Inflammation, Vol. 39, No. 1, February 2016 (© 2015)
DOI: 10.1007/s10753-015-0217-y

FOXO3 rs12212067: T > G Association with Active Tuberculosis in Han Chinese Population

Yanjun Lu,[1] Yaowu Zhu,[1] Xiong Wang,[1] Feng Wang,[1] Jing Peng,[1] Hongyan Hou,[1] and Ziyong Sun[1,2]

Abstract—It is well known that the human innate immune and adaptive immune response play important role in tuberculosis (TB) infection and progress. Emerging evidence shows that FOXO3 plays an important role in the human immune system. Recent research has shown that the FOXO3 genetic variants are associated malaria infection. In this study, 268 confirmed TB patients, 321 patients with latent tuberculosis infection (LTBI), and 475 TB-free controls were recruited; the single-nucleotide polymorphism (SNP) rs12212067: T > G in FOXO3 was genotyped using predesigned TaqMan® allelic discrimination assays. The results showed that the G allele of rs12212067 in FOXO3 was more common in health control and the latent TB group compared with the active TB group ($p=0.048$, odds ratio (OR) 95 % confidence intervals (CI)=1.37 (1.00–1.89); $p=0.042$, OR 95 % CI=1.42 (1.01–1.99), respectively); furthermore, within active TB patients, the G allele of rs12212067 in FOXO3 was more frequent in extra-pulmonary tuberculosis (EPTB) group compared to pulmonary tuberculosis (PTB) group ($p=0.035$, OR 95 % CI=0.57 (0.33–0.97). In conclusion, this study found that rs12212067 in FOXO3 was associated with increased risk of active TB. The minor G allele might be a protection factor which was found more common in latent TB patients and healthy controls than active TB patients.

KEY WORDS: tuberculosis; FOXO3; SNP; association.

INTRODUCTION

One third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), but less than 10 % of infected individuals will develop active disease in their lifetimes [1]. Whether an infected individual develops tuberculosis (TB) depends on the capacity of the host immune system to recognize and control the infection [2]. Genetic variability is an important determinant of the effectiveness of the host immune response to *M. tuberculosis* [3]. Several lines of evidence, including twin studies [4], genome-wide linkage studies [5], and genome-wide association studies [6, 7], have demonstrated that host genetic factors play a critical role in effecting the infected individuals eventually to develop TB.

Forkhead transcription factors of the O class (FOXOs), primarily identified as downstream targets of insulin/IGF-1 signaling pathway, consist of four members, FOXO1, FOXO3, FOXO4, and FOXO6 [8]. These transcription factors have been identified as important regulators involved in cellular differentiation, apoptosis, oxidative stress, glucose metabolism, and other cellular functions [9, 10]. Recently, there has been a surge in interest to elucidate the importance of FOXOs in regulating T cell biology. FOXO1 has been shown to control several aspects of T cells including development of regulatory T cells, the expression of adhesion molecules like L-selectin and CCR7, and cytokine receptors like the IL-7 receptor [11]. More recently, in the immune system, increasing evidence shows that FOXO3 is a critical regulator of T cell homeostasis [12, 13]. FOXO3 has been reported to have non-redundant roles in suppressing inflammatory cytokine production by dendritic cells and in limiting the inflammatory

Yanjun Lu and Yaowu Zhu contributed equally to this work.

[1] Department of Laboratory Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095# Jiefangdadao, Wuhan, 430030, China

[2] To whom correspondence should be addressed at Department of Laboratory Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095# Jiefangdadao, Wuhan, 430030, China. E-mail: tjszyong@163.com

sequelae of viral infections [14–16]. It has been reported that FOXO3 has been linked to the regulation of immune responses in systems biology and knockout mouse models; however, whether it is relevant in human disease remains unclear.

Previously, polymorphisms in FOXO3 have been independently associated with human longevity [17]; recent research has shown that a genetic variant in FOXO3 (rs12212067: T > G) associates with the prognosis, but not diagnosis, of three distinct diseases: Crohn's disease, rheumatoid arthritis, and malaria. The rs12212067: T > G in FOXO3 probably impairs parasite clearance and increases the risk of severe malaria which is associated with lesser inflammatory responses characterized with less TNF-α and more IL-10 production [18]. Whether FOXO3 in control of inflammatory cytokine production links TB infection and thereby influences TB progress is unknown. To test this, we genotyped rs12212067 in FOXO3 in active TB patients or latent TB patients and ethnically matched healthy controls; we found an association between the decreased minor (G) allele and active TB in this study.

MATERIALS AND METHODS

Study Population

In this study, diagnosis of active TB was based on clinical symptoms, the presence of acid-fast bacilli in sputum smears, and culture on Lowenstein-Jensen medium in all cases. Latent tuberculosis infection (LTBI) was diagnosed by the positive interferon-γ response assay (IGRA) in the absence of clinical signs and symptoms of full-blown disease [19]. Control individuals from healthy Han Chinese were randomly recruited from the same area and not related to the members of TB group, with none having a history of TB confirmed by X-ray and physical examinations, and the IGRA test was negative. Exclusion criteria: Patients with immunosuppressive condition such as HIV, diabetes, or on steroids for inflammatory conditions were excluded from the study.

In this study, 268 confirmed TB patients, 321 LTBI, and 475 TB-free controls were recruited from Wuhan Tongji Hospital in China from January 2013 to July 2014; the TB patients were then further divided into two subgroups: (1) pulmonary tuberculosis patients (PTB) ($n=144$) and (2) extra-pulmonary patients (EPTB) ($n=124$). All subjects signed informed consent forms voluntarily, and the research was approved by the ethics committee of the Tongji Medical College, Huazhong University of Science and Technology.

Genotyping

Genomic DNA was extracted from whole blood as previously described [20]. The single-nucleotide polymorphisms (SNPs) were genotyped using predesigned TaqMan® allelic discrimination assays in a ViiA 7 Real-Time Polymerase Chain Reaction (PCR) System from Applied Biosystems. All reagents required for the TaqMan assay including universal master mix, amplifying primers, and probes were obtained from Applied Biosystems (Foster City, CA, USA). One allelic probe was labeled with FAM dye and the other with the fluorescent VIC dye. PCR was run in the TaqMan universal master mix at a probe concentration of 20×. The reaction was performed in a 96-well format in a total reaction volume of 25 mL using 20 ng of genomic DNA. The reaction plates were heated for 2 min at 50 °C and for 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1.5 min. The fluorescence intensity of each well in the TaqMan assay plate was subsequently read, and fluorescence data files from each plate were analyzed by automated software.

Statistical Analysis

All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). A x^2 test was used to compare the distribution of genotypes among patients and controls. The associations between the FOXO3 SNP and the disease status were expressed in odds ratio (OR) and their 95 % confidence intervals (CI). A $p<0.05$ was considered to be statistically significant. The distributions of genotype for rs12212067 were analyzed for deviation from Hardy-Weinberg equilibrium (HWE) using x^2 analysis; a cutoff p value of 0.05 was set for HWE.

RESULTS

Demographics and Phenotypes of the Study Subjects

As shown in Table 1, the mean age of the 268 patients with active TB was 45.6 years, the mean age of 321 patients with LTBI was 51.7 years, and the mean age of 475 matched healthy controls was 44.8 years. Additionally, there were 268 patients with active TB including 144 PTB patients and 124 EPTB patients, which were composed of 14 with meningeal TB, 41 with pleural TB, 17 with bone

Table 1. Demographics and Phenotypes of the Study Subjects

Subgroup	Number	Male	Female	Age
Health control	475	245	230	44.8±18.7
LTBI	321	171	150	51.7±15.6
Active TB	268	153	115	45.6±18.6
PTB	144	89	55	48.3±19.2
EPTB				
All forms	124	64	60	42.5±17.3
Meningeal	14	8	6	38.3±17.6
Pleural	41	27	14	44.1±19.0
Bone	17	7	10	48.6±15.0
Abdominal	42	18	24	40.7±15.7
Other	10	4	6	38.3±18.9

TB, 42 with abdominal TB, and 10 others. There was no significant difference among the three groups in age and sex.

Association of the FOXO3 rs12212067 Polymorphism with TB

In this study, evaluation of HWE showed that the genotype frequencies of the FOXO3 rs12212067 polymorphism were in line with HWE test in the three groups ($p>0.05$) (data not shown). The allele and genotypic frequencies of rs12212067 in TB patients, latent TB patients, and controls are summarized in Table 2. Analysis of the contribution of FOXO3 gene allele frequencies revealed that the G allele of rs12212067 in FOXO3 was more common in health control and the latent TB group when compared with active TB group ($p=0.048$, OR 95 % CI=1.37 (1.00–1.89); $p=0.042$, OR 95 % CI=1.42 (1.01–1.99), respectively), whereas no significant difference was found between latent TB group and health control ($p=0.823$, OR (95 % CI)=0.97 (0.74–1.28)) and also no significant difference in latent TB group + active TB group compared to the health control (data no shown).

Although, no significant difference of FOXO3 rs12212067 genotype was found among these groups, analysis of the genotype frequencies of FOXO3 rs12212067 in a dominant inheritance mode showed that the genotypes GG + GT (*vs*. TT) was more common in health control (28.0 %) and the latent TB group (29.6 %) when compared to active TB group (18.1 %) ($p=0.001$, OR 95 % CI=0.57 (0.40–0.80); $p=0.001$, OR 95 % CI=0.53 (0.36–0.76), respectively). There was no significant difference in distribution of genotype frequencies in a recessive in heritance mode (GG *vs*. GT + TT) (Table 3). All these results suggested that decreased rs12212067

Table 2. Genotype Frequencies in Health Control, LTBI, and Active TB Subjects

	Healthy control	LTBI	Active TB	*P* value			OR (95 % CI)			
				L *vs*. H	A *vs*. H	A *vs*. L	L *vs*. H	A *vs*. H	A *vs*. L
Genotype									
GG	14 (2.9 %)	7 (2.2 %)	5 (1.9 %)	–	–	–	1 (referent)	1 (referent)	1 (referent)
GT	119 (25.1 %)	88 (27.4 %)	53 (19.8 %)	0.416	0.686	0.780	1.48 (0.57–3.82)	1.25 (0.43–3.64)	1.19 (0.36–3.93)
TT	342 (72 %)	226 (70.4 %)	210 (78.3 %)	0.552	0.300	0.657	1.32 (0.53–3.33)	1.72 (0.61–4.84)	0.77 (0.24–2.46)
Allele									
G	147 (15.4 %)	102 (15.9 %)	63 (11.8 %)	–	–	–	1 (referent)	1 (referent)	1 (referent)
T	803 (84.6 %)	540 (84.1 %)	473 (88.2 %)	0.823	0.048	0.042	0.97 (0.74–1.28)	1.37 (1.00–1.89)	1.42 (1.01–1.99)

Table 3. Association of the FOXO3A rs12212067 with TB in Dominant and Recessive Models

Model	health	LTBI	active TB	*P* value			OR (95 % CI)		
Dominant				L *vs*. H	A *vs*. H	A *vs*. L	L *vs*. H	A *vs*. H	A *vs*. L
GG + GT	133	95	58	–	–	–	1 (referent)	1 (referent)	1 (referent)
TT	342	226	263	0.625	0.001	0.001	1.08 (0.79–1.48)	0.57 (0.40–0.80)	0.53 (0.36–0.76)
Recessive									
GG	14	7	5	–	–	–	1 (referent)	1 (referent)	1 (referent)
GT+TT	461	314	263	0.508	0.370	0.788	0.73 (0.29–1.84)	0.63 (0.22–1.76)	0.85 (0.27–2.72)

minor allele G might be associated with the increased risk of active TB patients, but not susceptibility to TB infection.

Association Analysis of rs12212067 Polymorphism Within Subgroups of Active TB Patients

Also, association analysis of rs12212067 polymorphism between PTB and EPTB was performed. The results showed that the genotype frequency of FOXO3 rs12212067 was not significantly different in the PTB group when compared to EPTB group. However, analysis of the gene allele frequencies revealed that the G allele of rs12212067 in FOXO3 was more frequent in EPTB compared to PTB group (p=0.035, OR 95 % CI=0.57 (0.33–0.97) (Table 4).

In addition, in both dominant inheritance and recessive inheritance modes, the genotype frequencies of FOXO3 rs12212067 and gene allele frequencies showed no significant difference between PTB and EPTB (p=0.067, OR 95 % CI=0.58 (0.32–1.04; p=0.127, OR 95 % CI=0.21 (0.02–1.90) (Table 5).

DISCUSSION

It's well known that the human innate immune and adaptive immune response play important role in TB infection and progress [21]. FOXO transcription factors respond to growth factors, oxidative stress, inflammation, and nutritional abundance-physiological conditions that influence the magnitude and effectiveness of an immune response [22]. FOXO3 has been reported to contribute to the pathogenesis of several immune-associated disorders, including TB [23]; furthermore, the genetic variation at rs12212067 in FOXO3 is also associated with prognosis in malaria [18]. However, the genetic association of FOXO3 variations and TB is poorly understood.

In this study, we found that rs12212067 in FOXO3 was associated with active TB in dominant inheritance mode. The minor G allele of rs12212067 in FOXO3 was more frequent in health control and latent TB group compared to the active TB group. These data suggest that decreased rs12212067 minor allele G might be associated with the increase risk of active TB patients. In the study of *Lee et al.* [18], they revealed that the rs12212067 might be a functional SNP affecting FOXO3 expression, which the minor G allele promoting FOXO3 transcription when stimulated with lipopolysaccharide (LPS). Based on the above research, in active TB, the less G allele of rs12212067 in FOXO3 might result in decreased FOXO3 expression, subsequently affecting the TB progress.

Emerging evidence shows that FOXO3 plays an important role in the human immune system. FOXO3-

Table 4. Association of the FOXO3A rs12212067 with PTB and EPTB

	Active TB		*P* value	OR (95 % CI)
Genotype	PTB	EPTB		
GG	1	4	–	1 (referent)
GT	24	29	0.275	0.30 (0.03–2.89)
TT	119	91	0.103	0.19 (0.02–1.74)
Allele				
G	26 (9.0 %)	37 (14.9 %)	–	1 (referent)
T	262 (91.0 %)	211 (85.1 %)	0.035	0.57 (0.33–0.97)

Table 5. Association of SNPs with PTB and EPTB in Dominant and Recessive Models

Model	Active TB		*P* value	OR (95 % CI)
Dominant	PTB	EPTB		
GG + GT	25	33	–	1 (referent)
TT	119	91	0.067	0.58 (0.32–1.04)
Recessive				
GG	1	4	–	1 (referent)
GT + TT	143	120	0.127	0.21 (0.02–1.90)

deficient mice exhibit enhanced T cell proliferation in response to viral infections, and FOXO3 acts downstream of CTLA-4-induced signals to constrain IL-6 production by dendritic cells (DCs) [14]. Furthermore, Jeremy *et al.* [24] systematically examined the T cell-intrinsic role of FOXO3 in controlling the expansion, contraction, and memory phases of the polyclonal $CD8^+$ T cell response to an acute viral infection. FOXO3 regulates the clonal expansion of polyclonal $CD8^+$ T cells in a tissue-specific fashion by T cell intrinsic mechanisms. FOXO3 activity controls the difference in survival capacity between central memory and effector memory T cells in human [25]. Studies of human memory T cells have ascribed a negative regulatory role for FOXO3 in the persistence of memory $CD4^+$ T cells, and FOXO3 deficiency would be expected to increase the number of effector memory cells [26]. FOXO3 also has an important role in the innate immune; for example, FOXO3 is involved in migration in neutrophils and survival in the context of inflammation [27, 28]. Recently, FOXO transcription factors are involved in the cellular responses to bacterial stimuli and act as central regulators of innate immune functions in respiratory epithelial cells [29]. The detailed role of FOXO3 in TB deserved further investigation.

In addition, we attempted to reveal whether there is a difference between PTB and EPTB in active TB group. The results showed that the G alleles of rs12212067 in FOXO3 were more frequent in EPTB. Analysis of the genotype frequencies of FOXO3 rs12212067 and gene allele frequencies both in a dominant inheritance mode and in a recessive inheritance mode showed that there is no significant difference in the two groups. Whether rs12212067 in FOXO3 differentiated from PTB and EPTB needs further investigation with larger sample group.

This study also had several limitations. First, whether the LTBI will eventually keep the status needs to be followed for a longer time, and whether the healthy controls had the history of exposure to MTB is not clear. Second, the number of participants was relatively small. Third, functional studies are needed to clarify how the genotypes of FOXO3 affect the active TB.

In summary, for the first time, this study found that rs12212067 in FOXO3 was associated with the increase risk of active TB. The minor G allele might be a protection factor which was found more common in latent TB patients and healthy controls than active TB patients.

Conflict of Interest. All authors have no conflict of interest.

REFERENCES

1. Trajman, A., R.E. Steffen, and D. Menzies. 2013. Interferon-gamma release assays versus tuberculin skin testing for the diagnosis of latent tuberculosis infection: an overview of the evidence. *Pulmonary Medicine* 2013: 601737.
2. van der Eijk, E.A., E. van de Vosse, J.P. Vandenbroucke, and J.T. van Dissel. 2007. Heredity versus environment in tuberculosis in twins: the 1950s United Kingdom Prophit Survey Simonds and Comstock revisited. *American Journal of Respiratory and Critical Care Medicine* 176: 1281–1288.
3. Azad, A.K., W. Sadee, and L.S. Schlesinger. 2012. Innate immune gene polymorphisms in tuberculosis. *Infection and Immunity* 80: 3343–3359.
4. Comstock, G.W. 1978. Tuberculosis in twins: a re-analysis of the Prophit survey. *The American Review of Respiratory Disease* 117: 621–624.
5. Cooke, G.S., S.J. Campbell, S. Bennett, et al. 2008. Mapping of a novel susceptibility locus suggests a role for MC3R and CTSZ in human tuberculosis. *American Journal of Respiratory and Critical Care Medicine* 178: 203–207.
6. Thye, T., F.O. Vannberg, S.H. Wong, et al. 2010. Genome-wide association analyses identifies a susceptibility locus for tuberculosis on chromosome 18q11.2. *Nature Genetics* 42: 739–741.
7. Png, E., B. Alisjahbana, E. Sahiratmadja, et al. 2012. A genome wide association study of pulmonary tuberculosis susceptibility in Indonesians. *BMC Medical Genetics* 13: 5.
8. Hosaka, T., W.H. Biggs 3rd, D. Tieu, et al. 2004. Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proceedings of the National Academy of Sciences of the United States of America* 101: 2975–2980.
9. Huang, H., and D.J. Tindall. 2007. Dynamic FoxO transcription factors. *Journal of Cell Science* 120: 2479–2487.
10. Maiese, K., Z.Z. Chong, J. Hou, and Y.C. Shang. 2009. The "O" class: crafting clinical care with FoxO transcription factors. *Advances in Experimental Medicine and Biology* 665: 242–260.
11. Kerdiles, Y.M., D.R. Beisner, R. Tinoco, et al. 2009. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. *Nature Immunology* 10: 176–184.
12. Coffer, P.J., and B.M. Burgering. 2004. Forkhead-box transcription factors and their role in the immune system. *Nature Reviews Immunology* 4: 889–899.
13. Birkenkamp, K.U., and P.J. Coffer. 2003. FOXO transcription factors as regulators of immune homeostasis: molecules to die for? *Journal of Immunology* 171: 1623–1629.
14. Dejean, A.S., D.R. Beisner, I.L. Ch'en, et al. 2009. Transcription factor Foxo3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells. *Nature Immunology* 10: 504–513.
15. Watkins, S.K., Z. Zhu, E. Riboldi, et al. 2011. FOXO3 programs tumor-associated DCs to become tolerogenic in human and murine prostate cancer. *The Journal of Clinical Investigation* 121: 1361–1372.
16. Litvak, V., A.V. Ratushny, A.E. Lampano, et al. 2012. A FOXO3-IRF7 gene regulatory circuit limits inflammatory sequelae of antiviral responses. *Nature* 490: 421–425.

17. Willcox, B.J., T.A. Donlon, Q. He, et al. 2008. FOXO3A genotype is strongly associated with human longevity. *Proceedings of the National Academy of Sciences of the United States of America* 105: 13987–13992.
18. Lee, J.C., M. Espeli, C.A. Anderson, et al. 2013. Human SNP links differential outcomes in inflammatory and infectious disease to a FOXO3-regulated pathway. *Cell* 155: 57–69.
19. 2000. Diagnostic Standards and Classification of Tuberculosis in Adults and Children. This official statement of the American Thoracic Society and the Centers for Disease Control and Prevention was adopted by the ATS Board of Directors, July 1999. This statement was endorsed by the Council of the Infectious Disease Society of America, September 1999. *American Journal of Respiratory and Critical Care Medicine* 161: 1376–1395.
20. Loparev, V.N., M.A. Cartas, C.E. Monken, A. Velpandi, and A. Srinivasan. 1991. An efficient and simple method of DNA extraction from whole blood and cell lines to identify infectious agents. *Journal of Virological Methods* 34: 105–112.
21. Bozzano, F., F. Marras, and A. De Maria. 2014. Immunology of tuberculosis. *Mediterranean Journal of Hematology and Infectious Diseases* 6, e2014027.
22. Hedrick, S.M., R. Hess Michelini, A.L. Doedens, A.W. Goldrath, and E.L. Stone. 2012. FOXO transcription factors throughout T cell biology. *Nature Reviews Immunology* 12: 649–661.
23. Haoues, M., A. Refai, A. Mallavialle, et al. 2014. Forkhead box O3 (FOXO3) transcription factor mediates apoptosis in BCG-infected macrophages. *Cellular Microbiology* 16: 1378–1390.
24. Sullivan, J.A., E.H. Kim, E.H. Plisch, S.L. Peng, and M. Suresh. 2012. FOXO3 regulates CD8 T cell memory by T cell-intrinsic mechanisms. *PLoS Pathogens* 8, e1002533.
25. Dejean, A.S., S.M. Hedrick, and Y.M. Kerdiles. 2011. Highly specialized role of Forkhead box O transcription factors in the immune system. *Antioxidants & Redox Signaling* 14: 663–674.
26. van Grevenynghe, J., F.A. Procopio, Z. He, et al. 2008. Transcription factor FOXO3a controls the persistence of memory CD4(+) T cells during HIV infection. *Nature Medicine* 14: 266–274.
27. Crossley, L.J. 2003. Neutrophil activation by fMLP regulates FOXO (forkhead) transcription factors by multiple pathways, one of which includes the binding of FOXO to the survival factor Mcl-1. *Journal of Leukocyte Biology* 74: 583–592.
28. Jonsson, H., P. Allen, and S.L. Peng. 2005. Inflammatory arthritis requires Foxo3a to prevent Fas ligand-induced neutrophil apoptosis. *Nature Medicine* 11: 666–671.
29. Seiler, F., J. Hellberg, P.M. Lepper, et al. 2013. FOXO transcription factors regulate innate immune mechanisms in respiratory epithelial cells. *Journal of Immunology* 190: 1603–1613.