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Dectin-1 rs3901533 and rs7309123 Polymorphisms Increase Susceptibility to Pulmonary Invasive Fungal Disease in Patients with Acute Myeloid Leukemia from a Chinese Han Population*

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Summary: This study aimed to assess whether genetic variants of dendritic cell-associated C-type lectine-1 (*Dectin-1*), Toll-like receptor 2 (*TLR2*), Toll-like receptor 4 (*TLR4*), and myeloid differentiation primary response 88 (*MyD88*) influence the susceptibility to pulmonary invasive fungal disease (IFD) in patients with acute myeloid leukemia (AML) from a Chinese Han population. Eight single nucleotide polymorphisms (SNPs) of *Dectin-1* (rs16910526, rs3901533, and rs7309123), *TLR2* (rs5743708), *TLR4* (rs4986790 and rs4986791) and *MyD88* (rs4988453 and rs4988457) in the genomic DNA of 172 adult AML patients were genotyped. Pulmonary IFD was diagnosed as proven or probable according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) consensus guidelines. SNPs that were significant in the univariate analysis were further analyzed using the multiple logistic regression analysis to determine their association with the occurrence of pulmonary IFD. The mRNA expression of *Dectin-1* was detected according to the genotype by quantitative real-time PCR (qRT-PCR), and the correlation of this expression with the occurrence of pulmonary IFD in AML patients was analyzed. Two *Dectin-1* intron SNPs (rs3901533 and rs7309123) were found to be significantly associated with the susceptibility to pulmonary IFD in AML patients in a Chinese Han population. Significant associations were noted between pulmonary IFD and *Dectin-1* rs3901533 dominant model (G/T+G/G *vs.* T/T, OR: 2.158; 95% CI: 1.109–4.2, *P*=0.02), *Dectin-1* rs3901533 G allele (OR: 2.201; 95% CI: 1.206–4.019, *P*=0.01), or *Dectin-1* rs7309123 C allele (OR: 1.919; 95% CI: 1.047–3.518, *P*=0.03). There were no significant associations between pulmonary IFD and the remaining *Dectin-1* SNPs (rs16910526), *TLR2* (rs5743708), *TLR4* (rs4986790 and rs4986791) or *MyD88* (rs4988453 and rs4988457). In conclusion, two *Dectin-1* SNPs (rs3901533 and rs7309123) are associated with increased susceptibility to pulmonary IFD in AML patients in a Chinese Han population.
Key words: *Dectin-1*; polymorphisms; invasive fungal infection; acute myeloid leukemia

Invasive fungal disease (IFD) is a major cause of morbidity and mortality in patients with acute myeloid leukemia (AML) who tend to develop drug-induced neutropenia resulting from intense chemotherapy[1]. In 2004, a multicenter Italian study reported 373 (69%) patients with AML had IFD among 538 proven or probable IFD patients with hematologic malignancies[2]. Furthermore, a multicenter Chinese study by the China Assessment of Antifungal Therapy in Hematological Disease (CAESAR) reported that the IFD incidence was significantly higher in AML patients (11.8%)[1]. Severe prolonged neutropenia, impaired innate immunity, progression of the underlying malignancy, diabetes, and other conditions such as environmental exposure to fungal spores have for many years been considered to predispose patients with hematologic malignancies to IFD[3–5]. AML patients have multiple risk factors for developing IFD, such as advanced age, prolonged and profound neutropenia and monocytopenia, the presence of indwelling catheters, alimentary mucositis and individual genetic susceptibilities[6]. Previous studies

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have reported an association between IFD development and single nucleotide polymorphisms (SNPs) of host innate immune, thus, SNPs affect the susceptibility to invasive fungal infections[7–12]. Additionally, innate immune SNPs have been suggested to play an important role in the susceptibility to and severity of IFD[13–17].

Innate immunity is used by the host to respond to various fungal pathogens in a rapid and conserved manner[18]. Host cells express pattern recognition receptors (*PRRs*), such as Toll-like receptors (*TLRs*) and C-type lectin-like receptors (*CLRs*)[19, 20], that sense pathogen-associated molecular patterns (PAMPs) in fungi[18, 21]. *CLRs* play central roles in host defenses against fungal infections by coordinating the innate and adaptive immunity[22]. *Dectin-1* (also known as *CLEC7A*) was the first identified *CLR* and is best known for its ability to recognize fungal β-1, 3-glucans present in the cell wall of *Aspergillus*, *Candida* and many other fungal species[23, 24].

Although *CLRs* are considered to constitute the primary receptor class responsible for fungal recognition, members of the *TLR* family and their interactions with *CLRs* are critical for antifungal immunity. The *TLR* family is considered the most well defined of the four families of *PRRs*. The *TLR* family comprises 10 members (*TLR*1-10) in humans. *TLR2* and *TLR4* are two of the most extensively studied *TLRs* and play an important role in the recognition of both bacterial and fungal pathogens[13]. Myeloid differentiation primary response 88 (*MyD88*) is a key adaptor protein that interacts with the *TLRs* to activate transcription factors, leading to the production of proinflammatory cytokines and the subsequent induction of adaptive immunity[9].

Human and mouse studies have shown that genetic deficiencies in *Dectin-1*, *TLR2*, and *TLR4* confer high susceptibility to fungal infections[25–28]. Although *TLR* signaling molecule *MyD88*-deficient mice have been shown to be highly susceptible to various fungal infections[18], no associations of SNPs in *MyD88* with susceptibility to candidemia were observed[16]. Cunha *et al* found that the *Dectin-1* rs16910526 gene polymorphism increased susceptibility to invasive aspergillosis in hematopoietic stem cell transplantation (HSCT) patients[29]. Besides, Sainz *et al* found that two *Dectin-1* intron SNPs (rs3901533 and rs7309123) conferred a significantly increased risk of developing invasive pulmonary aspergillosis (IPA) infection in patients with hematological malignancies[14]. Polymorphisms of *TLR2* (rs5743708) and *TLR4* (rs4986790, rs4986791) have been shown to confer significantly increased susceptibility to pulmonary aspergillosis[7, 17] and bloodstream candidemia in HSCT patients[16, 18, 30].

According to two studies in Germany, polymorphisms of *Dectin-1* (rs7309123), *TLR2* (rs5743708) and *TLR4* (rs4986790, rs4986791) were associated with pulmonary IFD after chemotherapy in AML patients[13, 31]. However, these studies were limited to Caucasians. The correlation between SNPs and pulmonary IFD in the Han Chinese population is still unclear. Based on these findings, the objective of this study was to investigate the role of genetic variants of *Dectin-1*, *TLR2*, *TLR4*, and their adaptor *MyD88* in the susceptibility to pulmonary IFD in adult AML patient from a Chinese Han population.

1 SUBJECTS AND METHODS

1.1 Study Subjects

In total, 172 adult Chinese Han subjects with AML (excluding those with acute promyelocytic leukemia) were recruited from Union Hospital, Fujian Medical University (China) after informed consent was obtained. The local ethics committee of Fujian Medical University, China, provided institutional review board approval for this study. The study was performed in accordance with the Declaration of Helsinki.

1.2 Clinical Evaluation

Pneumonia was defined as a new infiltrate on a chest radiograph (X-ray and/or computed tomography) along with at least two of the following criteria: cough, sputum production, temperature >38°C or <35°C, hemoptysis, thoracic pain or auscultatory findings consistent with pneumonia. The clinical evaluation of pulmonary IFD was performed using clinical parameters and computerized tomography diagnostics[32, 33]. Additionally, the patients' histories of previous fungal colonization/infection were considered. The patients were monitored for the presence of signs and symptoms of invasive fungal infections[32].

1.3 Collection of DNA Samples and SNPs Typing

Peripheral blood samples were collected from all the adult AML patients, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using a Ficoll-Paque gradient[31]. Genomic DNA was extracted from PBMCs using a QIAamp Blood Mini Kit according to the manufacturer's instructions (QIAGEN, Germany)[31]. In total, 8 SNPs of *Dectin-1* (rs16910526, rs3901533, and rs7309123)[14, 31], *TLR2* (rs5743708)[13, 31], *TLR4* (rs4986790 and rs4986791)[13, 34] and *MyD88* (rs4988453 and rs4988457)[9] were selected based on their previously published associations with human IFD. The DNA products were purified, sequenced, and typed according to the human papillomaviruses (HPV) databases. The SNPs were determined using KASPar assays (KBiosciences, UK). The samples were used to analyze the association between the risk of pulmonary IFD and specific *PRRs* gene polymorphisms.

1.4 RNA Preparation and Quantitative Real-Time PCR (qRT-PCR) Analyses

RNA was extracted from the samples using TRIzol (Invitrogen, USA) according to the manufacturer's instructions, and the total RNA concentrations were determined using a NanoDrop[35]. Approximately 2 μg of total RNA was reverse-transcribed using oligo (dT) primers and avian myeloblastosis virus reverse transcriptase (Roche, Mannheim, Germany) to generate cDNA for the qRT-PCR analysis. qRT-PCR was performed on all samples using Power SYBR green master mix (ABI biosystems, USA). The sequences of the primers are listed in table 1.

The qRT-PCR samples were run in triplicate on an ABI 7500 Sequence Detection System (Applied Biosystems, USA), and the housekeeping gene β-actin was used to normalize the data[35]. The *Dectin-1*, *TLR2*, *TLR4*, and *MyD88* expression levels were calculated as follows: $2^{-[\Delta Ct\ (assayed\ gene) - \Delta Ct\ (\beta\text{-actin})]}$.

1.5 Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS) software, revision 20.0 for Windows (SPSS Inc., USA). Quantitative characteristics were expressed as median values. Student's *t*-tests (parametric, two-tailed) and χ^2 or Fisher exact tests (as appropriate) were used to analyze the baseline demographic and clinical categorical variables in the case and control groups. SNPs with a value of $P<0.05$ in the analysis of the association with susceptibility to infection were included as confounders in the multiple logistic regression (or exact logistic regression as appropriate). *P* values less than 0.05 were considered statistically significant. The odds ratio (OR) and their 95% confidence intervals (CIs) were calculated.

2 RESULTS

2.1 Demographics

Genetic and clinical data were collected from 172 adult Chinese Han AML patients in this analysis. The demographic and clinical characteristics of all study subjects are presented in table 2.

2.2 Frequency of *TLR2*, *TLR4*, *MyD88*, and *Dectin-1* Polymorphisms

The presence of the investigated SNPs of *TLR2* (rs5743708), *TLR4* (rs4986790 and rs4986791), *MyD88* (rs4988453 and rs4988457) and *Dectin-1* (rs16910526, rs3901533, and rs7309123) was evaluated in all 172

Table 1 Oligonucleotide primer sequences

Gene	Sequence (5′ to 3′)	Product (bp)
Dectin-1	Forward: 5′- CGA CTC TCA AAG CAA TAC CAG GA-3′	138
	Reverse: 5′- GTA CCC AGG ACC ACA GCT ATC AC-3′	
TLR2	Forward: 5′-TGG ACT TCT CCC ATT TCC GTC-3′	118
	Reverse: 5′-ATT ATC TTC CGC AGC TTG CAG A-3′	
TLR4	Forward: 5′-TGG TGG AAG TTG AAC GAA TGG-3′	115
	Reverse: 5′-AGG ACC GAC ACA CCA ATG ATG-3′	
MyD88	Forward: 5′-AGG CAC CAG CAT ACA CAC GTT-3′	100
	Reverse: 5′-TTT TGT TCA GGG ACA TGG TTA GG-3′	
β-actin	Forward: 5′-CTG TCC ACC TTC CAG CAA ATG T-3′	105
	Reverse: 5′-CGC AAC TAA GTC ATA GTC CGC C-3′	

Table 2 Demographic and baseline clinical characteristics of the adult patients

Variables	Pulmonary IFD (*n*=113)	Non-pulmonary IFD (*n*=108)	*P*
Age (year)			0.179
<60, *n* (%)	60 (47.2)	67 (52.8)	
≥60, *n* (%)	53 (56.4)	41 (43.6)	
Sex			0.152
Male, *n* (%)	60 (47.2)	67 (52.8)	
Female, *n* (%)	53 (56.4)	41 (43.6)	
Type of AML FAB			<0.001
M0, *n* (%)	2 (100.0)	0 (0.0)	
M1, *n* (%)	8 (80.0)	2 (20.0)	
M2, *n* (%)	25 (67.6)	12 (32.4)	
M4, *n* (%)	3 (75.0)	1 (25.0)	
M5, *n* (%)	74 (45.7)	88 (54.3)	
Others, *n* (%)	1 (16.7)	5 (83.3)	
WBC count at diagnosis ($\times10^9$/L), median (range)	26.5 (0.04–326.5)	17.3 (0.1–256.7)	<0.001
Hemoglobin level at diagnosis (g/L), median (range)	69.5 (36.0–135.0)	70.6 (39.0–125.0)	0.282
Platelet count at diagnosis ($\times10^9$/L), median (range)	38.7 (0–267.0)	30.6 (3.0–413.0)	0.173
Peripheral blood blasts at diagnosis (%), median (range)	51.5 (0–99.0)	25.6 (0–99.0)	<0.001

WBC, white blood cell

patients (table 3). In total, 64 (37.2%) patients had the *Dectin-1* rs3901533 polymorphism; of these patients, 60 (34.9%) were heterozygous and 4 (2.3%) were homozygous for the SNPs. Furthermore, the *Dectin-1* rs7309123 polymorphism was found in 61 patients (35.4%); of these patients, 59 (34.3%) were heterozygous and 4 (2.3%) were homozygous for the SNPs. In contrast, the *MyD88* rs4988453 A/C genotype and *MyD88* rs4988457 genotype were detected in only 5 (2.9%) and 6 (3.5%) patients, respectively. Additionally, no *TLR2* (rs5743708), *TLR4* (rs4986790 and rs4986791) and *Dectin-1* (rs16910526) polymorphisms were detected. All eight SNPs were in Hardy-Weinberg equilibrium.

Polymorphisms that were not present or had very low frequency were excluded from further analysis. We focused on the impact of the *Dectin-1* (rs3901533 and rs7309123) polymorphisms by comparing heterozygous, and homozygous genotypes to wild-type genotypes in AML patients.

2.3 Impact of *Dectin-1* Polymorphisms on the Occurrence of Pulmonary IFD

The data from all adult AML patients (n=172) were analyzed to determine the association between the *Dectin-1* polymorphisms and the susceptibility to pulmonary IFD according to the abovementioned criteria. We compared the occurrence of pulmonary IFD among all eight abovementioned SNPs and showed that *Dectin-1* rs3901533 and rs7309123 conferred a significantly higher risk of pulmonary IFD following induction chemotherapy in AML patients, while the remaining SNPs were not associated with pulmonary IFD.

Thus, both the *Dectin-1* rs3901533 and rs7309123 polymorphisms were identified as independent risk factors for the occurrence of pulmonary IFD. The association between the two *Dectin-1* polymorphisms and the risk of pulmonary IFD was again confirmed by a logistic regression analysis and by calculation of the ORs of a *Dectin-1* rs3901533 dominant model (G/T+G/G *vs.* T/T, OR: 2.158; 95% CI: 1.109–4.2, P=0.02), the *Dectin-1* rs3901533 G allele (OR: 2.201; 95% CI: 1.206–4.019, P=0.01), and the *Dectin-1* rs7309123 C allele (OR: 1.919; 95% CI: 1.047–3.518, P=0.03) (table 4).

2.4 Analyses of *Dectin-1* Expression

The *Dectin-1* mRNA expression levels in 131 samples were measured by RT-PCR to further understand the clinical impact of the *Dectin-1* polymorphism (fig. 1A, 1B). Patients harboring the risk allele T or G and those with the G/G (G/T) or C/C (C/G) genotypes of *Dectin-1* rs3901533 and rs7309123, respectively, exhibited comparable mRNA levels. No difference was observed in the mRNA expression level for the *Dectin-1* rs3901533 and *Dectin-1* rs7309123 genotypes (fig. 1A, 1B). In addition, no correlation was observed between *Dectin-1* mRNA expression and pulmonary IFD in AML patients (fig. 2).

3 DISCUSSION

In the last decade, more than 20 SNPs have been reported to influence the risk of IFD in patients with hematologic malignancies or after HSCT[12, 13]. However these studies have focused on patients receiving HSCT, and few studies have examined AML patients. Moreover, genetic differences between different ethnic groups have been observed, including white, African American and Caucasian populations[16, 31]. In this study, we investigated whether eight SNPs in four *PRR* genes, i.e., *TLR2* (rs5743708), *TLR4* (rs4986790 and rs4986791), *Dectin-1* (rs16910526, rs3901533, and rs7309123) and *MyD88* (rs4988453 and rs4988457), are associated with an increased risk of developing pulmonary IFD in Chinese Han AML patients who received intensive induction chemotherapy.

Table 3 Distribution of the *TLR2*, *TLR4*, *Dectin-1* and *MyD88* genotypes [*n* (%)]

Gene	SNPs	Genotype frequency			
Dectin-1 (CLEC7A)	rs16910526	T/T	T/T	T/T	T/T
		171 (99.4)	0 (0)	0 (0)	1 (0.6)
Dectin-1 (CLEC7A)	rs3901533	T/T	G/T	G/G	
		108 (62.8)	60 (34.9)	4 (2.3)	
Dectin-1 (CLEC7A)	rs7309123	G/G	C/G	C/C	Missing
		108 (62.8)	59 (34.3)	4 (2.3)	1 (0.6)
TLR2	rs5743708	G/G	A/G	A/A	Missing
		171 (99.4)	0 (0)	0 (0)	1 (0.6)
TLR4	rs4986790	A/A	G/A	A/A	
		172 (100)	0 (0)	0 (0)	
TLR4	rs4986791	C/C	C/T	T/T	
		172 (100)	0 (0)	0 (0)	
MyD88	rs4988453	C/C	A/C	A/A	
		167 (97.1)	5 (2.9)	0 (0)	
MyD88	rs4988457	C/C	C/G	G/G	
		166 (96.5)	6 (3.5)	0 (0)	

Table 4 Association between the *Dectin-1* polymorphisms and pulmonary IFD in AML patients after induction chemotherapy

Gene, SNP	Genotype	Pulmonary IFD (%)	Non-pulmonary IFD (%)	OR (95% CI)	P^*	*P*-trend
Dectin-1, rs3901533	T/T	26 (24.1)	82 (75.9)	1.000		
	G/T	23 (38.3)	37 (61.7)	1.960 (0.991–3.878)	0.05	
	G/G	3 (75.0)	1 (25.0)	9.462 (0.943–94.916)	0.05	
	G/T+G/G	26 (40.6)	38 (59.4)	2.158 (1.109–4.200)	**0.02**	
	T/T+G/T	49 (29.2)	119 (70.8)	1.000		
	G/G	3 (75.0)	1 (25.0)	7.286 (0.74–71.766)	0.08	
	per G allele			2.201 (1.206–4.019)		**0.01**
Decin-1, rs7309123	G/G	27 (25.0)	81 (75.0)	1.000		
	C/G	21 (35.6)	38 (64.4)	1.622 (0.808–3.255)	0.17	
	C/C	3 (75.0)	1 (25.0)	9.000 (0.898–90.191)	0.06	
	C/G+C/C	24 (38.1)	39 (61.9)	1.846 (0.945–3.606)	0.07	
	G/G+C/G	47 (28.5)	118 (71.5)	1.000		
	C/C	3 (75.0)	1 (25.0)	7.532 (0.764–74.249)	0.08	
	per C allele			1.919 (1.047–3.518)		**0.03**

IFD, invasive fungal disease; AML, acute myeloid leukemia; OR, odds ratio; CI, confidence interval. $^*P$ values were calculated at the 95% confidence interval. Significant *P* values are shown in bold.

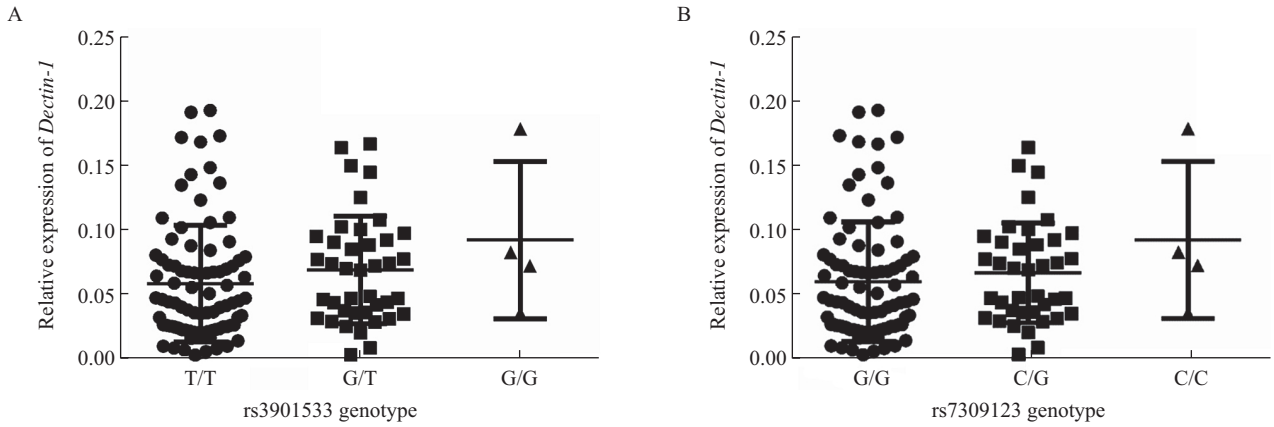

Fig. 1 mRNA expression of *Dectin-1* in individuals with the rs3901533 and rs7309123 genotypes
A: mRNA expression of *Dectin-1* according to the rs3901533 genotype. The results include 4 G/G homozygous, 40 G/T heterozygous and 87 T/T wild type individuals. No difference was observed among the three groups (P=0.58).
B: mRNA expression of *Dectin-1* according to the rs7309123 genotype. The results include 4 C/C homozygous, 39 C/G heterozygous and 88 G/G wild-type individuals, and no difference was observed among the three groups (P=0.63).

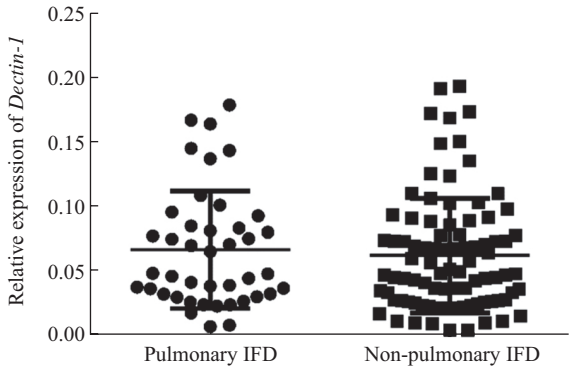

Fig. 2 mRNA expression of *Dectin-1* in pulmonary IFD and non-pulmonary IFD patients with AML
No significant difference was observed between the pulmonary IFD (n=42) and non-pulmonary IFD (n=89) patients (P=0.59).

We found that the *Dectin-1* rs3901533 and rs7309123 polymorphisms were associated with pulmonary IFD, and AML patients who were carriers of the *Dectin-1* rs3901533 G allele and rs7309123 C allele had a significantly increased risk of developing pulmonary IFDs. No difference was observed in the mRNA expression levels between patients harboring the T or G risk alleles and patients with the G/G (G/T) and C/C (C/G) genotypes of *Dectin-1* rs3901533 and rs7309123, respectively. Moreover, no significant difference was observed in the *Dectin-1* mRNA level between pulmonary IFD and non-pulmonary IFD AML patients. No influence of the *TLR2*, *TLR4*, or *MyD88* SNPs on the susceptibility to pulmonary IFD was observed among AML patients.

Several *TLRs* and *Dectin-1* have been reported to be involved in sensing fungal components, *TLR2*

and *TLR4* mediate the recognition of zymosan, phospholipomannan, and O-linked mannans, and *Dectin-1* recognizes β-glucans. Because genetic deficiencies in *TLR2*, *TLR4* and *Dectin-1* have been shown to be associated with the susceptibility to fungal infections in both human and mouse studies[18], we assessed the role of the SNPs of TLRs and their adaptor molecules, *MyD88* and *Dectin-1*, to obtain potential insight into the susceptibility to pulmonary IFD.

Among the genes and SNPs examined, two polymorphisms of *Dectin-1* were found to be associated with increased susceptibility to the development of pulmonary IFD in hospitalized AML patients from a Chinese Han population. The observation that both SNPs influence the susceptibility to pulmonary IFD strengthens the conclusion that *Dectin-1* plays an important role in the susceptibility to invasive fungal infection. However, no difference was observed in the mRNA expression levels between the alleles, i.e., heterozygous and homozygous, of these two polymorphisms of *Dectin-1*.

Recently, two studies in Germany demonstrated that *TLR2* (rs5743708) and *TLR4* (rs4986790 and rs4986791) are associated with IFD in AML patients[13, 31]. These data, however, could not be confirmed in our study. The *Dectin-1* rs7309123 polymorphism was reported to be a risk factor for developing IFD in AML patients in their studies. Our results also documented the similar effects of genetic susceptibility and found the important effects of the *Dectin-1* rs3901533 and rs7309123 polymorphisms on pulmonary IFD in patients with AML. In contrast to *Dectin-1*, no effects of the *TLR2*, *TLR4* or *MyD88* polymorphisms on the susceptibility to pulmonary IFD were apparent.

In conclusion, our study demonstrates that the *Dectin-1* rs3901533 and rs7309123 polymorphisms increase the susceptibility to pulmonary IFD in AML patients in a Chinese Han population, although no definite conclusions regarding the pathophysiological mechanism can be drawn from our data. Further studies are warranted to elucidate the exact role of *Dectin-1* signaling in host defenses against pulmonary IFD in AML patients. Knowledge regarding the polymorphisms of *Dectin-1* could be used in risk assessments and the development of prophylactic, empiric, or preemptive therapies.

Conflict of Interest Statement

The authors declare no conflict of interests.

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