Research Paper

Assessment of Human Tribbles Homolog 3 Genetic Variation (rs2295490) Effects on Type 2 Diabetes Patients with Glucose Control and Blood Pressure Lowering Treatment

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Abstract

Effects of human tribbles homolog 3 (TRIB3) genetic variation (c.251 A > G, Gln84Arg, rs2295490) on the clinical outcomes of vascular events has not been evaluated in patients with type 2 diabetes after blood pressure lowering and glucose controlling treatment. We did an analysis of a 2 × 2 factorial (glucose control axis and blood pressure lowering axis) randomized controlled clinical trial at 61 centers in China, with a follow-up period of 5 years. The major vascular endpoints were the composites of death from cardio-cerebral vascular diseases, non-fatal stroke and myocardial infarction, new or worsening renal and diabetic eye disease. A total of 1884 participants were included in our research with a 4.8 years median follow-up. For glucose lowering axis, patients with TRIB3 (rs2295490) AA (n = 609) genotype exhibited significantly reduced risk of major vascular events compared with AG + GG (n = 335) genotype carriers (Hazard ratio 0.72, 95% CI 0.55–0.94, p = 0.016). Paradoxically, the risk of vascular events were significantly increased in patients with AA (n = 621) compared to AG + GG (n = 319) genotype for intensive glucose control (Hazard ratio 1.46, 95% CI 1.06–1.96, 35 p = 0.018). For blood pressure lowering axis, a marginal significant difference was found between TRIB3 variant and coronary events. Our findings suggest that good glucose and blood pressure control exhibited greater benefits on vascular outcomes in patients with TRIB3 (rs2295490) G allele.

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1. Introduction

Recently, China faces an increasing burden of type 2 diabetes and its vascular complications with approximately 11.6% of overall prevalence in adults (Xu et al., 2013). People with diabetes impose about 3.38 times higher cost than those with normal glucose intolerance, while the cost will be 3.75 times higher for patients with longer duration of diabetes (≥10 years) compared with those with shorter duration (≤5 years) due to its complications (Yang et al., 2012). Therefore, it’s important to conduct precise strategies to treat diabetes with diabetes effectively. Actually, clinical trials demonstrated that more intensive blood pressure lowering and glucose control yielded evident clinical benefits for diabetest patients with vascular endpoints (Zoungas et al., 2014).

On the other hand, inter-individual variation of drug response was observed in prophylactic medications for diabetes and its comorbidities (Tkac, 2015), which may be partially explained by accumulating the pharmacogenetics evidence. However, the influence of common genetic variations on the individual difference in drug response or clinical endpoints need to be confirmed by large and long-term follow-up studies, because of the progressive feature of type 2 diabetes mellitus (T2DM), and the mechanisms in molecular levels that related to clinical heterogeneity phenotype remains unclear.

Human tribbles homolog 3 (TRIB3, also called NIPK, SINK, TRB3, SKIP3) is a pseudokinase that can inhibit Akt by physically occupying its phosphorylation sites, which plays a pivotal role on subsequent insulin signaling (Du et al., 2003). Previous reports indicated that TRIB3 has a ubiquitous cellular functions by interacting with a host of molecules establishing a mechanic link between metabolic phenotypes and cardiovascular risk traits (Wang et al., 2012; Ti et al., 2011), renal diseases (Ding et al., 2014), tumor progression (Hua et al., 2015) in T2DM. Interestingly, in vitro study data showed TRIB3 genetic variation (c.251 A > G, Gln84Arg, rs2295490) does not affect its protein level, but impacts on insulin-stimulated Akt (Thr308, Ser473) and eNOS (Ser1177) phosphorylation, which results in 2 to 3 times of decreased eNOS activity and nitric oxide production (Andreozzi et al., 2008). On another hand, valsartan (an antihypertensive drug), could

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significantly down-regulate the expression of TRIB3 mRNA levels and improve the cardiac functions in rats with diabetic cardiomyopathy (Zhang et al., 2006). Moreover, The presence of the gain-of-function mutation in TRIB3 (rs2295490) is one of a leading cause of TRIB3 over-activity and subsequent Akt inhibition, which has linked to increased risk of cardiovascular diseases by metabolic syndrome, endothelial and/or cardiac dysfunction (Prudente et al., 2012). Therefore, we considered that TRIB3 may have an irreplaceable role in mediating the development of the diabetic vascular complications by regulating insulin signal transduction pathway and (or) by regulating blood pressure homeostasis in patients with type 2 diabetes. And the relationship between TRIB3 genetic variation and diabetes and its vascular complications merits further attention.

The achievement of therapeutic goals for plasma glucose, lipid and blood pressure levels is important to decrease the vascular diseases risk in patients with diabetes (Li et al., 2015). Although some evidence showed that a common TRIB3 genetic variation (rs2295490) was a risk factor for diabetic nephropathy, atherosclerosis, and coronary artery disease (Prudente et al., 2005; De Cosmo et al., 2007; Formoso et al., 2011; Gong et al., 2009), some low-frequency genetic variants, which are detected by re-sequencing of TRIB3, can partially account for cardiovascular clinical outcomes in diabetes (Prudente et al., 2015). However, systematical study on the relationship between TRIB3 genetic variation (rs2295490) and antidiabetic or antihypertensive drug response, vascular complications, especially in a large and long-term follow-up T2DM clinical trial cohort have not been reported. The aim of this study is to explore the effects of TRIB3 genetic variation (rs2295490) on the clinical outcomes of vascular events after blood pressure lowering and glucose controlling treatment.

2. Methods

2.1. Patients

We conducted a retrospective study on the cohort from Action in Diabetes and Vascular disease: preterAx and diamicroN-MR Controlled Evaluation (ADVANCE) clinical trial at 61 centers in China, with a follow-up period of 5 years. Approval to conduct the trial was obtained from the ethics committee of each study center, and all participants provided written informed consent (registration number NCT00145925). Detailed rationale, design, follow-up schedule and clinical endpoints of ADVANCE trial have been described in previous studies (ADVANCE Management Committee, 2001a, 2001b). In brief, it was a 2 × 2 factorial randomized controlled trial. Patients with type 2 diabetes were randomly assigned (1:1) to receive perindopril-indapamide or matching placebo for blood pressure lowering, and modified-release gliclazide based intensive or local standard therapy for glycaemic control. For blood lowering cohort, participants were treated for 6 weeks as run-in period with combination of perindopril and indapamide, then randomly grouped into fixed combination regimen (perindopril/indapamide, initially 2.0/0.625 mg daily, increasing to 4.0/1.25 mg daily after 3 months) or matching placebo. For glucose controlling group, an open label, randomized protocol was implemented to an intensive glucose control or to local standard therapy based on local guidelines. The intensive glucose control was defined as the use of gliclazide modified release based regimen (30–120 mg daily) and other oral agents, then insulin aiming for a hemoglobin A1c (HbA1c) value of 6.5% or lower. The local standard treatment was defined as the patients who continue with their usual glucose control regimes, which may include any therapy except the use of gliclazide.

The major vascular endpoints include death from cardio-cerebral vascular diseases, nonfatal stroke or nonfatal myocardial infarction, and new or worsening renal or diabetic eye disease. Other vascular events such as cerebrovascular events (include death due to cerebrovascular disease, stroke, transient ischemic attack, and subarachnoid hemorrhage), coronary events (include myocardial infarction, angina pectoris, myocardial ischemia, and sudden death), heart disease (coronary heart disease, heart failure, atrial fibrillation), new or worsening microalbuminuria, and visual deterioration were also evaluated both jointly and separately. In our study, there was no other pre-specified criterion for the levels of blood pressure, HbA1c or other baseline clinical

Fig. 1. Outline of DNA sample collection, randomization, and follow-up information of participants.
characteristics at entry. A total of 1884 patients from 61 clinical centers were successfully genotyped in \textit{TRIB3} (rs2295490) by Sanger sequencing.

### 2.2. Genotyping Procedure for \textit{TRIB3}

Genotyping of \textit{TRIB3} rs2295490 was determined by PCR-direct sequencing. The PCR primers for \textit{TRIB3} (rs2295490) were as follows: the sense primer, 5′ GTGCCCTGAG-GCCACCTACT3′; and the antisense primer, 5′ TCCTTGATGCTTCCTCCACTA3′, with a production length of 286 bp. The reaction mixture (25 μl) contained: 10 × PCR buffer (2.5 μl), 10 × dNTP (2.5 μl), 10 μM of each of the sense and antisense primers (0.5 μl), H2O (16.8 μl), g - DNA (2 μl), Taq-ase (0.2 μl). Temperature cycling was proceeded as follows: initial denaturation for 5 min at 94 °C, followed by 25 cycles of 30 s at 94 °C, annealing at 57 °C for 30 s, and elongation at 72 °C for 30 s, and a terminal extension for 5 min. Genotypes were determined without knowledge of the status of patients and 10% blinded. Random DNA samples from the patients were used for quality control.
genotyped twice with 100% concordance. Sequencing was assisted by Shanghai Majorbio Bio-pharmTechnology Company.

2.3. Statistical Analysis

All the analyses were performed with SPSS (version 20.0 for windows; Chicago, IL). Allele frequencies were determined by the genotypes of all the participants. Continuous data are presented as mean values ± SD, and frequencies or percentages are for categorical variables. The Hardy–Weinberg equilibrium analysis was carried out for the study participants using the $\chi^2$-test. Differences in baseline characteristics among the phenotypes were assessed by independent-samples t-test or Wilcoxon rank sum test, appropriately. General linear model-multivariate ANOVA and linear regression analysis was performed in plasma glucose, lipid levels and blood pressure between TRIB3 (rs2295490) AA, AG + GG genotype with adjustment for sex, age, duration of the disease, body mass index (BMI), combined medication and drug dosage. Cox backward (LR: entry $p = 0.05$ and removal $p = 0.10$) regression model adjustment by history of vascular disease, baseline of the clinical biochemical data, sex, age, duration of the disease, combined medication and drug dosage was used to investigate the relationship between genetic variation and the risk of clinical outcomes of vascular event endpoints. A two-tailed $P$-value < 0.05 was considered significant.

Fig. 3. Effects of TRIB3 (rs2295490) genetic variation on the cumulative hazard function of clinical endpoints according to glucose control strategy. The major vascular endpoints include death from cardiovascular disease, stroke or myocardial infarction, and new or worsening renal or diabetic eye disease. Other vascular events such as cerebrovascular events (include death due to cerebrovascular disease, stroke, transient ischemic attack, and subarachnoid hemorrhage), coronary events (include myocardial infarction, angina pectoris, myocardial ischemia, and sudden death). Vertical broken lines indicate additional information on microvascular events (diabetes related nephropathy and retinopathy) were obtained at 24-month and 48-month visits. And the event times were recorded as the visit dates. The curves were truncated at Month 57, by which time 99% of events had occurred. The effects of treatment between TRIB3 (rs2295490) AA and AG + GG genotypes (hazard ratios and p-values) were estimated from survival-cox regression models with backward LR adjustment for baseline available data.
3. Results

3.1. Genotyping and Characteristics of Patients

A total of 1898 Chinese participants were enrolled from May 2002 to December 2002 in ADVANCE trial (China centers) from 61 hospitals, with an median of 4.8 years follow-up duration in 1714/1884 (91.0%) participants who were successfully completed at scheduled end of follow-up (Fig. 1), and 1884/1898 (99.3%) of DNA samples from these patients passed quality control for genotyping of TRIB3 (rs2295490). Genotyping data of TRIB3 (rs2295490) in our study cohort showed a minor allele (G) frequency of 17.1%, the (AA, AG and GG) genotype frequencies were 1230/1884 (65.3%), 581/1884(30.8%) and 73 (3.9%), respectively, which were in Hardy-Weinberg equilibrium across the randomized groups (Table S1 in the Supplementary Appendix). No significant difference was observed across the baseline characteristics at entry between AA and AG + GG genotype of TRIB3 (rs2295490) in blood pressure lowering or glucose controlling axis (Table 1).

3.2. Glucose Controlling Axis

For the intensive glucose controlling group, the mean glycosylated HbA1c level was 6.8% throughout the follow-up period, and it was higher in patients with AG + GG genotypes (6.7%) compared to AA genotype (6.6%) after a follow-up of 6 months, while the difference observed was not significant until the period between 15 and 30 months (AA vs AG + GG, 6.6% vs 6.8%) (Fig. 2a). For the standard glucose control group, the mean glycosylated HbA1c levels was 7.4% throughout the follow-up period, and it showed no significant difference between AA and AG + GG genotypes (AA vs AG + GG, 7.4% vs 7.4%) after randomized treatment (Fig. 2b). Additionally, in the intensive and standard glucose treatment groups, fast plasmid glucose and HbA1c levels between AA
and AG + GG genotypes showed a similar trend, although not significant (data not shown).

A total of 458 patients had major macrovascular or microvascular events during the visits: 152/621 (24.5%) patients with AA genotype and 61/319 (19.1%) with AG + GG genotype (hazard ratio, 1.46; 95% CI, 1.06 to 2.17; \( p = 0.018 \)) were observed in the intensive glucose control group (Fig. 3a); 143/609 (23.5%) patients with AA genotype and 102/335 (30.4%) with AG + GG genotype (hazard ratio, 0.72; 95% CI, 0.55 to 0.94; \( p = 0.016 \)) were observed in the standard glucose control group (Fig. 3b). Additionally, TRIB3 (rs2295490) genetic variation showed an inverse effect on the major macrovascular events (Fig. 3c and d) and microvascular events (Fig. 3e and f) when compared between the two groups, and the effects on the secondary clinical outcomes of coronary (Fig. 3g and h) and cerebrovascular events (Fig. 3i and j) are similar as well. There was no significant difference in other major and secondary macrovascular or microvascular events when analyzed jointly or separately, except for the secondary clinical endpoints of new or worsening microalbuminuria and death from any cause (Table 2).

### 3.3. Blood Pressure Controlling Axis

Over the duration of follow-ups, the mean systolic blood pressure (SBP) was 132.8 mm Hg and diastolic blood pressure (DBP) 74.6 mm Hg in perindopril–indapamide fixed tablet treatment group; while the mean SBP was 137.8 mm Hg and DBP 76.9 mm Hg in placebo matching treatment group. In the randomized active blood lowering treatment group, compared with TRIB3 (rs2295490) AA genotype carriers, patients with AG + GG genotype had a mean decrease of DBP of 0.8 mm Hg. Marginally significant difference (\( p = 0.063 \)) between the two groups at the end of follow-up was observed (Fig. 4a). Conversely, in matched placebo treatment group, the mean DBP was increased by

### Table 2

Effects of TRIB3 (c.251, A > G) genetic variation on patients with major and secondary outcomes according to glucose-control strategy.

| Subgroup                                      | Intensive, n (%) | HR (95% CI), P-value | Standard n (%) | HR (95% CI), P-value |
|-----------------------------------------------|------------------|----------------------|----------------|----------------------|
| Major clinical outcomes                       |                  |                      |                |                      |
| Combined major macro- and microvascular events| 152(24.5)        | 0.91(0.34–2.46), 0.86|                |                      |
| Major macrovascular events                    | 89(14.3)         | 1.14(0.72–2.42), 0.57|                |                      |
| MI                                            | 14(2.3)          | 1.27(0.91–1.56), 0.078|                |                      |
| Stroke                                        | 60(9.7)          | 0.76(0.57–1.05), 0.13|                |                      |
| Death from Cardio-cerebral vascular cause     | 27(4.3)          | 0.81(0.62–1.06), 0.034|                |                      |
| Major microvascular events                    | 75(12.1)         | 1.62(1.02–2.58), 0.040|                |                      |
| New or worsening nephropathy                  | 28(4.5)          | 1.57(0.70–3.54), 0.27|                |                      |
| New or worsening retinopathy                  | 57(9.2)          | 1.65(0.97–2.82), 0.065|                |                      |
| Secondary clinical outcomes                   |                  |                      |                |                      |
| Coronary events                               | 69(11.1)         | 1.80(1.09–2.98), 0.023|                |                      |
| Heart disease                                 | 76(12.2)         | 1.42(0.91–2.21), 0.12|                |                      |
| Cerebrovascular events                        | 68(11.0)         | 1.19(0.77–1.85), 0.43|                |                      |
| All macrovascular events                      | 144(23.2)        | 1.26(0.93–1.71), 0.14|                |                      |
| Visual deterioration                          | 127(20.5)        | 1.14(0.83–1.57), 0.42|                |                      |
| New or worsening microalbuminuria             | 23(3.7)          | 0.60(0.32–1.15), 0.12|                |                      |
| All microvascular events                      | 211(34.0)        | 1.25(0.97–1.62), 0.079|                |                      |
| Death from any cause                          | 45(7.2)          | 1.96(1.02–3.75), 0.044|                |                      |
| Neuropathy                                    | 29(4.7)          | 1.35(0.67–2.73), 0.41|                |                      |

**HR = Hazard Ratio**, the effects of treatment between TRIB3 (c.251, A > G) AA and AG/GG genotypes (hazard ratios and \( p \)-values) were estimated from survival-cox regression models with backward LR adjustment for baseline available data.
1.0 mm Hg respectively (Fig. 4b). However, SBP presented no remarkable difference between the AA and AG + GG groups (data not shown).

For blood pressure lowering axis, there was no significant effect of TRIB3 (rs2295490) genetic variation on the risk of any major and secondary clinical outcomes between the active and placebo treatment groups (Table S2 in the Supplementary Appendix). However, it is worth noting that the genetic variation was weakly correlated to the risk of total coronary events in the active treatment group (hazard ratio, 1.52; 95% CI, 0.98 to 2.35; \( p = 0.061 \)) (Fig. 4c), but not in the matched placebo treatment group (hazard ratio, 1.04, 95% CI, 0.67 to 1.02; \( p = 0.85 \)) (Fig. 4d).

4. Discussion

For the intensive glucose control axis, as data shown in Fig. 2, TRIB3 (rs2295490) genetic variation had a significant influence on patients that were assigned to the intensive glucose control group, but not on patients assigned to the standard glucose control group. For the intensive glucose control group, the gliclazide MR-based, along with other mainly prescribed drugs such as metformin, insulin preparations were required to make the glycated hemoglobin value target to 6.5% or lower. For the standard glucose control group, however, a routine administration of metformin and sulphonylureas (except gliclazide) glucose control strategies was recommended. The main difference in the clinical medication between the two groups was that the proportion of insulin combination, angina pectoris, myocardial ischemia, and sudden death. The effects of treatment on coronary events between TRIB3 (rs2295490) AA and AG + GG genotypes (hazard ratios and \( p \)-values) were estimated from survival-cox regression models with backward LR adjustment for baseline available data (panel c and d).
treatment, mainly as a consequence of a reduction in the major microvascular and all coronary clinical outcomes (Fig. 3e and g). For the patients in the standard glucose control group, however, the G allele was a risk factor of major macrovascular and cerebrovascular clinical outcomes (Fig. 3d and j). Our sub-center data illustrated that the incidence of major microvascular events was significantly reduced in the intensive glucose control when compared with the standard glucose control (hazard ratio, 0.72; 95% CI, 0.56 to 0.94; p = 0.016) (Fig. 1 in the Supplementary Appendix), and it was consistent with the results reported previously (Patel et al., 2008). Data from Zhang et al. have revealed that the induction of TRIB3 by high glucose was reversed to baseline by euglycemia achieved in vivo and in vitro (Zhang et al., 2015). It is suggested that the significant difference in blood glucose levels between intensive and standard glucose control groups may be a leading cause to the reverse role of TRIB3 (rs2295490) genetic variation between the two groups, while the exact mechanism needs to be confirmed by further research.

For the blood pressure lowering axis, we found that TRIB3 (rs2295490) genetic variation was not significantly associated with the blood pressure response after a fixed combination therapy of perindopril and indapamide. Interestingly, when taking TRIB3 (rs2295490) AA genotype as the baseline, patients carrying TRIB3 (rs2295490) AG + GG genotype had a flip effect on diastolic blood pressure response in a fixed combination of perindopril and indapamide treatment group compared with placebo matching treatment group (Fig. 4a and b). However, there was no significant difference between AA and AG + GG genotypes in the two groups on the incidence of major macrovascular, microvascular, coronary and cerebrovascular outcomes when analyzed jointly or separately. The more frequent use of perindopril, calcium channel blockers (CCBs) (57% and 33% at the end of follow-up, respectively) in the placebo group than the active treatment group (43% and 26% at the end of follow-up, respectively) might be relevant for such results. Details are shown in Fig. 4a and b.

However, in the continuous blood pressure lowering group, irrespective of initial blood pressure levels, TRIB3 (rs2295490) G allele carriers in the active treatment group got more benefit on coronary events when compared with placebo group, although the difference was not significant. Limited data (Andreozzi et al., 2008; Zhang et al., 2006; Leiris et al., 2013; Chan et al., 2007) and our findings indicate that TRIB3 mediated signaling pathway may be related to blood pressure regulation and its genetic variation can affect the response of blood pressure lowering drugs, subsequently affect cardiovascular event outcomes. At this point, it is necessary to conduct further study.

Recently, investigation of common genetic variability in type 2 diabetes patients with cardiovascular complications from VND study showed that the genetic variations in gatekeepers along the insulin signaling pathway (IRS1, ENPP1 and TRIB3) were associated with increased coronary heart disease (Trombetta et al., 2016). It is suggested that investigation of genetic variation in other new or key genes, which are related to insulin signaling pathway may have higher clinical value and significance (Menzaghi et al., 2014; Choi et al., 2016). However, up to now, the allele gene frequencies of the related genetic variations in TRIB3 (except rs2295490 mutation) are rare in Chinese population (<1%). In addition, results from large-scale sequencing did not support any major role of lower-frequency variants in type 2 diabetes neither (Fuchsberger et al., 2016). Therefore, we appropriately selected rs2295490 as candidate single nucleotide polymorphism (SNP) for our study.

5. Conclusion

In this study, our findings include: 1) during follow-up period, a partially difference in glucose and blood pressure response were observed after the intensive and the active treatment between TRIB3 (rs2295490) AA and AG + GG genotype, it suggests this gene polymorphism may have a plausible effect on antidiabetic and antihypertensive drug response in patients with T2DM. 2) TRIB3 (rs2295490) genetic variation was associated with the clinical outcomes of major macrovascular and microvascular complications in T2DM, while the effects of this genetic variation has a reverse role between the intensive and standard glucose control groups, which can be caused by the obvious differences in glucose levels between the two groups. 3) Our findings suggest that good glucose and blood pressure control exhibited greater benefits on vascular outcomes in patients with TRIB3 (rs2295490) G allele.

Conflicts of Interest

We declare: no competing interests within the submitted manuscript; no other relationships or activities that could appear to have influenced the submitted work.

Author Contributions

FZH, WZ, XYW were responsible for the study design. FZH, ZRC, GJL, ZMW, JQL were responsible for the experiments: FZH, RL, ZKC did the data processing and statistical analysis. FZH, MZL and JT wrote and interpreted the paper. HHZ, XL, ZQL, XYW, XPC, WZ were responsible for data management and contributed reagents and materials.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jbiom.2016.10.025.

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