RESEARCH ARTICLE

Serum BMP-2 Up-regulation as an Indicator of Poor Survival in Advanced Non-small Cell Lung Cancer Patients

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Abstract

Purpose: High levels of bone morphogenetic protein (BMPs) have been reported in patients with lung cancer. This study was conducted to assess correlations between serum BMP-2 levels and prognostic outcome in patients with non-small-cell lung cancer (NSCLC). Methods: Blood samples from 84 patients with advanced NSCLC and 42 healthy controls were analyzed and quantitated for serum BMP-2 levels before and after two cycles of chemotherapy using a commercially available ELISA kit. Results: The median level of BMP-2 was 146.9 pg/ml in patients with NSCLC vs. 87.7 pg/ml in healthy controls ($P<0.01$). A significant correlation was observed between pretreatment serum BMP-2 level and ECOG PS, disease stage and number of organs with metastases ($P<0.05$). Serum BMP-2 level decreased significantly in patients who achieved objective response after two cycles of chemotherapy. Multivariate analysis showed that increased BMP-2 level and advanced clinical stage were significantly correlated with poor prognosis. Conclusion: Thes erum BMP-2 level is positively correlated with clinical stage, ECOG PS and metastatic burden and may serve as an independent negative predictor for prognosis. Decreased BMP-2 after chemotherapy could be a reliable marker for efficacy of treatment.

Keywords: Non-small-cell lung cancer - bone morphogenetic protein-2-prognosis

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Introduction

Lung cancer is one of the most common malignant tumors worldwide and the leading cause of human cancer-related deaths for several decades (Siegel et al., 2013). Non-small-cell lung cancer (NSCLC) accounts for about 80% of lung cancer cases, of which about two-thirds present with stage IIIB/IV disease. For patients with advanced NSCLC, the therapeutic option is limited to combination chemotherapy, which is largely ineffective with a response rate ranging from 20% to 35% and a 1-year survival rate of 35% (Ohe et al., 2007). This poor prognosis emphasizes the urgent need for accurate prognostic and predictive factors to evaluate the treatment for patients with advanced NSCLC so as to know earlier whether the tumor responds to the treatment, or the therapeutic regimen should be changed in time (Reich, 2005).

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF)-α superfamily and play important roles in the regulation of various cellular processes such as migration, proliferation, apoptosis, differentiation and remodeling of the extracellular matrix (Hogan, 1996). BMP-2 was originally described as an osteoinductive cytokine that induces the entire cascade of endochondral bone formation. Many studies have demonstrated that BMP-2 is necessary for the development of the lung, heart, digits, limbs, central nervous system and epidermis (Yu et al., 2002; Dzietczenia et al., 2010). BMP-2 acts via two types of serine/threonine receptors. When ligands bind to either type I or type II serine/threonine kinase receptors, the receptor complex mediates intracellular signaling via phosphorylation of Smad 1, Smad 5 and/or Smad 8. Phosphorylated Smad 1/5/8 forms a complex with Smad 4 and translocates to the nucleus to activate the transcription of downstream targets, such as alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), and osteocalcin (Miyazono, 2002; Hocking and McFarlane, 2007).

In addition to the physiological roles of BMPs, dysregulation of BMPs has been reported in several types of cancers. BMPs are overexpressed in prostate, lung, colon, and breast cancer cells as compared with the corresponding normal tissues or cells (Dai et al., 2008; Katsuno et al., 2008; Bieniasz et al., 2009; Deng et al., 2009). BMP-2 increases the adhesion, motility and invasiveness of primary human ovarian cancer cells and induces epithelial mesenchymal transition. BMP-2 and 4 were reported to increase the motility and invasiveness of prostate cancer cell line PC-3 both in vitro and in vivo (Dai et al., 2008). Recent findings suggest that tumor-associated macrophages might promote gastric

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cancer cell invasion through enhancing the activation of invasion-related genes and BMPs signal pathway (Shen et al., 2013). Consequently, BMPs are considered an oncogenic activator that promotes tumorigenesis and tumor metastasis. However, some other studies showed that the effects of BMP-2 on cancer cells are inconsistent. For instance, BMP-2 clearly inhibited the proliferation of tumor cells including cancers arising from myeloma, gastric and breast cells. BMP-2 induced G1 arrest and promoted apoptosis by increasing p21 and cleaved caspase-3 in MCF-7 breast cancer cells (Chen et al., 2012). The current consensus is that BMPs are involved in both promotion and inhibition of cancer progression depending on the tissue and environment where they are expressed.

The aim of the present study was to assess in a prospective manner both the sensitivity and the specificity of the serum levels of BMP-2 and their correlation with the response to chemotherapy treatment, and evaluate their prognostic significance in patients with advanced NSCLC.

Materials and Methods

Patient selection

Serum samples were collected from two groups: 42 healthy adult volunteers as the control group, and 84 patients with advanced-stage NSCLC who were treated consecutively and received follow-up care at the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) between 2009 and 2011 as the study group,. The study was approved by the Ethics Committee on Human Research of the First Affiliated Hospital of Wenzhou Medical University, and written informed consent was obtained from all patients and subjects. Inclusion criteria were patients with cytologically or histologically confirmed NSCLC who met the following conditions: unresectable stage III-IV; measurable disease, Eastern Cooperative Oncology Group (ECOG) performance status 0-2; and no concomitant serious co-morbidity. Eligible patients were required to have received at least two cycles of platinum-based combination chemotherapy with a life expectancy > 3 months. Patients with a personal history of previous malignant neoplasm were excluded. The clinicopathologic characteristics of the NSCLC patients are shown in Table 1.

Tumor response was assessed by the Response Evaluation Criteria in Solid Tumors criteria. In this study, patients who achieved a complete response (CR) or a partial response (PR) were classified as objective response (OR), and all remaining patients with stable disease (SD) and progressive disease (PD) were considered non-responders.

Biochemical analysis

Peripheral blood samples (5 ml) were collected from 84 NSCLC patients before and 48 h after chemotherapy administration centrifuged at 1,000g for 10 min and stored at - 20°C until evaluation 4 months later. All samples were analyzed simultaneously.

BMP-2 expression was determined by a commercial ELISA kit (R&D, Wiesbaden, Germany). Briefly, serum samples were thawed on wet ice 3 h prior to assay. BMP-2 serum samples were pretreated with an acidic solution to promote dissociation of BMP-2 from abundant BMP-2 binding proteins and stabilized in buffer and preservatives. Samples were placed in a 96-well format in duplicate, after which conjugated BMP-2/horseradish peroxidase polyclonal secondary antibody was added. Substrate solution (hydrogen peroxide/tetramethylbenzidine) was then administered for 30 min, after which the reaction was quenched with sufuric acid. Plates were read at an absorbance of 450 nm on a Victor 3 plate reader (BIO-TEK, Vermont., USA). Extrapolated absorbance was analyzed using Masterplex Readerfit ELISA software (Hitachi, Waltham, Mass., USA), and the concentration was determined following a 4 Parameter Logistic curve fit as per the manufacturer's recommendation. Measurements were made by a single investigator blinded to the patient clinicopathological data.

Statistical Analysis

The SPSS software package (version 17.0, SPSS Inc., Chicago, IL, USA) was used for all data analyses. The compatibility test of Shapiro-Wilk was used for measurable features consistent with normal distribution. Differences in the values between the groups under study were determined by using the independent-samples t test. The paired-samples t test procedure was used to compare BMP-2 levels before treatment and after the second course of chemotherapy.

To evaluate the diagnostic value of BMP-2 in NSCLC, receiver operating curves (ROCs) were constructed. The area under the curve (AUC) was calculated to determine the most useful cut-off values of BMP-2 levels, which were derived from the first derivative of the curve, and the sensitivity and specificity at the cut-off point were determined.

Survival analysis and curves were established according to the Kaplan–Meier method and compared by the log-rank test. Overall survival (OS) was measured from the time of initial diagnosis until the date of the patient death or the termination of patient follow-up. Progression-free survival (PFS) was defined as the time from diagnosis until disease progression, the date of death, or the termination of patient follow-up. Univariate analyses were carried out to evaluate important prognostic factors. Then, multivariate analysis with the Cox proportional hazards model was performed to further analyze independent prognostic factors that were found in the univariate analysis to predict OS and PFS.

Research experience

We have enough experience in conducting medical researches, and have published some results elsewhere (Huang et al., 2004; Zhou et al., 2009; Jiang et al., 2010; Yan et al., 2010; Gao et al., 2011; Huang et al., 2011; Li et al., 2011; Li et al., 2011; Li et al., 2011; Xu et al., 2011; Xu et al., 2011; Xu et al., 2011; Yan et al., 2011; Zhang et al., 2011; Gong et al., 2012; Gong et al., 2012; Gu et al.,2012; Li et al., 2012; Yu et al., 2012; Zhan et al., 2012; Zhan et al., 2012; Deng et al., 2013; Huang et al., 2013; Liu et al., 2013; Liu et al., 2013; Lu et al., 2013; Wu et al., 2013; Yin et al., 2013; Yin et al., 2013).

Table 1. Patient Characteristics

Characteristics	n=	Median serum BMP-2 [range]	*P* value
Age, y			0.49
< 65	39	153.3 [119.2-181.3]	
≥ 65	45	146.4 [113.6-177.1]	
Gender			0.81
Male	51	145.6 [113.6-177.2]	
Female	33	146.9 [118.8-175.6]	
Stage			0.02
IIIB	22	110.3 [129.6-155.6]	
IV	62	147.4 [118.8-182.9]	
ECOG PS scores			0.01
0	41	136.9 [105.4-159.6]	
1-2	43	156.4 [125.0-203.5]	
Histology			
Adenocarcinoma	48	151.9 [123.1-186.6]	0.12
Squamous cell carcinoma	36	145.8 [109.1-163.3]	
Cell differentiation*			0.29
Well	23	133.6 [101.1-153.2]	
Moderate	36	155.3 [121.4-185.3]	
Poor	25	161.1[135.4-196.1]	
Metastatic organs			0.04
<3	57	163.9 [132.2-195.3]	
≥3	27	136.4 [114.3-164.8]	
Responders			0.03
Before treatment	38	137.1 [106.3-166.3]	
After treatment	38	126.1 [90.8-164.2]	
Non-responders			0.72
Before treatment	46	153.2 [126.5-179.3]	
After treatment	46	148.1 [108.2-184.7]	

*Use Kruskal-Wallis test

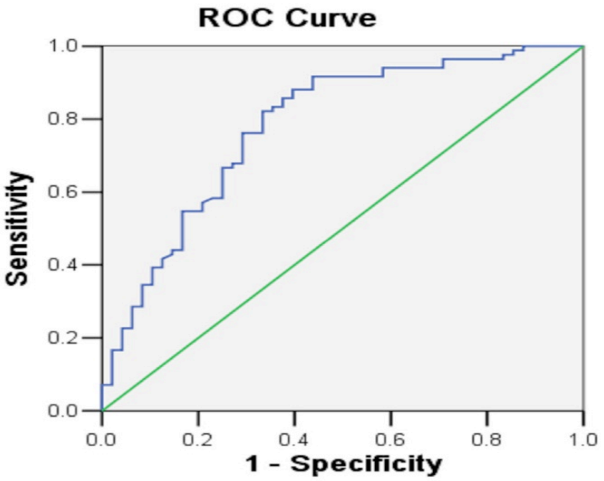

Figure 1. Receiver-operating Characteristic Curve for the Diagnosis of Serum BMP-2 for Advanced NSCLC

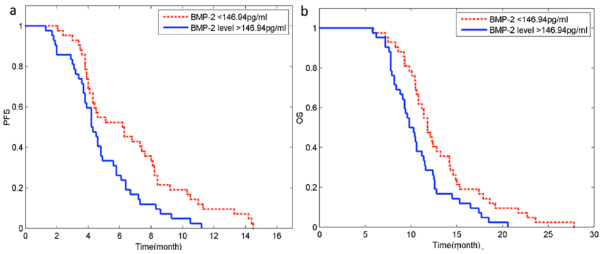

Figure 2. Kaplan-Meier Survival Curves for Non-small Cell Lung Cancer (NSCLC) Patients Based on Serum BMP-2 Levels. (a) Progression-free survival (PFS); (b) overall survival (OS)

Results

A total of 84 NSCLC patients (median age 64.0 years) and 42 control subjects (median age 66.0 years) were analyzed. The proportion of men was 60.7% in the control group and 67.4% in the patient group. The baseline patient characteristics are summarized within Table 1. The pathological type was identifiable in 48 (57.1%) patients as adenocarcinoma; 62 (73.8%) patients were diagnosed in the stage IV; 41 (48.9%) patients had ECOG PS 0 at the initiation of first-line chemotherapy; 27 (32.1%) patients had more than 3 metastatic organs. All patients had received more than two cycles of platinum-based combination chemotherapy including navelbine + platinum, gemcitabine+ platinum or docetaxel + platinum. The median serum BMP-2 level in the health control subjects was 87.7 pg/ml (interquartile range, 61.3 pg/ml -113.8 pg/ml) vs. 146.9 pg/ml (interquartile range,116.5–176.6 pg/ml) in the NSCLC group, showing a statistically significant difference ($P<0.01$). The pretreatment serum BMP-2 level was not correlated with age, sex, smoking history, tumor grade or histology. A significant difference was observed between pretreatment serum BMP-2 level and ECOG PS ($P=0.012$). Patients who had a PS of 0 had the lowest serum BMP-2 level. A significant difference was also observed between pretreatment serum BMP-2 level and disease stage ($P=0.02$) and number of metastatic organs $P=0.04$). There also was a trend toward an association with disease stage and patients with IIIB phase who had the lowest serum BMP-2 levels (Table 1).

ROC curve analysis was performed to determine the values of serum BMP2 levels that distinguished between NSCLC patients and healthy volunteers. AUC was 0.782 (95% CI, 0.698-0.867) for BMP-2. The best efficacy (combination between sensitivity and specificity) was observed at 106.6 pg/ml, with a sensitivity of 82.7% and a specificity of 66.7% (Figure1).

All patients were able to receive efficacy assessment after the second course of chemotherapy. Of the 84 NSCLC patients, 38 (45.2%) patients achieved a"OR" after 2 chemotherapy cycles, in whom the pretreatment serum BMP-2 level had no impact on response to chemotherapy. Patients who achieved a radiological OR had a median serum BMP-2 level of 137.1 pg/ml (interquartile range, 106.3 pg/ml -166.3 pg/ml), while patients who had stable or progressive disease (n = 46) had a median serum BMP-2 level of 153.2 pg/ml (interquartile range, 126.5 pg/ml -179.3 pg/ml, $P=0.19$). After the second course of chemotherapy, serum BMP-2 levels of patients who achieved a radiological OR decreased significantly as compared with the baseline levels ($P=0.03$). The median serum concentration of BMP-2 decreased to 126.1 pg/ml (90.8 pg/ml -164.2 pg/ml). However, there was no significant decrease in serum BMP-2 level in non-responders (median 148.1 pg/ml vs. 153.2 pg/ml, $P=0.72$) (Table 1).

Median PFS for all 84 NSCLC patients was 5.7 months (95% CI, 5.1-6.4 months). Univariate analysis showed that ECOG PS had statistically significant prognostic

Table 2. Univariate and Multivariate Analyses of Progression-free Survival in NSCLC Patients

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	*P*	HR	95% CI	*P*
Age	0.992	0.643-1.531	0.98			
Gender	1.147	0.742-1.772	0.53			
Histological subtype	0.965	0.627-1.487	0.87			
Cell differentiation	1.171	0.7555-1.816	0.48			
Metastatic organs	1.618	1.011-2.590	0.04	1.726	1.068-2.789	0.03
PS	1.872	1.189-2.946	<0.01	1.671	1.029-2.712	0.04
TNM stage	1.935	1.156-3.240	0.02	1.694	0.983-2.919	0.06
BMP-2	2.111	1.327-3.356	<0.01	1.77	1.099-2.849	0.02

Table 3. Univariate and Multivariate Analyses of Progression-free Survival in NSCLC Patients

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	*P*	HR	95% CI	*P*
Age	0.956	0.619-1.477	0.95			
Gender	1.142	0.736-1.772	0.55			
Histological subtype	0.965	0.627-1.487	0.87			
Cell differentiation	1.171	0.7555-1.816	0.48			
Metastatic organs	1.567	0.985-2.492	0.06			
PS	1.666	1.052-2.638	0.03	1.363	0.845-2.199	0.20
TNM stage	1.874	1.139-3.084	0.01	1.678	1.007-2.798	0.04
BMP-2	1.903	1.215-2.981	<0.01	1.686	1.067-2.662	0.03

impact on PFS, the number of metastatic organs, disease stage and BMP-2 level. Patients who had a pretreatment serum BMP-2 level >146.94 pg/ml (the median level for all patients) had significantly shorter PFS than patients who had a serum BMP-2 level <146.94 pg/ml (median progression-free survival, 4.8 months vs. 6.7 months; P=0 .005) (Figure 2a). The parameters that had significant correlations with survival as shown by univariate analysis were further evaluated by multivariate analysis. The results showed that BMP-2 level (HR: 1.77; 95 % CI: 1.09-2.84; p=0.02), the number of metastatic organs (HR: 1.72; 95%CI: 1.06-2.78; p=0.03) and ECOG PS (HR: 1.67; 95%CI: 1.02-2.71; p=0.04) were independent prognostic markers for assessing the PFS rate (Table 2).

The median OS of the 84 NSCLC patients was 11.9 months (95% CI, 11.0-12.8months). Univariate analysis showed that ECOG PS had statistically significant prognostic impact on OS, disease stage and BMP-2 level. Patients who had a pretreatment serum BMP-2 level >146.94 pg/ml had significantly shorter OS than patients who had a serum BMP-2 level <146.94 pg/ml (median overall survival, 10.8months vs.13.1 months, *P*=0.01) (Figure 2b). The parameters that had significant correlations with survival as shown by univariate analysis were further evaluated by multivariate analysis. The results showed that BMP-2 level (HR: 1.68; 95%CI: 1.06-2.66; *p*=0.03) and disease stage (HR: 1.67; 95%CI: 1.007-2.79; *p*=0.04) were independent prognostic markers for assessing the OS rate (Table 3).

Discussion

The aim of the present investigation was to assess the correlation of pretreatment serum levels of BMP-2 with clinicopathological parameters and prognostic outcomes in patients with advanced NSCLC (stage IIIB with malignant pleural effusion or stage IV). The results of analysis showed that serum BMP-2 in advanced NSCLC patients was significantly up-regulated as compared with the healthy control subjects. In addition, the median serum BMP-2 level in NSCLC patients with PS of 0, IV phase or large metastatic burden was higher than that in the other patients. After two cycles of chemotherapy, BMP-2 reduced markedly in patients who achieved objective response. More importantly, multivariate analysis showed that baseline BMP-2 levels strong and independent predictors of PFS and OS outcomes.

The exact role of BMPs in the pathogenesis of tumors remains unclear. Previous in vivo and in vitro studies showed that BMP-2/4 could promote the metastasis of melanoma and prostate cancer, and stimulate the proliferation of bladder cancer cells (Rothhammer et al., 2005; Dai et al., 2008; Zhang et al., 2013). BMP-2 is reported to be highly expressed in approximately 98% of human lung carcinomas, but little expression of BMP-2 has been reported in normal lung tissues. In addition, recombinant BMP-2 stimulated the migration and invasiveness of the A549 and H7249 human lung cancer cell lines in vitro, and enhanced the growth of tumors grown from A549 cells by subcutaneous injection into nude mice in vivo (Langenfeld et al., 2006). Hsu et al found that BMP-2 in the bone matrix cooperated in an enhanced effect on lung cancer epithelial-to-mesenchymal transition (EMT) and migration via activation of the MAPK/ Runx2/Snail signaling pathway (Hsu et al., 2011). EMT has been identified to lead to the degradation of the basement membranes and the extracellular matrix (ECM) at the primary tumor site and the establishment of metastatic colonies at distant organs (Yilmaz and Christofori, 2009). Our results indicate that serum BMP-2 levels were significantly up-regulated in NSCLC patients as compared with the healthy control subjects. Previous

studies suggested that disease stage or the number of metastatic organs was correlated with serum levels of BMP-2 (Choi et al., 2012). Consistent with their finding, we also observed an increased incidence of high serum BMP-2 levels in patients who had more than 3 metastatic organs and in patients at clinical IV phase. Furthermore, we firstly analyzed the relation between the ECOG PS and BMP-2 levels. Interestingly, patients with ECOG PS 0 had a lower level of BMP-2 as compared with those with PS 1 OR 2. All these results support the view that serum BMP-2 is associated with migration and invasion of advanced NSCLC. So BMP-2 level may prove to be a biomarker for assessing the progression of NSCLC.

Computed tomography (CT) is routinely used as an objective assessment of response to therapy in NSCLC patients. However, CT measurements of the lung tumor size are sometimes inconsistent in the case of patients with pleural effusions, diffuse nodules, or tumors with poorly defined margins (Jones et al., 2011). Thus it is necessary to develop novel strategies to improve the evaluation of response. Recent studies reported that some serum biomarkers that are closely related to treatment responses in other tumors could also be used for NSCLC (Hsu et al., 2011; Yu et al., 2011; Arrieta Rodriguez et al., 2013). If so, it would be more convenient and economical to predict the efficacy of chemotherapy and guide the clinician on the timing to request further studies. For instance, Qian et al reported that a ≥ 11.5% reduction in soluble intercellular adhesion molecule-1 (sICAM-1) in serum after two cycles of chemotherapy could be used as a surrogate marker for assessing the chemotherapy efficacy in NSCLC patients. In this study, we are the first to report serum BMPs as a marker for assessing the response to cisplatin-based chemotherapy in NSCLC patients. Our data show that there is a good correlation between objective response and the decrease in serum BMP-2 levels. BMP-2 levels decreased significantly in the responders after the second course of chemotherapy as compared with the baseline levels, while no significant change was observed in the non-responders, suggesting that continuous monitoring of serum BMP-2 may predict the efficacy of chemotherapy in advanced NSCLC.

The prognostic impact of BMP-2 levels in cancer patients has been evaluated in some studies (Park et al., 2008; Ma et al., 2010). Using immunohistochemical analysis, Ma et al. evaluated the influence of intratumoral BMP-2 on five-year survival in 100 patients with epithelial ovarian cancer, showing that the median five-year survival was significantly longer in the groups with a low intratumoral BMP-2 staining intensity (40.32%) than that in the group with tumors expressing high levels of BMP-2 (18.42%, P=0.023) (Qian et al., 2011). Liu et al. also found that the median survival of glioma patients with intensively positive BMP-2 expression was significantly shorter than that with negative expression (318 vs. 1197 days, $P < 0.0001$) (Liu et al., 2009). Our multivariate analysis on the covariates (age, stage, histology, cell differentiation, ECOG PS scores, the number of metastatic organs and BMP-2 levels) that might influence OS in the Cox proportional hazard model suggested that high serum BMP-2 levels may be an independent prognostic factor for PFS and OS in advanced NSCLC patients. Choi et al. (2012) found that serum BMP-2 was correlated with the clinical outcome in NSCLC patients as analyzed in an univariate model rather than in a multivariate model. The results might be due to some early stage NSCLC patients included in their report, while only advanced NSCLC patients were included in our study. Our data indicate that high serum BMP-2 levels may serve as a novel biomarker for predicting the prognosis of advanced NSCLC.

Our study has some limitations. For instance, the sample size was relatively small (n=84). As our study included a relatively homogeneous population with advanced NSCLC patients, the role of serum BMP-2 as reported in our study may not apply to patients with earlier stage NSCLC. We did not perform consecutive determinations of serum BMP-2 during the follow-up or for treatment response assessment. To resolve these problems, future studies in larger patient samples are needed to elucidate the role of BMP-2 expression in NSCLC at a molecular level.

In conclusion, our study demonstrated that serum BMP-2 level is higher in patients with advanced NSCLC than that in healthy people. In addition, measurements of serum BMP-2 level before and during first-line chemotherapy can be used to assess treatment efficacy in advanced NSCLC patients. In addition, our results also identified the pretreatment serum concentration of BMP-2 as an independent prognostic biomarker for survival in patients with advanced NSCLC. However, further collaborative studies in larger patient samples are needed to confirm our conclusion.

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