ABSTRACT

BACKGROUND
The prevalence of type-2 diabetes mellitus (T2DM) has been increasing rapidly in developed and developing countries. Many new loci associated with T2DM have been uncovered by genetic and genome-wide association studies, e.g. the transcription factor 7-like 2 (TCF7L2) gene variant rs7895340 has been associated with type-2 diabetes mellitus. The aim of this research was to find an association between polymorphisms of TCF7L2 gene variant rs7895340 with T2DM in ethnic Minangkabau.

METHODS
This was a case-control study using a consecutive sampling technique among ethnic Minangkabau patients who came for treatment to the Metabolic Endocrinology Polyclinic at Dr. M. Jamil Hospital. Analysis was performed on 132 subjects (66 T2DM patients and 66 controls). DNA was isolated from venous blood samples and amplified using the amplification refractory mutation system - polymerase chain reaction (ARMS-PCR) method to detect single nucleotide polymorphisms (SNPs) of TCF7L2 gene variant rs7895340. To determine the significance of association of SNPs of rs7895340 with T2DM in ethnic Minangkabau, data analysis was performed using chi-square test.

RESULTS
The ARMS - PCR for TCF7L2 gene variant rs7895340 found polymorphisms in 28 (42.42%) subjects with type 2 diabetes mellitus and in 15 (22.72%) non-diabetic subjects. There was a significant association between rs7895340 SNPs with the probability of T2DM in ethnic Minangkabau (OR=2.505, p=0.026).

CONCLUSIONS
Single nucleotide polymorphism rs7895340 of TCF7L2 gene can be used as genetic marker of type-2 diabetes mellitus in ethnic Minangkabau.

Keywords: TCF7L2 gene, rs7895340 SNP, type-2 diabetes mellitus, ethnic Minangkabau
Transcription factor 7-like 2 sebagai petanda diagnostik untuk diabetes melitus tipe-2 pada etnik Minangkabau

LATAR BELAKANG

METODE

HASIL
Hasil ARMS-PCR pada gen TCF7L2 varian rs7895340 pada subyek diabetes mellitus tipe-2 ditemukan polimorfisme sebanyak 28 orang (42,42%), dan pada subyek tidak DMT2 (kontrol) hanya 15 orang (22,72%). Hasil uji chi-square membuktikan adanya hubungan yang signifikan antara gen TCF7L2 varian rs7895340 dengan diabetes melitus tipe-2 (OR = 2,505, p=0,026).

KESIMPULAN
Gen TCF7L2 varian rs7895340 dapat dijadikan petanda genetik untuk diabetes melitus tipe-2 pada etnik Minangkabau.

Kata kunci: Gen TCF7L2, rs7895340, diabetes melitus tipe-2, etnik Minangkabau

INTRODUCTION
Diabetes mellitus (DM) is a complex metabolic disease caused by pancreatic cellular damage. It is clinically classified into four types: type 1 diabetes mellitus, type 2 diabetes mellitus (T2DM), gestational diabetes mellitus, and other types of diabetes mellitus. T2DM is characterized by the phenomenon of insulin resistance as a result of relative insulin deficiency. The prevalence of T2DM has been increasing in developed and developing countries. The prevalence of DM in Indonesia is 4.1%, while in West Sumatra for the year 2000 it was estimated to be 5.1%. Patients with diabetes commonly suffer from cardiovascular complications, which are the most common cause of death in patients with diabetes mellitus. The diagnostic methods for DM are still being developed, e.g. using DNA analysis. To perform DNA analysis, data are required on genes associated with T2DM. Several ethnic groups in the world have already gene banks for T2DM, e.g. in Denmark, USA, UK, France, and India.
Through DNA or gene analysis, patients with T2DM can be more rapidly and accurately diagnosed. Although there are not many monogenic causes of diabetes, such as in maturity onset diabetes in the young (MODY), several gene variants have been found through extensive research as causes of T2DM, among others PPARG (Pro12 Ala), KCNJ11, CAPN-10, and the genes for PC-1, PGC-1α, and IRS-2. (9) Recently the association of the transcription factor 7-like 2 (TCF7L2) gene with T2DM was mapped and consistent gene variants were found in several populations or ethnic groups. The TCF7L2 gene on chromosome 10q is strongly associated with T2DM (10) in ethnic Danes, Indians, and several Asian ethnic groups. (9,11,12) The human TCF7L2 gene consists of 224429 bp and encodes a high mobility group (HMG) box that is a transcription factor for the Wnt pathway. The protein transcription product is implicated in the homeostasis of glucose in the blood. Its gene variants are associated with an increased risk for T2DM. (13)

An appropriate marker or haplotype may furnish indications of increased susceptibility of an individual to T2DM, characterized by a minimum relative risk of 1.2-1.4. One variant of the TCF7L2 gene that is believed to be associated with T2DM is rs7895340, with ACAGTTCTAGACACCTAGAGAGTAAA[AG]TGAAGAAGCCTGTTTTCAGGTTTCC as sequence motif. (13) The presence of the G allele in rs7895340 is an indication of increased susceptibility to T2DM. (10,14,15)

The aim of the present study was to evaluate TCF7L2 gene variant rs7895340 polymorphisms as diagnostic marker for T2DM in ethnic Minangkabau.

METHODS

Design of the study

This was a case-control study on a population of ethnic Minangkabau patients with type-2 diabetes mellitus who attended the Metabolic Endocrinology Polyclinic at Central General Hospital Dr. M. Djamil. Controls were taken from healthy ethnic Minangkabau without T2DM. The study was carried out at the Biomedical Laboratory, Faculty of Medicine, Andalas University, from February 2013 until April 2014.

Subjects of the study

The subjects of the study were ethnic Minangkabau who were healthy as well patients with T2DM. The diagnostic criteria for cases and controls were fasting blood glucose (FBG) and 2-hour postprandial blood glucose (2HPPBG). The diagnosis of T2DM was based on FBG ≥126 mg/dL, 2HPPBG ≥200 mg/dL, that of nondiabetic controls FBG <100 mg/dL and 2HPPBG <140 mg/dL.

The inclusion criteria in this study were: ethnic Minangkabau domiciled in West Sumatra; both parents and all grandparents of Minangkabau ethnicity, and aged 40-60 years. The exclusion criteria were: type 2 prediabetes mellitus; malignancies; chronic disease such as hepatic, renal, pulmonary diseases, and pregnancy.

Recruitment was by consecutive sampling among ethnic Minangkabau patients with T2DM who came for treatment to the Metabolic Endocrinology Polyclinic at Central General Hospital Dr. M. Djamil. Healthy ethnic Minangkabau without T2DM were used as controls.

The sample size was determined from the formula; (16)

\[ n_1 = n_2 = \left( \frac{Z_{a/2} \sqrt{P_1 Q_1} + Z_{a/2} \sqrt{P_2 Q_2}}{P_1 - P_2} \right)^2 / \left( \frac{P_1 - P_2}{1 - P_1 - P_2} \right)^2 \]

where \( n \) = number of subjects per group; \( Z_{a/2} = 1.960 \); \( Z_{a/2} = 0.842 \); \( P \) = expected proportion of cases \( P_1 = \frac{1}{2} (P_1 + P_2) \); \( P_1 \) = estimated proportion of cases (42%) \(^3\); \( P_2 \) = proportion of controls (subjects without risk factors) (26%). \(^3\)

According to the formula, the sample size for each group was 62 (62 cases and 62 controls). In anticipation of drop-outs, each group was expanded by 5%, giving 66 subjects per group.
Assay for single nucleotide polymorphisms of TCF7L2 gene variant rs7895340

Blood samples of 2 mL were collected from each subject in tubes containing EDTA (0.1 mmol/L) and stored at –20 °C before use. For DNA extraction we used PureLink Genomic DNA kits (Invitrogen). Testing for success and effectiveness of genomic isolation was by electrophoresis on agarose gel 1.5% at 100 volts for 30 minutes. The DNA isolation products were then used as templates for in vitro reaction in the assay for single nucleotide polymorphisms (SNPs) of TCF7L2 gene variant rs7895340.

Primers for detection of TCF7L2 gene variant rs7895340 SNPs were constructed using a primer designer software. The TCF7L2 gene sequence used for primer construction was obtained from the NCBI gene bank. (13)

Before application of the primers on the clinical samples, the primer constructs were subjected to computerized testing, to find any mispriming of primers against other regions of the *Homo sapiens* genome. After no evidence of mispriming was found, the primer constructs were ready for synthesis into oligonucleotides.

To evaluate the ability of the primers to detect TCF7L2 gene variant rs7895340 SNPs, amplification was done using the Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) method. The PCR reaction was carried out with a PCR master mix using the Invitrogen RTG kit. Its principle consists of using three or more primers for simultaneous amplification of several DNA regions. One of the three primers is the specific primer for identification of SNP or mutating strains. The occurrence of an SNP in a specific region can be detected by constructing a specific primer that can recognize the SNP site.

To detect the ARMS-PCR DNA amplification products, electrophoresis was done on agarose 1.5%. A test sample without rs7895340 polymorphisms (wild type) produces two large fragments of ± 431 bp and ±209 bp, respectively. The presence of one fragment of ± 431 bp indicates a mutation or polymorphism in TCF7L2 gene variant rs7895340. All samples, consisting of 66 T2DM patients and 66 controls, were subjected to tests for TCF7L2 gene variant rs7895340 polymorphisms. Selected samples were sequenced for checking and confirmation of the ARMS-PCR results. Description of the primer and the assay for the ability to detect TCF7L2 gene rs7895340 SNPs were performed at the Biomedical Laboratory, Faculty of Medicine, Andalas University.

Ethical clearance

The present study obtained ethical clearance from the Faculty of Medicine, Andalas University.

Data analysis

The significance of the association of TCF7L2 gene variant rs7895340 SNPs was analyzed by the chi-square test.

RESULTS

Three primer constructs were made, i.e. RS78F, RS78R and RS78C. The primers RS78F and RS78C were forward primers, whereas RS78R was the reverse primer. Primers RS78F and RS78R were used for DNA amplification comprising a region of ± 431 bp (external primer). Primers RS78C and RS78R were used for amplification of the region that will directly recognize the rs7895340 SNP (internal primer, of ± 209 bp).

The RS78F primer sequence was: 5'-CCTTGTGATGTCTTCTCTCC-3', with GC= 50% and Tm C=64 °C. The RS78R primer sequence was: 5'-ACATCTTCTCTCCTCA CTC-3', with GC=50% and Tm C=61 °C. The RS78C primer sequence was: 5'-GAGACCTAG AGAGT GAAG-3', with GC=50% and Tm C=58 °C. Visualization of the electrophoresis results (electrophoregram) of the PCR products for the primer pairs RS78C, RS78F and RS78R is presented in Figure 1.
Figure 1. Visualization of ARMS-PCR assay using the primer pairs RS78C, RS78F and RS78R. D11 = sample code for T2DM patients; N11 = sample code for normal controls; L = 100 bp DNA ladder

In addition to the use of the ARMS-PCR method for recognizing rs7895340 SNPs, this study also used direct DNA sequencing. After having been submitted to a BLAST search, the direct sequencing results yielded a query score of 200, indicating that the PCR results with the RS78 forward and reverse primers indeed originated from a fragment of the TCF7L2 gene.

The sequencing results were then aligned with the rs7895340 SNP nucleotide profile. The alignment results proved that the PCR products with RS12 forward and reverse primers indeed originated from a fragment of the TCF7L2 variant rs7895340 SNP, so that they could be used to detect the SNP of interest. The sequencing results are shown in Figure 2.

The results of association analysis of TCF7L2 variant rs7895340 SNP in 66 subjects with T2DM and 66 non-T2DM subjects of Minangkabau ethnicity may be seen in Table 1.

**DISCUSSION**

In this study we found a significant association of rs7895340 SNP with the risk for T2DM in ethnic Minangkabau. Our results are consistent with those of several previous studies carried out on a number of ethnic groups in Asia, Iceland, and Finland. Table 2 illustrates that rs7895340 is highly correlated with T2DM in ethnic Minangkabau. This indicates that Minangkabau individuals with an rs7895340 SNP have a 2.5-fold probability to suffer from DM in comparison with individuals without the SNP.

Figure 2. Alignment sequencing results with TCF7L2 variant rs7895340 SNP motif; a. Sample with polymorphism resulting in A→G base change; b. Sample without polymorphism, with sequencing results identical to the rs7895340 SNP motif.
The TCF7L2 gene, also known as TCF-4, belongs to the T-Cell Factor (TCF)/Lymphoid-enhancing factor family of the HMG box that comprise transcription factors for the Wnt signaling pathway, a key component for the regulation of cell proliferation and differentiation. Wnt activity is also important for lipid and glucose metabolism, proliferation and functioning of pancreatic beta cells, and production of the glucagon-like peptide-1 (GLP-1) incretin hormone. Together with the TCF7L2 nuclear receptor, WNT signaling is an important factor in GLP-1 secretion by intestinal endocrine L-cells. A change in this pathway may trigger decreased GLP-1 secretion, which then affects insulin secretion. GLP-1, in close association with insulin, plays an important role in the homeostasis of glucose in the blood. It has been established that variation of the TCF7L2 gene may indirectly trigger susceptibility to T2DM through alterations in GLP-1 levels.

Diabetes mellitus type 2 is a degenerative disease that will appear in an individual carrying a genetic defect after long term genetic changes. With an appropriate diagnostic marker we may be able to predict the possibility of developing the disease at an early stage, which will aid in the prevention and treatment of the disease. On the basis of the obtained data, the TCF7L2 gene variant rs7895340 can be used as a diagnostic marker in ethnic Minangkabau. The diagnosis of T2DM susceptibility can be established by detection of polymorphisms in rs7895340. The detection may be done by ARMS-PCR that can specifically recognize the SNP-bearing region.

The success of an ARMS-PCR assay in detecting mutations/SNPs is highly dependent on the specificity of the constructed primer. The choice of the correct primer will facilitate recognition and analysis of an SNP in a sample. Errors in primer selection result in the amplification of a different region, which will influence the quality of the assay results. Nonspecificity of the selected primer for a given region or SNP will preclude detection. Therefore the primer construction process is an early and crucial step in SNP detection.

Before being applied to a clinical sample, the primer should subjected to computerized testing, involving not only the site of primer adherence, but also other criteria that may influence the PCR assay. Among the several criteria for primer construction to be considered are the Tm and G+C content, which will affect the annealing temperature of the primer. Other criteria are primer homology to template DNA, the possibility of primer dimer and hairpin formation, and various intra- and inter-molecular reactions capable of leading to erroneous PCR results. From the enumeration of primer criteria, it may be seen that all three primers were in relatively ideal condition.

Visualization of the ARMS-PCR assay using the primer pairs RS78C, RS78F and RS78R can be seen in Figure 1. The electrophoresis results show the presence of 2 DNA bands formed by amplification of the non-T2DM (control) blood samples. Both bands have estimated positions of ± 239 bp and ± 431 bp, respectively, while amplification of the T2DM templates yielded one band at a position of ± 431 bp. This indicates that in these conditions only the primers RS78F and RS78R were able to amplify the DNA target, whereas amplification of primers RS78C and RS78R could not be

### Table 1. Association of TCF7L2 variant rs7895340 SNP with T2DM subjects

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Type 2 diabetes mellitus</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs7895340 SNP</td>
<td>Yes (n=66)</td>
<td>28</td>
<td>63.1</td>
</tr>
<tr>
<td>Wild type</td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
</tbody>
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continued because of the prior occurrence of changes (SNPs) in the DNA sequence of the TCF7L2 gene. The occurrence of an SNP in a given region may be found with a primer construct that specifically recognizes the mutant site. This may be done by positioning the 3’ end of the primer exactly within the SNP/point mutation sequence. The primer could not amplify the proposed target DNA because of a nucleotide change in the template. As a result of the differing PCR target, the Taq DNA polymerase was unable to continue the amplification process in the region of interest.\(^{(17)}\)

On the basis of these data, it may be concluded that the ARMS-PCR assay may be used to detect SNPs in the TCF7L2 gene, particularly rs7895340 SNPs. However, this method has a limitation: the assay cannot possibly detect 100% of the SNPs in the TCF7L2 gene, which contains more than 25 types of SNP.\(^{(15)}\) In spite of this, its high specificity and sensitivity in detecting SNPs may be cited as one of the reasons why this method can be used. In comparison with other SNP detection methods, ARMS-PCR has several advantages, being more economic and easily applied. It is faster in execution, since the whole process, from the preparation of reagents and equipment, addition of genomic DNA (template), ARMS-PCR amplification, to agarose gel electrophoresis, can be completed in one day. Its rapid application and ease of execution for detection of rs7895340 SNPs are essential for the prevention of T2DM. This method is relatively ideal to be applied in Indonesia, since in contrast with other molecular mutant detection methods (such as the microarray technique), that use sophisticated equipment, are expensive, and need specialized expertise, the ARMS–PCR method is simpler. This method applied the principles of simultaneous PCR with a number of primers (multiplex PCR), which are generally available in almost every region in Indonesia. Although relatively simple, the ARMS–PCR technique still has a high sensitivity in detecting SNPs.

CONCLUSIONS

The TCF7L2 gene variant rs7895340 may be used as a genetic marker for type 2 diabetes mellitus in ethnic Minangkabau. In other words, the polymorphisms in the TCF7L2 gene variant rs7895340 can be used as an indicator of the risk factor contributing to individual susceptibility to type 2 diabetes mellitus in ethnic Minangkabau.

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