Upregulated TCR ζ Enhances Interleukin-2 Production in T-Cells from Patients with CML

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T-cell immunodeficiency is a common feature in patients with chronic myeloid leukemia (CML), and deficiency in CD3 levels was detected in T cells from these patients, which may represent a characteristic that is related to a lower T cell activation. In this study, we explored the possibility that forced TCR ζ gene expression may upregulate T cell receptor (TCR) signaling activation and reverse interleukin-2 (IL-2) production in T cells from patients with CML. A recombinant eukaryotic vector expressing TCR ζ was transfected into T cells by nucleofection. Phosphorylated TCR ζ , phosphorylated NF- κ B, and the IL-2 level in TCR ζ -transfected CD3+T cells that were activated with anti-CD3 and anti-CD28 antibodies were measured by Western blot and enzyme-linked immunosorbent assay (ELISA). Significantly increased TCR ζ levels were found in TCR ζ -transfected CD3+T cells. After CD3 and CD28 antibody stimulation, a significantly higher phosphorylated TCR ζ chain level was demonstrated, and an increased IL-2 production in TCR ζ -upregulated T cells was associated with the increased expression of the phosphorylated NF- κ B. In conclusion, TCR ζ gene transfection could restore TCR ζ chain deficiency and enhance IL-2 production in T cells from patients with CML. It is possible that TCR ζ chain reconstitution in leukemia-specific, clonally expanded T cells will effectively increase their activation of antileukemia cytotoxicity.

Introduction

CHRONIC MYELOID LEUKEMIA (CML) accounts for 15%–20% of newly diagnosed cases of leukemia in adults. CML is a clonal disease of hematopoietic stem cells that is characterized by the Philadelphia chromosome (Ph), which is generated by the reciprocal translocation t(9;22) (q34;q11) that results in the fusion of the c-abl oncogene 1 (*ABL1*) with the breakpoint cluster region gene (*BCR*) (Smahel, 2011). The constitutively upregulated tyrosine kinase activity of the chimeric BCR-ABL1 protein affects several intracellular signaling pathways that promote cell proliferation and survival and, thus, contribute to their malignant transformation (Quintas-Cardama and Cortes, 2009; Smahel, 2011). In most cases, the chronic phase (CP) CML can be suppressed by imatinib mesylate (IM), a BCR-ABL1 protein tyrosine kinase inhibitor. However, numerous patients with CML die due to an Abl mutation that is related to drug resistance and blast crisis. Because IM does not kill quiescent leukemia stem cells, these cells persist in a majority of patients and may cause disease relapse. Moreover, advanced-stage CML responds poorly to all therapies, including IM (Savona and Talpaz, 2008; Jabbour *et al.*, 2009; Smahel, 2011). Further studies are needed to better understand this disease and improve patient outcome.

In patients with leukemia, T-cell function becomes suppressed as the disease progresses. Such immune dysfunction, which has been demonstrated in many patients with leukemia, may be due to a disorder in the thymic output function, and the abnormal expression of the T cell receptor (TCR) repertoire and may, in part, be due to abnormal TCR signal transduction, possibly through altered expression of the CD3 genes (Rezvan *et al.*, 1999; Chen *et al.*, 2000; Torelli *et al.*, 2003; Li, 2008; Chen *et al.*, 2009; Li *et al.*, 2010; Li *et al.*, 2011b). In CML, decreased levels of recent thymic emigrants in both CD4+ and CD8+T cells may underlie the persistent immunodeficiency found in patients. Restricted expression of the TCR V β repertoire indicates T cell immunodeficiency in patients, although clonally expanded T cells suggest a specific immune response to leukemia-associated antigens. Deficiency in CD3 gene expression levels may represent a characteristic of lower T cell activation (Chen *et al.*, 2000; Li, 2008; Chen *et al.*, 2009).

The TCR complex is an octameric receptor composed of two chains, $\alpha\beta$ or $\gamma\delta$, that bind to specific ligands that are antigenic peptides presented on major histocompatibility complex molecules. These chains are noncovalently associated with CD3 subunits, which include four transmembrane proteins named CD3 γ , CD3 δ , CD3 ϵ , and CD3 ζ (TCR ζ), that form three distinct dimers (i.e., CD3 $\gamma\epsilon$, CD3 $\delta\epsilon$, and CD3 $\zeta\zeta$).

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and mediate TCR signal transduction (Clevers *et al.*, 1988; Call *et al.*, 2004; Call and Wucherpennig, 2005; Fischer *et al.*, 2005).

The absence of the TCR ζ chain not only influences TCR expression on the cell membrane and the number of single-positive (CD4+ or CD8+) circulating T cells, it also impairs the proliferative response and mature T cell activation level. T cells from patients with leukemia (Chen *et al.*, 2000; Mozaafari *et al.*, 2004; Li, 2008; Chen *et al.*, 2009) are functionally impaired, and this is indicated by decreased TCR ζ chain expression. Recently, our studies have shown the expression pattern of the four CD3 genes in patients with AML, CML, and MM (Li, 2008; Chen *et al.*, 2009; Chen *et al.*, 2011; Li *et al.*, 2011a). Aberrant TCR ζ expression in T-cells from patients with Systemic lupus erythematosus (SLE) may be associated with decreased stability and the translation of a TCR ζ mRNA with an alternatively spliced 3'-untranslated region (Chowdhury *et al.*, 2005). However, the mechanism of TCR ζ deficiency in T-cells from tumors and patients with leukemia remains unclear.

TCR ζ upregulation may improve T cell activation and recover their cytotoxicity function. TCR ζ chain upregulation was first investigated by Nambiar MP and colleagues who transferred a eukaryotic expression vector containing TCR ζ chain cDNA into freshly isolated SLE T cells and indicated that reconstitution of the deficient TCR ζ chain could reverse TCR/CD3-mediated signaling abnormalities and the defective interleukin-2 (IL-2) production in the T cells of patients with SLE (Nambiar *et al.*, 2003). However, little is known about whether TCR ζ upregulation in T cells from CML patients could improve T cell function, which may restore the immunodeficiency status of patients with CML to a certain degree. In this study, we investigated the T cell IL-2 production characteristics after the upregulation of TCR ζ by gene transfer in the T cells of patients with CML.

Materials and Methods

Construction of the TCR ζ -pIRES2-EGFP recombinant plasmid

The plasmid used in this study is based on the pIRES2-EGFP vector (Clontech Laboratories, Inc.), which encodes an internal ribosome entry site followed by the enhanced green fluorescent protein (EGFP). The sequence encoding the full-length TCR ζ gene was amplified from cDNA that was prepared from a healthy individual, and it was cloned between the *NheI* and *EcoRI* restriction sites. The recombinant plasmid was confirmed by double digestion using the *NheI* and *EcoRI* restriction enzymes, and the concentration was adjusted to 0.5 $\mu\text{g}/\mu\text{L}$ (Chen *et al.*, 2012).

Human CD3+ T cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) that were obtained from 12 newly diagnosed, untreated patients with CP CML were isolated from heparinized venous blood by Ficoll-Paque gradient centrifugation. All procedures were conducted according to the guidelines of the Medical Ethics Committee of the Health Bureau of the Guangdong Province of China. The cells were collected, washed twice in the Hank's balanced salt solution, and resuspended at a final concentration of 2×10^6 cells/mL in a complete RPMI 1640

medium (Invitrogen) that was supplemented with 10% heat-inactivated fetal calf serum (HyClone), 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. CD3+ T cells were positively purified from freshly isolated PBMCs from four CML cases using CD3+ microbeads (Miltenyi Biotec) according to the manufacturer's protocol. The purity of the collected CD3+ T cells was assessed by flow cytometry (FCM). More than 95% of the CD3+ T cells were collected using this technique. The peripheral blood from 10 healthy individuals served as control.

TCR ζ chain expression in T cells

TCR ζ chain expression was measured in permeabilized cells by FCM using a phycoerythrin (PE)-conjugated mouse anti-human TCR ζ (6B10.2) mAb (Santa Cruz Biotechnology). For each sample, 10,000 events were acquired, and all cells were included in the forward scatter/side scatter gate. The TCR ζ cell surface density was expressed as the average fluorescence intensity of the analyzed cell populations (mean fluorescence intensity [MFI]). The data were processed using the FlowJo software (Tree Star, Inc.).

TCR ζ gene transduction in T cells

Briefly, cells (5×10^6) were resuspended in 0.1 mL of supplemented Nucleofector solution (human T cell Nucleofector™ kit; Amaxa) at room temperature. TCR ζ -IRES2-EGFP and IRES2-EGFP (5 μg) were mixed with 0.1 mL of cell suspension, transferred to a 2.0-mm electroporation cuvette, and nucleofected using an Amaxa Nucleofector II apparatus according to the manufacturer's guidelines. Storage of the cell suspension in the human T cell Nucleofector solution for longer than 20 min was avoided because this reduces cell viability and gene transfer efficiency. The cells were transfected using the U-014 program. The transfected T cells were immediately transferred to a prewarmed complete culture medium and cultured in 12-well plates in a humidified incubator at 37°C and 5% CO₂ (Chen *et al.*, 2012).

Transfection efficiency determination

The transfection efficiency was estimated in each experiment by scoring the number of double-positive CD3+ and GFP cells stained with a PE-conjugated mouse anti-human CD3 antibody (eBioscience) 18 h post-transfection using FCM analysis. For analysis, the relative fluorescence density (Log transformed) of the live cells was measured using a multi-color FACScan flow cytometer (Calibur; BD Biosciences). The data were analyzed using the FlowJo software (Tree Star).

Western blot analysis

CD3+ T cells at $1 \times 10^6/0.2 \text{ mL}$ were stimulated with 10 $\mu\text{g}/\text{mL}$ OKT3 for 2 min at 37°C 18 h after transfection. The reaction was stopped by the addition of 1 mL of ice-cold stop buffer (PBS, 5 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, and 0.4 mM sodium vanadate), and the cell pellets were solubilized in RIPA lysis buffer (1 $\times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{mL}$ aprotinin, and 100 mM sodium orthovanadate) and incubated on ice for 30 min to isolate total protein. Proteins (20 μg) were separated using 12% SDS-PAGE and transferred to a PVDF membrane (Pall) using a damp-dry transfer device

(Bio-rad). After blocking for 1 h in 5% defatted milk powder in PBS, the membrane was washed and probed with 1:200 mouse anti-human TCR ζ or phosphorylated TCR ζ (Santa Cruz) or 1:1000 rabbit anti-human phosphorylated NF- κ B (p65 subunit) monoclonal antibody (CST). Similar studies were performed using 1:500 mouse anti-human β -actin (CST). The antibodies were detected using 1:10,000 horseradish peroxidase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch). A Western blotting luminol reagent (Lianke) was used to visualize the bands corresponding to each antibody (Nambiar *et al.*, 2003). Blot images were captured using an Alliance 4.7 Gel doc system (UVI), and densitometric analysis of the bands was performed using ImageJ software (<http://rsb.info.nih.gov/ij>).

Enzyme-linked immunosorbent assay

Cell supernatants were collected from the TCR ζ -IRES2-EGFP or IRES2-EGFP groups 24 h after OKT3 (CD3) (10 μ g/mL) and anti-human CD28 antibody (2.5 μ g/mL) stimulation, and the IL-2 levels were measured using the human IL-2 enzyme-linked immunosorbent assay (ELISA) Kit (Raybiotech) according to the protocol of the manufacturer.

Statistical analysis

Statistical analyses were performed using the Independent Samples t-test for TCR ζ , phosphorylated TCR ζ , and phosphorylated NF- κ B protein levels in the TCR ζ -IRES2-EGFP or IRES2-EGFP groups. Differences with a $p < 0.05$ were considered statistically significant.

Results

Transfection and expression of the TCR ζ chain in cell lines

The full-length TCR ζ gene was amplified, and its sequence was confirmed by restriction enzyme digestion analysis and sequencing. The TCR ζ gene sequence (537 bp) was confirmed by comparison with the TCR ζ gene sequence in the NCBI gene bank (data not shown). The pIRES plasmid, which contains the enhanced green fluorescent protein (IRES2-EGFP), was used as a positive control and to construct TCR ζ recombinant plasmids (TCR ζ -IRES2-EGFP). The TCR ζ recombinant vector and IRES2-EGFP were first transferred into K293 and Jurkat cell lines by Nucleofection. Indirect immu-

FIG. 1. Immunofluorescence detection (200 $\times$) of enhanced green fluorescent protein (EGFP) expression to measure the TCR ζ -IRES2-EGFP transfection efficiency in K293 (A) and Jurkat cells (B) and Western blot analysis of TCR ζ protein expression in K293 (C, 1–2) and Jurkat cells (C, 3–4) 24 h post-transfection. Color images available online at www.liebertpub.com/dna

nofluorescence and Western blot were used to verify the transfection efficiency and TCR ζ protein expression, respectively, 24 h post-transfection (Fig. 1A, B). The results indicated that the TCR ζ recombinant vector was successfully

FIG. 2. The TCR ζ expression level in peripheral blood mononuclear cells (PBMCs) from patients with chronic myeloid leukemia (CML) as determined by flow cytometry (FCM) analysis. (A) Comparison of the mean fluorescence intensity (MFI) of TCR ζ in PBMCs between healthy individuals (HI) (17.2 ± 5.7 , $n = 10$) and patients with CML (8.5 ± 4.9 , $n = 12$). (B) Example of the shift in the MFI of TCR ζ in PBMCs from a patient with CML relative to the MFI (PE-A) of TCR ζ in PBMCs of a healthy individual. PE-A = fluorescence intensity of PE. Color images available online at www.liebertpub.com/dna

constructed and could be used to upregulate the TCR ζ gene in primary T cells by gene transfer.

TCR ζ overexpression in T cells from patients with CML

The TCR ζ chain expression level was detected in PBMCs from patients with CP CML by counting the MFI using FCM, and a significantly lower MFI of TCR ζ in PBMCs from patients with CML (8.5 ± 4.9 , $n=12$) in comparison with those of healthy individuals (17.2 ± 5.7 , $n=10$; $p=0.001$) was found

(Fig. 2). Thus, CD3+ T cells were sorted from the PBMCs of four patients with CML that had TCR ζ deficiency. The CD3+ T cell purity was evaluated by fluorescent microscope detection. More than 95% of the isolated cells were CD3-positive (data not shown). The CD3+ T cells were transfected with TCR ζ -IRES2-EGFP and IRES2-EGFP by nucleofection (Fig. 3A). The transfection efficiencies were determined by FCM analysis, and the results demonstrated that the cells double-positive for CD3+ and GFP were $58.3\% \pm 19.2\%$ and $57.2\% \pm 19.4\%$ for TCR ζ -IRES2-EGFP and IRES2-EGFP

A

FIG. 3. The transfection efficiencies of TCR ζ -IRES2-EGFP or IRES2-EGFP transfected CD3+ T cells as determined by fluorescence microscopy (**A**) and FCM detection (**B**) 18 h post-transfection. FITC-A = the fluorescence intensity of FITC. Color images available online at www.liebertpub.com/dna

B

transfected CD3+T cells, respectively, 18 h post-transfection (Fig. 3B). After 18 h, the cells were lysed, and the TCR ζ level was measured by Western blot analysis. A significantly increased TCR ζ level was found in the TCR ζ -IRES2-EGFP transfected CD3+T cell group (gray value: 113.0 ± 19.9) compared to that of the controls (gray value: 17.8 ± 8.6 , $p=0.003$) (Fig. 4).

IL-2 production in TCR ζ chain upregulating T cells from patients with CML

After stimulation with anti-CD3 antibodies to activate the TCR ζ -transfected T cells, the phosphorylated TCR ζ chain expression level was measured by Western blot analysis. A significantly higher phosphorylated TCR ζ chain level was observed in the TCR ζ -transfected CD3+T cell group (gray value: 61.5 ± 11.7) than in the control group (gray value: 17.2 ± 12.9 ; $p=0.006$) (Fig. 4). A lower T cell activation level has been described in CML T cells, and to investigate whether upregulating TCR ζ in CML T cells could reconstitute IL-2 production, we measured the IL-2 levels in TCR ζ -transfected CD3+T cells that were activated with anti-CD3 and anti-CD28 antibodies using an ELISA. As shown in Figure 5, the amount of IL-2 secretion was significantly higher in the TCR ζ -transfected CD3+T cell group (180.4 ± 73.9 pg/mL) compared with that of the control group (78.4 ± 18.2 pg/mL) ($p=0.017$) (Fig. 5). To determine whether an increased IL-2 production was associated with restoration of the NF- κ B level, we further analyzed the phosphorylated NF- κ B level. In comparison with the control group (gray value: 27.57 ± 16.83), a significantly higher phosphorylated NF- κ B level was found in the TCR ζ -IRES2-EGFP-transfected CD3+T

FIG. 5. The interleukin-2 (IL-2) levels in TCR ζ -IRES2-EGFP or IRES2-EGFP transfected CD3+T cell groups ($n=4$).

cell group (gray value: 49.21 ± 20.55) ($p=0.02$) (Fig. 6). Thus, the increased IL-2 production in TCR ζ upregulated T cells may associate with increased phosphorylated NF- κ B (p65 subunit) in these T cells.

Discussion

T cell modification by the transfer of different target genes that enhance or suppress primary T cell function has been described in previous studies. The first TCR gene transfer

FIG. 4. The expression levels of TCR ζ and phosphorylated TCR ζ as determined by Western blot analysis. (A) Western blot results, P1–P4: CML samples, HI: healthy individual control, (B) Comparison of the expression levels of TCR ζ and phosphorylated TCR ζ between the TCR ζ -IRES2-EGFP and IRES2-EGFP transfected CD3+T cell groups ($n=4$).

FIG. 6. The expression level of phosphorylated NF- κ B by Western blot analysis. (A) Western blot results, P1–P4: CML samples, HI: healthy individual control, (B) Comparison of the expression levels of phosphorylated NF- κ B between the TCR ζ -IRES2-EGFP and IRES2-EGFP transfected CD3+T cell groups ($n=4$).

into primary human T lymphocytes was accomplished in a study of a melanoma antigen, and CD8⁺T cells transduced with a TCR that is specific for MART-1 were able to lyse an HLA-A2⁺ melanoma cell line *in vitro* (Clay *et al.*, 1999). The successful transfer of genes encoding TCR $\alpha\beta$ chains, which recognize a variety of virus-specific and tumor-associated antigens, into primary T cells has been demonstrated (Xue *et al.*, 2005; Engels and Uckert, 2007; Heemskerk, 2010; Yin *et al.*, 2011; Stroncek *et al.*, 2012). In comparison with the transfer of both the TCR α and TCR β genes, which leads to the expression of the TCR $\alpha\beta$ heterodimer in T-cells, the transfer of the TCR ζ gene into primary T cells appeared to be easier. Using nucleoporation, successful upregulated TCR ζ expression was achieved in SLE T cells that were transfected with a TCR ζ chain containing eukaryotic expression vector at high efficiency (Nambiar *et al.*, 2003). In this study, after the successful construction of a recombinant eukaryotic vector, we attempted to deliver the TCR ζ gene into freshly sorted CD3⁺T cells from patients with CP CML.

Previous studies have shown decreased expression in the level of the TCR ζ gene and impaired TCR ζ expression in patients with CP CML (Chen *et al.*, 2000; Chen *et al.*, 2009), and we further confirmed the surface expression of the TCR ζ chain in T cells from patients with CML by FCM, which is similar to the decreased TCR ζ chain expression result that was found in T-cells in 12 cases with CP CML. After TCR ζ transfection, TCR ζ expression was increased, and phosphorylated TCR ζ was increased in modified T cells after CD3 and CD28 monoclonal antibody stimulation. These data indicate that TCR/CD3 signaling may be reversed in CML T cells after upregulating TCR ζ .

It has been demonstrated that forced TCR ζ chain expression could reverse TCR/CD3-mediated signaling abnormalities and defective IL-2 production in SLE T cells (Nambiar *et al.*, 2003). In this study, similar results were found in T cells from patients with CP CML who had different immunodeficiency statuses. When TCR ζ was upregulated in T cells from patients with CML, their activation function in IL-2 production was enhanced, and this was associated with an increase in phosphorylated NF- κ B. NF- κ B plays a central role in regulating inflammatory and immune responses (Sakurai *et al.*, 2003). NF- κ B activated by T cell costimulation can induce the expression of IL-2 in T cells (Schmitz *et al.*, 2003). The low level of NF- κ B (p65 subunit) was associated with a decreased production of IL-2 in SLE T cells, and reconstitution of p65 NF- κ B can restore CD3-mediated IL-2 production in SLE T cells (Herndon *et al.*, 2002). In this study, we tried to prove that the reconstitution of TCR ζ chain could increase phosphorylated NF- κ B (p65 subunit), and then promote the IL-2 production, and it is possible that restoration of the TCR ζ chain in T cells from CML will increase their antileukemia cytotoxic ability. Further investigation is needed to evaluate the anti-CML cytotoxicity of TCR ζ -transfected T cells.

The upregulation of TCR ζ expression could be stimulated in different ways using different active factors, including cytokines, such as IL-2 and IFN- γ (Chen *et al.*, 2000; Frydecka *et al.*, 2001), and anti-CD3 and anti-CD28 monoclonal antibodies (Renner *et al.*, 1996). It has been reported that dexamethasone primarily acts at the transcriptional level, and it differentially modulates TCR ζ chain expression in human

T cells by the increased anti-CD3 antibody-induced tyrosine phosphorylation of the TCR ζ chain and the downstream signaling intermediates ZAP-70 and PLC γ , which increase and sustain the TCR/CD3-mediated [Ca(2⁺)] (i) response (Nambiar *et al.*, 2001). However, decreased TCR ζ chain expression in patients with T-cell acute lymphoblastic leukemia was not reversed by immunotherapy using IL-2 and anti-CD3 alone or in combination (Torelli *et al.*, 2003). Our previous study has shown that TCR ζ expression in T cells from patients with CML-CP could not be completely upregulated after IL-2 or PHA stimulation (Chen *et al.*, 2009). These findings suggested that stimulation plays a minor role in restoring this impaired T cell signal transduction in CML. Therefore, the establishment of a TCR ζ gene transfer technique is required for deficient TCR ζ chain reconstitution in T cells from patients with CML, and it may also be useful for restoring T cell function in different patients with cancer. Further studies are needed to investigate the mechanisms responsible for upregulating the TCR ζ chain in T cells from patients with CML.

Adoptive immunotherapy of malignant diseases using tumor-specific cytotoxic T cells showed remarkable efficacy in recent trials. Such cytotoxic T cells possess not only specific TCRs that recognize the tumor-associated antigens expressed on tumor cells, they also have active TCR signaling, which transduces the immune response and performs cytotoxic functions (Yin *et al.*, 2011; Stroncek *et al.*, 2012). The combined transfection of specific TCR and TCR ζ genes in T cells may be an ideal method to manufacture redirected T cells for immunotherapy. For example, the transgenic expression of the CD3 ζ signaling molecule chimeric antigen receptor, which is specific for CMV, recovered hyporesponsive T cells to full effector functions, released cytokines and mediated redirected cytotoxicity as efficiently as younger effector T cells (Rappl *et al.*, 2012). Clonally expanded T cells from patients with leukemia possess a leukemia-associated, antigen-specific TCR (Zha *et al.*, 2011), and these T cells have a specific cytotoxic ability against antileukemia cells; however, their activation was poor due to deficiencies in TCR signaling, including decreased TCR ζ expression (Chen *et al.*, 2000; Li, 2008; Chen *et al.*, 2009). Thus, restoring their activation function can enhance their antileukemia function. Transfecting the TCR ζ gene into T cells from such patients with leukemia may be an alternative approach to establish an ideal immunotherapeutic strategy.

In conclusion, this study described the construction of a TCR ζ recombinant vector that was successfully delivered and expressed in primary T cells from patients with CP CML. TCR ζ gene transfection could restore TCR ζ chain expression and enhance IL-2 production in T cells from patients with CML. It is possible that reconstitution of the TCR ζ chain in leukemia-specific, clonally expanded T cells will effectively increase their antileukemia cytotoxic ability.

Authors' Contributions

Y.Q.L. contributed to the concept development and study design. X.F.Z., S.H.C., L.J.Y., S.L., and B.L. performed the laboratory studies. X.L.W. and Y.H.L. were responsible for collection of clinical data. Y.Q.L. and X.F.Z. coordinated the study and helped draft the manuscript. All authors read and approved the final manuscript.

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Disclosure Statement

The authors declare that they have no competing interests.

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