RESEARCH ARTICLE

Overexpression of Twist and Matrix Metalloproteinase-9 with Metastasis and Prognosis in Gastric Cancer

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Abstract

Objective: Twist, a basic helix-loop-helix transcription factor, plays a key role in the metastatic progression of human cancer. Matrix metalloproteinase (MMP)-9 is an endopeptidase that digests basement membrane type IV collagen, therefore being possibly related to tumor progression. It has been reported that Twist and matrix metalloproteinase-9 (MMP-9) are expressed in gastric cancers. However, the exact roles of Twist and MMP-9 in tumor metastasis and prognosis remain unclear. The aim of this study was to casts light on this question. Methods:Twist and MMP-9 expression in tissue sections of 37 gastric carcinomas was evaluated with immunohistochemistry. The staining results were compared with clinicopatholgic features and to patients'outcome. Results: Twist positive expression was significantly increased in gastric cancer cases with lymph node metastasis (*P*=0.023). But no correlations were found between MMP-9 overexpression and clinicopathologic features, such as recurrence, TNM stage, and lymph node metastasis. Overall survival (OS) was significantly correlated with recurrence, serosa invasion, TNM stages, distant metastasis, and MMP-9 (*P*=0.027, 0.021, 0.000, 0.024 and 0.036, respectively). Disease-free survival (DFS) was prominently related to recurrence location, serosa invasion and TNM stages (*P*=0.000, 0.038 and 0.003, respectively). In the Cox regression multivariate analysis, TNM stage, distant metastasis and MMP-9 were significantly associated with prognosis of gastric cancer (*P*=0.002, 0.019, and 0.032, respectively). Conclusions: This study showed Twist positive expression to be significantly correlated with lymph node metastasis in gastric cancer. MMP-9 overexpression is associated with OS, suggesting that MMP-9 is a prognostic indicator for survival in patients with gastric cancer.

Keywords: Gastric cancer - twist - matrix metalloproteinase-9 - prognosis - survival

Asian Pac J Cancer Prev, **14 (9)**, 5055-5060

Introduction

Gastric cancer is the second most lethal cancer with approximately 800,000 deaths each year in the world (Jemal et al., 2011). The natural feature of gastric cancer is invasion and metastasis, which is the major cause of death (Weigelt et al., 2005; Mehlen et al., 2006). Although there are lots of studies focused on the cancer invasion and metastasis, the exact mechanism remains unclear. Tumor metastasis is a complex multistep process, including loss of cellular adhesion, increased motility and invasiveness, entry and survival in the circulation, exit into new tissue, and eventual colonization of a distant site (Fidler, 2003; Gupta et al., 2006; Steeg, 2006).

Epithelial-mesenchymal transition (EMT) is one of the critical events in such multistep process, a developmental process characterized by loss of epithelial markers, gain of mesenchymal markers and changes in cellular morphology and phenotype (Hugo et al., 2007; Yang et al., 2008). In this process, the adhesion structures between epithelial cells gradually disappear and cell-cell adhesion decreases; in addition, changes occur in cell polarity and the cytoskeleton (Lee et al., 2006; Thiery et al., 2009). Previous studies have found that EMT played a critical role in cancer progression, through increased ability to migrate and invade of cancer cells. In the process of tumor invasion and metastasis, cancer cells obtain the phenotype and invasive characteristics of mesenchymal cells through EMT to achieve the infiltration of surrounding tissue. Through EMT, tumor cells are seeded in a location, and finally form metastatic cancer tissues whose morphology is similar to that of the primary tumor (Thiery, 2002; Thiery et al., 2006). Recent researches indicated that the abnormal EMT is closely associated with epithelial tumors, such as breast cancer (Zhang et al., 2012), lung cancer (Nagathihalli et al., 2012), colon carcinoma (Bellovin et al., 2005), prostate cancer (Shiota et al., 2012), cervical cancer (Lei et al., 2012), and gastric cancer (Jia et al., 2013).

Twist, a basic helix-loop-helix transcription factor originally identified as being required for mesoderm induction in Drosophila (Thisse et al., 1987), plays an essential role in the metastasis of cancer. Twist overexpression results in loss of cell-cell adhesion,

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activation of mesenchymal markers, and induction of cell motility in the process of tumor metastasis, suggesting that Twist contributes to metastasis by promoting EMT (Kang et al., 2004; Yang et al., 2004).

The matrix metalloproteinases (MMPs) are a large group of secreted proteinases that require zinc for catalytic activity. MMP-9 is a member of this gene family (Nagase et al., 1999; Stamenkovic, 2000). MMP-9 predominantly degrades gelatin and type IV, V XI and XVI collagen, the major structural component of basement membrane, which appears to be very crucial in tumor cell invasion and metastasis (Egeblad et al, 2002). MMPs not only induced EMT with elevated levels of MMPs in the tumor microenvironment, but also cancer cells that undergo EMT can produce more MMPs, facilitating cell invasion and metastasis (Radisky et al., 2010; Ke et al.,2013). MMP-9 contributes to tumor metastasis via sonic hedgehog signaling pathway in gastric cancer (Yoo et al., 2011). Twist1 overexpression is able to increase the invasion ability of hepatocellular carcinoma cells and promote MMPs activation, specifically MMP-2 and MMP-9 in hepatocellular carcinoma metastasis (Zhao et al., 2011).

Here, we evaluated the expressions of Twist and MMP-9 immunoreactive protein in paraffin-embedded samples of patients with gastric cancer. The roles of Twist and MMP-9 proteins in metastasis and prognosis of gastric cancer were studied by correlating Twist and MMP-9 expressions to clinicopathologic features of the disease and prognosis of patients with gastric cancer.

Materials and Methods

Patients and Tumor Tissues

Tumor specimens were obtained from 37 patients with gastric cancer at the Department of Oncology, Zhongnan Hospital of Wuhan University (Wuhan, China) from January 2004 to January 2008. The mean age of the patients was 60 years (rang: 24 - 79 years) and the male/female ratio was 1.47. All patients were staged according to TNM classification system of American Joint Committee on Cancer (AJCC) staging criteria (version 6). The patients underwent curative gastrectomy with D2 lymph nodes dissection for stages I to III cases and palliative surgery for some stage IV cases. All patients beyond stage II received platinum and 5-flurouracil (5-FU) based adjuvant chemotherapy beginning 21 days after surgery. In the clinical follow-up, the patients were seen every 3-6 months to assess tumor control and survival. The last follow-up was on December 1, 2009. Major clinicopathological characteristics of these patients were listed in Table1. Written informed consent was obtained from the patients and the study protocol was approved by the ethics committee of Zhongnan Hospital of Wuhan University.

Immunohistochemical Staining

Immunohistochemical staining was used to detect the expression of Twist and MMP-9. Briefly, tissue slides were first deparaffinized in xylene, ethanol and water, then the slides were pretreated in 0.01 M citrate buffer (pH 6.0) for Twist and MMP-9, and heated in a microwave oven for 8 min (medium heat) and 7 min (medium-low heat). For staining, endogenous peroxidase activity was blocked by immersing the slides with 3% hydrogen peroxide for 10 min to prevent any nonspecific binding. After blocked with 2% BSA, the slides were incubated overnight at 4°C with the primary antibodies for Twist (ab50581, Abcam, England, dilution 1/300) and MMP-9 (sc13595, Santa Cruz, USA, dilution 1/300). Then the slides were incubated with corresponding HRP-labled secondary antibody for 50 min at 37°C. The slides were washed thoroughly with Tris-buffered saline between all stages of the procedure. The antibody reaction products were visualized with diaminobenzidine (DAKO, Denmark). The slides were counterstained with hematoxylin, dehydrated, and mounted in Permount TM Mounting Medium. For the negative controls, the primary antibody was replaced with Tris-buffered saline on slides that were proven to be positive for Twist and MMP-9 in preliminary experiments.

Evaluation of Immunohistochemical Variables

The slides were analyzed separately by two independent investigators blinded to clinical data. Positive cells were stained brownish granules. The staining score in gastric cancer cells in each slide was assessed according to the staining intensity and the percentage of the positive cells. The staining intensity was scored as 0 (negative), 1 (very weak), 2 (weak), 3 (medium) and 4 (strong). The extent of staining was scored as 0 (0-10%), 1 (10%-30%), 2 (30%-50%), 3 (50%-75%) and 4 (>75%) according to the percentage of positive-staining cells in relation to the total cancer cells. The expressions of Twist and MMP-9 in each slide were scored as the sum of intensity and extent of positive-staining cells. The slide with a final staining score of more than 3 was defined as positive expression.

Statistical Analysis

All statistical analyses were performed using the SPSS software system (SPSS Inc, version 13.0, Chicago, IL). Fisher exact tests were used to analyze the correlation of immunohistochemical variables with clinicopathological characteristics. Cumulative survival was calculated by the Kaplan-Meier method and analyzed by the Log-rank test. The Cox proportional hazards model was applied for multivariate analysis. Two-tailed $P < 0.05$ was judged to be significant.

Results

Immunohistochemical characteristics

Immunohistochemical analysis showed that in positive cases, the expression of Twist was prominent in tumor tissue and seemed mostly to localize in the cell cytoplasm as a diffuse staining (Figure 1 A). MMP-9 high expression mainly appeared in tumor tissue (Figure 1 C). The negative expression of these proteins was investigated in these tissuses (Figure 1 B, D).

Correlation of Immunohistochemical Variables with clinicopathologic features

Sex, age, pathological types, recurrence status, TNM

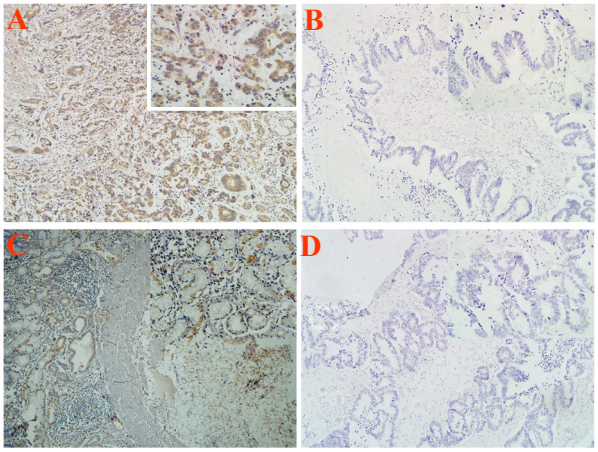
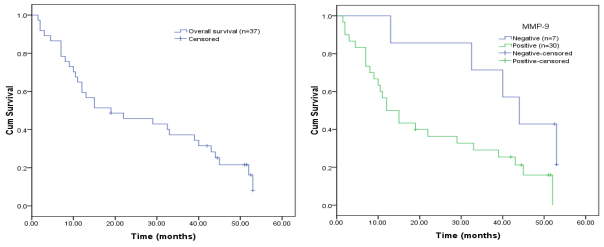


Figure 1. Positive Staining of Twist and MMP-9. A. Twist positive expression was prominent in tumor tissue and seemed mostly to localize in the cell cytoplasm as a diffuse staining. B. Tumor tissue with negative immunostaining for Twist. C. MMP-9 was mainly expressed in tumor tissues. D. Tumor tissue with negative immunostaining for MMP-9. Magnifications: A, B, C, D: 100×; Inserts in higher right corner show the sub-cellular localization of immunostaining at higher magnification (400×)



A B

Figure 2. Kaplan-Meier Analysis of Overall Survival (OS). The median OS for 37 patients overall and was 19.0 months (A). The OS was better in GC patients with MMP-9 negative expression (B, upper curve) than those with MMP-9 positive expression (B, lower curve) ($P = 0.036$, Log-rank test)

Table 1. Clinicopathological Characteristics in Relation to Twist and MMP-9

Variables	N	Twist Positive (%)	P*	MMP-9 Positive (%)	P*
Sex					
Male	21	13(61.9)	0.519	16(76.2)	0.439
Female	16	8(50.0)		14(87.5)	
Age(yr)					
≤58	18	10(55.6)	1.000	13(72.2)	0.232
>58	19	11(57.9)		17(89.5)	
Pathological types					
Adeno	25	16(64.0)	0.291	22(88.0)	0.183
Nonadeno	12	5(41.7)		8(66.7)	
Recurrence					
Yes	24	13(54.2)	0.739	20(83.3)	0.678
No	13	8(61.5)		10(76.9)	
Recurrence location					
Ovarian	7	4(57.1)	0.648	6(85.7)	0.600
Local	11	7(63.6)		8(72.7)	
Distant	6	2(33.3)		6(100.0)	
No	13	8(61.5)		10(76.9)	
Serosa invasion					
Yes	29	14(48.3)	0.104	23(79.3)	0.677
No	8	7(87.5)		7(87.5)	
Lymph node metastasis					
Yes	27	12(44.4)	**0.023**	22(81.5)	1.000
No	10	9(90.0)		8(80.0)	
Distant metastasis					
M0	29	16(55.2)	1.000	23(79.3)	0.677
M1	8	5(62.5)		7(87.5)	
TNM Stage					
I	5	4(80.0)	0.565	4(80.0)	0.746
II	6	4(66.7)		5(83.3)	
III	14	6(42.9)		10(71.4)	
IV	12	7(58.3)		11(91.7)	

*Fisher's exact test (two-tailed), bold face representing significant data ($P<0.05$); Adeno, Adenocarcinoma; Nonadeno, Nonadenocarcinoma

stage, recurrence location, serosa invasion, lymph node status and distant metastasis were the variables investigated in the study. Twist positive expression was significantly increased in cases with lymph node metastasis (P=0.023; Table 1). However, there were no associations between Twist and sex, age, pathological types, recurrence, TNM stage, recurrent location, serosa invasion, distant metastasis. No correlations were found between MMP-9 and sex, age, pathological types, recurrence, TNM stage, recurrence location, serosa invasion, lymph node metastasis, distant metastasis (Table 1).

Analysis of factors related to overall survival (OS) and disease-free survival (DFS)

At the time of last follow-up, 30 patients died, 6 survived free of disease and 1 survived with disease. The median OS and median DFS were 19.0 (range: 1.5-53.0) months and 15.0 (range: 1.0-53.0) months, respectively. In terms of traditional clinicopathological features, OS was significantly correlated with recurrence, serosa invasion, TNM stages, and distant metastasis (P=0.027, 0.021, 0.000 and 0.024, respectively); DFS was statistically related to recurrence area, serosa invasion and TNM stages (P=0.000, 0.038 and 0.003, respectively).

With regard to key molecular features in the study, the OS was longer in Twist positive group (29.0 months) than Twist negative group (15.0 months) (P=0.739) and in MMP-9 negative group (44.0 months) than MMP-9 positive group (13.5 months) (P=0.036, Table 2), although the differences in Twist expression did not reach statistical significance. In terms of DFS, there were no correlations between DFS and expression levels of Twist and MMP-9 in this study.

The Cox regression multivariate analysis was performed to evaluate whether the correlation with MMP-9 and survival depended on the four other major factors associated with prognosis: recurrence, serosa invasion, TNM stage, and distant metastasis. The analysis showed that TNM stage was the most significant prognostic factor for survival in gastric cancer. The patients with a stage IV disease had a 59.4-fold relative risk for death compared with the patients with a stage I disease (P=0.002, 95% confidence interval 4.314-817.743). Patients with distant metastasis had a 6.1-fold relative risk for death compared with the patients with no distant metastasis (P=0.019, 95% confidence interval 1.353-27.585). In the same analysis, patients with an extensively positive staining for MMP-9 had a 3.9-fold relative risk for death when compared

Table 2. The analyses of Factors Regarding OS and DFS

Variables	OS (months)			DFS (months)		
	N	Median (Range)	*P**	N	Median (Range)	*P**
Clinico-pathological data						
Pathological types						
Adeno	25	19.0(1.5-52.5)	0.860	20	14.0(1.0-52.5)	0.796
Nonadeno	12	22.0(3.0-53.0)		9	25.0(2.0-53.0)	
Recurrence						
Yes	24	14.0(1.5-53.0)	0.027			
No	13	32.5(7.0-53.0)				
Recurrence location						
Ovarian	7	13.0(2.0-52.0)	0.137	2	24.0(2.0-46.0)	0.000
Local	11	12.0(1.5-53.0)		9	8.0(1.0-49.0)	
Distant	6	12.0(1.5-53.0)		5	13.0(3.0-32.0)	
No	13	32.5(7.0-53.0)		13	25.0(4.0-53.0)	
Serosa invasion						
Yes	29	13.0(1.5-53.0)	0.024	22	9.3(1.0-53.0)	0.038
No	8	44.0(2.0-52.5)		7	45.0(13.0-52.5)	
Lymph node metastasis						
Yes	27	12.5(2.0-33.0)	0.213	20	11.3(1.0-53.0)	0.681
No	10	22.0(1.5-53.0)		9	38.0(6.0-52.5)	
Distant metastasis						
M0	29	22.0(1.5-53.0)	0.021			
M1	8	12.5(2.0-33.0)				
TNM stage						
I	5	51.0(42.0-52.0)	0.000	5	46.0(42.0-52.5)	0.003
II	6	41.0(11.0-51.5)		6	26.5(8.0-51.5)	
III	14	17.0(4.5-53.0)		14	11.3(3.0-53.0)	
IV	12	7.5(1.5-33.0)		4	4.0(2.0-6.0)	
Immunohistochemistry						
Twist						
Positive	21	29.0(2.0-52.5)	0.739	16	22.0(4.0-52.5)	0.512
Negative	16	15.0(1.5-53.0)		13	13.0(1.0-53.0)	
MMP-9						
Positive	30	13.5(1.5-52.0)	0.036	23	9.5(1.0-51.5)	0.171
Negative	7	44.0(13.0-53.0)		6	43.5(25.0-53.0)	

Adeno, Adenocarcinoma; Nonadeno, Nonadenocarcinoma; OS, overall survival; DFS, disease-free survivala

Table 3. The Cox Regression Multivariate Analysis

Covariates	B	SE	Relative risk	*P*	95%CI
Recurrence	0.450	0.473	1.568	0.341	0.621-3.960
Serosa invasion	0.064	0.801	1.066	0.936	0.222-5.127
TNM stage					
I	0				
II	1.186	1.061	3.273	0.264	0.409-26.187
III	1.799	1.119	6.044	0.108	0.674-54.222
IV	4.084	1.338	59.396	0.002	4.314-817.743
Distant metastasis	1.810	0.769	6.109	0.019	1.353-27.585
MMP9	1.363	0.635	3.907	0.032	1.125-13.572

with patients with a MMP9-negative immunostaining (*P*=0.032, 95% confidence interval 1.125-13.572; Table 3). TNM stage, distant metastasis and MMP-9 retained their roles as independent markers predicting shortened survival, but recurrence and serosa invasion did not have prognostic roles in this study.

Discussion

As there are tens of thousands patients died of gastric cancer every year all over the world, the research of gastric cancer deaths is critical nowadays. A great many studies found that the invasion and metastasis is essential in the process of gastric cancer (Mehlen et al., 2006). In the process of tumor metastasis, cancer cells lose their polarity and intercellular adhesions and acquire the phenotype and invasive characteristics of mesenchymal cells through EMT to achieve the infiltration of surrounding tissue (Sanchez-Tillo et al., 2012). MMPs can promote tumor metastasis through degradation proteins of the extracellular matrix and basement membrane (Egeblad et al., 2002). Therefore EMT is an important process for the invasiveness and metastasis of tumors, and the role of EMT in tumor metastasis is studied by many researchers recently (Christofori, 2006).

In this study, Twist and MMP-9's associations with clinical parameters have been extensively investigated. The result demonstrated that positive Twist expression was significantly correlated with lymph node metastasis. The result suggested that Twist overexpression is likely to play a role in the EMT process and progression, and metastasis of gastric cancer. In human breast cancer, increased Twist expression has been shown to correlate with metastasis (Martin et al., 2005). Our results showed no correlation between MMP-9 positive expression and clinicopathologic features such as sex, age, pathological types, recurrence, TNM stage, recurrence location, serosa invasion, lymph node metastasis or distant metastasis. These results were in line with those of Ruokolainen H et al., who did not see correlation between overexpression MMP-9 and clinicopathological factors in head and neck squamous cell carcinoma (Ruokolainen et al., 2004). However, Sampieri CL et al. found that MMP-9 mRNA is significantly enhanced in gastric cancer compared to normal mucosa (Sampieri et al., 2010). The positive rate of MMP9 expression in hepatocellular carcinoma with metastasis was higher than hepatocellular carcinoma without metastasis (Zhao et al., 2011). Zhang K et al. (2011) found that MMP9 positive expression was significantly associated with nodal TNM stage, metastases and nervous invasion in 60 cases of pancreatic cancer.

To assess the prognostic value of Twist and MMP-9 expressions in gastric cancer, we analyzed these proteins' expression in patients with gastric cancer using OS, DFS and Cox regression analysis. Our results demonstrated that recurrence, serosa invasion, TNM stages and distant metastasis were significantly correlated with OS and recurrence area, serosa invasion and TNM stages were closely related to DFS. However, no significant correlations were found between Twist expression and OS or DFS in this study. Up-regulation of Twist and its prognostic value have been described in several human cancers. In human breast cancer, increased Twist expression has been shown to correlate with metastasis development and shorter survival (Martin et al., 2005). Twist overexpression has also been suggested to associate with poor outcome and shorter patient survival in patients with melanoma (Hoek et al., 2004). A remarkable difference was found in OS between patient groups presenting with MMP-9 positive or negative tumors, but we did not find any correlation between DFS and expression levels of MMP-9 in this study. These results suggest that MMP-9 may be a strong indicator for prognosis in gastric cancer. MMP-9 upregulation has been associated with poor prognosis in various tumors (Sillanpaa et al., 2007; Wu et al., 2008; Lin et al., 2011).

In the Cox regression analysis, the most significant prognostic factor for survival in the present study was TNM stage of the disease. And distant metastasis could play a role as an independent marker predicting patients' survival. It was observed that MMP-9 positive expression also had prognostic value independent of recurrence, serosa invasion, TNM stage and distant metastasis. Therefore, the relative risk of dying is higher in the cases with MMP-9 positivity than in the cases with MMP-9 negativity. MMP-9, as a new prognostic marker in gastric cancer, could be to provide new treatment possibilities, especially in adjuvant treatment. For instance, synthetic MMP-9 inhibitors have been introduced as an option in gastric cancer treatment.

Invasion and metastasis of gastric cancer is a very complex process including many steps. In the process of invasion and metastasis, EMT can improve cancer cells mobility with loss of cell-cell adhesion and changes of cytokeratin structure. Twist, as a helix-loop-helix transcription factor and plays an essential role in the EMT. The new findings of Yang et al. have showed that Twist could play as the inducer in the EMT by binding DNA with similar E-box sequence motifs (Yang et al., 2004). Twist elevated levels results in loss of cell-cell adhesion, activation of mesenchymal markers, and induction of cell motility in the process of tumor metastasis, suggesting that Twist contributes to metastasis by promoting EMT (Kang et al., 2004). The positive expression of Twist has been observed in a variety of neoplasms, including hepatocellular carcinoma (Lee et al., 2006), breast cancer (Martin et al., 2005), lung cancer (Hui et al., 2013), bladder cancer (Yu et al., 2010), ovarian cancer (Yoshida et al., 2009) and gastric cancer (Yan-Qi et al., 2007). The process of cancer metastasis is undoubtedly a complex process, and the proteolysis of basement membranes and other extracellular matrix is thought to be very crucial, which could enhance the migration of tumor cells and penetration of tumor by blood vessels. MMPs are proteolytic enzymes which play important roles in tumor invasion and metastasis. MMPs have been associated with EMT in cancer progression through three distinct mechanisms: (a) elevated levels of MMPs in the tumor microenvironment can directly induce EMT in epithelial cells, (b) cancer cells that undergo EMT can produce more MMPs, facilitating cell invasion and metastasis, and (c) EMT can generate activated stromal-like cells that drive cancer progression via further MMP production (Radisky et al., 2010). MMPs can promote cancer cells invasion and metastasis by degrading proteins in the basement membrane and extracellular matrix. Previous studies have found that MMP-9 increased expression in metastasis of many tumors. In invasion and metastasis of gastric cancer, Twist and MMPs can promote cancer cells invasion and metastasis with enhancement of cell mobility and degradation proteins. Therefore we can understand invasion and metastasis of gastric cancer with investigation of Twist and MMP-9 expressions.

In summary, MMP-9 positive expression is a prognostic indicator for OS in patients with gastric cancer. Twist overexpression were significantly associated with lymph node metastasis in gastric cancer. These implied that we might observe Twist expression to learn clinical changes in patients with gastric cancer. Moreover we learn survival conditions according to MMP-9 expression in gastric cancer.

Acknowledgements

The authors thank members of their laboratory and their collaborators for their research work. This work was supported by Fund Program of Health Department of Hubei Province (QJX2012-14), and National Natural Science Foundation of China (grant No. 30901387). The author(s) declare that they have no competing interests.

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