simply determining BMP6 expression alone. Activation of Smad-1, a downstream factor of BMP and TGF-β, has been shown to correlate with poor prognosis in several cancer types,[40] suggesting that the transmission of the upstream ligand-receptor binding to the downstream signaling pathway might be an important determinant of cancer progression. Indeed, our *in vitro* analysis in the EC109 cells showed that knockdown of noggin resulted in Smad activation, which implies that BMP signaling can be activated by interfering with the expression of its antagonist.

Bone morphogenetic protein signaling was recently suggested to play an important role in stem cell biology.[41] BMP activate Id proteins via Smad signaling to maintain the self-renewal ability of mouse embryonic stem cells.[42] In the present study, an increase in BMP signaling led to an increase in Id proteins, thereby enhancing the aggressiveness of cancers through dedifferentiation and increased stem cell-like

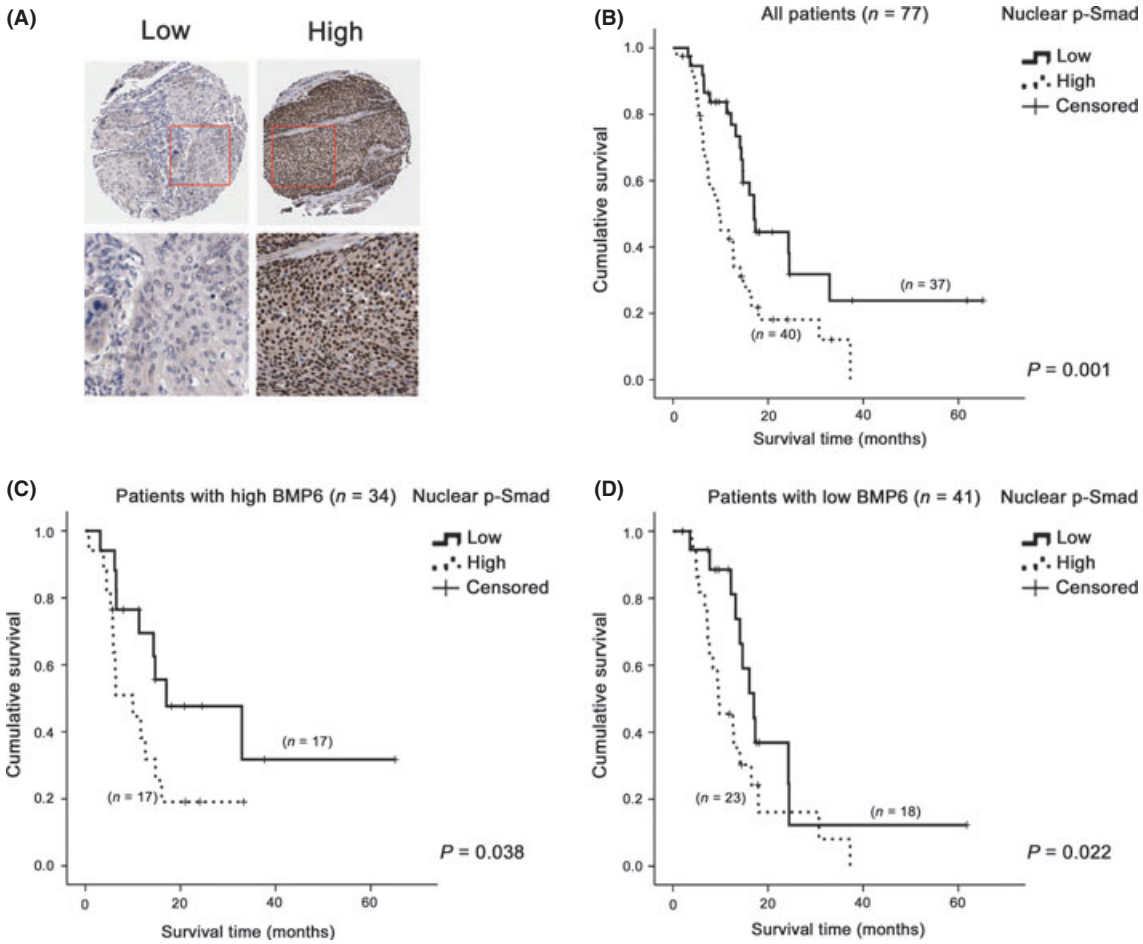

Fig. 5. Expression and prognostic significance of pSmad1/5/8 in esophageal squamous cell carcinoma (SCC) patients. (A) Representative images of immunohistochemical staining of pSmad1/5/8 in human esophageal SCC patients. (B) Kaplan–Meier analysis for pSmad1/5/8 expression in esophageal SCC patients. (C) Kaplan–Meier analysis for pSmad1/5/8 expression levels in bone morphogenetic protein 6 (BMP6)-high esophageal SCC patients. (D) Kaplan–Meier analysis for pSmad1/5/8 expression levels in BMP6-low esophageal SCC patients.

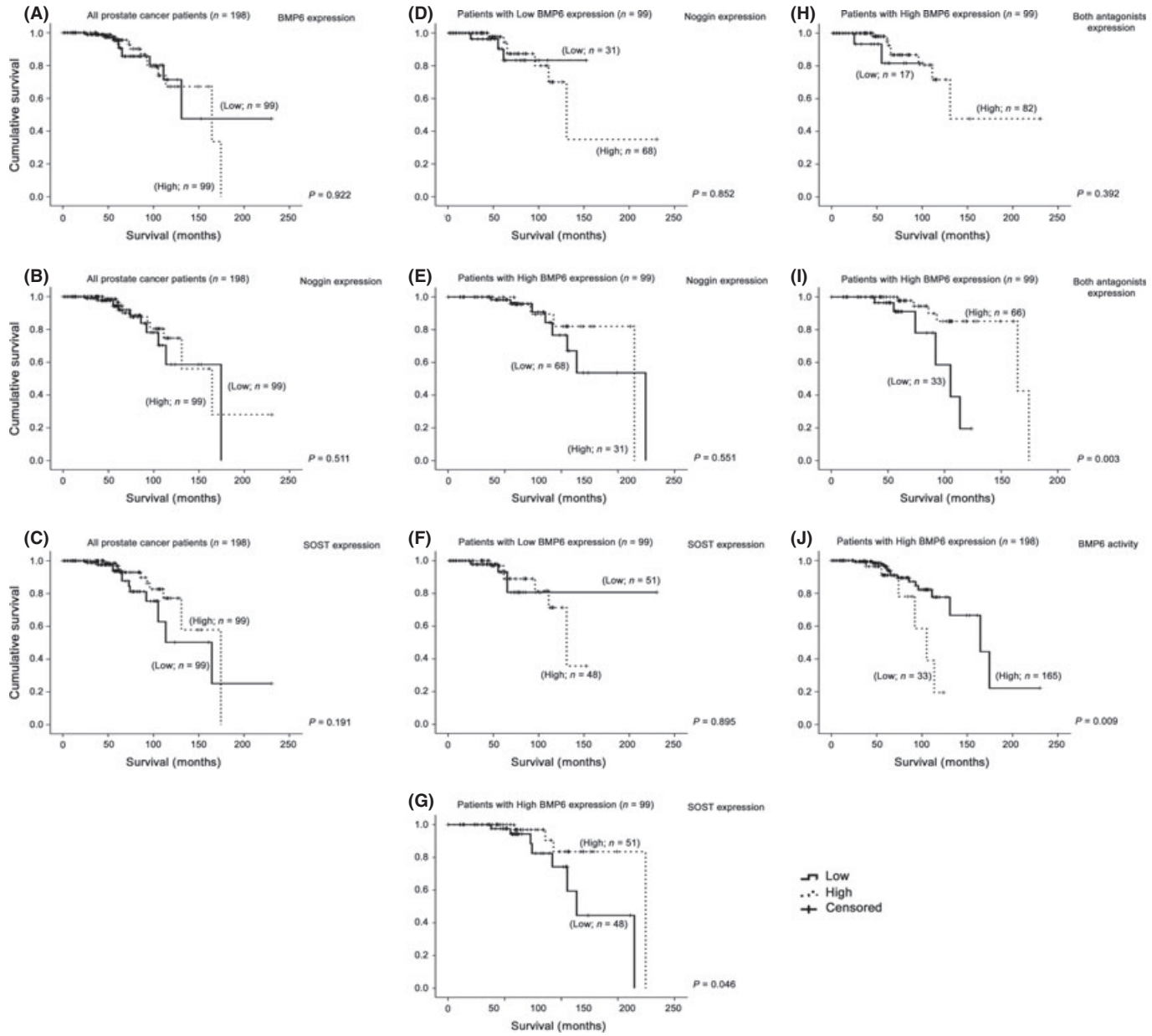

Fig. 6. Kaplan–Meier analysis of the prostate cancer patient cohort GSE 21035 in the Gene Expression Omnibus database. (A) bone morphogenetic protein 6 (BMP6), (B) noggin and (C) SOST mRNA expression levels were not significantly associated with patient survival in the whole patient cohort. Noggin mRNA expression was not significantly associated with survival in patients expressing (D) low levels of BMP6 mRNA or (E) high levels of BMP6 mRNA. (F) SOST mRNA expression was not significantly associated with survival in patients expressing low levels of BMP6. (G) High levels of SOST mRNA were significantly associated with a shorter survival time in patients expressing high levels of BMP6 mRNA. (H) Expression levels of noggin and SOST were not significantly associated with survival in patients with low BMP6 expression. (I) Low level expression of both noggin and SOST was significantly associated with a shorter survival time of patients expressing high levels of BMP6. (J) High BMP6 activity was significantly associated with a shorter survival time in the whole prostate cancer patient cohort.

properties of the tumor cells. In this study, we found that the expression of BMP6 and Id-1 were significantly positively correlated in our tumor specimens, while Id-1 expression was correlated with survival in patients with high-BMP6 tumors. We and others have previously shown that Id-1 promotes esophageal SCC progression and is overexpressed in human esophageal SCC.[22,43,44] Our results indicate that Id-1 might also be a downstream effector of BMP6 signaling in esophageal SCC, thereby promoting cancer progression. In contrast, although pSmad1/5/8 expression correlated with patient survival in our esophageal SCC patient cohort, pSmad1/5/8 expression did not correlate with BMP6 activity in the esophageal SCC specimens, suggesting that pSmad1/5/8 expression cannot replace BMP6 activity for cancer prognostication.

This study on BMP6, noggin and SOST in esophageal SCC, as with our previous study of the same factors in prostate cancer,[15] has highlighted the importance of considering functionally related proteins in a signaling pathway (i.e. positive and negative regulators) simultaneously for the prognosis of cancer patients. A combinatorial use of several cooperating factors might be more useful in predicting cancer progression and patient survival than individual use of the same factors. Most notably, our results show that protein expression of BMP6, noggin and SOST in combination can be used for prediction of

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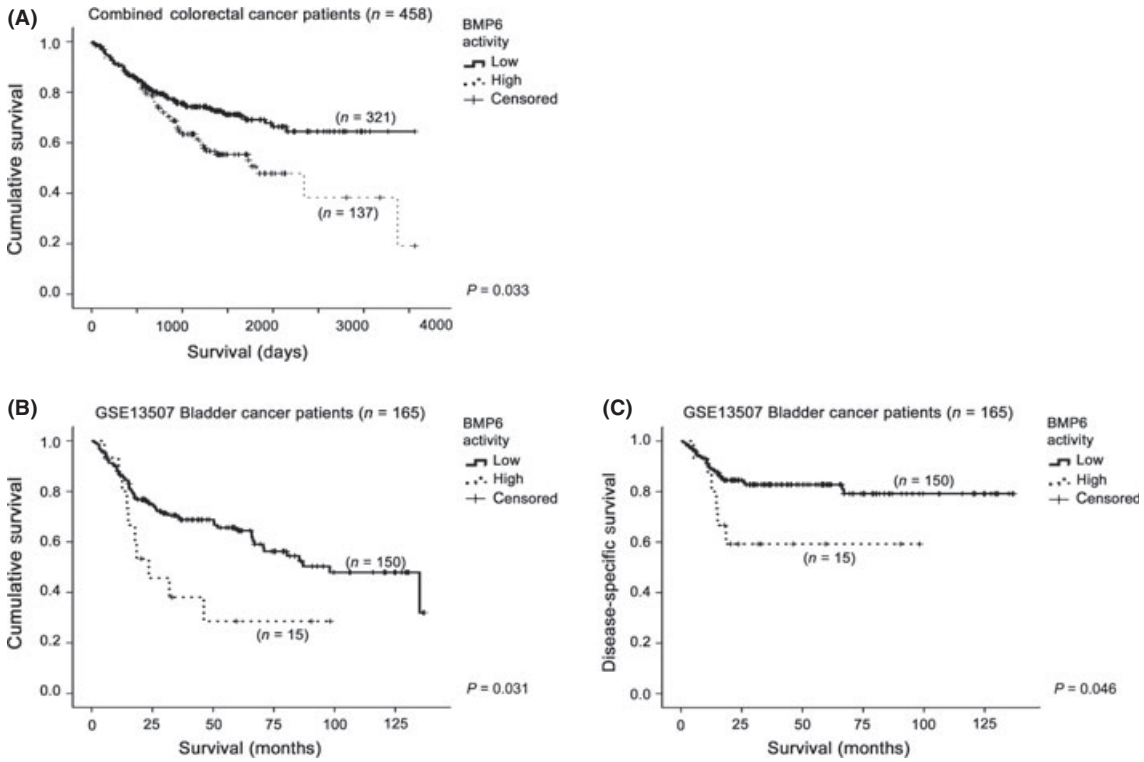

Fig. 7. Kaplan–Meier analysis of the colorectal and bladder cancers in the Gene Expression Omnibus database. Bone morphogenetic protein 6 (BMP6) activity is a prognostic indicator in (A) colorectal cancer patients and (B and C) bladder cancer patients.

prostate cancer metastatic progression and esophageal SCC patient survival, whereas mRNA expressions of these proteins in combination can be used for prediction of patient survival in several types of cancer. Noggin and SOST may play different roles in different types of cancer. More importantly, the combinatorial use of BMP6, noggin and SOST consistently shows positive correlation with cancer progression.

Deligezer *et al.*[45] has indicated that the plasma level of BMP6 mRNA might act as a biomarker to distinguish metastatic and non-metastatic prostate cancers. Whether the incorporation of the mRNA levels of noggin and SOST in plasma could improve the predictive power of this non-invasive method warrants further investigation.

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Disclosure Statement

The authors declare no conflict of interest.

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