miR-21, miR-17 and miR-19a induced by phosphatase of regenerating liver-3 promote the proliferation and metastasis of colon cancer

BACKGROUND: Phosphatase of regenerating liver-3 (PRL-3) is an oncogene known to promote tumour metastasis, especially in colorectal cancer (CRC). Here, we demonstrate that the miR-21, miR-17 and miR-19a expressions induced by PRL-3 are involved in the proliferation and metastasis of colon cancer.

METHODS: Microarray analysis and quantitative reverse-transcription polymerase chain reactions (qRT–PCR) were used to investigate the changes in miRNA expression due to the overexpression of PRL-3. Transwell chamber invasion assays, CCK-8 proliferation assays and RNA interference assays were used to explore the effects of PRL-3 on miR-21, miR-17 and miR-19a expression in colon cancer cells. Immunohistochemistry and qRT–PCR were performed in colon cancer tissues to evaluate the expression of PRL-3, signal transducer and activator of transcription 3 (STAT3), miR-21, miR-17 and miR-19a.

RESULTS: Our study demonstrated that the overexpression of PRL-3 in colon cancer cells induced the expression of miR-21, miR-17 and miR-19a by activating STAT3. Subsequently, these microRNAs contributed to the increased proliferation and invasiveness of the colon cancer cells. Positive correlations between PRL-3 and these microRNAs were also observed in matched primary colon cancer tissues and metastatic lesions.

CONCLUSION: miR-21, miR-17 and miR-19a induced by PRL-3 contribute to the proliferation and invasion of colon cancer.

Keywords: PRL-3; microRNA; STAT3; colon carcinoma

Tumour metastasis is the main cause of death in colorectal cancer (CRC) patients. Among numerous human oncogenes, phosphatase of regenerating liver-3 (PRL-3) has received significant attention because of its involvement in CRC metastasis. A member of the classic protein tyrosine phosphatase family, PRL-3 (also known as PTP4A3), is important in regulating many signal transduction pathways. The overexpression of PRL-3 was observed in various cancer types (Parker et al., 2004; Rouleau et al., 2006; Wang et al., 2007b; Fagerli et al., 2008), especially in metastatic lesions derived from primary CRC, regardless of the target organ (Bardelli et al., 2003); however, its expression was low or absent from normal colorectal epithelia, adenomas and primary lesions (Saha et al., 2001). It has also been widely reported that PRL-3 facilitates the motility, invasiveness and metastasis of different cancer cell lines (Zeng et al., 2003; Wu et al., 2004; Fiordalisi et al., 2006), whereas the silencing of PRL-3 by RNA interference abrogated the cell growth and peritoneal metastasis of gastric cancer (Li et al., 2006; Cai et al., 2008). Moreover, a high level of PRL-3 expression was directly linked to a poor outcome in gastric cancer and in early-stage colon cancer (Wang et al., 2009; Xing et al., 2009). These studies suggest that PRL-3 triggers cancer metastasis and also acts as a biomarker of poor prognosis. Despite the considerable evidence that PRL-3 has a causal role in tumorigenesis and tumour metastasis, the underlying mechanism of PRL-3 action remains unclear.

MicroRNAs are small non-coding RNAs that specifically inhibit the translation of mRNA or induce mRNA degradation. Micro-RNAs regulate various cell processes at the post-transcriptional level. Moreover, certain microRNAs have been shown to have important roles in the initiation and development of human cancers by inhibiting tumour suppressor genes. MicroRNAs that regulate tumorigenesis, by acting as oncogenes, are also called oncomiRs. To date, no study has investigated the relationship between PRL-3 and microRNA expression. Thus, we aimed to explore whether microRNAs were involved in the growth and metastasis of colon cancer induced by PRL-3 and to elucidate the signalling pathway(s) that connect PRL-3 and microRNAs. Our results indicate that PRL-3 upregulates the expression of miR-21, miR-17 and miR-19a in both colon cancer cells and tissues by activating signal transducer and activator of transcription 3 (STAT3) via the Csk-Src-STAT3 pathway, a finding that may partially explain the connection between PRL-3 and tumour metastasis.
MATERIALS AND METHODS

Cell culture and cell transfection

Human colon cancer cell lines LoVo, SW480 and caco2 were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a 37 °C incubator containing 5% CO₂. Human PRL-3 was cloned into the pAcGFP-C3 (Takara Biotechnology, Dalian, China) expression vector, and the colon cancer cells were stably or transiently transfected with the pAcGFP-C3-PRL-3 or pAcGFP-C3 plasmids using Lipofectamine 2000 (Invitrogen). Stable cell lines were established by selection with 0.4 mg ml⁻¹ G418.

Microarray analysis

The method used for the miRNA microarray assay was described previously (Yu et al, 2007).

Cell proliferation assay

Cells were seeded in 96-well plates (2000 cells per well) and incubated for 24 h; 10 μl WST-8 from the CCK-8 kit (Boster, Wuhan, China) was then added to each well. After incubation at 37 °C in 5% CO₂ for 1 h, the absorbance of each sample was measured at a wavelength of 450 nm.

Wound-healing assay

Cells were grown to 95% confluence in a 35-mm plate and scratched with a pipette tip. The floating cells were removed by washing with phosphate-buffered saline, and images of the wounds were obtained using a microscope.

RNA isolation and qRT–PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen). The PRL-3 miRNA level was determined using the SYBR PrimeScript RT–PCR Kit (Takara) with the following primers: PRL-3 forward, 5′-GG GAC TTT CAGGTTGTCG-3′ and PRL-3 reverse, 5′-AGCCCCGT ACT CCTCAGGT-3′. The quantitative reverse-transcription polymerase chain reactions (qRT–PCR) assays for miR-17, miR-19a and miR-21 were performed using the Hairpin-it miRNA qPCR Quantitation Kit (GenePharma, Shanghai, China). All quantitative real-time PCR was performed using a LightCycler 480 (Roche, Basel, Switzerland) according to the operating instructions.

MicroRNA transient transfections

All of the miRNA mimics and inhibitors were designed by and purchased from GenePharma. Because the mimics are duplexes, the effective concentration of active miRNA mimic (i.e., the strand loaded into RISC) within the cell was half of the total transfected concentration (100 nm). The inhibitors are single-stranded, special antisense miRNAs (anti-miRs), and the total transfected concentration is 200 nm. Transient transfections were performed using Lipofectamine 2000 (Invitrogen), according to the protocol provided with the reagent.

Western blotting

The cells were lysed with RIPA buffer (Beyotime Biotechnology, Haimen, China), and the extracted proteins were separated using SDS–PAGE gels and then transferred onto PVDF membranes (Millipore, Bedford, MA, USA) for western blotting. The following primary antibodies were used: anti-STAT3 (Cell Signaling, Beverly, MA, USA), anti-pSTAT3 (Tyr705) (Cell Signaling), anti-Csk (Cell Signaling), anti-pSrc (Tyr527) (Cell Signaling), anti-pSrc (Tyr416) (Cell Signaling), anti-PTEN (Cell Signaling), and anti-PRL-3 (Abcam, Cambridge, MA, USA). The antibody dilutions were 1:1000 for anti-STAT3, -pSTAT3, -Csk, -pSrc, -PTEN, and -PRL-3 and 1:2000 for anti-GAPDH.

Invasive assay

Transwell chambers precoated with Matrigel (BD Bioscience, San Jose, CA, USA) were used to perform the invasion assay. Cells were cultured in serum-free medium in the upper chambers of a Transwell (Corning, NY, USA) plate (1 × 10⁶ cells per chamber), which are separated from the lower chambers with permeable 8.0μm polycarbonate membranes; medium containing 10% FBS served as the attractant in the lower chambers. After 12 h, the cells were fixed with 75% ethanol and stained with crystal violet. Non-migrating cells on the upper side of the membrane were gently wiped off, and the stained cells on the lower side were observed under a microscope. The number of migrating cells in five fields per chamber were counted and average values were calculated.

Tumour tissues

Paraffin-embedded tissue samples and frozen fresh tissue were obtained from the surgical pathology archives of the gastrointestinal surgery department at Sun-Yat-Sen Memorial Hospital. Approval was obtained from the research ethics committee of Sun Yat-Sen Memorial Hospital.

Immunohistochemistry

Immunohistochemistry of the paraffin sections was performed using a two-step protocol according to the manufacturer’s instructions. The sections were deparaffinised with xylene, rehydrated through a graded ethanol solution series, immersed in 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity, and submitted to antigen retrieval by pressure cooking for 3 min in citrate buffer. The slides were pre-incubated with 10% normal goat serum at room temperature for 30 min to block nonspecific binding. The sections were incubated with primary rabbit antibodies against PRL-3 (Abcam, 1:100 dilution) and pSTAT3 (Cell Signaling, 1:100 dilution) overnight at 4 °C in a humidified chamber. The slides were then incubated with the PV-6000 secondary antibody (Zhongshan Golden-bridge, Beijing, China) for 1 h at room temperature and stained with DAB (3,3-diaminobenzidine). Finally, the sections were counterstained with Mayer’s haematoxylin, dehydrated and mounted. Negative control sections were prepared using normal mouse IgG instead of the primary antibodies. A semiquantitative immunohistochemical evaluation of the PRL-3 and pSTAT3 staining was conducted. Scores were ranked as follows: ‘−’, no immunoreactive tumour cells detectable; ‘+’, <10% of tumour cells positive; ‘++’, 10–50% of tumour cells positive; and ‘+++’, >50% of tumour cells positive, with strong staining intensity.

RESULTS

Overexpression of PRL-3 promoted the proliferation, migration and invasion of LoVo colon cancer cells

To evaluate how PRL-3 affects the proliferation and invasion of colon cancer cells, we established LoVo colon cancer cell lines that stably expressed PRL-3. The PRL-3 expression plasmid (pAcGFP-C3-PRL-3) or empty vector (pAcGFP-C3) was transfected into LoVo cells, and several stable cell lines were established. Both the LoVo-PRL-3 and LoVo-PRL-3-2.2 cells, derived from two independent clones selected by G418 and isolated by limited dilution, stably expressed PRL-3. The LoVo cells that were stably transfected with the empty vector (LoVo-VC) served as the vector control. The levels of ectopic PRL-3 mRNA and protein expression were considerably higher than those of endogenous PRL-3 in the vector control cells, as determined by qRT–PCR and western blotting (Figure 1A and B). Increased proliferation properties of the PRL-3-expressing cells were observed both by a colony
formation assay and CCK-8 assay. The cells were seeded in a 6-well plate at a very low density (500 cells per plate) for 2 weeks, after which obvious colony formation was observed for the LoVo-PRL-3 and LoVo-PRL-3–2.2 cells, whereas no visible colonies of the LoVo-VC cells were formed (Figure 1C). Similarly, we found that PRL-3 contributed to the growth of the LoVo-PRL-3 and LoVo-PRL-3–2.2 cells in the CCK-8 assay (Figure 1D). Wound-healing assays were performed to investigate the motility of these cell lines. The LoVo-PRL-3–2.2 cells had migrated significantly faster than the LoVo-VC cells at 36 h after wounding (Figure 1E). In addition, we used Transwell chambers precoated with Matrigel to perform an invasion assay. As shown in Figure 1F, PRL-3 enhanced the cell invasive ability by 2.86-fold relative to the vector control cells. Although consistent with previous reports using other cell lines, we found that PRL-3 promotes the proliferation, migration and invasion of LoVo colon cancer cells, which provides a firm basis for the involvement of PRL-3 in tumour metastasis.

PRL-3 elevated the expression of miR-21, miR-17 and miR-19a

Using miRNA array analysis, we found that PRL-3 altered the expression of numerous microRNAs (data not shown) in LoVo cells, including the oncomiRs miR-21, miR-17 and miR-19a (Figure 2A). The aberrant expression of miR-21, miR-17 and miR-19a in association with tumorigenesis, tumour growth and tumour metastasis has been reported in different malignancies (Asangani et al, 2008; Takakura et al, 2008; Todoerti et al, 2010; Cioffi et al, 2011). Therefore, we validated the expression of these miRNAs in LoVo colon cancer cells that had been stably or transiently transfected with PRL-3. Using real-time quantitative PCR with RNU6B as an endogenous control, we found that miR-17, miR-19a and miR-21 were upregulated 2–3.6-fold by PRL-3 (Figure 2B and C). To explore whether the PRL-3-induced expression of these miRNAs was reproducible in other CRC cell lines, we employed SW480 and caco2 cells that had been transiently transfected with the PRL-3 expression plasmid or the control vector. As shown in Figure 2D and E, PRL-3 also significantly increased the expression of miR-17, miR-19a and miR-21 in SW480 and caco2 cells. This is the first report demonstrating that miR-17, miR-19a and miR-21 are positively regulated by PRL-3 in CRC cells. Interestingly, PTEN, which is a suppressor of the tumorigenic PI3K pathway and also a target of miR-21, miR-17 and miR-19a (Meng et al, 2007; Olive et al, 2009), was downregulated at the protein level in the LoVo-PRL-3 cells (Figure 2F), reflecting the tumorigenic potential of PRL-3.

Figure 1 Overexpression of PRL-3 promoted the proliferation, migration and invasion of LoVo colon cancer cells. (A) qRT–PCR and (B) western blot analysis confirmed the expression of PRL-3 in the PRL-3 and PRL-3–2.2 cells; cells transfected with the empty vector were used as a negative control. (C) PRL-3 promoted the proliferation of LoVo cells in a colony formation assay and (D) a CCK-8 proliferation assay. (E) PRL-3 promoted the migration of PRL-3–2.2 cells in a wound-healing assay. (F) PRL-3 cells exhibited more invasive potential (versus the control) in a Transwell chamber invasion assay (*p < 0.05, **p < 0.01, ***p < 0.001; Student’s t-test; n = 3).
**miR-21, miR-17 and miR-19a promoted the proliferation and invasion of LoVo cells and directly targeted PTEN**

Accumulating evidence shows that the aberrant expression of oncomiRs, in particular miR-21, miR-17, miR-19a and other microRNAs, is involved in carcinogenesis, tumour growth and tumour metastasis (Kong et al., 2007; Zhang et al., 2010; Osada and Takahashi, 2011). In our study, miR-21, miR-17 and miR-19a also exhibited oncogenic potential; the LoVo-VC cells over-expressing miR-21, miR-17 and miR-19a exhibited significantly increased proliferative and invasion capabilities (Figure 3A, B, G and H). Furthermore, miR-21, miR-17 and miR-19a inhibitors antagonised the proliferation and invasion of the LoVo-PRL-3 cells (Figure 3D, E, J and K). As a known target of miR-21 and miR-17–92, the PTEN protein was significantly downregulated by miR-21 and miR-19a mimics (Figure 3C and I), but miR-21 and miR-19a inhibitors restored the expression of the PTEN protein in the LoVo-PRL-3 cells (Figure 3F and L). Among the miR-17–92 family members, miR-19 is a key oncogene, and its oncogenic activity is at least partially exerted through its suppression of PTEN (Olive et al., 2009). This may explain why miR-17 had a less potent effect on the expression of the PTEN protein (Figure 3I and L). To assess the role of these miRNAs in targeting PTEN, we cloned the human PTEN mRNA, excluding its 3’UTR, into the pc-DNA3 plasmid (GenePharma) and overexpressed PTEN in LoVo-PRL-3 cells (Figure 3O). Interestingly, PTEN antagonised the phenotypes caused by these miRNAs in both proliferation and invasion assays (Figure 3M and N).

**PRL-3 activated STAT3, leading to the upregulation of miR-17, miR-19a and miR-21**

To explore the mechanism underlying the upregulation of oncomiRs induced by PRL-3, we referred to previous reports and found that PRL-3 can activate the Src kinase via the downregulation of Csk. This initiates a number of signalling pathways and culminates in the phosphorylation of ERK1/2, STAT3, and p130 Cas (Liang et al., 1997). Thus, we sought to evaluate the expression of STAT3 in the LoVo-PRL-3 cells. As shown in Figure 4A, pSTAT3 (Tyr705) was elevated, Csk and pSrc (Tyr527) were downregulated, and STAT3 and pSrc (Tyr416) were unaffected at the protein level in the LoVo-PRL-3 cells compared with the vector control cells. The phosphorylation of ERK1/2, STAT3, and p130 Cas (Liang et al., 1997). Thus, we sought to evaluate the expression of STAT3 in the LoVo-PRL-3 cells. As shown in Figure 4A, pSTAT3 (Tyr705) was elevated, Csk and pSrc (Tyr527) were downregulated, and STAT3 and pSrc (Tyr416) were unaffected at the protein level in the LoVo-PRL-3 cells compared with the vector control cells. This initiates a number of signalling pathways and culminates in the phosphorylation of ERK1/2, STAT3, and p130 Cas (Liang et al., 1997). Thus, we sought to evaluate the expression of STAT3 in the LoVo-PRL-3 cells. As shown in Figure 4A, pSTAT3 (Tyr705) was elevated, Csk and pSrc (Tyr527) were downregulated, and STAT3 and pSrc (Tyr416) were unaffected at the protein level in the LoVo-PRL-3 cells compared with the vector control cells. The phosphorylation of ERK1/2, STAT3, and p130 Cas (Liang et al., 1997). Thus, we sought to evaluate the expression of STAT3 in the LoVo-PRL-3 cells. As shown in Figure 4A, pSTAT3 (Tyr705) was elevated, Csk and pSrc (Tyr527) were downregulated, and STAT3 and pSrc (Tyr416) were unaffected at the protein level in the LoVo-PRL-3 cells compared with the vector control cells. This initiates a number of signalling pathways and culminates in the phosphorylation of ERK1/2, STAT3, and p130 Cas (Liang et al., 1997). Thus, we sought to evaluate the expression of STAT3 in the LoVo-PRL-3 cells. As shown in Figure 4A, pSTAT3 (Tyr705) was elevated, Csk and pSrc (Tyr527) were downregulated, and STAT3 and pSrc (Tyr416) were unaffected at the protein level in the LoVo-PRL-3 cells compared with the vector control cells.
phosphorylation of Tyr705 (Yu et al., 1995). To determine the effects of STAT3 on miR-17, miR-19a and miR-21 expression, we knocked-down STAT3 in the LoVo-PRL-3 cells using a specific siRNA. After 48 h of suppression, we found that the downregulation of the STAT3 protein was accompanied by reduced expression levels of miR-17, miR-19a and miR-21 (Figure 4B and C).

PRL-3 was elevated in metastatic colon cancer lesions and positively correlated with pSTAT3, miR-17, miR-19a, and miR-21

We used immunohistochemistry to detect PRL-3 and pSTAT3 expression in paraffin sections of 13 primary colon cancer samples.
and corresponding metastatic lesions. Four normal colon tissue samples collected from a site in the proximity to the tumour were used as the controls. As shown in Figure 5A and B, strong PRL-3 and pSTAT3 expression was observed in the metastatic lesions, whereas no positive staining was found in the paired primary tumour or normal colon tissues. A summary of the PRL-3 and pSTAT3 expression data is shown in Table 1. The positive expression rates of PRL-3 and pSTAT3 were significantly higher in the metastatic lesions (11/13 and 10/13, respectively) than in the paired primary tumours (2/13, \( P < 0.01 \) and 0/13, \( P < 0.001 \), respectively; Fisher’s exact test) and normal colon tissues (0/4, \( P < 0.01 \) and 0/4, \( P < 0.05 \), respectively; Fisher’s exact test). Using qRT–PCR, we determined the expression levels of PRL-3, miR-17, miR-19a and miR-21 in freshly frozen primary colon cancer samples and their corresponding metastatic lesions. As shown in Figure 6, miR-17, miR-19a and miR-21 were elevated in almost all of the metastatic tissues upon the upregulation of PRL-3.

**DISCUSSION**

PRL-3 has an important role in carcinogenesis and tumour metastasis, especially in CRCs; however, the mechanism underlying its activity remains unclear. It is reported that PRL-3 participates in multiple signalling pathways and regulates several cellular components, including pERK, Ras homologue (Rho), intermediate-conductance Ca\(^{2+}\)-activated K\(^+\) channels (KCNN4), PTEN, E-cadherin, and matrix metalloproteinases; thus, PRL-3 exerts its tumorigenic activity by enhancing the proliferation, motility, invasion, angiogenesis and epithelial mesenchymal transition of cancer cells (Kong et al, 2007; Wang et al, 2007a; Liu et al, 2009; Ming et al, 2009; Mizuuchi et al, 2009; Peng et al, 2009; Zhang et al, 2010; Lai et al, 2011). Many recent reports have focussed on the functional role of non-coding RNAs, and microRNAs, which regulate cell processes at the post-transcriptional level, are widely reported to participate in tumorigenesis. However, it has not yet been reported whether microRNAs are implicated in PRL-3-related carcinogenic activity.

**Table 1** Summary of PRL-3 and pSTAT3 expression in colon cancer samples and normal colon tissues detected by IHC

|                | PRL            | pSTAT3         |
|----------------|----------------|----------------|
| Normal colon tissue | - ++ +++++++ | - ++ +++++++  |
| Primary tumour   | 11 2 0 0        | 13 0 0         |
| Lymph node metastasis | 1 2 1 1       | 2 2 1 0       |
| Liver metastasis | 1 1 3 3         | 1 4 3 0       |

\(+\), <10% of tumour cells positive; \(+ +\), 10–50% of tumour cells positive; and \(+ + +\), >50% of tumour cells positive, with strong staining intensity.

**Figure 4** PRL-3 activated STAT3, leading to the upregulation of miR-17, miR-19a and miR-21. (A) PRL-3 downregulated Csk and pSrc (Tyr527), which is an inactive form of Src, culminating in the phosphorylation of Tyr705 of STAT3; By contrast, total STAT3 and pSrc (Tyr416), which is an active form of Src, were not affected. (B) The knockdown of STAT3 with a specific siRNA in LoVo-PRL-3 cells lowered the expression of the STAT3 protein. (C) The expression levels of miR-17, miR-19a and miR-21 were significantly reduced upon STAT3 downregulation (*\( P < 0.05 \); Student’s t-test; \( n = 3 \)).

**Figure 5** PRL-3 and pSTAT3 were elevated in metastatic colon cancer lesions. (A) Strong PRL-3 expression can be observed in metastatic lesions, mainly in the cytoplasm, whereas no positive staining was observed in the paired primary tumour or normal colon tissues. (B) pSTAT3 was strongly expressed in metastatic lesions, no positive staining was observed in the corresponding primary tumour or normal colon tissue.
miR-21, miR-17, and miR-19a. In addition, the downregulation of PTEN by these oncomiRs suggests that the PI3K pathway might be partly involved in the downstream signalling of PRL-3. To explore the signal transduction of PRL-3, we selected STAT3, a transcription factor that is activated by PRL-3. In fact, the activation of STAT3 has been widely implicated in the proliferation, migration and invasion of different cancer cells (Bollrath and Greten, 2009; Barbieri et al., 2010; Chiu et al., 2011; Fukuda et al., 2011). In accordance with previous reports (Liang et al., 2007), we showed that PRL-3 can activate the Src kinase via the downregulation of Csk, culminating in the phosphorylation of STAT3. The down-regulation of STAT3 by siRNA decreased the expression of these oncomiRs in LoVo-PRL-3 cells. Recently, a positive correlation between STAT3 and miR-21 was observed in colon adenocarcinomas (Iliopoulos et al., 2010). Moreover, STAT3 was able to activate the transcription of the miR-17–92 gene (C13orf25) by binding to its promoter (Brock et al., 2009), and it mediated the overexpression of miR-17 in lung cancer cell lines (Dai et al., 2011). Consistent with these data, our results suggest that STAT3 has a crucial role in the induction of oncomiRs. A previous report found that the frequency of PRL-3 upregulation in cases of human primary CRCs associated with liver (84.4%) or lung (88.9%) metastasis was statistically higher than in cases without either type of metastasis (liver, 35.9%; lung, 42.3%); in addition, high PRL-3 expression was frequently detected in metastatic CRC lesions (liver, 91.3%; lung, 100%) (Kato, et al., 2004). In the present study, we found that PRL-3 was elevated in metastatic lesions of colon cancer tissues and was positively correlated with pSTAT3, miR-17, miR-19a, and miR-21 expression. Thus, the novel signalling pathway PRL-3—STAT3—miR-21/miR-17–92 described here may partly explain the oncogenic effects of PRL-3. For the first time, we report the connection between PRL-3 and the specific oncomiRs miR-21, miR-17, and miR-19a, the downregulation of which may be a useful therapeutic strategy for targeting colon cancer metastasis.

ACKNOWLEDGEMENTS

This work was partly supported by the National Natural Science Foundation of China (81071761), the Natural Science Foundation of Guangdong Province (10151008901000044) and the Medical Science Research Foundation of Guangdong Province (A2011171).
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