Drug-Related Genomics in Cancer and Immunological Diseases

Guest Editors: Ji-Fu Wei, Yong-Qing Wang, and Huai-Rong Luo
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Contents

Drug-Related Genomics in Cancer and Immunological Diseases, Ji-Fu Wei, Yong-Qing Wang, and Huai-Rong Luo
Volume 2014, Article ID 306980, 2 pages

High Expression of Polo-Like Kinase 1 Is Associated with Early Development of Hepatocellular Carcinoma, Wei Sun, Qi Su, Xiankui Cao, Bin Shang, Aishan Chen, Hongzhuan Yin, and Baolin Liu
Volume 2014, Article ID 312130, 9 pages

Genetic Variations of Cytokines and Cytokine Receptors in Psoriasis Patients from China, Xiao-Lan Li, Chun-Feng Wu, and Gui-Sheng Wu
Volume 2014, Article ID 870597, 5 pages

In Silico Prediction of T and B Cell Epitopes of Der f 25 in Dermatophagoides farinae, Xiaohong Li, Hai-Wei Yang, Hao Chen, Jing Wu, Yehai Liu, and Ji-Fu Wei
Volume 2014, Article ID 483905, 10 pages

Association between NFKB1−94ins/del ATTG Promoter Polymorphism and Cancer Susceptibility: An Updated Meta-Analysis, Xiao Yang, Pengchao Li, Jun Tao, Chao Qin, Qiang Cao, Jinbao Gu, Xiaoheng Deng, Jun Wang, Xuzhong Liu, Zijie Wang, Bian Wu, Min Gu, Qiang Lu, and Changjun Yin
Volume 2014, Article ID 612972, 8 pages

Upregulated PD-1 Expression Is Associated with the Development of Systemic Lupus Erythematosus, but Not the PD-1.1 Allele of the PDCD1 Gene, Qingqing Jiao, Cuiping Liu, Ziliang Yang, Qiang Ding, Miaomiao Wang, Min Li, Tingting Zhu, Hua Qian, Wei Li, Na Tu, Fumin Fang, Licai Ye, Zuo Tao Zhao, and Qihong Qian
Volume 2014, Article ID 950903, 6 pages

A Promoter Region Polymorphism in PDCD-1 Gene Is Associated with Risk of Rheumatoid Arthritis in the Han Chinese Population of Southeastern China, CuiPing Liu, JueAn Jiang, Li Gao, XiaoHan Hu, FengMing Wang, Yu Shen, GeHua Yu, ZuoTao Zhao, and XueGuang Zhang
Volume 2014, Article ID 247637, 8 pages

Molecular Evolution of the Vertebrate FK506 Binding Protein 25, Fei Liu, Xiao-Long Wei, Hao Li, Ji-Fu Wei, Yong-Qing Wang, and Xiao-Jian Gong
Volume 2014, Article ID 402603, 9 pages
Drug-related genes are defined as the genes which encode enzymes, transporters, and targets related to drug absorption, distribution, and metabolism to excretion. The studies on drug-related genomics in cancer and immunological diseases could enhance the understanding of the role of drug-related genes in the occurrence and development of cancer or immunological diseases. Pharmacogenomics research can help clinicians predict the efficacy of drugs in the body and avoid some side effects. In this respect, this special issue will add a few new points in the picture of drug-related genomics in cancer and immunological diseases.

C. Liu et al. in “A promoter region polymorphism in PDCD-1 gene is associated with risk of rheumatoid arthritis in the Han Chinese population of Southeastern China” provide an association between PDCD-1 polymorphism rs36084323 and rheumatoid arthritis (RA) risk. The potential mechanism might be related to the association between unregulated expressions of PDCD-1 on activated CD4+T cells in RA patients. They showed that the SNP rs36084323 could be a biomarker of early diagnosis of RA and a suitable indicator of utilizing PDCD-1 inhibitor for the treatment of RA.

In the study “Upregulated PD-1 expression is associated with the development of systemic lupus erythematosus, but not the PD-1 allele of the PDCD1 gene,” Q. Jiao et al. found that the PD-1 expression levels of systemic lupus erythematosus (SLE) patients were significantly increased compared with those of the healthy controls. Furthermore, the unregulated PD-1 expression levels in SLE patients were greatly associated with SLEDAI scores. These results suggest that increased expression of PD-1 may correlate with the pathogenesis of SLE, unregulated PD-1 expression may be a biomarker for SLE diagnosis, and PD-1 inhibitor may be useful for SLE treatment.

The association between cytokines and cytokine receptors and psoriasis is discussed in “Genetic variations of cytokines and cytokine receptors in psoriasis patients from China” by X.-L. Li et al. They genotyped seven SNPs in candidate genes of six ILs and only the rs3212277 in the IL12B gene was found to be associated with psoriasis at genotypic level in the studied population. Furthermore, the studied Chinese population has extremely low minor allele frequency for IL23R. Together, the author discussed the unique genetic patterns in the Chinese population that may be in part responsible for the lower risk for psoriasis in this population.

Nuclear factor-kB was found to be associated with the pathogenesis of numerous malignancies. In the review article “Association between NFKB1 -94ins/del ATTG promoter polymorphism and cancer susceptibility: an updated meta-analysis,” X. Yang et al. showed that the SNP was significantly associated with cancer risk in four genetic models. The stratified analyses revealed that the polymorphism can exert race- and cancer-specific effects on cancer risk.

Polo-like kinase 1 (PLK1), one of serine/threonine-protein kinases, has been demonstrated to play pivotal roles in malignant transformation. “High expression of polo-like kinase 1 is associated with early development of hepatocellular carcinoma” by W. Sun et al. showed that expression of PLK1 was increased significantly in HCC tissues than that of corresponding normal liver tissues and PLK1 was related to the HCC cell differentiation or capsule invasion. Their results also indicated that the potential mechanisms of PLK1 inhibition regulated cell growth involving the dysregulated
expression of caspase, Bcl, and p53. Therefore, the PLK1 expression might be an independent prognostic factor for HCC and targeting PLK1 might be a useful strategy for diagnosis and treatment of human HCC.

In the study "Molecular evolution of the vertebrate FK506 binding protein 25," F. Liu et al. calculated the nonsynonymous and synonymous substitution rates, suggesting that FKBP25 undergoes purifying selection throughout the whole vertebrate evolution. Moreover, the result of site-specific tests showed that no sites were detected under positive selection. Only one PPIase domain was detected by searching FKBP25 sequences at Pfam and SMART domain databases. The result of this study suggests that the purifying selection triggers FKBP25 evolutionary history, which allows us to discover the complete role of the PPIase domain in the interaction between FKBP25 and nuclear proteins.

The house dust mites are major sources of indoor allergens for humans. X. Li et al. discussed the relation between Der f 25 and dermatophagoides farinae in "In silico prediction of T and B cell epitopes of Der f 25 in dermatophagoides farinae." The sequence and structure analysis by them identified that Der f 25 belongs to the triosephosphate isomerase family and exhibited a triosephosphate isomerase pattern (PS001371). Eight B cell epitopes (11–18, 30–35, 71–77, 99–107, 132–138, 173–187, 193–197, and 211–224) and five T cell epitopes including 26–34, 38–54, 66–74, 142–151, and 239–247 were predicted in this study. These results can be used to benefit allergen immunotherapies and reduce the frequency of mite allergic reactions.

Ji-Fu Wei
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High Expression of Polo-Like Kinase 1 Is Associated with Early Development of Hepatocellular Carcinoma

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Polo-like kinase 1 (PLK1), one of serine/threonine-protein kinase, has been demonstrated to play pivotal roles in malignant transformation. Here we illustrated the clinicopathological significance of PLK1 expression in hepatocellular carcinoma (HCC) in more detail. Immunohistochemistry was performed to detect the expression of PLK1 in 67 HCC patients as well as corresponding noncancerous liver tissues. In addition, the correlation of PLK1 expression with clinicopathological factors or prognosis of HCC was analyzed. Results showed that the expression of PLK1 was increased significantly in HCC tissues than that of corresponding normal liver tissues. The correlation between PLK1 and HCC cell differentiation or capsule invasion was also revealed. We found that PLK1 inhibition promoted cell arrest in G2/M phase of cell cycle and cell apoptosis. Our results also indicated that the potential mechanisms of PLK1 inhibition regulating cell growth involved enhancing expression of caspase3, caspase8, and Bax and decreasing expression of Bcl-2. Furthermore, we also found that PLK1 downregulation inducing inhibition of cell growth was associated with enhancing expression of p53. Thus, we presume that the status of PLK1 expression might be an independent prognostic factor for HCC and targeting PLK1 might be a useful strategy for diagnosis and treatment of human HCC.

1. Introduction

Cancer is a complicated disease that develops slowly due to gradual accumulation of genetic and epigenetic alterations over time [1, 2]. Tumor cells often harbor mutations followed by activated oncogene expression and inactivated tumor-suppressor expression. These alterations combined with dysregulation of cell division, one of the hallmarks of the cancer phenotype [1]. In spite of the universal property of tumors, many important issues remain to be illustrated, including whether the order of the unsuccessful alterations is critical to cellular malformation and how mutations of cancer pathways are involved in the development of cancer disease.

Polo-like kinases (PLKs) as a family of proteins that has a significant role in the maintenance of mitotic integrity have attracted attention. Currently, five members of the polo-like kinase family have been identified in human (PLK1-5) [3]. Among them, PLK1 is best studied which is essential mitotic kinase that controls mitotic entry, centrosome maturation, bipolar spindle formation, cohesion dissociation, and chromosome congression and segregation, as well as cytokinesis [4–6]. The levels of PLK1 rise in G2 phase and arrive at the peak in M phase. The expression of PLK1 is increased in many types of tumor, including cancer in brain, breast, colon, head and neck, lung, pancreas, bile duct, bladder, and prostate as well as hepatocellular carcinoma (HCC). Moreover, its expression often correlates with poor diagnosis [7]. Its overexpression results in multinucleation and overrides the G2 arrest checkpoint which is induced by DNA damage. Meanwhile, the p53 tumor-suppressor protein is phosphorylated by PLK1, which can inhibit the proapoptotic function of p53. The inhibition of PLK1 leads to a failure to complete mitosis, eventually resulting in cell death [8]. Therefore, PLK1 expression shows a close relationship with cancer development and its small molecule inhibitors have been used in clinical trials in advanced cancer patients.

HCC is the third most common cancer in the world with more than 500,000 deaths a year [9]. It is reported that
Table 1: The relationship of PLK1 expression and HCC clinicopathological variables.

| Clinicopathological factors                  | n   | PLK1 expression | Positive incidence |
|----------------------------------------------|-----|-----------------|--------------------|
|                                              |     | None | Low | High |                |                    |
| Age (yr)                                      |     |      |     |      |                |                    |
| >50                                          | 39  | 8    | 12  | 19   | 78.5%           |                    |
| ≤50                                          | 28  | 8    | 9   | 11   | 71.4%           |                    |
| Clinical stage                                |     |      |     |      |                |                    |
| Phases I + II                                 | 46  | 12   | 14  | 20   | 73.9%           |                    |
| Phase III                                    | 21  | 4    | 7   | 10   | 81.0%           |                    |
| Tumor nodule                                 |     |      |     |      |                |                    |
| 1                                            | 48  | 12   | 12  | 24   | 75.0%           |                    |
| ≥2                                           | 19  | 4    | 9   | 6    | 78.9%           |                    |
| Tumor diameter                                |     |      |     |      |                |                    |
| >5 cm                                        | 44  | 12   | 14  | 18   | 72.7%           |                    |
| ≤5 cm                                        | 23  | 4    | 7   | 12   | 82.6%           |                    |
| Lymphatic metastasis                         |     |      |     |      |                |                    |
| Yes                                          | 5   | 2    | 2   | 1    | 60.0%           |                    |
| No                                           | 62  | 14   | 19  | 29   | 77.4%           |                    |
| Extrahepatic metastasis                      |     |      |     |      |                |                    |
| Yes                                          | 10  | 4    | 3   | 3    | 60.0%           |                    |
| No                                           | 57  | 12   | 18  | 27   | 78.9%           |                    |
| Capsule invasion                              |     |      |     |      |                |                    |
| Yes                                          | 27  | 10   | 8   | 9    | 63.0%**         |                    |
| No                                           | 40  | 6    | 13  | 21   | 85.0%           |                    |
| Tumor thrombi in portal vein                 |     |      |     |      |                |                    |
| Yes                                          | 15  | 4    | 5   | 6    | 73.3%           |                    |
| No                                           | 52  | 12   | 16  | 24   | 76.9%           |                    |
| Histological differentiation (Sugihara grading criteria) |     |      |     |      |                |                    |
| Well-                                        | 26  | 1    | 7   | 18   | 96.2%*          |                    |
| Moderate/poor-                               | 41  | 15   | 14  | 12   | 63.4%           |                    |
| Total                                        | 67  | 16   | 21  | 30   | 76.1%           |                    |

The P value is less than 0.01, when HCC with capsule invasion is compared with HCC without capsule invasion. The P value is less than 0.05, when HCC in well differentiation is compared with HCC in moderate or poor differentiation.

*P < 0.05, **P < 0.01.

the number of HCC cases increases every year [10]. Although several factors for HCC have been revealed, including infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), there is still no effective treatment for this cancer type at present, for the potential mechanisms in both molecular level and cellular level of HCC pathogenesis remained poorly clarified [11–13]. Previous studies have revealed that centrosome abnormality and upregulation of PLK1 were observed in HCC [14, 15]. So far, the correlation of PLK1 and HCC in clinicopathological variable is not well described and the corresponding potential mechanism should be illustrated.

In this study, we demonstrated that PLK1 was expressed in human multiple HCC cell lines (HepG2 and BCL-7402) and liver samples from HCC patients. We found that increased PLK1 expression not only is an adverse prognostic factor for HCC but also is associated with HCC early development. Our further studies demonstrated that PLK1 inhibition by siRNA can significantly downregulate hepatocellular cell cycle progression and promote cell apoptosis. Therefore, we presume that PLK1 expression can be the important marker for HCC diagnosis; moreover, PLK1 may be the target for cancer treatment.

2. Methods

2.1. Human Tissue Samples. Normal livers (n = 5), 67 HCCs patients (n = 67, which including 27 cases). Normal livers were from autopsy cases of healthy individuals. The clinical data of patients involved in this study was shown in Table 1. Liver tissues were kindly provided by Department of Pathology, Shengjing Hospital, China Medical University. The protocol of this study was approved by the ethics committee of the institution involved, and informed consent was obtained from all the subjects.

2.2. Cell Line and Treatment. HepG2 and BCL-7402, two human HCC cell lines, were provided by Department of Cell Biology, Institute of Basic Medical Science, China Medical
University. The sequences of small interfering RNA (siRNA) were as follows:

- siRNA1: sense: 5'-UGAAGAGAAGAUCCCCC-UDTTdT-3';
- antisense: 5'-AGGAGGGUGAUCUUCUCA-UTdT-3';
- siRNA2: sense: 5'-GCACCGAACCAGGUUAU-UDdTdT-3';
- antisense: 5'-AATAACTCGGTTTCGCTGdCdTdT-3';
- siRNA negative control: sense: 5'-UUCUCCGAA-CGUGUCACGUTT-3';
- antisense: 5'-ACGUGACACGUGUCGAGATT-3'.

2.3. Cell Apoptosis and Cell Cycle Analysis. For apoptosis assay, apoptotic cells were measured by staining with annexin V-PI provided in an apoptosis detection kit (BD Pharmingen, USA), according to the manufacturer's instructions. Stained cells were analyzed with a flow cytometry. Analysis of cell cycle distribution was performed by means of flow cytometry of 2,4-diamidino-2-phenylindole-stained nuclei with BD FACScalibur using the multicycle program.

2.4. Western Blot Analysis. For western blot testing, cells were lysed with RIPA (150 mM NaCl, 10 mM Tris-HCl [pH 8.0]), 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS], and 5 mM EDTA containing 0.7% phenylmethylsulfonyl fluoride and proteinase inhibitor mixture. Protein aliquots (30 μg) for detection of PLK1 and p53 were mixed with an equal amount of 2× SDS sample buffer, boiled at 98°C for 5 min, centrifuged, and separated by 10% SDS-PAGE. The gels were then transferred onto polyvinylidene fluoride membranes (Millipore, MA). Membranes were blocked with 5% nonfat milk powder in Tris-buffered saline (TBS)/Tween 20 (TBS/T) and washed three times with TBS/T. Primary anti-p53 antibody (Santa Cruz Biochemicals, USA), anti-PLK1 antibody (Merck Drugs & Biotechnology, USA) were added in TBS/T containing 5% bovine serum albumin at suitable dilution and kept overnight at 4°C. Membranes were washed with TBS/T, and horseradish peroxidase- (HRP-) conjugated secondary IgG (Cell Signaling Technology, USA) were added at a dilution of 1/2,000 in TBS/T for 1h at room temperature (RT). For control of equal protein loading, membranes were incubated overnight with primary antibody to β-actin (Sigma, USA) (1/1,000; diluted in TBS/T containing 5% bovine serum albumin), again washed extensively with TBS/T, and incubated for 2 h with blocking solution containing HRP-conjugated rabbit-anti-mouse IgG, and signal was detected by ECL (Invitrogen, USA).

2.5. Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction. Total RNA was isolated from cell pellets with the RNeasy Mini Kit (Qiagen, Germany). Genomic DNA was removed from total RNA before cDNA synthesis with the RNase-free DNase set for DNase digestion during RNA purification (Qiagen). RNA was stored at −80°C. First-strand cDNA synthesis was performed for each RNA sample with the Sensiscript RT Kit (Qiagen). Random hexamers were used to prime cDNA synthesis.

mRNA expression was detected by real-time polymerase chain reaction (PCR) with the SYBR Green master mix (Applied Biosystems, USA). Thermocycler conditions comprised an initial holding at 50°C for 2 min and then at 95°C for 10 min. This was followed by a two-step PCR program consisting of 95°C for 15 sec and 60°C for 60 sec for 40 cycles. Data were collected and quantitatively analyzed on an ABI Prism 7900 sequence detection system (Applied Biosystems). The β-actin gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. All quantities were expressed as number of folds relative to the expression of β-actin. Primers used in this study were as follows:

- β-actin: sense 5'-GAGAAGAGCTACGAGCTGCC TGA-3';
- antisense: 5'-ATCTTCTATTGCTGGTGTCGCC-3';
- PLK1: sense 5'-CTGCTGGCATCCCCCATCTTC-3';
- antisense: 5'-CACCATAGTGCGGGCGTAC-3';
- p53: sense 5'-TGACTGTACCACATCCACTACA ACTA-3';
- antisense: 5'-GGCGGGAGGTAGACTGCC-3'.

2.6. Immunohistochemistry. Immunohistochemistry was used to determine the expression of PLK1. Deparaffinized sections (4 μm) were treated with 3% hydrogen peroxide in methanol for 10 min at RT to block endogenous peroxidase activity. Then the sections were incubated in citrate buffer for 10 min at 121°C and cooled to RT. After blocking with 10% bovine serum albumin for 1 h at RT, the slides were subsequently incubated overnight with anti-PLK1 at a dilution of 1:75. After extensive washing with PBS, the slides were incubated with secondary Ab for 30 min. The sections were then counterstained with DAB (Maxim, China).

A semi quantitative evaluation was performed by two independent observers, who were blinded to the clinical and pathological stage of the patients, on two separate occasions. The intensity of specimen staining was scored as follows: ++: strong; +: moderate; ±: weak; and −: not detectable. The extent of positive staining was further categorized into four groups (0, <30%, 30–60%, and >60%), on the basis of the proportion of the positively stained area [16].

2.7. Transmission Electronic Microscope. After human HCC cell line BCL-7402 was transfected with PLK1 siRNA for 48 h, the cells were washed and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 4°C. Samples were then rinsed in 0.1 M cacodylate buffer and postfixed for 1 h in 1% osmium tetroxide in the same buffer. This procedure was followed by dehydration and embedding in araldite resin. Ultrathin sections were double-stained with uranyl acetate-lead citrate and observed under a JEM-1200EX transmission electron microscope at 80 kV.
2.8. Statistical Analysis. The $\chi^2$ and Fisher exact probability tests were used to examine associations between PLK1 expression and various other parameters including clinicopathological characteristics. Difference between two groups or among more than two groups was performed by Student's $t$-test and one-way ANOVA after analyzing the variance. The levels of significance were set to $\alpha \leq 0.05$.

3. Results

3.1. PLK1 Expression Is Significantly Upregulated in Human HCC. To investigate the role of PLK1 in human hepatocarcinogenesis, we determined its expression in normal human livers, HCCs, and surrounding cirrhosis tissues using immunohistochemistry. There was no PLK1 expression in normal liver (Figure 1). However, PLK1 expression in HCC samples was significantly increased compared with normal liver (Figure 1). To facilitate interpretation of the results, the patient was evaluated in different criteria. We found that PLK1 expression has no correlation with patient age, clinical stages, tumor nodules, tumor diameter, lymphatic metastasis, extrahepatic metastasis, with or without tumor thrombi in portal vein, HBV positive, HCV positive, and Child-Turcotte grading system for liver function evaluation. Interestingly, PLK1 expression was related to histological grading of tumor and capsule invasion. According to Sugihara grading criteria, HCCs were classified into well differentiated tumor, moderate differentiated tumor, and poor differentiated tumor. Notably, the expression of PLK1 in well differentiated tumor cells was significantly higher than moderate and poor tumor cells, but there was no significant difference between moderate tumor cells and poor tumor cells. In addition, PLK1 expression in different cells was different. This may be due to the different status of tumor cells. Therefore, we presume that PLK1 is associated with HCC development. Also, PLK1 expression in HCC without capsule invasion was more than that with capsule invasion (Table 1), which suggested less correlation of PLK1 expression with malignancy of HCC. More interesting is that, in 27 cases of cirrhosis from HCC, the expression of PLK1 in cirrhosis was positive either; moreover, 22 cases showed higher levels of PLK1 than that of cancer tissues (data not shown). We found that there is similar expression of PLK1 in cirrhosis with 3 paired HCC tissues and only 2 cases were lower than paired HCC tissues (data not shown). The difference of PLK1 expression in HCC nidus and surrounding cirrhosis tissues indicated that PLK1 may play an important role in HCC early development and its inhibition might contribute to the control of HCC.

3.2. PLK1 Inhibition Induces Growth Arrest in Hepatocellular Cell Lines. Using western blot, HepG2 and BCL-7402 cell lines were assayed for PLK1 expression. The assay on the above two cell lines revealed higher PLK1 expression (Figure 2(a)). In view of similar expression of PLK1 in both of the cell lines, we further select BCL-7402 cell lines for knockdown study. After interfering PLK1 expression with siRNA1 in BCL-7402, 24 h and 48 h later, we detected the PLK1 expression in PLK1 siRNA transfected BCL-7402, negative control, and blank control by real-time PCR. As shown in Figure 2(b), 24 h after siRNA1 transfection, PLK1 expression was decreased nearly 64% and 65%, which was compared with negative control and blank control, respectively, while, 48 h later, PLK1 expression decreased even more significantly, nearly 78% and 81%. Though PLK1 siRNA2 transfection in BCL-7402 also inhibited PLK1 expressions, the inhibitory efficiency is lower than that of PLK1 siRNA1 (Figure 2(b)). Thus, the role of PLK1 in HCC cell growth is investigated by assessing the consequence of PLK1 inactivation by siRNA1 in HCC cell lines.

Since PLK1 is important during mitosis, we determined the effects of PLK1 siRNA on the cell cycle profile of BCL-7402 by flow cytometry. After 48 h PLK1 siRNA transfected BCL-7402, the proportion of cells in G2/M phase is lower than that of negative control and blank control; however, there was no significant difference between G0/G1 phase and M phase (Figure 3). All these results indicated that PLK1 knockdown prevents cells from undergoing mitosis. Therefore, we think PLK1 inhibition will be beneficial for HCC treatment.

3.3. PLK1 Downregulation Promotes Cell Apoptosis in Hepatocellular Cell Lines. We then examined whether PLK1 has an important role in controlling cell apoptosis, for it can decide the tumor status. After knockdown of PLK1 in BCL-7402 for 48 h, cell apoptosis was assessed by annexin V and PI
Figure 2: Polo-like kinase 1 (PLK1) expressions in hepatocellular carcinoma cell lines. (a) Western blot was used to determine the levels of PLK1 in HepG2 and BCL-7402 cell lines; beta-actin serves as the loading control. (b) Two designed siRNA for PLK1 were transfected to BCL-7402, respectively, and, 24 h and 48 h later, cells were collected. The expressions of PLK1 were examined by real-time PCR. Results are shown as mean ± SD. Data are representative of at least 3 independent experiments. *P < 0.05; **P < 0.01.

Figure 3: Knockdown of PLK1 in HCC promotes cell cycle arrest. BCL-7402 cells were transfected with PLK1 siRNA. 48 h later, cells were harvested and subjected to flow cytometry analysis for cell cycle progression. Data are representative of at least 3 independent experiments.

staining. Flow cytometry analysis showed that the apoptosis of PLK1 siRNA transfected BCL-7402 was significantly higher than that of negative control and blank control (Figure 4(a)). These results indicated that inhibition of PLK1 expression in tumor cells promotes cell apoptosis. Also, we performed the transmission electron microscopy to detect the morphology of PLK1 siRNA transfected BCL-7402. Consistent with the enhanced cell apoptosis in PLK1 siRNA transfected BCL-7402, these cells exhibited microvilli formation in cell membrane, chromatin condensation, karyorrhexis, and nuclear fragmentation, which happened in both early and late phases of apoptosis (Figure 4(b)). However, negative control and blank control hardly observed these apoptotic phenomena (data not shown). This result suggested that PLK1 knockdown in HCC cells can improve HCC development.

Based on the important role of caspase dependent signaling pathway in cell apoptosis, we further investigated caspase 3, caspase 8, and caspase 9 expression in PLK1 inhibited BCL-7402 by real-time PCR. Compared to control groups, PLK1 siRNA transfected BCL-7402 revealed significantly increased
Figure 4: Continued.
Figure 4: Knockdown of PLK1 in HCC accelerates cell apoptosis. BCL-7402 cells were transfected with PLK1 siRNA. 48 h later, (a) cells were harvested and subjected to annexin V and PI staining for apoptosis analysis by flow cytometry; (b) cells were observed using transmission electronic microscope (TEM); (c) cells were analyzed for caspase 3, caspase 8, and caspase 9 mRNA expression by real-time PCR; (d) cells were analyzed for Bcl-2 and Bax mRNA expression by real-time PCR. Results are shown as mean ± SD. Data are representative of at least 3 independent experiments. *P < 0.05; **P < 0.001.

3.4. p53 Is Involved in PLK1 Regulating Cell Cycle and Cell Apoptosis. Since p53 is important in the control of cell cycle progress and cell apoptosis, previous studies also have pointed that PLK1 can regulate the growth of cells in a p53-dependent manner in multiple types of carcinoma [17]. Therefore, we further detected the p53 expression in PLK1 siRNA transfected BCL-7402 by real-time PCR. We found lower Bcl-2 expression and higher Bax expression in PLK1 siRNA transfected BCL-7402, which were compared to those of negative control and blank control (Figure 4(d)). Thus, the effect of PLK1 inhibition on cell apoptosis may be associated with mitochondria mediated signaling pathway.

4. Discussion

Human HCC is one of the common and lethal tumors in the world. Though new strategies are available, the survival of unresectable HCC patients is still poor. Thus, new molecules for diagnosis and therapeutic targets should be identified and targeted to improve HCC patients outcome. In this study, we found upregulated expression of PLK1 in human HCCs. Meanwhile, our data further indicated that PLK1 knockdown inhibited the growth of HCC cell lines and promoted the apoptosis of HCC cell lines. All these effects were associated with p53 signaling pathway. Therefore, we presume that PLK1, as the potential target, will be beneficial for the diagnosis and treatment of HCC.

In consistence with previous studies, we demonstrated that PLK1 upregulation in the liver was found in HCC patients [18, 19]. After evaluating by different clinicopathological variables, age, tumor size, tumor nodule, clinical stage, HBV positive, HCV positive, liver function, and with or without tumor thrombi in portal vein showed less correlation with PLK1 expression. However, the higher expression of PLK1 in HCC was found in well differentiated tumor cells and tumor without capsule invasion, but not in moderate and poor differentiated cells and tumor with capsule invasion. Moreover, though PLK1 expression was correlated with metastasis in many types of carcinoma, there was less relationship between PLK1 expression and HCC metastasis, including lymphatic metastasis and extrahepatic metastasis [14, 18]. Therefore, contrary to the positive relationship of PLK1 and some types of tumor malignance, less correlation of PLK1 and HCC malignancy was observed in the present study. This may be due to the special tissue microenvironment where tumor happened. Meanwhile, we found that, even in the same HCC sample, different levels of PLK1 distributions in different subgroup of tumor cells were detected. This finding indicated that PLK1 may play a different role in regulating HCC stem cells to differentiate into different subtypes of tumor. Thus, in controlling well differentiated HCC cells development, PLK1 may be important, while, in moderate or poor differentiated HCC cells, PLK1 maybe need to collaborate with other
factors. Also, given that the distinct sites of phosphorylation in PLK1 will influence different stage of cell growth [19, 20], further studies are still needed to investigate whether the different sites of phosphorylation in PLK1 will affect the differentiation of HCC stem cells. In addition, the similar elevated levels of PLK1 expression in HCCs and surrounding cirrhosis suggested that PLK1 can serve as the prognostic standard to judge the possibility of canceration of cirrhosis tissue. Thus, PLK1 may be an important diagnostic marker to evaluate HCC development.

Furthermore, we explored the effect of PLK1 on cell cycle modulation in human HCC cell lines. Consistent with previous studies, our data demonstrated the regulatory role of PLK1 in both G2/M phase of the cell cycle and the apoptotic process [21, 22]. In the present study, through inhibiting Bcl-2 expression, promoting Bax expression and PLK1 downregulation promoted cell apoptosis. It has been demonstrated that the knockdown of PLK1 leads to an increased expression of Bax in Hela cells, which contribute to the apoptosis of cells [23]. Bax is a key component for cellular induced apoptosis through mitochondrial stress. With apoptotic stimulation, oligomers of Bax interact with mitochondrial membrane, which at last initiates the caspase activation pathway for apoptosis [24]. We also detected the enhanced levels of caspase 3 and caspase 8 in PLK1 knockdown cells. Therefore, we put forward a hypothesis that PLK1 might be a crucial therapeutic target in HCC, due to the activation of proapoptotic pathway. We found that PLK1 inhibition can promote p53 expression, which in turn can contribute to cell cycle arrest and cell apoptosis. Recently, some new findings have pointed that PLK1 is able to inhibit apoptosis in a p53-dependent manner in a variety of carcinomas [8, 25]. PLK1 can interact with the DNA binding domain of p53, thereby decreasing its stability and transcriptional activity [17]. Thus, p53 is a major target for PLK1 controlling the growth of carcinoma cells. Besides p53, many researchers have found that p73 is another target of PLK1 and its expression was elevated in PLK1 silenced HCC cell lines with or without p53 expression [26]. The enhancement of p73 was also observed in MCF7 breast cancers expressing the p53; however, p53 is independent of the process of p73 induction by PLK1 [27]. Though some targets have been verified, we cannot exclude other molecules involved in PLK1 regulating the growth of HCC cell lines, such as Orc2, centrobin/NIP2, and HsCYK-4. All of them have been indicated to be targeted by PLK1, their activation following by promoting microtubule stabilization or regulating the onset of cell division. Therefore, it is still necessary to investigate whether these molecules were involved in PLK1 pathway modulating the growth of HCC.

5. Conclusions

Taken together, in the present study, we have demonstrated that upregulation of PLK1 expression may be an early diagnostic marker for the canceration of cirrhosis and the development of HCC, but less correlation between PLK1 and metastasis was observed. Inhibiting PLK1 expression in HCC cell line can significantly decrease cell proliferation and increase cell apoptosis. In addition, we found that these processes may be involved in p53 mediated signaling pathway. Our study provides new evidence for the involvement of PLK1 in HCC development process and puts forward the possibility of PLK1 serving as a target for HCC diagnosis and treatment.
Conflict of Interests
The authors declare that they have no conflict of interests regarding the publication of this paper.

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Research Article

Genetic Variations of Cytokines and Cytokine Receptors in Psoriasis Patients from China

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Psoriasis is a chronic inflammatory and hyperproliferative skin disease affected by both genetic and environmental factors. The aim of the present study was to investigate polymorphisms in a candidate gene family of interleukin (IL) in unrelated Chinese patients with psoriasis and control subjects without psoriasis. In this case-control study, 200 unrelated Chinese psoriasis patients and 298 age- and sex-matched control subjects were enrolled. Genomic DNA was prepared from peripheral blood obtained from all psoriasis patients and control subjects. We genotyped seven single-nucleotide polymorphisms (SNPs) in candidate genes of six ILs: IL4, IL10, IL12B, IL13, IL15, and IL23R, which have been shown in the literature to be associated with psoriasis in other ethnic groups. Among the seven SNPs in the six IL genes studied, only the rs3212227 in the IL12B gene was found to be associated with psoriasis at genotypic level in the studied population. The C/C genotype in the IL12B gene is a protective factor of psoriasis ($P = 0.0218$; OR = 0.51; 95% CI: 0.27–0.96) in Chinese. Furthermore, the studied Chinese population has extremely low minor allele frequency for IL23R. Together, the data reveal unique genetic patterns in Chinese that may be in part responsible for the lower risk for psoriasis in this population.

1. Introduction

Psoriasis is an immunologically mediated chronic inflammatory and hyperproliferative skin disease affected by both genetic and environmental factors. The prevalence of psoriasis varied among populations with different genetic backgrounds and habitats, from 3% in Northern Europe and 2% in North America and the UK to 0.1–0.3% in American Indians and East Asia [1, 2]. Psoriasis is proposed to be associated with other immune diseases, such as arthritis and Crohn’s disease [3]. Initial causative research has identified strong association between psoriasis and the interleukin genes (IL4, IL10, IL12B, IL13, and IL23R) in the northern European from US and UK [4–7]. Furthermore, the SNP rs56245420 in the IL15 gene has been found to be associated with psoriasis in the Chinese Han population but not in any of the UK, German, or US Caucasian populations investigated [8–10], since the minor allele frequency for this SNP and others across IL15 differs quite strikingly between the populations, suggesting heterogeneity in the genetic susceptibility to psoriasis.

In this study, we aimed to determine if psoriasis is associated with six IL genes that have been strongly associated with psoriasis in Europeans but not well studied in Chinese. The seven included SNPs are rs2243250 in the IL4 gene, rs1800872 in the IL10 gene, rs3212227 in the IL12B gene, rs1800925 and rs20541 in the IL13 gene, rs56245420 in the IL15 gene, and rs11209026 in the IL23R gene.

2. Materials and Methods

2.1. Study Population. A total of 200 psoriasis patients and 298 healthy controls were recruited in this study (see Table 2). All participants did not suffer from any other diseases and belonged to Han nationality in Yunnan Province, China. The study was performed according to the Helsinki Declaration
| SNP       | Gene | Position of SNP in genomic sequence | Forward primer<sup>a</sup> | Reverse primer<sup>a</sup> | Anneal temperature (°C) | Product length (bp) | Restriction endonucleases | Fragments of frequent allele genotype (bp) | Fragments of heterozygous genotype (bp) | Fragments of rare allele genotype (bp) | Reference |
|-----------|------|-------------------------------------|-----------------------------|-----------------------------|-------------------------|-----------------------|---------------------------|------------------------------------------|------------------------------------------|----------------------------------------|-----------|
| rs2243250 | IL4  | 132009154:C/T                       | TAAACTTGGAGAACATGGT         | TAGGGGAAAGATAGAATATA        | 49                      | 195                   | AvaII                    | 22 + 173 + 195               | 22 + 173                               | [1]        |
| rs1800372 | IL10 | 206946407:A/C                       | AGGTGATGAATATCTTGTT        | TAAATACCTCAGAAGTTCC         | 57                      | 303                   | Rsal                     | 65 + 238                   | 65 + 238 + 303                        | 303       |
| rs3212227 | IL12B| 158743950:A/C                       | TTCTATCTGATTGGTTA          | TGGAAACATTGCTACATCC         | 51                      | 233                   | TaqI                     | 233                       | 68 + 165 + 233                       | 68 + 165   |
| rs1800925 | IL13 | 13092889:G/T                        | GTGGGCTTCTCTGCTCTCCGCC     | GGAATCCAGCATGGCTTGGAGG      | 65                      | 247                   | BshI236I                 | 23 + 224                   | 23 + 224 + 247                       | 247       |
| rs20541   | IL13 | 131955964:C/T                       | TAGGCTGAAGACGGCGAGCA       | AACAGAATTITTTGCCGGAGGC     | 63                      | 199                   | MspI                     | 22 + 177                   | 22 + 177 + 199                       | 199       |
| rs6245420 | IL15 | 142873720:AT                        | TTATCGTATAAACAACATACCTTG   | CAACACTCTGTACATATTITTTATCAAT | 54                     | 274                   | SphI                     | 27 + 247                   | 27 + 247 + 274                       | 274       |

<sup>a</sup>Mismatch is shown in bold and underlined font.
### Table 2: Genotyping of seven studied SNPs in psoriasis patients ($n=200$) and controls ($n=298$).

| SNP       | Gene | Population | Genotype (%) | Minor allele (%) | Genotype (%) | Minor allele (%) |
|-----------|------|------------|--------------|------------------|--------------|------------------|
| rs2243250 | IL4  | Controls   | T/T 189 (63.4) | T/C 98 (32.9)   | C/C 11 (3.7) | C 120 (20.1)     |
|           |      | Psoriasis  | T/C 127 (63.5) | T/C 61 (30.5)   | C/C 12 (6.0) | C/C 85 (21.3)    |
| rs1800872 | IL10 | Controls   | A/A 138 (46.3) | A/C 123 (41.3)  | C/C 37 (12.4)| C/C 197 (33.1)   |
|           |      | Psoriasis  | A/C 93 (46.5)  | A/C 86 (43.0)   | C/C 21 (10.5)| C/C 128 (21.3)   |
| rs3212227 | IL12B| Controls   | A/A 119 (39.9) | A/C 128 (43.0)  | C/C 51 (17.1)| C/C 230 (38.6)   |
|           |      | Psoriasis  | A/C 77 (38.5)  | A/C 104 (52.0)  | C/C 19 (9.5)  | C/C 142 (35.5)   |
| rs1800925 | IL13 | Controls   | A/A 222 (74.5) | A/C 72 (24.2)   | C/C 4 (1.3)  | C/C 80 (13.4)    |
|           |      | Psoriasis  | A/C 140 (70.0)| A/C 52 (26.0)   | C/C 8 (4.0)  | C/C 68 (17.0)    |
| rs20541   | IL13 | Controls   | G/G 146 (49.0) | G/A 126 (42.3)  | T/T 26 (8.7) | T/T 178 (29.9)   |
|           |      | Psoriasis  | G/A 100 (50.0)| G/A 80 (40.0)   | T/T 20 (10.0)| T/T 120 (30.0)   |
| rs56245420| IL15 | Controls   | A/A 139 (46.6) | A/T 135 (45.3)  | T/T 24 (8.1) | T/T 183 (30.7)   |
|           |      | Psoriasis  | A/T 78 (39.0)  | A/T 97 (48.5)   | T/T 25 (12.5)| T/T 147 (36.8)   |
| rs11209026| IL23R| Controls   | G/G 298 (100)| G/A 0 (0)       | A/A 0 (0)   | A/A 0 (0)        |
|           |      | Psoriasis  | G/A 200 (100)| G/A 0 (0)       | A/A 0 (0)   | A/A 0 (0)        |

*with approval of the institutional review boards of the Affiliated Yan’an Hospital of Kunming Medical College and the Kunming Institute of Botany. Informed consent was obtained from each participant before inclusion in this study.

#### 2.2. Determination of Genotype.

Genomic DNAs were isolated from whole blood using regular phenol/chloroform method. The SNP rs11209026 in the IL23R gene was genotyped by the TaqMan allelic discrimination method (Applied Biosystems). New PCR-RFLP methods were generated to genotype the SNP rs56245420 in the IL15 gene. Primers 5’-TTTCTGGTTAACAACACACACCTCTG-3’ and 5’-CAA CAC TTG TAC ATA TTT TTA TTC AAt AT-3’ (mismatch is shown in bold lower case) were used for rs56245420. Other five SNPs were genotyped by PCR-RFLP methods described previously with slight modification [11–15]. PCR reaction was carried out in a total volume of 20 μL containing 20 ng of genomic DNA, 1 × PCR buffer, 1.5 mM MgCl₂, 200 μM of each dNTP, 30 ng of each primer, and 1 unit of Taq DNA polymerase (TakaRa). Samples were denatured at 95°C for 2 min followed by 30 cycles of 94°C for 45 sec, 61°C (rs2395029) or 54°C (rs56245420) for 45 sec, and 72°C for 45 sec and ended with a final extension for 7 min at 72°C. PCR products were digested with 4 U of appropriate restriction endonuclease and electrophoresed on 3% agarose gels and stained with ethidium bromide. The restriction endonucleases, PCR product lengths, and restriction patterns are shown in Table 1.

#### 2.3. Data Analysis.

Statistics analysis was performed by SPSS software for windows (SPSS Inc.). The frequencies of genotypes and alleles for all the six studied loci were determined assuming codominant inheritance. The Hardy-Weinberg equilibrium (HWE) for six loci in psoriasis patients and controls was tested by means of chi-square tests. The statistical significance of the genotype and allele frequency variables between the psoriasis patients and control group was evaluated by chi-square test with Yates correction for small numbers. Relative risk associated with the significant genotype was estimated by the odds ratio (OR). OR with 95% confidence intervals (95% CI) was tested using a chi-square distribution and the null hypothesis being tested is $OR = 1$. $P$ values < 0.05 were considered as statistically significant.

#### 3. Results

Only one of the fourteen Hardy-Weinberg tests (seven polymorphic loci each in the psoriasis patient and control groups) had $P$ values smaller than 0.05 ($P = 0.01$ at rs3212227 in controls). All nine remaining genotype frequencies fit Hardy-Weinberg expectations according to chi-square tests in psoriasis patients and controls ($P > 0.05$). Therefore, there is no meaningful deviation from WHE, and our population is derived from random mating.

Polymorphism (minor allele frequency > 1%) has been found for all studied SNPs except for rs11209026 in the IL23R gene (Table 2). Table 2 shows that rs3212227 in the IL12B gene
Table 3: Correlation of psoriasis symptoms with SNPs in the IL genes.

| SNP     | Genotype | Psoriasis | Controls | OR (95% CI) | Allele | Psoriasis | Controls | OR (95% CI) |
|---------|----------|-----------|----------|-------------|--------|-----------|----------|-------------|
| rs2243250 | TT       | 127       | 189      | 0.93 (0.63–1.37) | T      | 315       | 476      | 1.07 (0.78–1.46) |
|         | TC       | 61        | 98       | 1.00 (0.69–1.45)  | C      | 85        | 120      | 0.93 (0.63–1.37) |
|         | CC       | 12        | 11       | 1.62 (0.69–3.78)  | C      | 142       | 230      | 1.14 (0.88–1.48) |
|         | TC-CC    | 73        | 109      | 1.00 (0.69–1.45)  | C      | 142       | 230      | 1.14 (0.88–1.48) |
| rs1800872 | AA       | 93        | 138      | 1.04 (0.71–1.52)  | A      | 272       | 399      | 0.95 (0.72–1.25) |
|         | AC       | 86        | 123      | 1.07 (0.78–1.46)  | C      | 128       | 197      | 0.95 (0.72–1.25) |
|         | CC       | 21        | 37       | 0.84 (0.46–1.53)  | C      | 142       | 230      | 1.14 (0.88–1.48) |
|         | AC-CC    | 107       | 160      | 0.99 (0.69–1.42)  | C      | 142       | 230      | 1.14 (0.88–1.48) |
| rs3212227 | CC       | 19        | 51       | 2.18 (1.21–3.92)  | C      | 142       | 230      | 1.14 (0.88–1.48) |
|         | AC       | 104       | 128      | 1.71 (0.96–3.17)  | A      | 258       | 366      | 1.14 (0.88–1.48) |
|         | AA       | 77        | 119      | 1.97 (1.12–3.45)  | A      | 258       | 366      | 1.14 (0.88–1.48) |
|         | AC-AA    | 181       | 247      | 1.71 (0.96–3.17)  | A      | 258       | 366      | 1.14 (0.88–1.48) |
| rs1800925 | CC       | 140       | 222      | 0.99 (0.69–1.42)  | C      | 332       | 516      | 1.14 (0.88–1.48) |
|         | CT       | 52        | 72       | 1.25 (0.84–1.86)  | C      | 332       | 516      | 1.14 (0.88–1.48) |
|         | TT       | 8         | 4        | 3.17 (0.94–10.72) | C      | 332       | 516      | 1.14 (0.88–1.48) |
|         | CT-CC    | 60        | 76       | 1.25 (0.84–1.86)  | C      | 332       | 516      | 1.14 (0.88–1.48) |
| rs20541  | CC       | 100       | 146      | 0.93 (0.64–1.36)  | T      | 120       | 178      | 1.01 (0.77–1.33) |
|         | CT       | 80        | 126      | 1.12 (0.59–2.12)  | T      | 120       | 178      | 1.01 (0.77–1.33) |
|         | TT       | 20        | 26       | 0.99 (0.67–1.37)  | T      | 120       | 178      | 1.01 (0.77–1.33) |
|         | CT-TT    | 100       | 152      | 1.28 (0.87–1.87)  | T      | 147       | 183      | 1.28 (1.00–1.71) |
| rs56245420| AA       | 78        | 139      | 1.36 (0.94–1.96)  | T      | 147       | 183      | 1.28 (1.00–1.71) |
|         | AT       | 97        | 135      | 1.85 (0.99–3.46)  | T      | 147       | 183      | 1.28 (1.00–1.71) |
|         | TT       | 25        | 24       | 1.85 (0.99–3.46)  | T      | 147       | 183      | 1.28 (1.00–1.71) |
|         | AT-TT    | 122       | 159      | 1.36 (0.94–1.96)  | T      | 147       | 183      | 1.28 (1.00–1.71) |

(P = 0.0218) was associated with psoriasis at genotypic level in the studied population. Other SNPs examined were not associated with psoriasis considered from single locus. As shown in Table 3, while the A/C genotype (OR = 1.48; 95% CI: 0.95–2.30) and the alleles (OR = 0.84; 95% CI: 0.63–1.13) at rs3212227 in the IL12B were not a risk factor of psoriasis, the C/C genotype was a protective factor of psoriasis (OR = 0.51; 95% CI: 0.27–0.96).

4. Discussion

The etiology of psoriasis is a complex interaction of environmental and biological factors. Genetic factors may play a significant role in the risk of psoriasis in Chinese [10, 16]. Recent genetic studies indicate that the location of these genes varies considerably among populations and families. We are interested to know if psoriasis is associated with the genes that have been strongly associated with psoriasis in Europeans but not well studied in Chinese.

Our results showed that the IL12B gene was associated with psoriasis in Chinese at genotypic level (P < 0.05), which is in line with the findings from European studies [17]. The C/C genotype for rs3212227 in IL12B was a protective factor (OR = 0.51) from psoriasis. Similar result from studying SNP rs6887695 in IL12B showed that the minor allele C was a protective factor from psoriasis [5].

The nonsynonymous SNP in IL23R, rs11209026, widely thought to be the primary psoriasis-associated SNP in IL23R in Europeans, was found not to be polymorphic in Chinese, which is in agreement with the findings of others [18]. The low frequencies of variant in IL23R are accordingly of low risk for psoriasis in Chinese. With single SNP analysis, no association is found between the psoriasis and the IL4, IL10, IL13, and IL15 genes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

**In Silico Prediction of T and B Cell Epitopes of Der f 25 in Dermatophagoides farinae**

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The house dust mites are major sources of indoor allergens for humans, which induce asthma, rhinitis, dermatitis, and other allergic diseases. Der f 25 is a triosephosphate isomerase, representing the major allergen identified in *Dermatophagoides farinae*. The objective of this study was to predict the B and T cell epitopes of Der f 25. In the present study, we analyzed the physiochemical properties, function motifs and domains, and structural-based detailed features of Der f 25 and predicted the B cell linear epitopes of Der f 25 by DNAStar protean system, BAP, and BepiPred 1.0 server and the T cell epitopes by NetMHCIIpan-3.0 and NetMHCII-2.2. As a result, the sequence and structure analysis identified that Der f 25 belongs to the triosephosphate isomerase family and exhibited a triosephosphate isomerase pattern (PS001371). Eight B cell epitopes (11–18, 30–35, 71–77, 99–107, 132–138, 173–187, 193–197, and 211–224) and five T cell epitopes including 26–34, 38–54, 66–74, 142–151, and 239–247 were predicted in this study. These results can be used to benefit allergen immunotherapies and reduce the frequency of mite allergic reactions.

1. Introduction

The house dust mites (HDM) are major sources of indoor allergens for humans, which induce asthma, rhinitis, dermatitis, and other allergic diseases [1]. Their major allergens (*Dermatophagoides pteronyssinus* [Der p] and *Dermatophagoides farinae* [Der f]) coexist in most geographical regions with a high proportion (up to 85%) of asthmatics being typically HDM allergic; hence, sensitization is attributed as a risk factor for developing asthma. Recently, a birth cohort study showed that sensitization to HDM at age of 2 years was associated with current wheeze at age of 12 years in both monosensitized and polysensitized HDM-sensitive children [2]. In previous studies, fourteen *D. farinae* allergens (Der f 1–3, 6, 7, 10, 11, 13–18, and 22) were reported before other seventeen allergens belonging to twelve different groups were identified by a procedure of proteomics combined with two-dimensional immunoblotting from *D. farinae* extracts [3, 4]. Among the novel identified *D. farinae* allergens, Der f 25 is a triosephosphate isomerase (TPI) with a molecular weight of 34 kDa, showing 75.6% by immunoblotting and 60% by skin prick positive reaction to dust mite allergic patients, respectively. It represented the major allergen in *D. farinae* [4].

TPI is an enzyme (EC 5.3.1.1) that catalyzes the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate [5]. It has been found in nearly every organism searched for the enzyme, including animals such as mammals and insects as well as in fungi, plants, and bacteria. Moreover, some TPIs have been identified as an allergen in fish, midges, crustaceans, and various plants [6–12].

Currently, specific immunotherapy is the only allergen-specific approach for its treatment of mite allergy. The administration of increasing doses of allergen extracts to patients is the method most commonly applied. However, the use of crude extracts has several disadvantages. It could induce severe anaphylactic side reactions or lead to
sensitization towards new allergens present in the mixture [13, 14]. Different strategies have been designed to try to overcome these negative effects, as the use of allergen-derived B cell peptides, allergen-derived T cell epitopes containing peptides, or vaccination with allergen-encoding DNA [15]. Known epitopes for some of these mite allergens are described in detail in Cui’s review [16]. However, there is no report about the epitope of Der f 25 allergen. In the present study, we firstly identified the B and T cell epitopes of Der f 25 allergen by *in silico* approach. It implied their potential utility in a peptide-based vaccine design for mite allergy.

2. Methodology

2.1. Sequence Retrieval and Phylogenetic Analysis. The complete amino acid sequence of Der f 25 was acquired from the Nucleotide database of NCBI (http://www.ncbi.nlm.nih.gov/) with the accession number of KC305500.1. The amino acid sequence was also used as query to search for homologous sequences through the Swiss-Prot/TREMBL (Uniprot) (http://www.uniprot.org/) and tBLASTn in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The homologous amino acid sequences were retrieved and aligned using Clustal X 2.1 [17]. Phylogenetic tree was obtained by using ML (maximum-likelihood) method on the basis of the JTT amino acid sequence distance implemented in MEGA 5.1 [18]; the reliability was evaluated by the bootstrap method with 1000 replications.

2.2. Domain Architecture Analyses. The possible domains and characteristic motifs and patterns contained in Der f 25 were investigated by Pfam v27.0 (http://pfam.sanger.ac.uk/) [19], Prosite (http://prosite.expasy.org/scanprosite/) [20], InterPRO v46.0 (http://www.ebi.ac.uk/interpro/), and Superfamily v1.75 (http://supfam.cs.bris.ac.uk/SUPERFAMILY/index.html) [21].

2.3. Physiochemical Analysis and Posttranslational Patterns and Motifs. Physiochemical analysis including molecular weight, theoretical pI, amino acid composition, instability index, aliphatic index, and grand average of hydropathicity (GRAVY) of Der f 25 was performed by using ProtParam tool (http://web.expasy.org/protparam/). Der f 25 characteristic pattern was checked for original sequence and further analysis was performed to highlight the presence of functional motifs by using the Prosite database (http://prosite.expasy.org/) [20]. Biologically meaningful motifs and susceptibility to posttranslational modifications were derived from multiple alignments and the ScanProsite tool. Phosphorylation motifs with more than 80% of probability of occurrence were analyzed by using NETPhos v2.0 (http://www.cbs.dtu.dk/services/NetPhos/) and NETPhosK v1.0 (http://www.cbs.dtu.dk/services/NetPhosK/) [22].

2.4. Secondary Structure Prediction. Der f 25 secondary structural elements were predicted by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) [23], which threads sequence segments through protein data bank (PDB) library (http://www.rcsb.org/) to identify conserved substructures. Furthermore, the secondary structure elements were also identified with the result obtained with NetSurfP ver. 1.1 (http://www.cbs.dtu.dk/) [24].

2.5. Homology Modeling and Validation. The Der f 25 protein sequence was searched for homology in the PDB. As well, the homologous templates suitable for Der f 25 were selected by PSI-BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and SWISS-MODEL server (http://swissmodel.expasy.org/) [25, 26]. The best template was retrieved from the results of previous methods and used for homology modeling. Der f 25 modeled protein structure was built through alignment mode in SWISS-MODEL using the complete amino acid sequence. An initial structural model was generated and checked for recognition of errors in 3D structure by PROCHECK [27], ERRAT [28], and VERIFY3D [29] programs in structural analysis and verification server (SAVES) (http://nihserver.mbi.ucla.edu/SAVES/). The final model structure quality of Der f 25 was assessed by QMEAN [30], by checking protein stereology with ProSA program [31] and the protein energy with ANOLEA (http://protein.bio.puc.cl/cardex/servers/anolea/) [32]. The Ramachandran plot for all the models was generated, showing the majority of the protein residues in the favored regions.

2.6. Conservation Analysis and Poisson-Boltzmann Electrostatic Potential. Der f 25 model was submitted to Consurf server (http://consurf.tau.ac.il/) in order to generate evolutionary related conservation scores helping to identify functional regions in the proteins. Functional and structural key residues in Der f 25 sequence were confirmed by ConSeq server [33].

APBS molecular modeling software implemented in PyMOL 0.99 was used to investigate the electrostatic Poisson-Boltzmann (PB) potentials of Der f 25 model structure. AMBER99 in PDB2PQR server (http://mbcr-222.ucsd.edu/pdb2pqr_1.8/) was used to assign the charges and radii to all of the atoms (including hydrogens) [34]. Fine grid spaces of 0.35 Å were used to solve the linearized PB equation in sequential focusing multigrid calculations in a mesh of 130 points per dimension at 310.00 K. The dielectric constants were 2.0 and 80.0 for the protein and water. The output mesh was processed in the scalar OpenDX format to render isocontours and maps onto the surfaces with PyMOL 0.99. Potential values are given in units of $kT$ per unit charge ($k$ Boltzmann’s constant; $T$ temperature).

2.7. *In Silico* Prediction of B Cell Epitopes. Three immuninformatics tools including DNAStar protean system, bioinformatics predicted antigenic peptides (BPAP) system (http://imed.med.ucm.es/Tools/antigenic.pl), and BepiPred 1.0 server (http://www.cbs.dtu.dk/services/BepiPred/) were used to predicate the B cell epitopes of Der f 25. The ultimate consensus epitope results were obtained by combining the results of the three tools together with the
method published earlier [35]. In the DNAStar protean system, four properties (hydrophilicity, flexibility, accessibility, and antigenicity) of the amino acid sequence were chosen as parameters for epitopes prediction. The BPAP system and the BepiPred 1.0 server only need the amino acid sequence and provide more straightforward results which are combined with physicochemical properties of amino acids such as hydrophilicity, flexibility, accessibility, turns, and exposed surface [36].

2.8. In Silico Prediction of T Cell Epitopes. T cell epitopes are principally predicted indirectly by identifying the binding of peptide fragments to the MHC complexes. The binding significance of each epitope to the given MHC molecule is based on the estimated strength of binding exhibited by a predicted nested core peptide at a set threshold level. For HLA-DR-based T cell epitope prediction, the artificial neural network-based alignment (NN-align) method NetMHC1pan-3.0 (http://www.cbs.dtu.dk/services/NetMHC1pan/) [37] was applied. For HLA-DQ alleles, NetMHCII-2.2 (http://www.cbs.dtu.dk/services/NetMHCII/) [38] was used. In this study, HLA-DR 101, HLA-DR 301, HLA-DR 401, and HLA-DR 501 were used to predict HLA-DR-based T cell epitope prediction. The ultimate HLA-DR-based T cell epitope results were obtained by combining those four results together that if three of them showed epitope, then the consensus result was epitope. This method was also used in HLA-DQ-based T cell epitope prediction. HLA-DQA10101-DQB10501, HLA-DQA10501-DQB10201, HLA-DQA10501-DQB10301, and HLA-DQA10102-DQB10602 were used to predict HLA-DQ-based T cell epitope prediction. As a result, the ultimate consensus epitope results were obtained by combining the results of the HLA-DR-based T cell epitope and HLA-DQ-based T cell epitope.

B cell and T cell epitopes identified by computational tools were mapped onto linear sequence and on the three-dimensional model of Der f 25 to determine their position and secondary structure elements involved.

3. Results

3.1. Sequence Retrieval and Sequence Analysis. The amino acid sequence of Der f 25 was obtained from the Nucleotide database of NCBI. Uniprot and tBLASTn were used to search the homologous sequences of Der f 25. As a result, thirty-six sequences were obtained and in order to determine the relationships between Der f 25 and its homologous sequences, phylogenetic analysis was performed and the evolutionary tree inferred by the ML method was showed in Figure 1. Phylogenetic analysis result showed that there are proteins including Der f 25 clustered into the same group, belonging to TIPs. Moreover, domain analysis results showed that Der f 25 belongs to the TIM phosphate binding superfamily (SUPERFAMILY number SSF51351 and InterPro number IPR016040) and TPI family (SUPERFAMILY number SSF51352 and InterPro number IPR000652).

After searching for characteristic motifs or patterns, we found that Der f 25 exhibited a TPI pattern, PS00171 (162–172, AYEPVWAIGTG) (Figure 2). Phosphorylation sites including two Ser (95 and 221) and two Thr (146 and 171) residues were predicted and showed in Figure 2. Two types of kinases (PKC for 95, 146, and 171 and DNAPK for 221) were predicted to be phosphorylated for Der f 25 complete sequence.

The primary structure of Der f 25 contained 247 amino acids and the molecular weight is 27134.1. The theoretical pI is 6.24 and the aliphatic index is 95.06. The GRAVY is −0.103 meaning that Der f 25 exhibited hydrophilic character. The instability index is 30.57 meaning that the sequence of Der f 25 is stable.

3.2. Homology Modeling and Validation. Searching for the proteins with known tertiary structure in the PDB yielded Tenebrio molitor TPI (PDB accession number: 2I9E) showing the highest sequence identity (74%) with Der f 25. The SWISS-MODEL server was also used to identify the best possible template and found a high score of 365 and very low E-value of e-101 for 2I9E template. Hence, the 2I9E template was used for homology modeling. As indicated by the Ramachandran plot (Figure 3(b)), 93% residues in Der f 25 model were within the most favored regions, 7% residues in the additional allowed region, 0% residues in the generously allowed regions, and 0% residues in the disallowed region; 93.4% residues in 2I9E template were within the most favored allowed regions, 6.6% residues in the additional allowed region, 0% residues in the generously allowed regions, and 0% residues in the disallowed region. The goodness factor (G-factor) based on the observed distribution of stereochemical parameters (main chain bond angles, bond length, and phi-psi torsion angles) returned accurate values for a reliable model (Table 1), in comparison with the template 2I9E (Table 1). As indicated by the ERRAT program, the result showed that the overall quality factor is 97.034 for Der f 25 and 97.789 for 2I9E meaning that both of the two structures have good high resolution. As indicated by the VERIFY 3D program, the result showed that 99.6% and 99.8% of Der f 25 and 2I9E structures are 0.772 and 0.798, respectively. Q values for Der f 25 and 2I9E structures are 0.772 and 0.798, respectively. Root mean square deviations (RMSD) between Der f 25 structural model and 2I9E template Ca backbone are 0.062 Å (Table 1). Based on these validations, it is shown that the homology model was adopted for this study.

3.3. Structure Analyses. Secondary structure prediction of Der f 25 with PSIPRED identified ten α-helices and seven β-sheets (Figure 2) in Der f 25. Alternatively, NetSurfP v1.1 predicted nine α-helices and eight β-sheets. These results were predicated by different servers and have subtle distinction. The best template 2I9E was used for homology modeling; the overall 3D structure of Der f 25 was shown in Figure 3(a). Sequence polymorphism was responsible for the changes in the spatial distribution of the skeleton alpha carbons, which is reflected in differences between the structures of Der f 25 and 2I9E. A superposition of the Der f 25 with the 2I9E template...
is shown in Figure 3(b) and the values for superimposed Ca are 0.062 Å. As a TPI protein, Der f 25 has two active sites; His in the 94th position is an electrophile while Glu in the 164th position is the proton acceptor. It has two substrate binding sites, the Asn in the 10th position and the Lys in the 12th position. Moreover, the characteristic pattern predicted by ScanProsite tool is shown in Figure 3(c).

3.4. Conservational Analysis and Electrostatic Potential. Consurf conservational analysis of structural and functional key amino acids showed that the Der f 25 protein surfaces were not well conserved, with almost forty high variability residues in different superficial areas. All of the amino acids in the TPI pattern (AYEPVWAIGTG) are conserved. Surface electrostatic potential analysis reveals several prominent charged residues, with half of the side exhibiting large positive values (blue regions) and the other half showing predominantly negative values (red regions) (Figure 3(d)).

3.5. B Cell Epitopes Prediction. Surface accessibility and fragment flexibility are important features for predicting antigenic epitopes. In addition, the existence of regions with high hydrophobicity also provides strong evidence for epitope identification. Antigenic index directly showed the epitope forming capacity of the Der f 25 sequence. Based on these sequence properties, the final predicting regions of DNAStar were 11–19, 28–35, 68–77, 95–111, 130–139, 173–187, 193–197, and 211–225. Also, the predicted results of the BPAP system were 20–26, 32–52, 54–70, 86–93, 138–168, 175–209, and 218–242 and BepiPred 1.0 server were 11–18, 30–34, 71–78, 99–107, 132–138, 168–183, 194–199, and 210–224. Furthermore, the final potential B cell epitopes of Der f 25 were selected on the basis of the results of these three
tools. The ultimate results of the three immunoinformatics tools finally predicted eight peptides (11–18, 30–35, 71–77, 99–107, 132–138, 173–187, 193–197, and 211–224) (Table 2) and these peptides were also shown in Figures 2 and 4.

3.6. T Cell Epitopes Prediction. NetMHCIIpan-3.0 and NetMHCII-2.2 were used to identify the T cell epitope of Der f 25. For HLA-DR-based T cell epitope prediction, the final predicting regions of HLA-DR 101, HLA-DR 301, HLA-DR 401, and HLA-DR 501 were shown in Table 1 and the ultimate

Figure 2: Sequences and second structure analysis of Der f 25 allergen. Ten α-helices and seven β-sheets were identified in Der f 25.

Figure 3: Structural analysis of Der f 25. (a) Protein structure of Der f 25 homology model. (b) Superimposition between Der f 25 and 2I9E template. Der f 25 is depicted in red and 2I9E template is depicted in cyan. (c) Distribution of characteristic pattern in Der f 25. (d) Electrostatic potential representation on the Der f 25 protein surface.
Table 1: Parameters used for proteins structural assessment.

| Protein | Structural assessment methods | Ramachandran plot (%) | G-factor | MCBL (%) | CBA (%) | Z-score | Q value | RMSD |
|---------|-----------------------------|-----------------------|-----------|----------|---------|----------|----------|-------|
| Der f 25 | PROCHECK analysis | 93.0^E | 0.10^F | 100.0 | 99.0 | 93.4%^E | 0.25^F | 100.0 | 98.9 | -10.24 | 0.798 | 0.05^L |
|         | ProSa | 0.0^G | 0.23^K | 6.6%^F | 0.55^J | 0.0^G | 0.37^K | 0.0^H | 0.0| -10.1 | 0.772 | 0.0^L |
|         | QMEAN | | | | | | | | | | | |
|         | RMSD | | | | | | | | | | | |

MCBL: distribution of the main chain bond lengths; CBA: distribution of the covalent bond angles.

^E Residues in favorable regions; ^F residues in allowed regions; ^G residues in generally allowed regions; ^H residues in disallowed regions; ^I G-factor score of the dihedral bonds; ^J G-factor score of the covalent bonds; ^K overall G-factor score; ^L root mean square deviation between Ca Der f 25 structure and 2I9E template.

Figure 4: B cell and T cell epitopes superimposition on the surface of Der f 25 allergen structure.

results of HLA-DR-based T cell epitope prediction finally predicted five peptides (26–34, 38–54, 66–74, 142–151, and 239–247). For HLA-DQ alleles, the final results of HLA-DQA10101-DQB10501, HLA-DQA10501-DQB10201, HLA-DQA10501-DQB10301, and HLA-DQA10102-DQB10602 were also shown in Table 3 and the ultimate results of these four methods finally predicted one peptide, 39–48. As a result, Der f 25 was predicted to have five T cell epitope sequences including 26–34, 38–54, 66–74, 142–151, and 239–247 (Table 2) as shown in Figures 2 and 4.

4. Discussion

The prevalence of human atopic disorders including allergic rhinitis, asthma, and atopic dermatitis is increasing during the past several decades. House dust mite allergies constitute more than 50% of allergic patients and often have severe forms of respiratory allergy, such as asthma [1]. Characterization of mite allergens will be beneficial in the diagnosis and treatment of mite-induced atopic illnesses.

Among the identified allergens, Der f 25 is a new protein with a molecular weight of 34 kDa, representing the major allergen in D. farinae [4]. The objective of this study was to predict the B and T cell epitopes of Der f 25. Firstly, in order to better understand the structure and function of Der f 25, we analyzed the basic sequence properties and studied the 2D and 3D structures of Der f 25. Phylogenetic analysis result showed that Der f 25 protein clustered into the TIPs group; domain analysis also proved a strong evidence illustrating that Der f 25 belongs to the TPI family. In 2D structure analysis, it is clearly shown that Der f 25 composed of ten α-helices and seven β-sheets. The 3D structure of Der f 25 was performed by homology modelling which was widely used in many areas of structure-based analysis and study [39]. PDB
and the working time [44]. Recently, many algorithms for selecting epitopes from immunological relevant position are the completely conserved sites. Substrate binding sites Asn in 10th position and Lys in 12th different superficial areas of protein surface. The active sites 25, it is found that almost forty high variability residues sit in different superficial areas of protein surface. The active sites His in 94th position and Glu in 164th position as well as the substrate binding sites Asn in 10th position and Lys in 12th position are the completely conserved sites.

\textit{In silico} prediction has already become a familiar and useful tool for selecting epitopes from immunological relevant proteins, which can save the expense of synthetic peptides and the working time [44]. Recently, many algorithms have been developed to predict B cell epitopes on a protein sequence based on propensity values of amino acid properties of hydrophilicity, antigenicity, segmental mobility, flexibility, and accessibility [45]. In the present study, we used three algorithms (DNAStar protean system, BPAP, and BepiPred 1.0 server) to predict the B cell epitopes. The previous study showed that the use of bioinformatics approach to predict B cell epitopes correlated well with the experimental approach [46]. Earlier study showed that allergen epitopes were comprised of a high proportion of hydrophilic amino acids [47]. The amino acids Ala, Ser, Asn, Gly, and particularly Lys play a key role in the IgE binding allergic epitopes [48]. In our results, nearly half of the total residues lying in B cell epitopes were hydrophilic (Table 2). Moreover, each predicted B cell epitope has one or more special five amino acids and the common residues in all B cell epitopes were Gly and Lys (Table 2). Electrostatic interactions are known to determine the orientation of the molecules and stabilize antigen-antibody complexes [49]. Surface electrostatic potential analysis result showed that a great part of Der f 25 side exhibits large positive values (blue regions). Most parts of B cell epitopes are distributed in the blue regions and showed a strong negative potential. As a result, eight peptides (11–18, 30–35, 71–77, 99–107, 132–138, 173–187, 193–197, and 211–224) were predicted as the B cell epitopes. However, these B cell epitopes need further investigation in clinical samples.

In the last several years, some methods have substantially improved their accuracy to predict T cell epitopes such as NetMHCpan-3.0 and NetMHCII-2.2. NetMHCpan-3.0 is based on artificial neural networks and is trained on 52,062 quantitative peptide binding data covering all HLA as well as two mouse molecules. In this study, it was used to predict the HLA-DR-based T cell epitopes. For HLA-DQ-based T cell epitopes prediction, NetMHCII-2.2 was used. Although limited binding-affinity data are available for HLA-DQ, it was recently reported to provide the best performance in predicting this locus [50]. As a result, NetMHCIIpan-3.0 and NetMHCII-2.2 were used to predict the T cell epitopes in Der f 25 allergens and predicted 7 potential T cell epitope sequences including 26–34, 38–54, 66–74, 142–151, and 239–247. Despite the high accuracy of these predictions, this approach has not yet been applied to peptide-based vaccine development for allergic diseases.

Allergen-specific immunotherapy (SIT) represents the only allergen-specific and disease-modifying approach with long lasting effects for the treatment of allergic patients [51]. However, SIT can induce side effects, ranging from mild and local to severe and life-threatening symptoms, such as anaphylactic shock [52]. Severe side effects are frequently observed in patients with house dust mite (HDM) allergy [53]. The continuous exposure to HDM allergens further complicates the treatment of patients with HDM allergy. Additionally, the quality of natural HDM allergen extracts and vaccines based on these extracts is often poor. Attenuated allergenic molecules, that is, hypoallergens or synthetic peptide fragments, have been used as high dose and safer alternatives to conventional extract-based SIT [54]. Vaccination with a combination of small peptides that together extend across the entire native allergenic protein theoretically could preserve T cell activation while avoiding IgE-based immune responses. IgE recognizes conformational epitopes of larger peptides (B cell epitopes) and proteins while T cell receptors recognize small linear peptides of 8 to 10 amino acids (T cell epitope). By immunizing with small peptides, T cell activation could occur while IgE binding would be lost [55, 56]. Then, we predicted B and T cell epitopes of Der f 25 allergen, the major allergen in HDM, using \textit{in silico} method which can be used to benefit allergen immunotherapies and reduce the frequency of allergic reactions. However, their accuracies need to be confirmed in the further experiments.

5. Conclusion

In this study, we have a better understanding of the 2D and 3D structures of Der f 25 and have predicted eight B cell
Table 3: The results of B and T cell epitopes predictions.

| Tools                  | Location of the prediction results                                                                 |
|------------------------|--------------------------------------------------------------------------------------------------------|
| **B cell epitope prediction** |
| DNAStar protean        | 11–19, 28–35, 68–77, 95–111, 130–139, 173–187, 193–197, 211–225.                                      |
| BPAP                   | 20–26, 32–52, 54–70, 86–93, 138–168, 175–209, 218–242.                                                |
| BepiPred               | 11–18, 30–34, 71–78, 99–107, 132–138, 168–183, 194–199, 210–224.                                        |
| **T cell epitope prediction** (HLA-DR) |
| DRB1* 01:01            | 5–13, 11–19, 10–18, 24–32, 26–34, 39–47, 46–54, 50–58, 53–61, 57–65, 58–66, 60–68, 66–74, 73–81, 82–90, 89–97, 90–98, 104–112, 107–115, 114–122, 119–127, 122–130, 143–151, 142–150, 156–164, 157–165, 161–169, 163–171, 167–175, 187–195, 190–198, 191–199, 195–203, 197–205, 203–211, 205–213, 227–235, 228–236, 232–240, 235–243, 239–247. |
| DRB3* 01:01            | HLA-DQA1/01-DQB1/0501, 153–161, 154–162, 159–167, 160–168.                                              |
| DRB4* 01:01            | HLA-DQA1/02-DQB1/0602, 33–41, 40–48, 42–50, 57–65, 77–85, 117–125, 154–162, 192–200, 206–214, 213–221, 230–238, 237–245. |
| DRB5* 01:01            | HLA-DQA1/05-DQB1/0201, 19–27, 39–47, 37–45, 82–90, 100–108, 112–120, 122–130, 126–134, 158–166, 195–203, 220–228. |
| DRB5* 01:01            | HLA-DQA1/05-DQB1/0301, 6–14, 34–42, 38–46, 39–47, 58–66, 66–74, 69–77, 72–80, 74–82, 77–85, 82–90, 85–93, 107–115, 117–125, 121–129, 133–141, 161–169, 166–174, 167–175, 169–177, 172–180, 192–200, 195–203, 203–211, 205–213, 206–214, 207–215, 213–221, 215–233, 228–236, 238–238, 238–240, 237–245. |

epitopes (11–18, 30–35, 71–77, 99–107, 132–138, 173–187, 193–197, and 211–224) and five T cell epitope including 26–34, 38–54, 66–74, 142–151, and 239–247 of this TPI. All these results can be used to benefit allergen immunotherapies and reduce the frequency of mite allergic reactions.

Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution
Xiaohong Li, Hai-Wei Yang, and Hao Chen contributed to this paper equally as first author.

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Association between \textit{NFKB1} \textasciitilde 94ins/del ATTG Promoter Polymorphism and Cancer Susceptibility: An Updated Meta-Analysis

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1. Introduction

Cancer is a major public health problem worldwide; it is the primary and secondary causes of death in economically developed and developing countries, respectively [1]. The global concern on cancer continues to intensify as a result of the aging and expanding world population and the increasing adoption of cancer-causing habits. The mechanism of carcinogenesis remains largely unknown although genetic susceptibility is a known possible explanation for the interindividual variation in cancer risk [2].

Nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) was initially identified in 1986 as a transcription factor which binds to a 10 bp DNA element in kappa immunoglobulin light-chain enhancer in B cells [3]. The NF-\(\kappa\)B family consists of p50 (NF-\(\kappa\)B1), p52 (NF-\(\kappa\)B2), p65 (RelA), c-Rel (Rel), and RelB. The major form of NF-\(\kappa\)B is a heterodimer of the p50 and p65/RelA subunits which are encoded by the \textit{NFKB1} and \textit{NFKB2} genes, respectively [4]. The human \textit{NFKB1} gene is mapped to chromosome 4q24 and encodes a 50 kDa DNA-binding protein (p50) that can act as a master regulator of inflammation and cancer development [5–7].

A common insertion/deletion polymorphism (\textasciitilde 94ins/del ATTG, rs28362491) in the promoter region of the \textit{NFKB1} gene elicits a regulatory effect on the \textit{NFKB1} gene [8]. A previous meta-analysis concluded that the deletion allele serves as a risk or protective allele for cancer susceptibility in Caucasian or Asian populations, respectively; however, it revealed no association between the polymorphism and cancer risk [9]. An increasing number of studies have assessed the association between the \textit{NFKB1} promoter \textasciitilde 94ins/del ATTG polymorphism and cancer risk [10–12]. However, these studies obtained conflicting results. Therefore, we collected all available data to perform an updated meta-analysis.
that generates a precise estimation to comprehensively and objectively investigate the association between the NFKB1 promoter −94ins/del ATTG polymorphism and cancer risk.

2. Materials and Methods

2.1. Search Strategy and Identification of Relevant Studies. A comprehensive literature search for relevant articles published (last search updated in September 15, 2013) in PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) was performed with the following key words: ("genetic polymorphism," “polymorphism,” “SNP,” “single nucleotide polymorphism,” “gene mutation,” or “genetic variant”), (“neoplasm,” “cancer,” “tumor,” “carcinoma,” or “carcinogenesis”), and (“NFKB1,” “NF-κB1,” “nuclear factor kappa B1,” “NF kappa B1,” or “nuclear factor κB1”). The search was limited to human studies in English. All eligible studies were retrieved. The reviews and references of eligible studies were hand-searched for additional relevant publications. The most recent or complete study was selected when more than one publications contain overlapping data. A flow diagram of the study selection process is presented in Figure 1.

2.2. Inclusion Criteria. Case-control studies that evaluated the association of the NFKB1 promoter −94ins/del ATTG polymorphism with cancer risk and described in detail the genotype distributions of the polymorphism in cases and controls were included in this meta-analysis.

2.3. Exclusion Criteria. Studies that were not for cancer research, were only case population, and were duplication of previous publication were excluded in this meta-analysis.
2.4. Data Extraction. Information was carefully extracted from eligible studies independently by two investigators (Xiao Yang and Pengchao Li) according to the inclusion criteria listed above, and the result was reviewed by a third investigator (Jun Tao). The following data were collected from each study: surname of first author, year of publication, ethnicity, genotyping method, source of controls, frequencies of the genotypes in cases and controls, cancer type, and ethnicity. Studies that investigated more than one type of cancer were regarded as individual datasets only in subgroup analyses according to cancer type. No minimum number of patients was required for this meta-analysis. Articles that reported different ethnic groups or countries or locations were considered different study samples for each category cited above.

Table 1: Main characteristics of these studies included in this meta-analysis.

| First Author | Year | Ethnicity | Genotyping method | SC | Genotyping cases | Controls | Cancer type | HWE |
|--------------|------|-----------|-------------------|----|-----------------|----------|-------------|-----|
| Lin [16]     | 2006 | Asian     | PCR-RFLP          | HB | 59              | 103      | 50          | 43  | 100 58 | OSCC | 0.993 |
| Riemann [17] | 2007 | Caucasian | Pyrosequencing    | HB | 88              | 124      | 30          | 118 | 141 48 | Bladder cancer | 0.586 |
| Bu [18]      | 2007 | Caucasian | PCR-RFLP          | HB | 67              | 84       | 34          | 116 | 255 67 | Melanoma | <0.001 |
| Lewander [11]| 2007 | Caucasian | PCR-RFLP          | HB | 63              | 323      | 81          | 116 | 256 67 | Colorectal cancer | <0.001 |
| Lo [19]      | 2008 | Asian     | PCR-RFLP          | HB | 62              | 89       | 31          | 20  | 66 34 | Gastric cancer | 0.361 |
| Zhang [20]   | 2009 | Asian     | PCR-RFLP          | HB | 46              | 57       | 14          | 44  | 68 31 | Prostate cancer | 0.624 |
| Burnik [21]  | 2009 | Caucasian | PCR-RFLP          | HB | 18              | 30       | 2           | 30  | 58 12 | GNT | 0.047 |
| Zhou [22]    | 2009 | Asian     | PCR-RFLP          | HB | 74              | 67       | 22          | 71  | 90 42 | NC | 0.177 |
| Tang [23]    | 2009 | Asian     | PCR-RFLP          | HB | 89              | 92       | 26          | 74  | 108 46 | Bladder cancer | 0.565 |
| Andersen [24]| 2010 | Caucasian | Taqman            | PB | 121             | 195      | 62          | 307 | 347 102 | Colorectal cancer | 0.801 |
| Zhou [25]    | 2010 | Asian     | PCR-RFLP          | HB | 108             | 105      | 20          | 135 | 166 64 | CSCC | 0.297 |
| Fan [26]     | 2011 | Asian     | PCR-RFLP          | HB | 78              | 84       | 17          | 76  | 103 44 | Ovarian cancer | 0.396 |
| Lin [27]     | 2011 | Asian     | Taqman            | HB | 116             | 246      | 100         | 81  | 271 168 | OSCC | 0.099 |
| Vangsted [28]| 2012 | Caucasian | Taqman            | PB | 110             | 163      | 55          | 665 | 778 253 | Multiple myeloma | 0.303 |
| Cai [10]     | 2012 | Asian     | Taqman            | HB | 401             | 473      | 153         | 379 | 562 153 | Renal cell Carcinoma | 0.015 |
| Huo [29]     | 2013 | Asian     | MassARRAY         | HB | 83              | 82       | 22          | 71  | 103 47 | Ovarian cancer | 0.399 |
| Cheng [30]   | 2013 | Asian     | Taqman            | HB | 42              | 64       | 29          | 81  | 271 168 | HC | 0.099 |
| Mohd Suzairi [31]| 2013| Asian    | PCR-RFLP          | PB | 35              | 127      | 75          | 16  | 138 83 | Colorectal cancer | <0.001 |
| Kopp [32]    | 2013 | Caucasian | Taqman            | PB | 128             | 152      | 54          | 109 | 161 64 | Prostate cancer | 0.741 |
| Li [12]      | 2013 | Asian     | Taqman            | HB | 189             | 269      | 151         | 223 | 324 93 | Bladder cancer | 0.156 |

GNT: Gastroenteropancreatic neuroendocrine tumors; OSCC: oral squamous cell carcinoma; CSCC: cervical squamous cell carcinoma; NC: nasopharyngeal carcinoma; HC: hepatocellular carcinoma; HB: hospital-based study; PB: population-based study; SC: source of controls; HWE: Hardy Weinberg equilibrium.

2.5. Statistical Analysis. The strength of association between the NFKB1 promoter −94ins/del ATTG polymorphism and cancer risk was estimated through pooled odds ratio (OR) with its corresponding 95% CI. Pooled ORs were calculated for insertion allele versus deletion allele, ins/ins versus del/del, ins/del versus del/del, ins/ins + ins/del versus del/del, and ins/ins versus ins/del + del/del. Subgroup stratification analyses by ethnicity and cancer type were conducted to identify the association of the −94ins/del ATTG polymorphism with cancer susceptibility.

The between-study heterogeneity of the studies included in this meta-analysis was evaluated using the Q and I² statistic tests, where I² > 50% indicated heterogeneity [13]. The random-effects model was selected when I² was significant (>50%); otherwise, the fixed-effects model was selected. The allele frequencies of the NFKB1 promoter −94ins/del ATTG polymorphism from the respective study were determined by allele counting. In addition, a chi-square test was used to determine whether or not the observed frequencies of genotypes conform to HWE. Pooled OR in the current meta-analysis was performed by weighting individual ORs by the inverse of their variance. The significance of the pooled OR was determined by the Z-test. In addition to the comparison among all subjects, we performed stratification analyses by cancer type (if one cancer type contained only one study, it was combined into the “other cancers” group) and ethnicity. Begg’s funnel plot and Egger’s test were adopted to evaluate the publication bias in our meta-analysis [14, 15]. All statistical analyses were performed by STATA 10.0 software (StataCorp, College Station, TX, USA).

3. Results

3.1. Eligible Studies and Meta-Analysis Databases. A total of 21 case-control studies involving 6127 cases and 9239 controls were analysed. The characteristics of all studies are presented in Table 1. The allele and genotype frequencies of the NFKB1 promoter −94ins/del ATTG polymorphism were extracted...
from all eligible studies. In total, this meta-analysis included 3 bladder cancer studies, 4 colorectal cancer studies, 2 ovarian cancer studies, 2 oral cancer studies, 2 prostate cancer studies, and 8 studies with the “other cancers.” Of the 21 studies, 14 were conducted among Asians and 7 were conducted among Caucasians. All cases were clinically pathologically confirmed.

The results of HWE test for the genotype distribution in the control population are shown in Table 1. Six of the eligible studies were not in HWE [10, 11, 18, 21, 31].

3.2. Quantitative Synthesis. The pooled ORs of the included case-control studies revealed a statistically significant association between the NFKB1 promoter −94ins/del ATTG polymorphism and cancer risk across the four genetic models ins/ins versus del/del, OR=1.47, 95%, CI=1.11−1.93; dominant model, OR=1.26, 95% CI=1.03−1.53; recessive model, OR=1.26, 95% CI=1.05−1.51; and ins allele versus del allele, OR=1.19, 95%, CI=1.05−1.35 (Table 2, Figure 2). Stratified analyses also revealed a significant association between the polymorphism and ovarian, oral, and prostate cancers in the various models. Ethnic subgroup analyses revealed significant increases in cancer risk in the four models among Asians but not among Caucasians. The results became prominent when the six studies that deviated from HWE were excluded (see Supplementary Table 1 and Supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/612972).

3.3. Evaluation of Publication Bias. Publication bias was evaluated by Begg’s funnel plot and Egger’s test, and the visual asymmetry was determined in the funnel plot analysis (Figure 3). We further evaluated the publication bias in the subgroups. The results of Egger’s tests for all genetic models are shown in Supplementary Table 2 (ins allele versus del allele, P = 0.004).

4. Discussion

NF-κB serves important functions in pathogenetic regulation and influences cancer development and aggressiveness by enhancing tumour angiogenesis, antiapoptosis, and proliferation and by repressing immune response [7, 33, 34]. Several investigators reported the constitutive activation of NF-κB in various malignancies [35, 36], including nonsmall cell lung carcinoma and colon, prostate, breast, bone, and brain cancers. p50 overexpression is frequently observed in various tumour tissues; hence, p50 is potentially involved in tumorigenesis. A polymorphism in the promoter region of NFKB1 encoding the p50 subunit of NF-κB modulates gene activity. This polymorphism has been recently reported to influence cancer risk.

A meta-analysis of all eligible studies in 2010 suggested that the deletion allele serves as a protective or risk allele for cancer susceptibility among Asians or Caucasians, respectively [9]. However, no significant association was detected for the overall population [9]. After the reported study, numerous studies further assessed the relationship between the NFKB1 promoter −94ins/del ATTG polymorphism and cancer among Asians and Caucasians [10, 12, 32]. However, the association remains inconclusive because of the inconsistent results from the published studies. Li et al. [12] found an association between del/del genotype and bladder cancer risk but none between the polymorphism and hepatocellular carcinoma susceptibility [30].

In this study, we analysed 21 eligible case-control studies with 6127 cases and 9239 controls. The results of this meta-analysis revealed a significant association between insertion allele careers and enhanced cancer risk. The probable mechanism behind the observed association may be linked to the enhanced expression and activity of p50 (NF-κB1). The insertion allele is reportedly associated with the increased promoter activity and enhanced NFKB1 mRNA expression [8, 12, 17]. This association might influence cancer development.

The major effect of p50 (NF-κB1) is mediated by its function as a component of the transcription factor NF-κB, which is among the major signalling pathways involved in the cellular response to environmental stress [7]. p50 serves an important function in inhibiting cell apoptosis by modulating the expression levels of several survival genes, such as bcl-2 homologue A1 [37], PAI-2 [38], and IAP gene family [39]. Certain antiapoptosis proteins, such as Bcl-xL and Fas-associated death domain-like IL-1-converting enzyme inhibitor protein, are upregulated through the NF-κB signalling pathway [40–42]. In addition, accumulated evidence illustrated that the p50 (NF-κB1) signalling pathways participate in cellular proliferation by increasing IL-5 [43], promoting MAPK phosphorylation [7, 44], and modulating cyclin D1 expression [45]. Therefore, the observed association between the −94ins/del ATTG polymorphism and cancer risk can be accounted for by the insertion allele that can inhibit apoptosis and promote cellular proliferation by upregulating the expression of p50 (NFKB1) [8, 12, 17], which was implicated in the abovementioned mechanism.

In the stratified analyses, the increased cancer risk remained in subgroups of Asians but not in those of Caucasians. The ethnic differences in the allele frequencies may be caused by natural selection or balance to other related genetic variants. Possible differences in genetic backgrounds and gene environment may also interact with the etiology. The increased cancer risk also remained in the subgroups of ovarian, oral, and prostate cancers. This result suggested that the NFKB1 gene might function as a prominent factor in these cancers. Therefore, further investigations are warranted to validate ethnic difference and cancer specificity in the effect of this functional polymorphism on cancer susceptibility.

This study has several limitations. First, significant between-study heterogeneity was detected in some comparisons and may be distorting the meta-analysis. Second, the genotype distribution among controls did not completely agree with HWE. However, the association between the insertion allele and cancer risk in the overall population and in the Asian population became pronounced when the six studies that deviated from HWE were excluded. Third, the studies included in the analysis used different genotyping methods with different quality control issues that may have also influenced the results. Fourth, publication bias was observed
Table 2: Meta-analysis of the NFKB1 –94ins/del ATTG promoter polymorphism and cancer risk.

| Variables          | n | Cases/Controls | ins/ins versus del/del | ins/del versus del/del | ins/ins + ins/del versus del/del (dominant) | ins/ins versus ins/del + del/del (recessive) | ins allele versus del allele |
|--------------------|---|----------------|------------------------|------------------------|---------------------------------------------|---------------------------------------------|-------------------------------|
|                    |   | (95% CI)       | (95% CI)               | (95% CI)               | (95% CI)                                    | (95% CI)                                    | (95% CI)                      | (95% CI) |
| Total              | 21 | 6127/9239      | 1.47 (1.11–1.93)       | 1.15 (0.97–1.37)       | 1.26 (1.03–1.53)                            | 82.3                                       | 1.19 (1.05–1.35)              | 84.0    |
| Cancer types       |   |                |                        |                        |                                             |                                             |                               |          |
| Bladder cancer     | 3  | 1058/1175      | 1.07 (0.45–2.53)       | 1.00 (0.46–2.18)       | 1.04 (0.46–2.33)                            | 90.7                                       | 1.04 (0.73–1.48)              | 72.7    |
| Colorectal cancer  | 4  | 1275/1890      | 0.84 (0.47–1.50)       | 0.93 (0.77–1.13)       | 0.88 (0.73–1.06)                            | 0                                          | 0.89 (0.51–1.55)              | 88.9    |
| Ovarian cancer     | 2  | 366/444        | 2.57 (1.66–3.98)       | 1.88 (1.23–2.89)       | 2.17 (1.45–3.25)                            | 0                                          | 1.59 (1.19–2.11)              | 0       |
| Oral cancer        | 2  | 674/721        | 2.10 (1.54–2.87)       | 1.42 (1.10–1.83)       | 1.59 (1.25–2.03)                            | 3.9                                        | 1.67 (1.29–2.17)              | 0       |
| Prostate cancer    | 2  | 451/477        | 1.59 (1.09–2.33)       | 1.28 (0.89–1.84)       | 1.40 (1.00–1.98)                            | 35.8                                       | 1.33 (1.01–1.74)              | 0       |
| Other cancers      | 8  | 2303/4532      | 1.72 (1.13–2.61)       | 1.16 (0.88–1.53)       | 1.34 (0.99–1.83)                            | 72.4                                       | 1.46 (1.12–1.90)              | 78.0    |
| Ethnicities        |   |                |                        |                        |                                             |                                             |                               |          |
| Asian              | 14 | 4143/5169      | 1.83 (1.30–2.57)       | 1.23 (0.97–1.58)       | 1.42 (1.08–1.86)                            | 82.5                                       | 1.50 (1.26–1.78)              | 66.8    |
| Caucasian          | 7  | 1984/4070      | 0.90 (0.64–1.27)       | 1.00 (0.85–1.18)       | 0.95 (0.81–1.10)                            | 24.0                                       | 0.90 (0.66–1.23)              | 83.7    |

aNumber of comparisons.
bRandom effects estimate.
in our study, which may affect the validity of conclusion. In the stratified analysis, we found that the publication bias was significant among the Asian groups and other cancer groups but not significant among the Caucasian, bladder, and colorectal cancer groups. The sample sizes of the included studies were diverse, and most of them were insufficiently large. These conditions might partly interpret the publication bias. Finally, only three controls were population based; thus, they may not represent the general population. Therefore, the results of this study should be interpreted with caution.

In conclusion, the NFKB1 promoter −94ins/del ATTG polymorphism is associated with cancer risk. Well-designed studies with representative sample sizes are necessary to validate these findings.

**Conflict of Interests**

The authors have declared that no conflict of interests exist.

**Authors’ Contribution**

Xiao Yang, Pengchao Li, and Jun Tao contributed equally to this work.

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Upregulated PD-1 Expression Is Associated with the Development of Systemic Lupus Erythematosus, but Not the PD-1.1 Allele of the PDCD1 Gene

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Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease with complicated genetic inheritance. Programmed death 1 (PD-1), a negative T cell regulator to maintain peripheral tolerance, induces negative signals to T cells during interaction with its ligands and is therefore a candidate gene in the development of SLE. In order to examine whether expression levels of PD-1 contribute to the pathogenesis of SLE, 30 patients with SLE and 30 controls were recruited and their PD-1 expression levels in peripheral blood mononuclear cells (PBMCs) were measured via flow cytometry and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). Also, whether PD-1 expression levels are associated with the variant of the SNP rs36084323 and the SLE Disease Activity Index (SLEDAI) was studied in this work. The PD-1 expression levels of SLE patients were significantly increased compared with those of the healthy controls. The upregulated PD-1 expression levels in SLE patients were greatly associated with SLEDAI scores. No significant difference was found between PD-1 expression levels and SNP rs36084323. The results suggest that increased expression of PD-1 may correlate with the pathogenesis of SLE, upregulated PD-1 expression may be a biomarker for SLE diagnosis, and PD-1 inhibitor may be useful to SLE treatment.

1. Introduction

Systemic lupus erythematosus (SLE) is a progressive autoimmune disease with a wide range of immunological abnormalities [1]. It is characterized by an immune response against nucleus components, but the etiopathology is not clearly understood yet. Multiple genetic factors relating to SLE have been identified [2–4], which suggest that both immune disorder and genetic factors may play important roles during the SLE process.

The protein programmed death 1 (PD-1), a negative costimulatory molecule, belongs to the CD28 superfamily and is expressed on the surface of activated human CD4+ and CD8+ T cells, B cells, natural killer (NK) cells, activated monocytes, myeloid cells, and CD4−CD8− T cells from the thymus [5, 6]. As an immune inhibitory receptor, PD-1 interacts with its ligands, PD-L1 and PD-L2, which can suppress lymphocyte activation and cytokine production [7]. Current concepts regarding PD-1/PD-L pathway are categorized into immune dysfunction associated with SLE in humans [8]. In addition, it was reported that PD-1 gene polymorphisms were involved in the development of autoimmune diseases, such as SLE, rheumatoid arthritis, and Graves’ disease [9]. However, until now, only few studies have reported a possible link between PD-1 gene polymorphisms and SLE [8, 10–12]. Due to the existence of racial and regional differences in SNPs in PD-1, it is very important to study the relevance of PD-1 to SLE susceptibility in the Chinese Han population, which could
also bring more evidence to the connections among alleles and disease in SLE.

Among single nucleotide polymorphisms (SNPs) in the human PD-1 gene (PDCD1) region, PD-1.1 G/A (rs36084323) was reported to have connection with autoimmune diseases [13]. Previous studies suggested that nonfunctional SNPs could affect gene function through haplotype tagging [14]. In this work, PD-1.1 G/A (rs36084323), a nonfunctional SNPs in PDCD1, was studied in a Chinese Han population, aiming to explore whether PD-1 expression was related to the variant of the SNP PD-1.1 G/A (rs36084323) and SLE Disease Activity Index (SLEDAI).

2. Materials and Methods

2.1. Patients and Controls. This study was performed on 30 cases of Chinese Han population fulfilling the revised criteria for SLE from American College of Rheumatology 1997 [15,16] (27 women; 3 men; mean ± SD age, 43.432 ± 14.675) and compared with 30 age-matched controls (27 women; 3 men; mean ± SD age, 41.520 ± 10.478). SLEDAI [17] was taken for each patient at the time of recruitment. The protocol of this study was approved by the ethics committee of the institution involved, and informed consents for genetic studies were obtained from all subjects.

2.2. Flow Cytometry Analysis. Flow cytometry was performed using 50 μL of EDTA-treated peripheral blood incubated for 30 min at 4°C with fluorochrome-labeled monoclonal antibodies (mAbs): anti-CD4-FITC (Beckman), anti-CD8-FITC (Beckman), anti-CD56-FITC (Beckman), and anti-PD-1-PE (BioLegend). Erythrocyte lysis and cell fixation were carried out using OptiLyse C Lysing Solution (Beckman). Treated blood samples passed through the Coulter Epics XL Flow cytometer (Beckman), and the relevant data were acquired and accordingly examined. Data analysis was accomplished by FlowJo software (Tree Star, Ashland).

2.3. Real-Time RT-PCR Analysis. PBMCs were separated from fresh blood of patient and control groups in order to analyze the mRNA of the PD-1. Total cellular RNA was isolated by Trizol (Invitrogen, USA). After quantification, 1μg of total cellular RNA was used to conduct reverse transcription with Promega RT kit (A3800) and an oligo (dT) primer. PCR was performed in a 50 μL reaction system containing 200 nM PD-1 primers (Table 1), 120 nM TaqMan probe, and premix Ex Taq (Takara, Dalian, China). Samples were amplified in the Applied Biosystems 7900 HT Fast real-time PCR System (CA, USA) for 40 cycles under the following conditions: denaturation for 10 s at 95°C, anneal

| Table 1: Primers of SNP PD-1.1 used for sequencing. |
|-----------------------------------------------|
| Maker name | SNP | rs number | Location | PCR primers |
|---|---|---|---|---|
| PD-1.1 | −538 G/A | rs36084323 | promoter | Forward: 5’-CCCGTCAGGCTGTTGCA-3’ |
| | | | | Reverse: 5’-CCCTTCTTCCACATCCAC-3’ |

2.4. Genotyping. Blood DNA was extracted using Flexi Gene DNA kits (QIAGEN, Germany) according to the manufacturer’s instructions, and the DNA samples were then stored at −20°C. DNA fragments spanning PD-1.1 G/A (rs36084323) were amplified by polymerase chain reaction (PCR), and the products were gel-purified and sequenced. Then the sequencing data were applied for Genotyping of SNP (rs36084323).

2.5. Statistics. The data of PD-1 mRNA expression levels in PBMCs from all subjects were compared by the Mann-Whitney U-test. The genotype frequency of SNP rs36084323 was tested for Hardy-Weinberg equilibrium separately for SLE patients group, while the P values > 0.05 for all subjects in control group. Genotypes were compared using the Mann-Whitney U-test, and the relation between PD-1 mRNA expression level and the SLEDAI score was examined by Spearman’s correlation coefficient rank test. All analyses were processed using SPSS16.0. Data were presented as mean ± SD. P value < 0.05 (two-tailed) was considered as statistically significant.

3. Results

3.1. PD-1 Level Is Upregulated in PBMCs from SLE Patients Compared with Controls. Initially PD-1 protein and mRNA expression levels in all PBMCs samples were examined by flow cytometry and real-time RT-PCR. Flow cytometry analysis results demonstrated that the mean fluorescence intensity (MFI) of PD-1 was higher on CD4+ T cells, CD8+ T cells, and CD56+ T cells from PB samples of SLE patients compared to those of controls (Figures 1(a) and 1(b)). In addition, it is shown that the mean PD-1 mRNA expression levels increased in SLE patients’ samples compared with controls’ (Figure 1(c)).

3.2. SLEDAI Is Significantly Related to the Upregulated PD-1 Expression. In order to determine whether upregulated PD-1 expression is related to SLEDAI, correlation analysis was carried out. The results have shown that SLEDAI scores were significantly related to upregulated PD-1 expression on CD4+ T cells, CD8+ T cells, and CD56+ T cells, and increased PD-1 mRNA expression levels in PBMCs from PB samples of SLE patients (Figure 2), which indicates that upregulated PD-1 expression may be involved in the pathogenesis of SLE.

3.3. The Variant of the SNP rs36084323 Is Not Related to Upregulated PD-1 Expression. To explore whether the variant
of SNP rs36084323 was related to the upregulated PD-1 expression or not, the variant of SNP rs36084323 was genotyped. Results indicate that there was no connection between the variant of SNP rs36084323 and upregulated PD-1 expression (Figure 3).

4. Discussion

In this study, we demonstrated that PD-1 expression levels in PBMCs from SLE patients were significantly higher than those in control group. Also, significant relationship was found between SLEDAI scores and upregulated PD-1 expression in PBMCs from PB samples of SLE patients. However, no obvious difference was revealed between PD-1 expression levels and SNP rs36084323. Results show that increased level of PD-1 expression in PBMCs rather than SNP rs36084323 is associated with the development of SLE, and this discovery is presented for the first time. These findings provide more evidence to support the theory that upregulated PD-1 expression may be involved in the pathogenesis of SLE.

SLE is a chronic inflammatory disease of generalized autoimmunity and is characterized by B cell hyperactivity and abnormally activated T cells [1]. PD-1 can be expressed on activated T cells, B cells, and myeloid cells and is considered to play an important role in the regulation of peripheral tolerance [18]. Mice deficient for PD-1 have developed a lupus-like syndrome, with arthritis and glomerulonephritis as phenotypes [19]. In this study, increased expression of PD-1 in PBMCs is found to have significant relationship with SLEDAI scores, and the results suggest that PD-1 is involved in the development of SLE. Although the detailed etiology is still unclear, many genes are considered to have connections with the pathogenesis of SLE. At present, the programmed cell death 1 gene (PDCD1, also called PD-1) was
Figure 2: Correlation of upregulated PD-1 expression levels with SLEDAI in PBMCs. The association of SLEDAI with upregulated PD-1 expression on CD4⁺ T cell (a), CD8⁺ T cell (b), CD56⁺ T cell (c), and mRNA expression of PD-1 in PBMCs (d).

Figure 3: Analysis of PD-1 expression levels in SLE patients with different genotypes of rs36084323. Increased levels of PD-1 levels in PBMC of SLE (n = 30) patients, as compared with those from NC (n = 30). The patients with GG genotype (n = 11) exhibit higher PD-1 expression levels than those with AG (n = 15) and AA genotype (n = 4). Horizontal bars indicate the mean ± SD.
one of the top candidates linking to the disease [20]. Thus, it is therefore necessary to study the interconnection between polymorphisms in PDCD1 and SLE.

135 SNPs (found in the National Center for Biotechnology Information (NCBI) Entrez SNP database) have been identified in the human PDCD1 region. Among them, PD-1.1, PD-1.3, PD-1.5, and others are considered to have connection to autoimmune diseases [21]. PD-1.1 polymorphism is located in the promoter region (position −538 from transcription start site). Previous studies have shown that PD-1.1 G/A (rs36084323) is common in the Chinese Han population (49%), but it is very rare in Europeans (1%) [20], which may indicate that Chinese Han population is more susceptible to SLE. In this study, no connection was found between SNP PD-1.1 G/A (rs36084323) and increased expression of PD-1 in expression of PBMCs from SLE patients (P > 0.05), and the reason might be the limited sample size used in this study or some yet unidentified reason. However, it is observed that frequencies of the GG and AG genotype allele in SNP PD-1.1 were higher in SLE patients when compared with AA in our patients’ population. In addition, PD-1.1 is located within the promoter region of PD-1. This SNP has no function, and further study is required to explore its exact role in the development of SLE.

5. Conclusions

In conclusion, increased expression of PD-1 in PBMCs from SLE patients was significantly related to SLEDAI scores rather than SNP rs36084323. The presented results provide more evidence to support that upregulated expression of PD-1 might be correlated with the pathogenesis of SLE.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Qingqing Jiao, Cuiping Liu, and Ziliang Yang contributed equally to this work.

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A Promoter Region Polymorphism in \textit{PDCD-1} Gene Is Associated with Risk of Rheumatoid Arthritis in the Han Chinese Population of Southeastern China

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Objective. Programmed cell death 1 (PD-1) induces negative signals to T cells during interaction with its ligands and is therefore a candidate gene in the development of autoimmune diseases such as rheumatoid arthritis (RA). Herein, we investigate the association of \textit{PDCD-1} polymorphisms with the risk of RA among Chinese patients and healthy controls.

Methods. Using the PCR-direct sequencing analysis, 4 \textit{PDCD-1} SNPs (rs36084323, rs11568821, rs2227982, and rs2227981) were genotyped in 320 RA patients and 309 matched healthy controls. Expression of PD-1 was determined in peripheral blood lymphocytes by flow cytometry and quantitative real-time reverse transcriptase polymerase chain reaction. Results. We observed that the GG genotype of rs36084323 was associated with an increased risk for developing RA (OR = 1.70, 95% CI = 1.11–2.61, \(P = 0.049\)). Patients carrying G/G genotyped displayed an increased mRNA level of PD-1 (\(P = 0.04\)) compared with A/A genotype and healthy controls. Meanwhile, patients homozygous for rs36084323 had induced basal PD-1 expression on activated CD4+ T cells. Conclusion. The \textit{PDCD-1} polymorphism rs36084323 was significantly associated with RA risk in Han Chinese population. This SNP, which effectively influenced the expression of PD-1, may be a biomarker of early diagnosis of RA and a suitable indicator of utilizing PD-1 inhibitor for treatment of RA.

1. Introduction

Rheumatoid arthritis (RA) is a common chronic inflammatory autoimmune disease characterized by significant disability and early mortality, which affects \(\sim 1\%\) of adult population worldwide [1, 2]. It is generally accepted that RA is a complex autoimmune disorder, characterized by a chronic T-cell response that evaded normal control mechanisms [3, 4]. Therefore, the genes involved in the regulation of T-cell responses may be primary determinants of susceptibility to RA.

Programmed death-1 (PD-1, also called CD279) is a novel costimulatory member of B7/CD28 family, which is inducibly expressed on CD4+ T cells, CD8+ T cells, natural killer T cells, B cells, and activated monocytes [5]. PD-1 receptor has two ligands: PD-L1 (also known as B7-H1 or CD274) and PD-L2 (also called B7-DC or CD273). PD-L1 is expressed on T cells, B cells, dendritic cells (DC), macrophages, and some tumor cells and is further upregulated upon activation. PD-1 engagement by PD-L1 dephosphorylates proximal signaling molecules and augments PTEN expression, inhibiting PI3K and AKT activation [6, 7]. The critical role of PD-1 in immune regulation is highlighted by gene disruption studies demonstrating strain-specific autoimmune phenotypes [8, 9]. In addition, genetic studies revealed that there is an association between \textit{PDCD-1} gene polymorphism and susceptibility to...
autoimmune diseases, such as systemic lupus erythematosus (SLE) [10,11], rheumatoid arthritis [12,13], multiple sclerosis [14], and diabetes mellitus [15, 16].

There is mounting evidence that PD-1 is linked to human autoimmunity. In view of the pivotal role of PD-1/PD-L pathway in autoimmune immunology, it is worth considering of PDCD-1 functional SNPs, PDCD-1+606A/G (rs36084323, also known as PD1.1) in promoter, PDCD-1+7146A/G (rs11568821, also known as PD1.3) in intron 4, and PDCD-1+7625G/A (rs2227982, also known as PD1.9) and PDCD-1+7786G/C (rs2227981, also known as PD1.5) in exon 5 as prospective candidates for individuals susceptibility to RA in the Han Chinese population. Therefore, in the present study, we sought to determine the association between functional genetic variations in the PDCD-1 gene and RA risk in a Chinese population in mainland.

2. Materials and Methods

2.1. Patients and Controls. This study was approved by the Ethics Committee of Soochow University and all subjects gave informed consent for the genetic analyses. A total of 320 unrelated Chinese RA patients were recruited from the Outpatient Departments of Rheumatology in the First and the Third Affiliated Hospital of Soochow University. They were composed of 72 men and 248 women, whose mean age was 55.3 years (SD = ±12.6). Individual patients with RA were diagnosed according to the diagnosis criteria established by the American College of Rheumatology and the disease severity of individual patients was evaluated using the disease activity score 28 (DAS28) [1]. A total of 20 patients with new-onset RA (<6 months of disease duration) were recruited for expression of PD-1 protein on activated T cells. Individual RA patients were excluded if she/he received treatment with DMARDs, corticosteroids, or immunosuppressive for any reason during the past 6 months or had other chronic inflammatory and autoimmune diseases, such as diabetes, multiple sclerosis, inflammatory bowel disease, metabolic syndrome, hypertension, cardiovascular diseases, cancer, or recent infection. The controls were gender, age, and ethnically matched unrelated healthy people obtained from the checkup population in the above two hospitals (Table I).

2.2. DNA Extraction and Polymorphism Genotyping. Peripheral venous blood samples of 2 mL were drawn from each individual by standard venepuncture and stored at −20°C. Genomic DNA was isolated from peripheral blood leucocytes by standard procedures. The reference sequence is the human PDCD-1 sequence (GeneBank accession number AF363458). The PDCD-1+606A/G (rs36084323) in promoter, PDCD-1+7146A/G (rs11568821) in intron 4, and PDCD-1+7625G/A (rs2227982) and PDCD-1+7786G/C (rs2227981) in exon 5 polymorphisms were determined by direct sequencing in an Applied Biosystems sequencer (ABI Prism, Model 3100, Avant).

2.3. RNA Isolation and Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction. Total RNA from PBMC of 30 RA patients and 24 healthy controls were extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Total RNA (1μg) was used for cDNA synthesis with oligoRT primers (Invitrogen, Karlsruhe, Germany) and superscript II reverse transcriptase (Takara Bio, Shiga, Japan). PCR was performed using PCR Master (Roche, Mannheim, Germany) with the following primers: for PD-1 mRNA, 5’-TCAGG-GTGACAGAGAGAAG-3’ (forward) and 3’-GACACCA-AACCACAGGTTT-3’ (reverse) and for GAPDH, 5’-GTGAAGGTCGGAGTCAACG-3’ (forward) and 3’-TGAGGGTCATGGAAGGGGTC-3’ (reverse). Fold changes were normalised based on GAPDH expression, and each assay was conducted in a 96-well ABI 7900HT real-time PCR system (Applied Biosystems, Foster City, CA, USA). This procedure was performed in triplicate.

2.4. Antibodies and Flow Cytometry. All antibodies were mouse anti-human monoclonal antibodies (mAb). PE-Cy5-conjugated anti-CD4 (OKT4) were from Beckman Coulter (Miami, FL). PE-conjugated anti-PD-1 (MIH4), fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (M-A251), anti-CD69 (FN50), and anti-HLA-DR (G46-6) were from BD Pharmingen (Franklin Lakes, NJ). PE- or PE-Cy5-conjugated IgG1 (679.1Mc7) (Beckman Coulter), PE-conjugated IgG2a (eBMA2a; eBio-science), and FITC-conjugated IgG1 (P3) (eBioscience) were used as IgG isotype controls. PBMCs (0.5 x 10⁶) were incubated in wash buffer (PBS/2.5% fetal bovine serum [FBS]) with appropriate amounts of mAb on ice for 30 minutes. Cells were washed and were immediately analyzed on an EPICS XL-MCL flow cytometer (Beckman Coulter).

2.5. Statistics Analysis. The deviation from Hardy-Weinberg equilibrium (HWE) was examined in controls by the χ² test. The following statistical analyses were performed using SNPstats software (Availability: http://bioinfo.iconcologia.net/SNPstats) [17]. Based on the logistic regression method, the case-control association of genotypes was
3. Results

3.1. Single Nucleotide Polymorphism Analysis. A total of four SNPs were successfully genotyped in 320 RA patients and 309 healthy controls. Table 2 shows the allele and genotype distribution of these four SNPs. P values for Hardy-Weinberg proportions of the SNPs are shown in Table 2 as well. For all four SNPs, the genotypic distribution in controls conformed to HWE. Among the four SNPs, the genotype and allele distributions of rs36084323 differed significantly between RA patients and controls (P < 0.05). When logistic regression was used for association analysis after modeling the SNPs' effects as additive, dominant, or recessive, rs36084323 showed significant difference in codominant (OR, 1.70; 95% CI, 1.11–2.61), recessive (OR, 1.50; 95% CI, 1.05–2.14), and log-additive (OR, 1.30; 95% CI, 1.05–1.61) models (Table 3). The log-additive model was accepted as the best inheritance model because it showed the smallest Akaike information criterion value (869.4). The rs11568821 nucleotide is not polymorphic among Chinese population. The other two SNPs showed no association with RA in all 5 inheritance models (data not shown).

3.2. Linkage Disequilibrium and Haplotypes Association Analysis. Because rs11568821 was nonpolymorphic in our population, it was excluded from the haplotype construction and LD analysis. Pairwise LD between the SNPs of the PDCD1 (rs36084323, rs2227982, and rs2227981) was calculated for the cases and controls in the Han Chinese. We found strong LD (D’ > 0.75) between some pairs of markers in the PDCD1 gene including rs36084323/rs2227982 (D’ = 0.7729) and rs2227982/rs2227981 (D’ = 0.7841).

The association analysis of the haplotypes with RA was similar to that of genotypes by logistic regression (Table 4). We found that the haplotype of rs36084323/rs2227982/rs2227981 showed significant association with the disease (P = 0.00015); ACT and ACC haplotypes were less frequently observed in cases than in controls (OR: 0.23, 95% CI: 0.10–0.53; OR: 0.32, 95% CI: 0.11–0.96, resp.), indicating that these two haplotypes act as protective phenotype in RA. The association analysis of the haplotypes was adjusted by sex too.

3.3. Association between rs36084323 Polymorphism and PD-1 mRNA Expression. To evaluate the association between rs36084323 polymorphism and RA, we examined whether or not the rs36084323 polymorphism was associated with an altered PD-1 mRNA expression. Significant difference was observed in the relative PD-1 mRNA expression level between patients with GG and AA genotypes (Figures 1(a) and 1(b)). We concluded that PDCD1 polymorphisms were associated with RA, and the GG genotype and G allele of rs36084323 that associated with increased PD-1 mRNA expression might be involved in RA development in Han Chinese population.

3.4. Upregulated Expression of PD-1 on Activated CD4+ T Cells in RA Patients Was Associated with rs36084323. We
Table 2: The 4 SNPs of *PDCD-1* gene investigated in the cases (*n* = 320) and controls (*n* = 309).

| SNP        | dbSNP ID and position | Allele | Frequency (%) | Genotype | HWE |
|------------|------------------------|--------|---------------|----------|-----|
|            |                        | P      |               | A/A      |     |
| 1 rs36084323 | promoter (~606A/G)     | A      | 47.2          | 24.7     |     |
|            |                        | G      | 52.8          | 45.1     | 30.3|
|            |                        | Controls | 54.1          | 45.9     | 0.014* |
|            |                        |        |               | A/A      |     |
|            |                        |        |               | A/G      | 31.1 |
|            |                        |        |               | G/G      | 46.5 |
|            |                        |        |               | 22.5     | 0.049* |
|            |                        |        |               | 0.12     |     |
| 2 rs11568821 | Intron 4 (+7146A/G)   | A      | 54.1          | 24.7     | 0.014* |
|            |                        | G      | 45.9          | 53.5     | 21.8|
|            |                        | Controls | 54.1          | 45.9     | 0.049* |
|            |                        |        |               | A/A      |     |
|            |                        |        |               | A/G      | 29.6 |
|            |                        |        |               | G/G      | 45.8 |
|            |                        |        |               | 24.6     | 0.049* |
|            |                        |        |               | 0.21     |     |
| 3 rs2227982 | Exon 5 (+7625G/A)     | C      | 51.5          | 24.8     |     |
|            |                        | T      | 48.5          | 53.5     | 21.8|
|            |                        | Controls | 52.5          | 47.5     | 0.74 |
|            |                        |        |               | C/C      | 29.6 |
|            |                        |        |               | C/T      | 45.8 |
|            |                        |        |               | T/T      | 24.6 |
|            |                        |        |               | 0.19     | 0.21|
| 4 rs2227981 | Exon 5 (+7786G/C)     | C      | 73.2          | 54.8     |     |
|            |                        | T      | 26.8          | 36.8     | 8.4 |
|            |                        | Controls | 68.0          | 32.0     | 0.18 |
|            |                        |        |               | 10.9     | 0.18|
|            |                        |        |               | 0.65     |     |

HWE indicates Hardy-Weinberg equilibrium; *P* < 0.05.

Table 3: The association analysis of rs36084323 with RA (adjusted by sex) by using logistic regression.

| Model      | Genotype  | Cases (%) | Controls (%) | OR (95% CI)   | P value | AIC   | BIC   |
|------------|-----------|-----------|---------------|----------------|----------|-------|-------|
| Codominant | A/A       | 24.7      | 31.1          | 1              | 0.049*   | 871.3 | 884.6 |
|            | A/G       | 45.1      | 46.5          | 1.22 (0.84–1.78)| 0.049*   | 871.3 | 884.6 |
|            | G/G       | 30.3      | 22.5          | 1.70 (1.11–2.61)| 0.073    | 871.3 | 881   |
| Dominant   | A/A       | 24.7      | 31.1          | 1              | 0.073    | 872.1 | 881   |
|            | A/G-G/G   | 75.3      | 68.9          | 1.38 (0.97–1.96)| 0.026*   | 870.3 | 879.2 |
| Recessive  | A/A-A/G   | 69.7      | 77.5          | 1              | 0.026*   | 870.3 | 879.2 |
|            | G/G       | 30.3      | 22.5          | 1.50 (1.05–2.14)| 0.073    | 875.2 | 884   |
| Overdominant | A/A-G/G  | 54.9      | 53.5          | 1              | 0.015*   | 869.4 | 878.3 |
|            | A/G       | 45.1      | 46.5          | 0.95 (0.69–1.29)| 0.015*   | 869.4 | 878.3 |
| Log-additive |         | —         | —             | 1.30 (1.05–1.61)| —        |       |

OR: odds ratio; 95% CI: 95% confidence intervals; AIC: Akaike’s information criterion; BIC: Bayesian information criterion; *P* < 0.05.

next examined the expression of PD-1 on PBMCs in RA patients. Flow cytometry analyses of PBMC samples from 20 RA patients demonstrated the upregulated PD-1 expression on CD4+CD25+T cells (26.44±2.43 versus 16.3±1.54, *P* = 0.0014), CD4+CD69+T cells (22.95±2.68 versus 15.10±1.43, *P* = 0.0185), and CD4+HLA-DR +T cells (51.26±3.31 versus 26.46±2.469, *P* < 0.0001) compared with healthy controls (Figures 2(a) and 2(b)). Although we found no difference between wild-type (G/G) and heterozygous (A/G) patients, the 4 patients who were homozygous for the SNP rs36084323 (A/A) had minimal PD-1 expression on freshly isolated CD4+T cells, including the activated CD25+ and CD69+ T-cell subsets (Figure 2(c)). Collectively, these results suggest that the rs36084323 polymorphism confers increased basal PD-1 expression at early-to-intermediate stages of CD4+ T-cell activation and that this is associated with increased risk of RA.

4. Discussion

Rheumatoid arthritis is a chronic inflammatory disease that may involve extra-articular organs in addition to joints. Genetic and environmental factors are related to the pathogenesis of RA [1, 2]. PD-1 has been implicated as a critical pathway for tolerance since the first discovery of spontaneous autoimmune disease in PD-1 knockout mice. Studies using the NOD mouse model for spontaneous type I diabetes have shown that PD-1 not only is critical during the early phases of T-cell activation and expansion, but also plays a critical role in regulating T-cell effector functions and T-cell tolerance at late time points in peripheral tissue sites [16–19]. Strategies to selectively target the PD-1 pathway using antagonists have been effective in CIA model. Administration of PD-L1-Ig significantly ameliorated autoimmunity as assessed by clinical arthritis score and histology in the joints [20]. Similarly, PDL-1.Fc treatment ameliorated the severity of CIA and reduced joint swelling as well as antigen-specific T-cell proliferative responses [21]. PD-1 knockout mice have increased susceptibility and develop severe arthritis following immunization with type II collagen [21]. Collectively, these models demonstrate the importance of PD-1 in the pathogenesis of RA.

Greater than 30 single nucleotide polymorphisms (SNPs) have currently been identified in the human *PDCD-1* gene,
Figure 2: Increased basal programmed death 1 (PD-1) expression on activated CD4+ T cells in RA patients. (a) A representative flow cytometry analysis of PD-1 expression on CD4+ T cells in RA patients and healthy controls is shown. (b) Upregulated expression of PD-1 on CD4+CD25+, CD4+CD69+, and CD4+HLA-DR+ T cells from RA patients, as compared with those from healthy controls. (c) RA patients homozygous for the rs36084323 SNP (G/G) have significantly increased percentages of PD-1+ activated CD4+CD25+, CD4+CD69+, and CD4+HLA-DR+ T cells compared with healthy controls and rs36084323 A/A RA patients (* = \( P < 0.05 \), ** = \( P < 0.01 \), by Mann-Whitney U test). Horizontal bars indicate the mean ± SD.
and several SNPs have been linked to development of autoimmune in various populations [10]. Understanding how these SNPs impact PD-1 functions in human cells would be important for exploiting this pathway clinically. In our research, we selected four potentially functional polymorphisms (The PDCD-1−606A/G (rs36084323) in promoter, PDCD-1+7146A/G (rs11568821) in intron 4, and PDCD-1+7625G/A (rs2227982) and PDCD-1+7786G/C (rs2227981) in exon 5) of PDCD-1 gene and identified the association between the four polymorphisms and the risk of RA in Southeastern Chinese population.

A region of PDCD-1 intron 4 was described as an enhancer-like structure containing binding sites for several transcription factors [22]. PD-1.3 SNP, a regulatory SNP located in intron 4, showed to be involved in susceptibility to RA in Swedish, European American, and Mexican families and in sporadic cases. However, we found that this nucleotide is not polymorphic in our Chinese people, and this was also verified among other Chinese populations [13, 23, 24].

As it is known, mutations in the promoter region may influence the engagement between sequence motifs and transcription factors and further disrupt the activation of gene and the initiation of transcription [25]. Accordingly, as a polymorphism located in the promoter of PDCD-1 gene, rs36084323 may also affect the transcription and activation of PDCD-1 gene, thereby influencing the development of RA. These promoter polymorphisms have been considered risk factors for RA. One group observed that the frequency of SNP PD-1.1 (rs36084323) A allele is a risk allele in Italian RA patients [26]. Another group observed that the AA genotype of SNP PD-1.1 was associated with a decreased risk for developing RA in Hong Kong Chinese RA patients [13]. Previous reports also have shown that PD-1.1 was associated with risk of sporadic breast cancer [27]. We observed reduced frequencies of both the AA genotype and the A allele in RA patients compared with controls and a compensatory increase of the GG genotype in RA patients, which indicated that AA genotype might be a protective factor to decrease the risk of RA. This was consistent with the results from Hong Kong Chinese population [13]. Hence, rs36084323 may be a functional polymorphism which can influence the risk of diseases.

PD-1 is thought to be important for the “fine tuning” of lymphocyte activation at the level of synovial tissue, considering the wide pattern of expression of one of its ligands, PDL-1, in activated endothelial and epithelial cells [17, 28–30]. Furthermore, CD4+PD-1+ T cells accumulate as unique anergic cells in rheumatoid arthritis synovial fluid [31]. It is also reported that the negative costimulatory PD-1/PDL-1 pathway regulates peripheral T-cell responses in both human and murine RA [21]. In this study we found upregulated PD-1 expression on CD4+CD25+T cells, CD4+CD69+T cells, and CD4+HLA-DR+T cells compared with healthy controls. We also found that patients carrying G/G genotype displayed an increased mRNA level of PD-1 and increased basal PD-1 expression on activated CD4+ T cells. We concluded that PD-1 polymorphisms were associated with RA, and the GG genotype and G allele of rs36084323 that associated with induced PD-1 mRNA expression and PD-1 expression might be risk factor in RA development in the Han Chinese population.

In conclusion, the present study provides strong evidence that rs36084323 functional polymorphisms may contribute to the risk of RA. This SNP, which effectively influenced the expression of PD-1, may be a biomarker of early diagnosis of RA and a suitable indicator of utilizing PD-1 inhibitor for treatment of RA. However, our results were obtained from a sample-sized sample, and therefore this is a preliminary conclusion. Validation by a larger prospective study from a more diverse ethnic population is needed to confirm these findings.

Disclose

All authors declare no commercial association, such as consultancies, stock ownership, or other equity interests or patent licensing arrangements.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.
Authors’ Contribution

CuiPing Liu, JueAn Jiang, and Li Gao contributed equally to this work.

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Molecular Evolution of the Vertebrate FK506 Binding Protein 25

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FK506 binding proteins (FKBPs) belong to immunophilins with peptidyl-prolyl isomerases (PPIases) activity. FKBPs are named for binding to the immunosuppressive drug FK506, characterized by one or more PPIase domains. The 15 identified members of human FKBPs are divided into 4 groups: cytoplasmic, TPR domain, endoplasmic reticulum (ER), and nucleus. FKBP25 and FKBP133 locate in the nucleus, containing a single PPIase domain [1].

FKBP25 (also known as FKBP3) is the first mammalian FKB with a calculated molecular mass of 25 kDa found in the nucleus, which plays a role in regulating transcription and chromatin structure. The FKB25 comprises a conserved PPIase domain at its C-terminus with a 43% sequence identity to FKBP12 and a helix-loop-helix (HLH) motif at its unique hydrophilic N-terminal [2,3]. This conserved PPIase domain functions in binding to the immunosuppressive agent FK506 or rapamycin. Unlike another FKBPs, FKBP25 shows a strong affinity for binding rapamycin (Ki = 0.9 nM) over FK506 (Ki = 200 nM) [4]. The FKB25 was reported to be associated with nuclear proteins including transcription factor Yin-Yang1 (YY1), mouse double minute 2 (MDM2), and histone deacetylases (HDACs) [5]. FKBP25 binds to YY1 at N-terminal and increases its DNA-binding activity without the involvement of the FK506/rapamycin binding domain [6]. In addition, the level and activity of the tumor suppressor protein p53 are negatively regulated by MDM2. The HLH motif of FKBP25 mediates protein-protein interaction to enhance ubiquitination and degradation of oncogene MDM2, increasing the expression of tumor suppressor p53 and its downstream effector p21 [7]. Moreover, the protein-protein interaction contributes to form HDAC complexes, which is critical for the chromatin structure [2].

In 1992, Jin et al. reported the molecule cloning of human FKBP25 and performed a homology comparison between FKBP25 and FKBP12/FKBP13 [8]. Furthermore, Mas et al. showed the molecule cloning of mouse FKBP25 and expression pattern of FKBP25 gene during cerebral cortical neurogenesis [9]. However, the relationships between nuclear functions and evolution in FKBP25 are seldom reported.

1. Introduction

Immunophilins include three families with peptidyl-prolyl isomerases (PPIases) activity, FK506 binding proteins (FKBPs), cyclophilins, and parvulins. FKBPs are named for binding to the immunosuppressive drug FK506, characterized by one or more PPIase domains. The 15 identified members of human FKBPs are divided into 4 groups: cytoplasmic, TPR domain, endoplasmic reticulum (ER), and nucleus. FKBP25 and FKBP133 locate in the nucleus, containing a single PPIase domain [1].
In this study, we exhibit an evolutional analysis not only on selective pressure but also on intron-exon conversion among vertebrate FKBP25 genes.

2. Materials and Methods

2.1. Sequence Data Collection. All the FKBP25 gene and amino acid sequences were obtained from the ENSEMBL (http://www.ensembl.org/index.html) [10], based on orthologous and paralogous relationships. The gained FKBP25 sequences were applied as queries to search known FKBP25 genes using BLAST at the National Center for Biotechnology Information (NCBI), in order to confirm whether their best hit was an FKBP25 gene [11].

Incomplete sequences of FKBP25 genes in four species (tree shrew, horse, platypus, and turkey) were retrieved from both ENSEMBL and NCBI. After eliminating these incomplete sequences, 28 sequences were applied for this study. The 28 sequences from 23 species comprised human (ENSG000001000442), chimpanzee (ENSPTRG00000006305), gorilla (ENSGGOG0000013322), orangutan (ENSSPYYG0000005778), macaque (ENSMUMG00000016512), marmoset (ENSCJAG00000015972), mouse (ENSMUSG0000020949), rat (ENSRNOG00000004629), guinea pig (ENS CPG00000001444), rabbit1 (ENSOUCUG00000007535), rabbit2 (ENSOUCUG00000026892), dog1 (ENSCAFG000000014018), dog2 (ENSCAFG000000014093), dog3 (ENSCAFG00000024192), dog4 (ENSCAFG0000000578), cow (ENSBTAG00000002610), elephant1 (ENSLAFG00000003572), elephant2 (ENSLAFG00000027553), opossum (ENSMODG00000007352), chicken (ENSGALG000000012466), zebra finch (ENSTGUG00000013231), anole lizard (ENSCACG0000000480), xenopus (ENSTXETG00000003052), fugu (ENSTRUG0000001887), medaka (ENSORLG00000005070), stickleback (ENSGACG00000012834), tetraodon (ENSTNIG00000010980), and zebrafish (ENSDARG000000079018).

2.2. Molecular Phylogenetic Analyses. The protein coding sequences of FKBP25 were aligned using CLUSTAL W program in MEGA 5.05. We constructed a maximum likelihood (ML) tree of FKBP25 amino acid sequences by MEGA 5.05 with the optimal model (Kimura 2-parameter model). The relative support of internal node was performed by bootstrap analyses with 1000 replications for ML reconstructions [12].

2.3. Selection Pressure Analyses. The numbers of nonsynonymous substitutions per nonsynonymous site (dN) and the numbers of synonymous substitutions per synonymous site (dS) were computed by MEGA 5.05 with the modified Nei-Gojobori method. The dN/dS <1, =1 and >1 demonstrate purifying selection, neutral selection, and positive selection, respectively [13]. The dN is the numbers of nonsynonymous substitutions per nonsynonymous site, and the dS is the numbers of synonymous substitutions per synonymous site. The transition/transversion ratio was 1.55 estimated using the ML method by MEGA 5.05 [14].

The FASTA format of FKBP25 sequences was converted to the PAML format using DAMBE software for subsequent site analyses [13]. The CODEML program implemented in the PAML 4.7 package was used to detect positive selection of individual sites. The site-specific model was exerted using

| Species | Models | Estimates of parameters | lnL | 2ΔI | Positively selected sites |
|---------|--------|-------------------------|-----|-----|--------------------------|
| Vertebrate | M7 | \( p = 0.91900 \) \( q = 8.19764 \) | -5463.938465 | 0.003806 | NA |
| | M8 | \( p = 0.99999 \) \( p = 0.91899 \) \( q = 8.19758 \) \( p = 0.00001 \) \( w = 1.86072 \) | -5463.940368 | None |
| Mammalian | M7 | \( p = 0.33823 \) \( q = 1.62046 \) | -2182.244789 | 0.000258 | NA |
| | M8 | \( p = 0.99999 \) \( p = 0.33824 \) \( q = 1.62055 \) \( p = 0.00001 \) \( w = 1.00000 \) | -2182.244918 | None |
| Primate | M7 | \( p = 4.13016 \) \( q = 99.00000 \) | -997.077389 | 0.000012 | NA |
| | M8 | \( p = 0.99999 \) \( p = 4.12942 \) \( q = 99.00000 \) \( p = 0.00001 \) \( w = 1.00000 \) | -997.077440 | None |
| Mammalian excluding primate | M7 | \( p = 0.28229 \) \( q = 1.41420 \) | -2242.306222 | 0.000160 | NA |
| | M8 | \( p = 0.99999 \) \( p = 0.28230 \) \( q = 1.41430 \) \( p = 0.00001 \) \( w = 1.00000 \) | -2242.306302 | None |
| Rodent | M7 | \( p = 0.13287 \) \( q = 1.19764 \) | -1372.902164 | 0.000058 | NA |
| | M8 | \( p = 0.99999 \) \( p = 0.13287 \) \( q = 1.19764 \) \( p = 0.00001 \) \( w = 1.00000 \) | -1372.902193 | None |
| Teleost | M7 | \( p = 0.38691 \) \( q = 4.30540 \) | -2354.923181 | 0.004080 | NA |
| | M8 | \( p = 0.99999 \) \( p = 0.38690 \) \( q = 4.30545 \) \( p = 0.00001 \) \( w = 3.90806 \) | -2354.923385 | None |

In all the species, 2ΔI < 9.21, the P-value is more than the significance level 0.05, indicating that M8 model is not better than M7 model; NA: not allowed; NS: not shown (it means the sites under positive selection but not reaching the significance level of 0.9).
Table 2: Exon and intron lengths of FKBP25.

| Species       | Exon1 | Intron1 | Exon2 | Intron2 | Exon3 | Intron3 | Exon4 | Intron4 | Exon5 | Intron5 | Exon6 | Intron6 | Exon7 | Intron7 | Exon8 | Intron8 | Length (bp) |
|---------------|-------|---------|-------|---------|-------|---------|-------|---------|-------|---------|-------|---------|-------|---------|-------|---------|-------------|
| Human         | 108   | 3548    | 102   | 797     | 108   | 8173    | 136   | 530     | 68    | 2761    | 98    | 1775    |       |         |       |         | 675          |
| Chimpanzee    | 108   | 3524    | 102   | 797     | 108   | 8898    | 136   | 530     | 68    | 2725    | 98    | 1789    |       |         |       |         | 675          |
| Gorilla       | 108   | 3538    | 102   | 796     | 108   | 8214    | 136   | 530     | 68    | 2753    | 98    | 1778    |       |         |       |         | 675          |
| Orangutan     | 108   | 3498    | 102   | 793     | 108   | 8395    | 136   | 533     | 68    | 2457    | 98    | 1432    |       |         |       |         | 675          |
| Marmoset      | 108   | 3496    | 102   | 786     | 108   | 8273    | 136   | 531     | 68    | 2845    | 98    | 1818    |       |         |       |         | 675          |
| Mouse         | 108   | 3762    | 102   | 841     | 108   | 2224    | 136   | 837     | 68    | 1961    | 98    | 937     |       |         |       |         | 675          |
| Rat           | 108   | 3528    | 102   | 816     | 108   | 2030    | 136   | 942     | 68    | 1667    | 98    | 1118    |       |         |       |         | 675          |
| Guinea pig    | 108   | 3232    | 102   | 772     | 108   | 3600    | 136   | 1416    | 68    | 1346    | 98    | 1340    |       |         |       |         | 675          |
| Rabbit1       | 108   | 2189    | 102   | 1082    | 108   | 4634    | 136   | 1115    | 68    | 1826    | 98    | 1266    |       |         |       |         | 675          |
| Rabbit2       | 620   | 40      | 55    |         |       |         |       |         |       |         |       |         |       |         |       |         | 675          |
| Dog1          | 108   | 2573    | 102   | 1076    | 108   | 2088    | 136   | 468     | 68    | 1823    | 98    | 1216    |       |         |       |         | 675          |
| Dog2          | 296   | 13      | 229   | 4       | 129    |         |       |         |       |         |       |         |       |         |       |         | 654          |
| Dog3          | 30    | 2       | 195   | 2       | 252    | 4       | 129    |         |       |         |       |         |       |         |       |         | 645          |
| Dog4          | 427   | 190     | 248   |         |       |         |       |         |       |         |       |         |       |         |       |         | 675          |
| Cow           | 108   | 2332    | 102   | 603     | 108   | 2835    | 136   | 484     | 68    | 1706    | 98    | 1309    |       |         |       |         | 675          |
| Elephant1     | 108   | 3176    | 102   | 1089    | 108   | 4756    | 136   | 483     | 68    | 1580    | 98    | 1725    |       |         |       |         | 675          |
| Elephant2     | 675   |         |       |         |       |         |       |         |       |         |       |         |       |         |       |         | 675          |
| Opossum       | 108   | 2560    | 102   | 1484    | 108   | 2807    | 136   | 1051    | 68    | 1261    | 98    | 554     |       |         |       |         | 675          |
| Chicken       | 111   | 76      | 102   | 75      | 114    | 408     | 136   | 1040    | 68    | 1011    | 98    | 829     |       |         |       |         | 684          |
| Zebra finch   | 111   | 112     | 102   | 76      | 108    | 494     | 136   | 892     | 97    | 2054    | 16    | 69      | 53    | 49      | 55          |
| Arole lizard  | 186   | 1699    | 108   | 1333    | 136    | 1078    | 68    | 824     | 98    | 610     | 55    |         |       |         | 651          |
| Xenopus       | 111   | 239     | 102   | 403     | 108    | 418     | 136   | 129     | 68    | 186     | 98    | 787     | 55    |         |       |         | 678          |
| Frugu         | 105   | 375     | 102   | 78      | 105    | 65      | 136    | 82      | 68    | 98      | 106   | 55      |       |         |       |         | 669          |
| Medaka        | 105   | 109     | 102   | 71      | 99     | 738     | 136    | 75      | 68    | 70      | 98    | 804     | 55    |         |       |         | 663          |
| Stickelback   | 105   | 294     | 102   | 76      | 102    | 93      | 136    | 135     | 68    | 81      | 98    | 96      | 55    |         |       |         | 666          |
| Tetraodon     | 105   | 305     | 102   | 80      | 102    | 75      | 136    | 91      | 68    | 70      | 98    | 75      | 55    |         |       |         | 666          |
| Zebra fish    | 105   | 2527    | 102   | 447     | 16     | 1117    | 20    | 244     | 16    | 359     | 19    | 904     | 28    | 108     | 11          | 666 |

|        | Intron8 | Exon9 | Intron9 | Exon10 | Intron10 | Exon11 | Intron11 | Exon12 | Intron12 | Exon13 | Intron13 | Exon14 | Intron14 | Exon15 | Intron15 |
|--------|---------|-------|---------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|
|        | 1042    | 24    | 316     | 26     | 708      | 14     | 99       | 10     | 98       | 31     | 107      | 15     | 26       | 76     | 1784     |
| Exon16 | Intron16 | Exon17 |
| 98     | 118     | 55     |
| Species       | Sequence | Structural Alignment |
|--------------|----------|----------------------|
| Human        |          |                      |
| Chimpanzee   |          |                      |
| Gorilla      |          |                      |
| Orangutan    |          |                      |
| Macaque      |          |                      |
| Marmoset     |          |                      |
| Mouse        |          |                      |
| Rat          |          |                      |
| Guinea       |          |                      |
| Rabbit1      |          |                      |
| Rabbit2      |          |                      |
| Dog1         |          |                      |
| Dog2         |          |                      |
| Dog3         |          |                      |
| Dog4         |          |                      |
| Cow          |          |                      |
| Elephant1    |          |                      |
| Elephant2    |          |                      |
| Opossum      |          |                      |
| Chicken      |          |                      |
| Zebra        |          |                      |
| Anole        |          |                      |
| Xenopus      |          |                      |
| Medaka       |          |                      |
| Fugu         |          |                      |
| Stickleback  |          |                      |
| Tetraodon    |          |                      |
| Zebrafish    |          |                      |

**Figure 1:** Sequence and structural alignment of FKBP25.
Figure 2: Phylogenetic tree and motif distributions of FKBP25.

Figure 3: Pairwise comparisons of \(dN\) and \(dS\) among 28 vertebrate FKBP25 sequences.

Figure 4: The average nonsynonymous \((dN)\) and synonymous \((dS)\) in FKBP25 from different vertebrate groups. The value of average \(dN\) was in blue, and the value of average \(dS\) was in red.

Likelihood ratio tests (LRT) to compare M7 (null model) with M8 model. M7 is a null model that does not allow for any codons with \(\omega > 1\), whereas M8 model allows for positively selective sites \((\omega > 1)\). When the M8 model fitted the data significantly (\(P\)-value < 0.05) better than the null model (M7), the presence of sites with \(\omega > 1\) is suggested. On the contrary, the results of \(P\) value > 0.05 proved the absence of sites with \(\omega > 1\). The twice log likelihood difference between the two compared models \((2\Delta L)\) is compared against \(\chi^2\) with
critical values 5.99 and 9.21 at 0.05 and 0.01 significance levels, respectively [15].

2.4. Protein Domain and Motif Analyses. Protein domain analyses of FKBP25 were shown at Pfam domains database (http://pfam.sanger.ac.uk) [16]. SMART (http://smart.embl-heidelberg.de/) was used to make sure the presence of FKBP25 domains [17]. The motifs of FKBP25 were analyzed by the MEME software (http://meme.sdsc.edu/meme/web-site/intro.html) with a maximum of 10 motifs to find [18].

2.5. Exon-Intron Conservation Analyses. We collected elaborate information about FKBP25 exon and intron from ENSEMBL (http://www.ensembl.org/index.html) [19]. The number and length of FKBP25 exon and intron in 28 sequences were investigated for exon-intron conservation analyses.

3. Results

3.1. Phylogenetic Analyses of FKBP25. All the FKBP25 gene and protein sequences were collected from the ENSEMBL and checked by BLAST at NCBI. The sequence and structural alignment of FKBP25 was shown in Figure 1. The phylogenetic tree was constructed according to the protein coding sequences of FKBP25 using the maximum likelihood method (Figure 2, left panel). The FKBP25 genes from the primate lineage and teleost lineage form a species-specific cluster, respectively. Four FKBP25 isoforms of dog exhibited a close relationship and clustered together, according to the phylogenetic tree. There were similar phenomena in rabbit and elephant.

3.2. Selection Pressure Analyses. The nonsynonymous to synonymous rate ratio ($dN/dS$) may demonstrate the selective pressures of involved protein. We calculated the pairwise distance of FKBP25 sequences using MEGA 5.05. There was a significantly lower $dN$ than $dS$ in the pairwise comparisons of these sequences. Most values of $dN/dS$ in these sequences were distributed blow the diagonal, showing that the presence of a purifying selection existed in the FKBP25 (Figure 3). The comparisons of average $dN$ and $dS$ in various vertebrate groups were shown in Figure 4, respectively. Furthermore, site-specific tests were performed for searching the positive selection sites in vertebrate, mammalian, primate, and mammalian excluding primate, rodent and teleost lineages. Although some positive selection sites were computed, each $2\Delta \ell$ of M7 and M8 < 5.99 indicated that the M8 model was not significantly better than the M7 model to fit the data. Consequently, we concluded that the site-specific analyses also compute no positive selection sites acting on FKBP25 using PAML4.7 (Table 1).

3.3. Protein Domain and Motif Analyses. Early studies reported that mammalian FKBP25 have two portions: one is a putative helix-loop-helix motif within N-terminal unique sequence (Figure 5(a)) and the other is the PPIase domain at its C-terminus (Figure 5(b)) [20].

The domain distribution of FKBP25 was investigated using FKBP25 to search amino acid sequences at the Pfam database firstly. Only one domain (PPIase domain) was found in the Pfam database. The PPIase domain within FKBP25 sequences generally started at position 122 and ended at position 221. Similarly, we further make sure that the FKBP25 domain is at SMART, resulting in the single PPIase domain at position 119 to 221.
We then performed a detailed domain and motif analyses using the MEME software. Except two dog isoforms, dog2 and dog3, the FKBP25 sequences used in this study contain a conversed PPIase domain within motif 1 (shown in Figure 2) at its C-terminus. In addition, the result implied that motif 2 located in the N-terminal contained an HLH motif [6], which was associated with DNA binding and dimerization [21]. However, HLH motif was not found in dog3, anole lizard, and teleost lineage, implying that these FKBP25 proteins may function on gene expression in another pathway.

3.4. Exon-Intron Conservation Analyses. The exon-intron information collected from the ENSEMBL database was shown in Table 2 and Figure 6. Most of the FKBP25 genes have 7 exons with similar length in different species (Table 2). Mammalian FKBP25 shows exon-intron conservation with 6 introns and similar sizes of each intron. Intron deletions existed in several isoforms of species. The rabbit2 isoform had 2 exons, and elephant2 isoform had only one exon. The exon numbers of dog2, dog3, and dog4 isoforms were less than seven. Except mammalian FKBP25 genes, anole lizard reduced one exon compared with mammalian and birds, but the xenopus and teleost maintained 7 exons. The intron deletions of FKBP25 genes may happen in the evolutionary process from amphibian to reptile. Then, a subsequent intron insertion occurred in the evolution from reptile to more advanced animals. The FKBP25 genes also had intron insertion in zebra finch and zebra fish.

4. Discussion

FKBP25 is a nuclear member of the FKBPs family that is associated with transcription and chromatin structure [2]. The interactions of FKBP25 with nuclear proteins are closely associated with HLH motif at the N-terminal of FKBP25. However, whether the PPIase domain at C-terminus is important for these interactions remains uncertain. The selection pressure analyses revealed that the purifying selection triggered a whole evolutionary history of FKBP25 in vertebrates, even in each lineage of vertebrates. Purifying selection is one
of the natural selections that resist deleterious mutations with negative selective coefficients [22]. The mutations that disrupt the correct folding of the FKBP25 domain can weaken PPIase activity and may be the deleterious mutations [5]. It was hypothesized that the mutations of PPIase domain were one of explanations behind the purifying selection throughout FKBP25 evolution. Therefore, although the PPIase domain of FKBP25 was not found to be involved in the protein interactions previously, the PPIase domain might have some associations with the YY1 DNA-binding, MDM2 autoubiquitination and degradation, and HDACs complex formation. These inferences will become a potent direction for exploring the relationship between nuclear proteins and PPIase domain in the future.

The protein-coding sequence length of vertebrate FKBP25 is highly conserved that almost all the taxa are 224 bp; nevertheless the original gene length and exon-intron status are tremendously various among vertebrate species. However, mammalian FKBP25 exhibit exon-intron conservation with 6 introns and similar sizes of each intron. Chicken FKBP25 maintains 6 introns, but zebra finch has one more intron that inserts in the gene. Similarly, a large variability of intron number and sizes among all the taxa shown in Figure 6 revealed that intron insertion and deletion events happened frequently during the FKBP25 evolutionary history from teleost to birds. In particular, zebrafish demonstrated the maximum number of introns in this study, and the size of exon is much smaller than other teleost species (Figure 6(g)). The intron loss of FKBP25 gene from species more advanced than zebrafish is likely to induce alterations of gene expression due to the absence of specific intron splicing. Under the purifying selection, the FKBP25 gene expression event continuously removes the pernicious mutations that may associate with intron splicing regulation [23].

FKBP25 gene knockdown declined the expression levels of p53 and p21, which emphasized the significance of FKBP25 in regulating p53 and subsequently p21 expression through controlling the ubiquitination of MDM2. Both the FKBP25 PPIase domain and its N-terminal portion were critical for the ubiquitination and degradation of MDM2 [2]. Moreover, Jin et al. reported that FKBP25 prefers to bind to rapamycin rather than FK506, implying that FKBP25 may be an important target molecule for immunosuppression by rapamycin [8]. All the evolution analyses indicated the conservation of FKBP25 gene in vertebrates. Therefore, FKBP25 possesses some basic functions in vertebrate species, like regulating p53 and p21 expression and binding to rapamycin for immunosuppression, reinforcing the suggestion that the purifying selection triggered the evolution of vertebrate FKBP25.

In conclusion, FKBP25 as a nuclear FKBP subjects to the purifying selection throughout the whole evolution, which implied the complete role of the PPIase domain involved in the interaction between FKBP25 and the nuclear proteins that are needed to be discovered continually. Additionally, incomplete exon-intron conservation of FKBP25 meets the vertebrate lineage. The intron gain or loss among the taxa is likely to be involved in the purifying selection.

Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution
Fei Liu and Xiao-Long Wei contributed to this paper equally.

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