

# Expression of CRYAB with the angiogenesis and poor prognosis for human gastric cancer

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## Abstract

Alpha-B crystallin (CRYAB), as a small heat shock protein, has been found to be highly expressed in various human cancers and significantly associated with the unfavorable prognosis of these tumor. Nevertheless, the clinical significance of CRYAB in gastric cancer (GC) angiogenesis remains to be elucidated. In this study, we evaluated the expression of CRYAB and CD34 in GC tissues and corresponding normal gastric specimens to explore whether high level CRYAB is related with the angiogenesis and the poor prognosis in GC.

In this study, the expression of CRYAB and CD34 were detected in GC tissues and corresponding normal gastric tissues by immunohistochemical (IHC) technique. Furthermore, the relationship of CRYAB with CD34-evaluated microvessel density (MVD) and poor prognosis was also investigated.

CRYAB expression level was significantly higher in GC tissue than in normal gastric mucosa tissue, and clearly mean higher MVD was observed in tumor tissues compared with non-cancerous tissues. Besides, higher MVD value was observed in positive CRYAB expression group than in negative CRYAB expression group. Statistical analysis showed that CRYAB and MVD are associated with clinicopathological features including lymph node metastasis (LNM), tumor differentiation, invasion depth, and TNM stages. Kaplan-Meier method and multivariate survival analysis indicated that high expression of CRYAB, MVD, invasion depth, TNM stages, and tumor differentiation, as well as LNM significantly correlate with poor prognosis of GC patients.

High expression of CRYAB may contribute to angiogenesis, invasion and metastasis of GC. These results indicated that CRYAB was expected to be a promising molecular marker for poor prognosis and potential therapeutic target in patients with GC.

**Abbreviations:** CRYAB = alpha-B crystallin, GC = gastric cancer, LNM = lymph node metastasis, MVD = microvessel density, OS = overall survival, TNM = tumor node metastasis.

**Keywords:** angiogenesis, CRYAB, gastric cancer, MVD, prognosis

## 1. Introduction

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related mortality worldwide.<sup>[1]</sup> China, as a GC endemic region, it is estimated that approximately 40% of patients progress to advanced or metastatic GC,<sup>[2,3]</sup> as a result presenting with poor prognosis. Over the last 2 decades, despite the progress in the numerous therapeutic modalities in GC treatment, recurrence and metastasis remain the 2 primary challenging faced with these patients. The 5-year survival rate was 90% for post-resection early-stage GC patients, while it was

only 10% for advanced-stage patients.<sup>[4]</sup> Therefore, it is necessary to find novel and specific biomarkers to improve overall survival (OS) for GC patients with poor prognosis, which can facilitate early detection and predict early recurrence.

CRYAB, a principal member of the small molecule heat shock protein family,<sup>[5]</sup> was first discovered as a major structural protein in the lens of the eye.<sup>[6]</sup> It is widely accepted that CRYAB functions primarily as a molecular chaperone to promote cell survival.<sup>[7]</sup> Apart from being a molecular chaperone, CRYAB is suggested to play a crucial role in apoptosis inhibition.<sup>[8]</sup> The recent studies that CRYAB might play an important role in tumorigenesis and progression has drawn great attention. Aberrantly overexpressed CRYAB has been reported to be significantly associated with the poor prognosis of these tumors, such as breast carcinoma,<sup>[9]</sup> head and neck cancer,<sup>[10]</sup> and colorectal cancer.<sup>[11]</sup>

Microvessel density (MVD) is considered to be a valuable parameter for evaluating tumor angiogenesis and is significantly associated with tumor metastasis, recurrence and prognosis.<sup>[12–14]</sup> CD34 is a commonly used marker for tumor MVD,<sup>[15]</sup> and its high expression can predict risk of tumor progression. As a result, we speculate that CRYAB expression may correlate with MVD in GC.

## 2. Methods

### 2.1. Patients and tissue samples

Formalin-fixed and paraffin-embedded tumor samples from 100 GC cases and corresponding matched normal tissue specimens were collected in GC patients who received surgical gastric

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**Table 1****Patients characteristics.**

Patients characteristics	Frequency (n)	Percentage (%)
Ages		
<60 yr	28	28.0
≥ 60 yr	72	72.0
Gender		
Male	72	72.0
Female	28	28.0
Size (cm)		
<5	49	49.0
≥5	51	51.0
Grade		
Well	11	11.0
Moderate	49	49.0
Poor	40	40.0
LNM stage		
N0	32	32.0
N1 + N2 + N3	68	68.0
TNM stage		
I + II	47	47.0
III + IV	53	53.0
T stage		
T1 + T2	21	21.0
T3 + T4	79	79.0

CRYAB = alpha-B crystallin, GC = gastric cancer, LNM = lymph node metastasis, MVD = microvessel density, OS = overall survival, TNM = tumor node metastasis.

resection at the First Affiliated Hospital of Bengbu Medical College from January 2012 to December 2013. All patients have not been treated systematically, such as chemotherapy, radiotherapy, and immunotherapy prior to operation. Diagnosis with GC accompanied by other organ tumors, cancer of unknown primary origin, and history of previous cancer treatment were excluded from this study. All patients were provided written informed consent with complete clinical data and follow-up until December 2018. The clinicopathological data of patients are listed in Table 1. This study was approved by Ethics Committee of Bengbu Medical College and conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

## 2.2. Immunohistochemistry

Immunohistochemistry was conducted according to the guideline of Elivision Plus detection kit instructions (Lab Vision). Paraffin-embedded tissues were serially sectioned at 4 μm thick. All paraffin sections were dewaxed in xylene and dehydrated in a graded series of alcohol. Then it was incubated at 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes at room temperature to eliminate the activity of endogenous peroxidase. They were then placed in citrate buffer (pH 6.0) for antigen repair. After several washes with phosphate buffered saline (PBS, PH7.2), subsequently we blocked all slices with 10% goat blood serum for the prevention of non-specific binding with the time of 20 minutes at room temperature. Then we incubated with a rabbit anti-alpha-B crystallin monoclonal antibody (Abcam, USA) at 1:50 dilution and mouse monoclonal antibody CD34 (LabVision) for 1 hour at 37°C.

## 2.3. Evaluation of staining

All immunohistochemical stained tissue slides were carried out blindly by 2 experienced pathologists. The expression of CRYAB presented with appearance of brownish yellow particles in the

cytoplasm were evaluated by the proportion of positively stained cells, with a division into four grades: 0 points (<5%), 1 point (6%–25%), 2 points (26%–50% positive), and 3 points (>50%). Additionally, expression level was also scored based on staining intensity (0 point = negative; 1 point = weak intensity; 2 points = moderate intensity; 3 points = strong intensity). Grades for the samples were obtained by adding two scores. The sum scores with ≥3 points were regarded as positive, while the sum scores with <3 points were considered as negative.

CD34 expression in the cytoplasm and membrane of vascular epithelial cells was selected for the MVD counting. Observers selected 5 representative fields and counted individual microvessel on a 400 field after the area of highest was identified, and then take the average value as the MVD value. As a blood vessel, any brown-stained endothelial cell or endothelial cell layer in the tumor is counted as a blood vessel as long as the structure is not connected. Where the lumen is larger than the red blood cell size, the blood vessels with the muscular layer are not counted.

## 2.4. Statistical methods

All data were analyzed using the SPSS 25.0 (IBM, City of New York) statistical analysis software. Relationships among clinicopathological parameters, CRYAB and MVD status were analyzed by Chi-square test. The role CRYAB plays in survival was assessed by Kaplan–Meier method. Independent prognostic factors were determined by the Cox regression model for multivariate analysis. The results were considered statistically significant when  $P < .05$ .

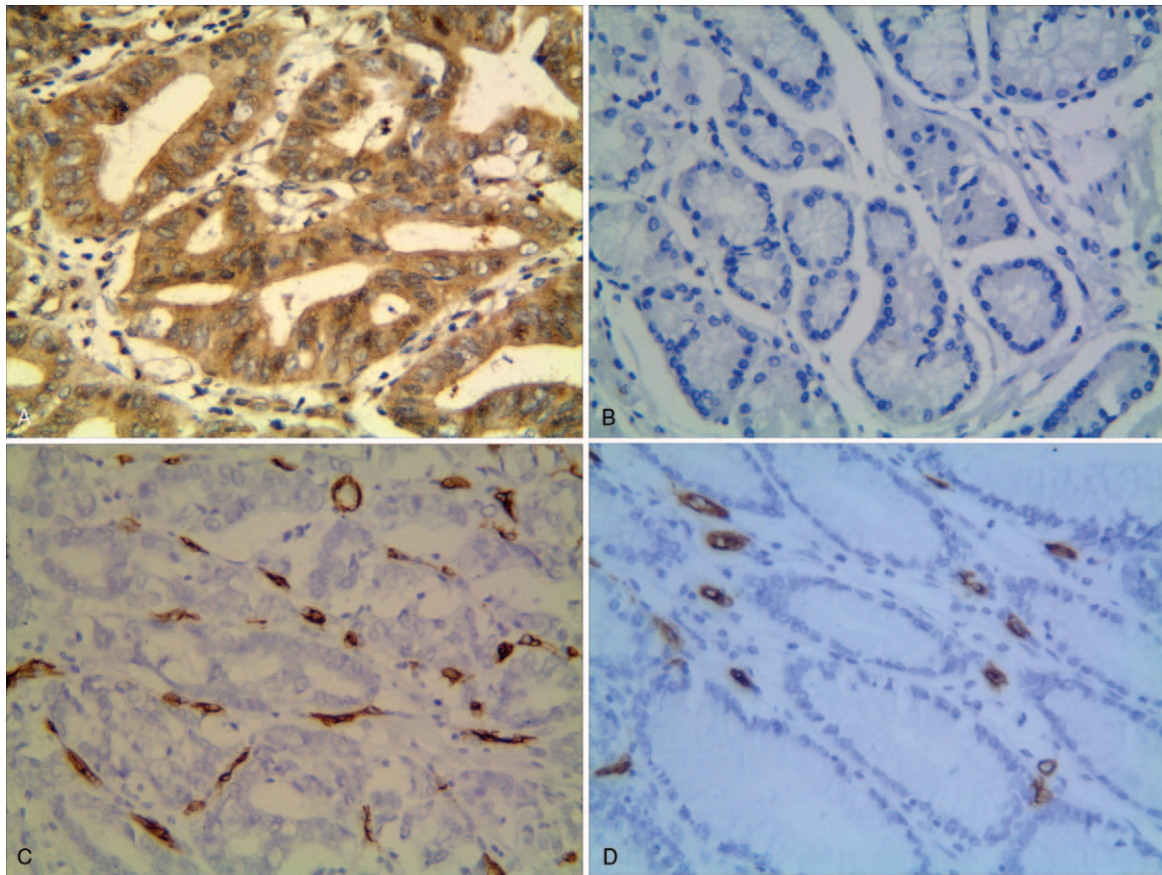
## 3. Results

### 3.1. CRYAB is overexpressed in human GC tissues and is closely correlated with clinicopathological characteristics as well as MVD

The positive staining of CRYAB protein was mainly located in the cytoplasm of cancer cells, and the staining color was brownish yellow or tan. Significantly higher level of CRYAB protein was observed in tumor tissues (57/100, 57.0%) than that in corresponding normal tissues (28/100, 28.0%) by IHC staining (Fig. 1A, B). The positive expression rate of CRYAB protein in patients with lymph node metastasis (LNM) (54/68, 79.4%) was higher than that without metastasis (3/32, 9.4%), and the difference between 2 groups was also statistically significant ( $P < .001$ ). Furthermore, the clinicopathological association analysis demonstrated that CRYAB expression in tumor tissues significantly correlated with tumor differentiation, T stage, pTNM stage ( $P < .05$ ; Table 2). However, there was no statistically significant difference among the expression of CRYAB protein and the age, patient gender, tumor size ( $P > .05$ ; Table 2). In addition, the average MVD count in the GC group and the normal group was  $25.14 \pm 8.67$ ,  $17.23 \pm 4.99$ , respectively (Fig. 1C,D). In the CRYAB positive expression group ( $29.9 \pm 6.4$ ) the MVD was higher than the CRYAB negative expression group ( $18.9 \pm 7.2$ ;  $P < .001$ ), and the association between MVD and clinicopathological parameters was listed in Table 2.

### 3.2. Univariate and multivariate analysis

The survival time of the CRYAB negative expression group ( $50.5 \pm 2.2$ ) was significantly higher than that of the positive expression



**Figure 1.** Immunostaining of CRYAB, and CD34 in GC or the control tissue. (A) Positive staining of CRYAB in GC tissue (400 × magnification); (B) Negative staining of CRYAB in normal tissue adjacent to GC (400 × magnification); (C) Expression of CD34 in GC tissue (400 × magnification); (D) Expression of CD34 in normal tissue adjacent to GC (400 × magnification). CRYAB=alpha-B crystallin, GC=gastric cancer.

**Table 2**

The correlation between CRYAB, or MVD and clinicopathological characteristic-s in GC.

Variable	CRYAB		P	MVD (/LP)	P
	Negative	Positive			
Ages			.359		.572
<60y	10	18		26.0 ± 9.2	
≥60y	33	39		24.8 ± 8.5	
Gender			.359		.248
Male	33	39		24.5 ± 8.3	
Female	10	18		26.8 ± 9.5	
Size (cm)			.707		.381
<5.0	22	27		26.0 ± 8.5	
≥5.0	21	30		24.4 ± 8.8	
Grade			.038		.001
Well	6	5		18.6 ± 10.0	
Moderate	26	23		23.4 ± 7.4	
Poor	11	29		28.6 ± 8.4	
T stage			.003		<.001
T1 + T2	15	6		15.3 ± 5.6	
T3 + T4	28	51		27.8 ± 7.3	
TNM stage			<.001		<.001
I + II	34	13		17.9 ± 5.0	
III + IV	9	44		31.6 ± 5.5	
LNM stage			<.001		<.001
N0	29	3		16.8 ± 5.7	
N1 + N2 + N3	14	54		29.1 ± 6.8	

CRYAB=alpha-B crystallin, GC=gastric cancer, LNM=lymph node metastasis, MVD=microvessel density, OS=overall survival, TNM=tumor node metastasis.



**Table 3****Results of univariate analyses of overall survival (OS) time.**

Variable	n	Mean OS (months)	Log-rank	P value
CRYAB			59.421	<.001
Negative	43	50.5±2.2		
Positive	57	26.0±1.6		
MVD			64.605	<.001
<25	48	49.4±2.1		
≥25	52	24.5±1.5		
Ages			0.097	.755
<60	28	34.6±4.0		
≥60	72	37.6±2.0		
Gender			1.074	.300
Male	73	38.0±2.1		
Female	27	33.2±3.7		
Size			2.198	.138
<5	49	34.8±2.4		
≥5	51	38.4±2.7		
Grade			25.916	<.001
Well	11	53.3±6.3		
Moderate	49	39.7±2.3		
Poor	40	28.3±2.4		
LNM stage			52.077	<.001
N0	32	54.2±2.2		
N1 + N2 + N3	68	28.1±1.6		
TNM stage			79.200	<.001
I + II	47	50.9±1.8		
III + IV	53	23.2±1.3		
T stage			22.126	<.001
T1 + T2	21	55.1±3.1		
T3 + T4	79	31.3±1.7		

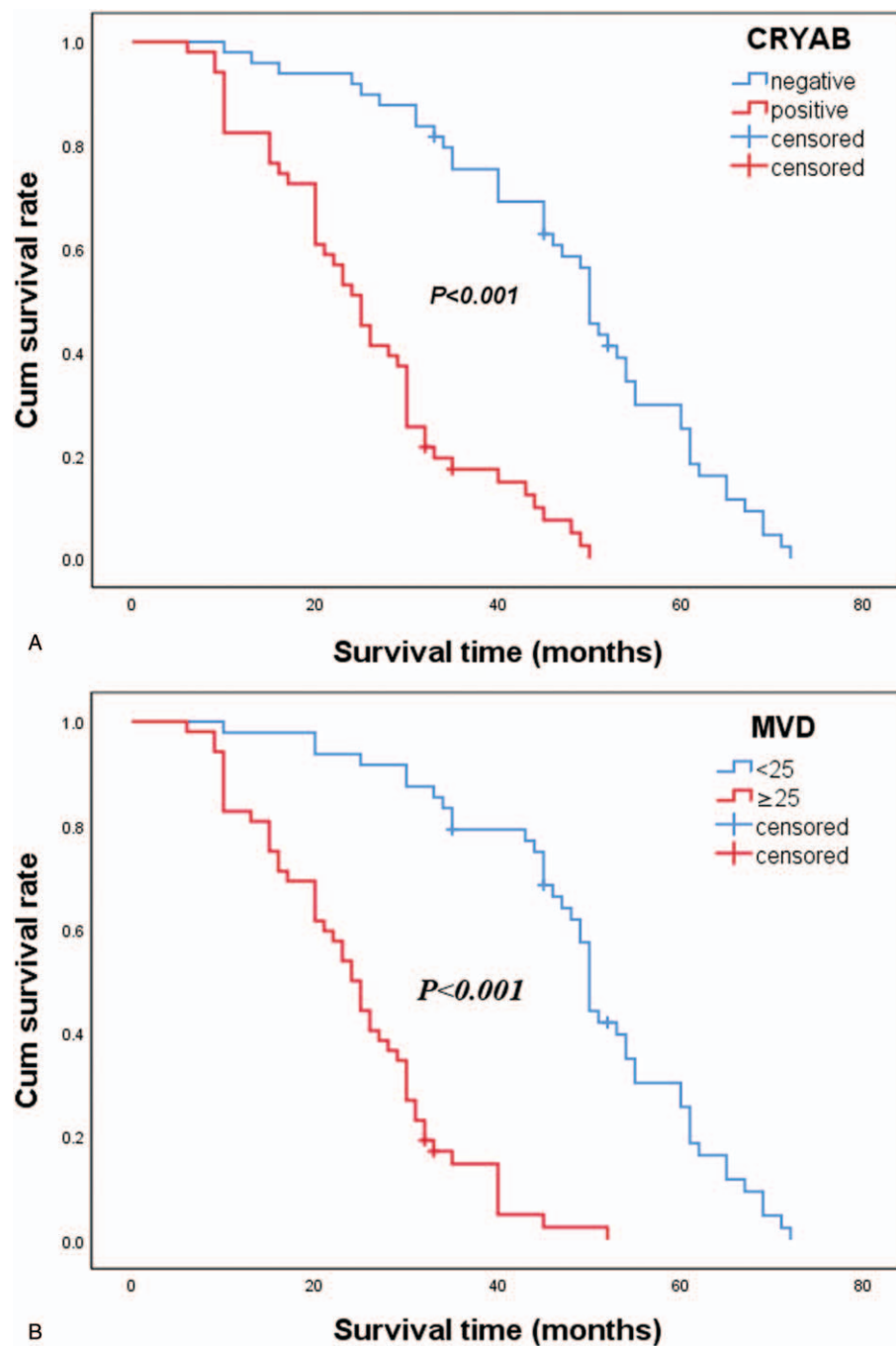
CRYAB = alpha-B crystallin, GC = gastric cancer, LNM = lymph node metastasis, MVD = microvessel density, OS = overall survival, TNM = tumor node metastasis.

group ( $26.0 \pm 1.6$ ,  $P < .001$ , Table 3, Fig. 2A) and the survival time of the MVD  $\geq 25$  group ( $24.5 \pm 1.5$ ) was significantly lower than that of the MVD  $< 25$  group ( $49.4 \pm 2.1$ ,  $P < .001$ , Table 3, Fig. 2B). Multivariate analysis showed that LNM, pTNM stage, invasion depth, tumor differentiation, CRYAB expression, and MVD count were independent predictors of GC patients ( $P < .05$ , Table 4).

#### 4. Discussion

CRYAB, a principal member of the small molecule heat shock protein family,<sup>[5]</sup> was first discovered as a major structural protein in the lens of the eye. It is widely accepted that CRYAB performs primarily as a molecular chaperone, preventing other proteins from stress-induced damage including heat shock, radiation, oxidative stress by selectively binding.<sup>[16]</sup> In recent years, which high expression level of CRYAB were detected in various cancers and significantly associated with the unfavorable prognosis arose much attention. For example, alpha-B crystallin (CRYAB), a validated prognostic factor for poor prognosis in squamous cell carcinoma of the oral cavity<sup>[5]</sup> and CRYAB is a novel oncoprotein that predicts poor clinical outcome in breast cancer.<sup>[16]</sup> It also correlates with poor prognosis in colorectal cancer and prompts epithelial-mesenchymal transition through ERK signaling.<sup>[11]</sup> In this study, coincidentally, we observed obviously up-regulated level of CRYAB in GC cancer samples compared with in that adjacent non-tumor tissues. However, we failed to determine whether CRYAB expression gradually increased from normal mucosa to atypical hyperplasia tissue and finally to cancer tissue, because we show few atypical

hyperplasia area (a kind of precancerous lesion). In addition, we also analyzed that high level of CRYAB significantly related with tumor differentiation, LNM, pTNM stages and T stage, and found that CRYAB expression in the areas of GC where MVD is very high was uniformly higher than that in normal tissues, presented with positive correlation between them. Whether high levels of CRYAB affect the tumor angiogenesis is unknown and our present findings provide evidence proving the crucial role of CRYAB in increasing the angiogenic potential of GC, thus promoting invasion and metastasis. Consistently, a growing number of studies suggested that high CRYAB expression level in tumor tissues have been closely associated with invasion and metastasis. Chen et al demonstrated that NF- $\kappa$ B signaling pathway as a mediator involved in CRYAB-induced EMT that related to invasion and metastasis in GC.<sup>[17]</sup> Furthermore, recent studies show that CRYAB can regulate tumor angiogenesis, invasion, and metastasis via vascular endothelial growth factor (VEGF)<sup>[18]</sup> Additional, CRYAB is suggested to play a crucial role in apoptosis inhibition by suppressing the autocatalytic maturation of caspase-3,<sup>[19]</sup> interacting with the proapoptotic Bcl-2 family proteins, such as Bax and Bcl-xs to prevent the mitochondrial translocation.<sup>[20]</sup> Resistance to apoptosis has been defined as one of the hallmarks of malignant disease.<sup>[21]</sup> One of novel discoveries was that CRYAB regulated tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis as well as cisplatin induced apoptosis in human ovarian cancer cells, decreasing the sensitivity of a tumor to cancer treatments.<sup>[22]</sup> Besides, CRYAB is a key downstream effector that mediates the effects of miR-491 on osteosarcoma lung metastasis and chemo-resistance.<sup>[23]</sup> On the basis of its potential to predict



**Figure 2.** Kaplan-Meier analysis of the survival rate of patients with gastric cancer. (A) Overall survival of all patients in relation to alpha-B crystallin expression (log-rank=59.421,  $P < .001$ ); (B) Overall survival of all patients in relation to microvessel density (log-rank=64.605,  $P < .001$ ).

**Table 4**

Results of multivariate analyses of overall survival (OS) time.

Variable	B	SE	P	RR	95.0% CI	
CRYAB	0.907	0.369	.014	2.478	1.202	5.107
MVD	1.369	0.437	.002	3.932	1.669	9.263
TNM	1.178	0.545	.031	3.249	1.116	9.462
LNm	1.160	0.401	.004	3.191	1.454	7.002
T stage	1.112	0.452	.014	3.039	1.253	7.371
Grade	0.597	0.190	.002	1.817	1.251	2.637

CRYAB=alpha-B crystallin, GC=gastric cancer, LNm=lymph node metastasis, MVD=microvessel density, OS=overall survival, TNM=tumor node metastasis.

chemotherapy and radiotherapy responses, CRYAB might be a promising target for anti-cancer treatment, which might facilitate the development of precise treatment selection and improve the outcome for GC patients.

## 5. Conclusions

High expression CRYAB was correlated with MVD-evaluated angiogenesis and poor prognosis in human GC. We argue that CRYAB would be a promising target and an independent predictor for poor prognosis of GC patients.

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**Project administration:** Jing Xu.

**Resources:** Lili Cheng.

**Software:** Yijie Li.

**Validation:** Yisheng Tao.

**Writing – original draft:** Xiaoying Tao.

**Writing – review & editing:** Yisheng Tao.

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