Molecular studies of the congenital malformation induced by Largehead Atractylodes Rhizome, the most commonly used Chinese medicine for threatened miscarriage

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Submitted on March 27, 2012; resubmitted on August 3, 2012; accepted on August 9, 2012

ABSTRACT: Largehead Atractylodes Rhizome (LAR) is the most commonly used Chinese herbal medicine for threatened miscarriage. Potential reproductive toxicity of LAR was identified in early pregnancy in animals. Skeletal anomalies including loss of ulna and distal digits, shortening of humerus and radius were observed in higher clinical dose groups. Here, we aimed to study the molecular mechanism of the congenital malformation induced by LAR. In vitro whole mouse embryo culture was used to confirm the embryotoxicity effects of LAR on developing limb buds during early organogenesis. A pregnant mouse model was employed to study the developmental gene expression by quantitative PCR and whole hybridization and apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling staining, in the forelimbs and hindlimbs during development in vivo. Severe growth retardation, multiple embryonic malformations and delayed limb bud development were observed. Limb-specific Tbx gene expressions in both developing forelimbs and hindlimbs were significantly decreased. Increased developmental apoptosis in apical ectodermal ridge and mesenchymal mesoderm of the developing limb buds was identified. Overexpressions of Tbx2 and Tbx3 in embryos in vitro rescued LAR-induced abnormal limb development and reduced apoptosis in the developing forelimb buds. In conclusion, LAR affects limb development by suppressing the expression of limb developmental genes and disturbing programmed cell death during limb formation in mice.

Key words: Chinese medicine / Largehead Atractylodes Rhizome / pregnancy / congenital malformation

Introduction

Chinese medicines have commonly been used in pregnancy (Sionneau, 1995; Westfall, 2001). In our systematic literature research, Largehead Atractylodes Rhizome (LAR) is the most commonly used Chinese medicine to prevent early pregnancy loss due to threatened miscarriage (Li, 2011; Li et al., 2012b). Over 40% of Chinese medicine regimes for threatened miscarriage included LAR in the treatment. According to Traditional Chinese Medicine, LAR can improve the conditions of ‘Spleen’ and ‘Kidney’ and improve ‘Blood’ circulation to relieve edema due to excessive ‘Damp’ due to ‘Spleen’ deficiency during pregnancy (National Phamacopoeia Committee, 2010). In clinical practice, Chinese medicine practitioners prescribe LAR to promote maternal and fetal health in normal pregnancy (Sionneau, 1995), prevent early pregnancy loss in miscarriage (Liang, 2008) and inhibit uterine contraction in premature delivery (Ma et al., 2006).

Although adverse outcomes of Chinese medicines used for threatened miscarriage cannot be confirmed in our latest meta-analysis (Li et al., 2012a), in our recent animal studies significant reduction of embryonic growth and development was observed in maternal exposure to LAR during pregnancy (Li et al., 2011; Wang et al., 2012). Congenital skeletal anomalies were also identified in higher clinical dose. Affected pups exhibited limb malformations, including oligodactyly in early pregnancy, congenital shoulder and hip dislocation in...
lower limbs and missing ulna and distal digits and shortening of humerus and radius in upper limbs in late pregnancy. These results suggested that LAR might have potential embryonic toxicity that hindered the limb development.

In this study, we investigated the molecular mechanisms of the congenital malformation induced by LAR in order to better understand its embryotoxic effects.

Materials and Methods

LAR extract

Concentrated LAR extract was obtained from a local pharmaceutical company (PuraPharm Nongs, Hong Kong), with the accreditation of Good Manufacturing Practice certification for patient use. The quality of each medicine was controlled by recognized organoleptic authentications in Chinese Pharmacopoeia as described in our previous study (Li et al., 2011). Molecular authentication by PCR, direct amplification of length polymorphism or amplified fragment length polymorphism was employed to confirm the unique genetic composition for each taxon. Chemical authentications by thin-layer chromatography or high-performance liquid chromatography was conducted to confirm the quality and quantity of chemical components in each medicine and to ensure the quality and to avoid any pesticide, mineral and other biological contaminations. Each crude extract was weighed and dissolved in distilled water before experimentations.

Animals

Pregnant Institute of Cancer Research (ICR) mice were obtained from the Laboratory Animal Services Centre in The Chinese University of Hong Kong. Virgin female and male mice were mated at 1800 h, and the female mice were examined for a copulation plug in vagina in the next morning. The day on which vaginal plug was identified was regarded as gestation day 0 (E0). Guidelines were followed for the use and care of all laboratory animals as set in the university. All the mice were housed in a room with temperature maintained at 25°C, a 12/12 h light–dark cycle, and with standard water and rodent chow provided ad libitum. Animal Ethics approvals (06/073/MIS, 09/073/MIS, 09/015/GRF) were obtained for the animal experimentations. Mouse embryos at E7.5 were harvested at E9.5 as previously described (Wang et al., 1993). The total morphological score (TMS) of each embryo at the test concentration was calculated by summing up all the scores. To determine the embryotoxic potential of LAR, a dose–response curve was plotted by percentage changes of TMS against logarithmic concentrations of LAR. IC50 was the concentration of LAR at which these was 50% reduction of TMS in the developing embryos after 48 h of culture. Embryotoxic effects were compared with control groups. Embryotoxicity was evaluated on the basis of protocol from the European Center for the Validation of Alternative Methods (ECVAM, Geschow et al., 2000) using a combination of tests on whole rat embryos, limb bud cells and embryonic stem cells to determine functions. Test substance is classified as non-embryotoxic if prediction function I >functions II and III; weakly embryotoxic if prediction function II >functions I and III; or strongly toxic if prediction function III >functions I and II.

Pregnant mouse model in vivo

According to our previous in vivo study (Li et al., 2011), limb malformations were observed at three times the clinical dosage (group receiving 3.54 g/kg). The test dosages were calculated based on the recommended dose formula for Chinese herbal medicines: \[d_0 = d_w \times (W_b/W_a)^b\] (You, 2007). \(d_w\) stands for the known dose for animal A (mg/kg), while \(d_0\) stands for the unknown dose for animal B. \(W_b\) and \(W_a\) are the body-size factors, i.e. 59 for mouse and 100 for human (You, 2007), while \(W_b\) and \(W_a\) are the weights of animal A and B. The median clinical dose of LAR for human is 12 g/day, which is regarded as single (1×) clinical dose for human (National Pharmacopoeia Committee, 2010). The I× clinical dose of LAR for mice was 1.18 g/kg. In the present study, pregnant mice were daily administered a single bolus oral dose of 3.54 g/kg of LAR from E7.5 before limb bud formation for 3 days as treatment groups, and vehicle (0 g/kg) was included as a negative control group for comparison. Animals were sacrificed at E10.5 after limb bud formation, uteri were removed and embryos were harvested for quantitative real-time PCR, whole mount in situ hybridization and terminal deoxynucleotidyl transferase dUTP nick end labeling staining assay.

Quantitative real-time PCR

Developing forelimb and hindlimb buds of the mouse embryos were dissected in diethylpyrocarbonate-treated PBS solution under microscope. Total RNA from pooled limb buds was isolated using the RNeasy Mini kit (Qiagen) and cDNA were subsequently prepared using Superscript II Reverse Transcriptase (Invitrogen). The expression levels of T-box genes, Tbx1–5, for limb development were determined by real-time quantitative PCR. Gpdpd was used as the internal control for normalization. The validated TaqMan® primers specific to Tbx1–5 were obtained from Applied Biosystems Gene Expression Assays (Applied Biosystems). The assay ID for mouse Tbx1–5 and Gpdpd were Mm00449489_ml, Mm00436915_ml, Mm00809779_sl, Mm00550372_ml, Mm00803518_ml and Mm9999915_ml respectively. All reactions were carried out in triplicate. Differences in gene expression, expressed as fold-change, were calculated using the 2−ΔΔCt method. Data were analyzed with one-way analysis of variance (ANOVA) with P < 0.05 considered to be statistically significant.

Whole mount in situ hybridization

Whole mount in situ hybridization in the mouse embryos was performed as described previously (Wilkinson, 1992). Tbx2 and Tbx3 consisting of ~1 kb portion of the T-box were amplified by PCR and cloned in plasmid vectors. Antisense and sense RNA probes of mouse Tbx2 and Tbx3 mRNA including part of the T-box coding region were transcribed from linearized plasmids using SP6 and T7 RNA polymerase, respectively. Embryos were incubated with digoxigenin-labeled riboprobes, and then exposed to the BM Purple AP substrate (Roche) in darkness at 4°C.
Spatial expressions of Tbx2 and Tbx3 in developing limb buds were examined under a microscope.

**Tbx overexpression in mouse embryos**

LAR-treated embryos were overexpressed by empty pCMV6 vector as control or either Tbx2 or Tbx3 overexpression vector (OriGene Technologies, Inc., MD) as previously described (Ufer et al., 2008). Ten nanograms per microlitre of overexpression vector was microinjected into the amniotic cavity of E7.5 mouse embryos. The injected mouse embryos were cultured as discussed earlier above in heat-inactivated full rat serum supplemented with 1 mg/ml of LAR extracts for 72 h, and harvested at about the E10.5 developmental stage. Then the embryos were either subjected to whole mount Nile blue staining to detect the dead cells in the developing limb buds as previously described (Wang et al., 2007) or fixed in 4% P-formaldehyde for whole mount in situ hybridization to confirm the overexpression of Tbx2 and Tbx3 in the developing limb buds as seen earlier. Apoptotic cells in the forelimb buds were stained in tissue sections by standard TUNEL technique as is explained going to be.

**TUNEL analysis**

Paraffin sections (5 μm) of paraformaldehyde fixed mouse embryos were deparaffinized and apoptotic nuclei were stained using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Chemicon) according to the manufacturer’s instructions. Diaminobenzidine was used as substrate for the TUNEL labeling, and apoptotic cells in the distal and proximal regions of developing limb buds were examined under a microscope.

**Results**

**Embryotoxicity of LAR**

In embryo culture in vitro, E9.5 embryos from the control group had normal embryonic growth and development (Fig. 1A). Abnormal embryonic growth and development were identified in LAR groups at 0.5 mg/ml and higher doses. With LAR at 0.5 mg/ml, exencephaly and underdeveloped forelimb and hindlimb buds were observed. The growth restriction, absence of limb buds and viscera bareness were found in 1 mg/ml LAR group. With LAR at 2 mg/ml, developmental arrest and craniorachischisis (entire opened cranial neural tube defects) were identified. The morphological score of the forelimb and hindlimb buds was significantly reduced in 1 and 2 mg/ml groups (Table I). The severity of the developmental anomalies was increased with increased LAR concentrations. The dose–response curve indicated that embryotoxicity IC₅₀ of LAR was 2.31 mg/ml (Fig. 1B). According to the biostatistical prediction model (Genschow et al., 2000), LAR was classified as weak embryotoxicity (prediction function II = functions I and III).

**Dysregulation of apoptotic events in limb buds**

Developmental apoptosis in the developing limbs was examined by TUNEL staining. In control group, no apoptosis was detected in the distal and proximal regions of developing limb buds as seen earlier. Apoptotic cells in the forelimb buds were stained in tissue sections by standard TUNEL technique as explained going to be.

Spatial expressions of Tbx2 and Tbx3 mRNA in the limbs were confirmed by whole mount in situ hybridization. Tbx2 and Tbx3 mRNA were expressed not only in the developing limb buds, but also in the forebrain, first brachial arch and tails (data not shown). The mRNA expression of Tbx2 was highly expressed in the whole forelimb and hindlimb buds of the control groups (Fig. 2). However, the mRNA expression of Tbx2 was reduced in both forelimb and hindlimb buds of the treatment group (Fig. 3). The expression levels of Tbx1 and Tbx5 mRNA were significantly lower in forelimb buds of the treatment group only. Significant reduction of the mRNA expression level of Tbx4 was observed in hindlimb buds of the treatment group only.

Spatial expressions of Tbx2 and Tbx3 mRNA in the limbs were confirmed by whole mount in situ hybridization. Tbx2 and Tbx3 mRNA were expressed not only in the developing limb buds, but also in the forebrain, first brachial arch and tails (data not shown). The mRNA expression of Tbx2 was highly expressed in the whole forelimb and hindlimb buds of the control groups (Fig. 2). However, the mRNA expression of Tbx2 was reduced in both forelimb and hindlimb buds of the treatment group, which was consistent with the quantitative real-time PCR results. In the forelimb buds, Tbx2 expression cannot be detected at proximal limb plate, and was less expressed in the peripheral limb plate. In hindlimb buds, the expression of Tbx2 could hardly be detected. Tbx3 was also highly expressed in forelimb buds and hindlimb buds (Fig. 3). The expression of Tbx3 was hardly detected in the treatment group, either in the forelimb or hindlimb buds.

**Figure 1** Embryotoxicity of LAR in mouse embryo culture. (A) Abnormal embryonic development and anomalies were observed in treatment groups from 0.5 to 2 mg/ml of LAR. Representative embryos are presented. Closed arrows represent the normal structures. Open arrows represent the malformed structures, including microencephaly, opened neural tube defects and sacral regression. Magnification × 25. (B) Dose–response curve of LAR on embryo development. The minimum concentration of LAR which resulted in a 50% reduction in relative TMS in the embryos was indicated. n = 10 embryos per each LAR concentration.
apical ectodermal ridge (AER) of the forelimb buds. Significant increase in cell death was found in the AER of forelimb buds in the treatment group (Fig. 4, Table II). Significant increase in cell death was also observed in the mesenchymal mesoderm of both forelimb buds and hindlimb buds in the treatment group when compared with the control group (Fig. 4, Table II).

Rescue of developing limb buds by Tbx overexpression. Overexpressions of Tbx2 and Tbx3 in embryos were employed to confirm the molecular mechanism of Tbx genes in the LAR-induced skeleton malformations during early forelimb development in vitro. Overexpression of Tbx2 and Tbx3 in embryos rescued the development of forelimb buds under LAR treatment (Fig. 5A). Tbx2 and Tbx3 overexpressions were confirmed by high in situ hybridization expressions in the developing forelimb buds (Fig. 5B). Reduction of dead cells by whole mount Nile blue staining and cellular apoptosis by in situ TUNEL staining in the developing forelimb buds were observed under Tbx2 and Tbx3 overexpression (Fig. 6).

Table I Means of morphological score for forelimb buds and hindlimb buds of embryos in cultures with LAR interventions.

<table>
<thead>
<tr>
<th>Concentrations of LAR</th>
<th>Forelimb buds</th>
<th>Hindlimb buds</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>2.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>0.5 mg/ml</td>
<td>1.5 ± 0.3</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>1 mg/ml</td>
<td>0.0 ± 0.0*</td>
<td>0.0 ± 0.0*</td>
</tr>
<tr>
<td>2 mg/ml</td>
<td>0.0 ± 0.0*</td>
<td>0.0 ± 0.0*</td>
</tr>
</tbody>
</table>

n = 20 for each dose group.

*P < 0.001 (one-way ANOVA).

Figure 2 Differential expressions of Tbx gene family in forelimb and hindlimb buds in control and LAR treatment groups by quantitative real-time PCR. Significant reduction of Tbx1, Tbx2, Tbx3 and Tbx4 was observed in forelimb buds of the treatment group. Significant reduction of Tbx3 and Tbx4 was observed in hindlimb buds of the treatment group. ***P < 0.001, n = 5 dams for each group, one-way ANOVA.
Whole embryo cultures confirmed the embryotoxic effects of LAR on the developing mouse embryos. Increased dose of LAR resulted in exencephaly, underdeveloped or absence of forelimb and hindlimb buds, viscera bareness and severe growth retardation in the embryos. Significantly reduced morphological scores of the forelimb and hindlimb buds were observed in higher doses. The embryotoxicity IC50 of LAR was 2.31 mg/ml, which was much smaller than the clinical dose. Taken together with our previous animal study in vivo (Li et al., 2011), LAR does hinder the growth and development of embryo in early pregnancy. According to the classification of embryotoxicity for pharmaceuticals, LAR is classified as having weak embryotoxicity in mice.

**Discussion**

Whole embryo cultures confirmed the embryotoxic effects of LAR on the developing mouse embryos. Increased dose of LAR resulted in exencephaly, underdeveloped or absence of forelimb and hindlimb buds, viscera bareness and severe growth retardation in the embryos. Significantly reduced morphological scores of the forelimb and hindlimb buds were observed in higher doses. The embryotoxicity IC50 of LAR was 2.31 mg/ml, which was much smaller than the clinical dose. Taken together with our previous animal study in vivo (Li et al., 2011), LAR does hinder the growth and development of embryo in early pregnancy. According to the classification of embryotoxicity for pharmaceuticals, LAR is classified as having weak embryotoxicity in mice. *In vitro* LAR extracts were supplemented directly to the serum.
Figure 5 Rescue of limb development by Tbx2 and Tbx3 overexpression. (A) Development of the forelimb buds was examined under bright field (blue arrows) after LAR treatment and either empty pCMV6 vector, Tbx2 or Tbx3 overexpression vector injection in vitro. Closed arrows represent under-developed limb buds under control treatment, open arrows represent rescued and normal limb buds under Tbx overexpression. Magnification X1000, n = 5 embryos for each group. (B) Overexpression of Tbx2 or Tbx3 mRNA in the forelimb buds was confirmed by whole mount in situ hybridization (Tbx2 red arrows, Tbx3 yellow arrows) after LAR treatment and either empty pCMV6 vector, Tbx2 or Tbx3 overexpression vector injection in vitro. Dark purple colorations represent positive in situ hybridization signals. Closed arrows represent under-developed limb buds under control treatment and open arrows represent rescued and normal limb buds under Tbx overexpression. Magnification ×1200, n = 5 embryos for each group.

Figure 6 Reduced apoptosis in developing limb buds by Tbx2 and Tbx3 overexpression. (A) Dead cells were observed in forelimb buds by whole mount Nile Blue staining (red arrows) after LAR treatment and either empty pCMV6 vector or Tbx2 or Tbx3 overexpression vector injection in vitro. Dark blue colors represent positive dead cells in the embryos. Closed arrows represent under-developed limb buds under control treatment and open arrows represent rescued and normal limb buds under Tbx overexpression. Magnification ×1200, n = 5 embryos for each group. (B) Reduced apoptotic cells in AER (yellow arrows) and mesenchymal mesoderm of sections of the forelimb buds were examined by TUNEL staining after LAR treatment and either empty pCMV6 vector, Tbx2 or Tbx3 overexpression vector injection in vitro. Closed arrows represent the normal AER structures; open arrows represent the malformed AER structures. Magnification ×1000, n = 5 embryos for each group.
for culture of the whole mouse embryos, whereas the presence of a placental barrier and the maternal drug metabolism mechanism could limit in vivo the transfer of the Chinese medicine extracts and its biological components from maternal circulation, and therefore protect embryos from embryotoxic effects. Further pharmacokinetic study is necessary to confirm the bioavailability and embryotoxicity of LAR in human pregnancy.

Congenital skeletal anomalies were identified under LAR treatment in vivo (Li et al., 2011). The skeletal abnormalities included missing ulna, distal digits and shortening of humerus and radius. The abnormalities in upper limb and lower limb closely resemble the phenotype seen in Tbx2 and Tbx3 homozygous mutant in mice. The T-box gene family plays a critical role in the embryonic development of vertebrae (Naiche et al., 2005), which encodes for transcription factors important for patterning and development of limbs (Gibson-Brown et al., 1996). Severe human congenital malformations, involving skeletal, craniofacial and cardiovascular structures resulted from haploinsufficiency of multiple T-box proteins (King et al., 2006). Tbx1 and Tbx5 play an essential role in cardiac development. Tbx1 haploinsufficiency in the DiGeorge syndrome region causes aortic arch defects in mice (Lindsay et al., 2001). Mutations in the Tbx5 cause heart septal defects found in human Holt–Oram syndrome and the failure of heart formation in Xenopus embryos (Horb and Thomsen, 1999). Tbx2 and Tbx3 are expressed in similar spatiotemporal expression patterns in developing limbs in mice (Chapman et al., 1996; Gibson-Brown et al., 1996). Tbx2 and Tbx3 are expressed at the posterior margin of the forelimb bud of E9.5 embryos. At E10.5, their expressions are observed in the anterior and posterior proximal mesenchyme of both forelimb and hindlimb buds. Tbx3 is expressed in the AER of both limb buds, while Tbx2 is expressed in the mesenchyme underlying the posterior half of the ridge. In humans, Tbx3 mutations are responsible for causing ulna-mammary syndrome (OMIM 181450), including loss of the fifth digit and reduction or loss of ulna (Bamshad et al., 1997). In mice, Tbx3 homozygous mutant lacked an ulna and carpals as well as metacarpals and phalanges of fourth and fifth digits (Todd et al., 2003), while in Tbx2 homozygous mutant, heart and limb abnormalities were found (Harrelson et al., 2004; Aanhaanen et al., 2009). In our study, significant reduction of the expression levels of Tbx2 and Tbx3 was confirmed in both forelimb and hindlimb buds of the treatment group. It suggested that LAR affects the limb bud development by dysregulating the expression of Tbx2 and Tbx3.

Expression of Tbx is upstream of Sonic hedgehog signaling (Shh) in the initiation and/or maintenance of Shh expression (Todd et al., 2003). Shh is involved in digit identities in mammalian development (Suzuki et al., 2004). Inhibition of Shh resulted in increased cell death (Sanz-Ezquerro and Tickle, 2000). Cell death plays an important role in regulating limb formation and the control of apoptosis in specific cells during limb development is genetically programmed (Gilbert, 2000). The effects of LAR on programmed cell death in developing limbs are still unknown. In our study, significant increase in the number of TUNEL-positive cells in the AER and mesenchymal mesoderm was seen in the forelimb and hindlimb buds in treatment groups. The AER is a specialized epithelium that runs along the distal limb bud tip. AER is responsible for the sustained outgrowth and development of the limb (Zwilling, 1955; Saunders et al., 1957; Krabbenhoft and Fallon, 1989). AER maintains the high proliferation rates in the progress zone and prevents them from forming cartilage (Saunders, 1948; Summerbell et al., 1973). A series of interactions between the limb bud mesenchyme and the AER leads to the proximal–distal growth and differentiation of the limb bud (Harrison, 1918; Saunders, 1948). In chicken embryo, loss of AER resulted in hindrance in the development of distal limb skeletal elements (Wessells, 1977). Mesenchymal mesoderm is a zone of differentiation. Formations of the skeletal elements result from the tight aggregation of mesenchymal cells called mesenchymal condensation (Tamayuki and Koji, 1990).

Besides, the developing limb can be divided into three principal zones including stylopod, zeugopod and autopod (Zeller et al., 2009). The autopod region will develop into digits; zeugopod regions will develop into ulna and radius in the forelimb, tibia and fibula in the hindlimb; while stylopod will develop into humerus in the forelimb and femur in the hindlimb. Since AER and mesenchymal mesoderm are found with autopod and zeugopod regions respectively, the increased apoptosis in AER and mesenchymal mesoderm might account for skeletal anomalies, including the missing of distal digits and ulna as seen in our previous in vivo animal models (Li et al., 2011).

Induced cellular apoptosis by reduction of Tbx genes is demonstrated in Tbx Knockout models (Todd et al., 2003; Harrelson et al., 2004; Aanhaanen et al., 2009). Unfortunately, our current study cannot confirm if LAR really target Tbx genes directly. The direct target of LAR to Tbx genes requires further studies, such as LAR active components, structural conformation and receptor-ligand interaction.

In conclusion, LAR affects the mouse limb development by suppressing the expression of limb developmental genes and increasing the programmed cell death in limb formation.

**Acknowledgements**

We thank the help of the laboratory members in Department of Obstetrics and Gynaecology, and the research team in Institute of Chinese Medicine, from The Chinese University of Hong Kong.

**Authors’ roles**

L.Y.T. and L.L. performed experiments, analyzed data and drafted the manuscript. A.B. advised the molecular study and prepared the reagents for experiments. C.B.S.L. and P.C.L. prepared the extracts, obtained the grant and commented on the manuscript. C.C.W. conceived the project, designed and performed experiments, obtained the grant and revised the manuscript.

**Funding**

This work was supported by Health and Health Services Research Fund (06070511 and 08090581) from Food and Health Bureau, Hong Kong SAR. The study was partially supported by Hop Wai Scholarship and Zi Ying Scholarship from the Institute of Chinese Culture; Postgraduate Student Grants for Overseas Academic Activities from the Graduate School, The Chinese University of Hong Kong and DAAD Short-term Scholarship for Foreign Doctoral Candidates/Young Academics to study in Germany; and the Yu To Sang Memorial Scholarship 2008/2009 and 2009/2010 from The Chinese University of Hong Kong to pursue PhD for L.L.
**Conflict of interest**

None declared.

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