

Original Article

Screening of *SMAD7* in Malay patients with ventricular septal defect

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Abstract: Ventricular septal defect (VSD) is the most common form of cardiac malformations accounting approximately 20% of all congenital heart defects. *SMAD7* is an inhibitory protein that antagonizes the signalling of TGF- β family member and has been found in the development and function of mouse heart models. This study aims to screen and identify the polymorphisms of *SMAD7* exonic regions in Malay population with VSD. Peripheral blood samples were collected and extracted from 30 clinically diagnosed VSD patients. PCR amplification was performed using 12 sets of designed primers encompassing seven exons of *SMAD7*. Re-sequencing was conducted to characterize the polymorphisms of *SMAD7*. Observed polymorphisms were then genotyped in 30 healthy individuals using both re-sequencing and allele-specific PCR techniques. A total of 10 variants were identified in the patient population located in the upstream (rs7236774), exonic (rs368427729, rs145686330, rs3764482, rs3809922, rs780863704 and rs3809923), intronic (rs3736242) and 3'UTR regions (rs375444823 and rs16950113). No significant difference of genotype and allele frequency was observed among these SNPs. Two synonymous variants (rs3809922 and rs3809923) were found in complete linkage disequilibrium ($r^2=1.0$) with each other indicate a strong correlation of these SNPs. The identification of these SNPs provides a new perspective of the VSD causation.

Keywords: Ventricular septal defect, *SMAD7*, MH2 domain, linkage disequilibrium

Introduction

Ventricular septal defect (VSD) is the most common form of congenital heart malformations that account for 20% and 10% in children and adults respectively [1]. VSD is defined as an opening in the ventricular septum and classified according to its location in the septum of the heart; perimembranous defects, muscular defects and subarterial infundibular defects [2]. The pathogenesis of VSD is still unknown and it is hypothesized as multifactorial inheritance model implicating both genetic and environmental factors in disease development [3].

SMAD7 is a member of SMAD protein that inhibits the signalling of transforming growth factor- β (TGF- β) family member through multiple mechanisms; one of the methods is by forming a stable complex with type I receptor

(T β RI), thus prevent the phosphorylation of SMAD complex [4]. *SMAD7* is responsible in recruiting E3 ubiquitin ligase Smurfs 1 and 2 to T β RI and target them for degradation [5,6]. *SMAD7* also recruits protein phosphatase 1 to inactive T β RI [7].

SMAD7 is mapped to chromosome 18 and consists of two highly conserved MH1 and MH2 domains that are located at the C-terminal and N-terminal region respectively. These two termini are separated by a proline-rich divergent and less conserved linker region. The MH2 domain of *SMAD7* is believed to be involved in the inhibition of TGF- β signaling through its four amino acid residues (Lys312, Lys316, Lys401 and Arg409) to the type I receptors [8].

A study by Zwijsen and colleagues demonstrated that *SMAD7* is expressed in the cardiovascu-

lar system in mouse embryos [9]. Another study by Chen and colleagues showed that majority of *SMAD7* knockout mice with targeted deletion of MH2 domain died *in utero* due to multiple defects in cardiovascular development including ventricular septal defect, ventricular non-compaction and outflow tract malformation [10]. Recently, Wang *et al.* reported an association of two variants (rs3809922 and rs3809923) of *SMAD7* with the risk of septation defects in the Han Chinese population [11]. This study was conducted to screen the *SMAD7* in Malay population with VSD whether it contribute to the associated risk in VSD patients as compared to normal controls.

Materials and methods

Sample collection

The subjects enrolled in this study were of Malay ethnicity from Malaysia. A total of 30 clinically diagnosed VSD patients and 30 unrelated controls were recruited from Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, Kelantan, Malaysia between February 2013 and March 2014. Echocardiography test was conducted prior to blood or saliva sample collection to determine the status and type of VSD in patients with the age at diagnosis ranged from newborns to adults (mean age 13 ± 8.39 years old). Individuals with syndromes and genetic disorders were excluded from this study. Written informed consent and family history was obtained by interviewing the patients and their family members. The controls cohort consisted of normal healthy individuals with unrelated age (mean age 23 ± 3.69 years old) and gender-matched individuals who were not diagnosed with CHD and no known genetic disorders. Ethical approval (FWA Reg. No: 00007718; IRB Reg. No: 00004494) was obtained from the Research and Ethics Committee, USM, Kelantan, in line with declaration of Helsinki.

Mutational screening of *SMAD7* exonic regions in cases

Genomic DNA was extracted from peripheral blood and saliva samples using two commercially available kits (Exgene™ Blood SV Mini Kit, Gene All Biotechnology, Korea and PSP® Saliva Gene DNA kit, STRATEC) and was subjected to PCR amplifications using 12 sets of designed

primers encompassing all seven exons of *SMAD7*. The primer sequences were obtained from Ensembl version 78 and verified using Sequence Manipulation Suite version 2. Forward and reverse primers were designed at approximately 30-80 nucleotides from the start or at the end of the exonic regions. The concentration and the purity of the DNA were determined using NanoQuant plate reader (DKSH, TECAN). The PCR products were purified using Illustra™ ExoStar (GE Healthcare) and subsequently were sent for sequencing (Centre for Chemical Biology, Penang) to characterize the polymorphisms in *SMAD7* of the cases. All variants identified in this study were confirmed by repeating PCR and sequencing.

Genotyping in controls group

The identified SNPs were genotyped in 30 healthy controls using allele-specific PCR and sequencing techniques. Allele-specific PCR was carried out using three sets of primers consisting of one common primer and two sets of other primers which represent different alleles based on its nucleotide changes. The allele-specific primers were designed using Yaku Bonczyk principle [12]. Sequencing was applied for those amplicons that could not successfully discriminate between the wild type and variant allele using the allele specific PCR method.

Statistical analysis

The data from this study was analyzed using Statistical Packages for Social Software (SPSS) version 22.0 (SPSS Inc. Chicago, USA). The allele and genotype frequencies among the groups were determined using Chi-square or Fisher's exact test with odds ratio and 95% confidence interval (CI). A *P*-value of < 0.05 was considered as statistically significant. Linkage disequilibrium and reconstructed haplotype blocks were analyzed using Haploview version 4.2 software [13] based on the expectation-maximization (EM) algorithm. The standardized correlation coefficient, r^2 was used to represent LD value and color scheme where $r^2=0$, $0 < r^2 < 1$ and $r^2=1$ was shown by white, shades of grey and black respectively.

Results

VSD classifications

The recruited VSD patients were classified into two subtypes, namely, perimembranous VSD

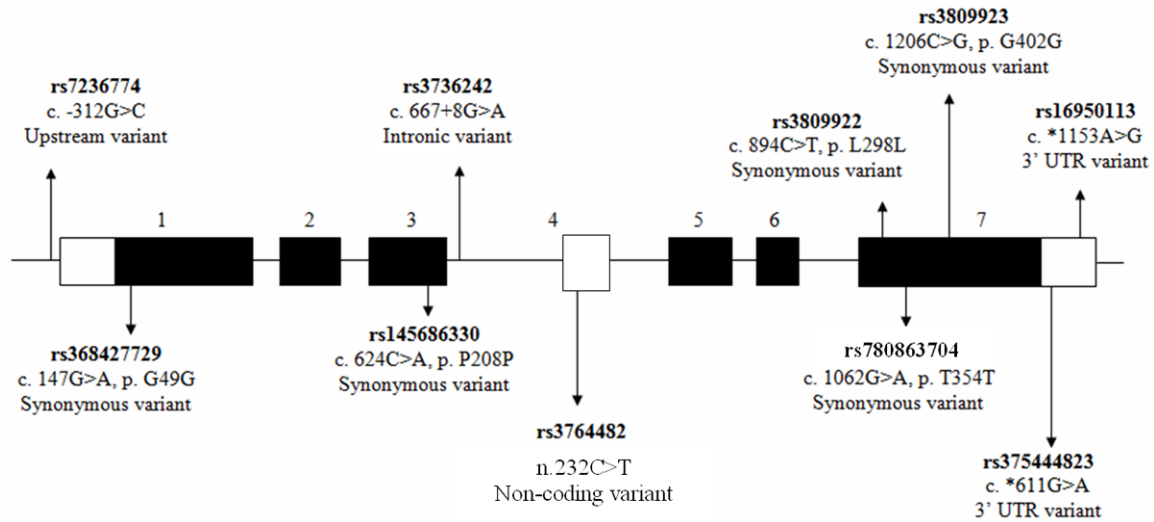


Figure 1. Distributions of identified SNPs in SMAD7. The black boxes represent the protein-coding region; the open boxes represent the untranslated regions and lines indicate introns. Arrows mark the locations of the SNPs.

Table 1. SMAD7 variants identified in cases and controls

Position	SNPs	Nucleotide change	Amino acid change	Protein domain	Patients n=30	Controls n=30
Upstream	rs7236774	c. -312G>C			1	2
Exon 1	rs368427729	c. 147G>A	p. G49G	MH1	2	8
Exon 3	rs145686330	c. 624C>A	p. P208P		1	0
Intron 3	rs3736242	c. 667+8G>A			14	11
Exon 4	rs3764482	n. 232C>T			7	5
Exon 7	rs3809922	c. 894C>T	p. L298L	MH2	6	6
Exon 7	rs780863704	c. 1062G>A	p. T354T	MH2	1	0
Exon 7	rs3809923	c. 1206C>G	p. G402G	MH2	6	6
3'UTR	rs375444823	c. *611G>A			1	1
3'UTR	rs16950113	c. *1153A>G			7	12

c. = coding DNA; n. = non-coding RNA (gene producing RNA transcript but not protein).

and outlet VSD. A total of 19 patients (63.3%) were diagnosed with perimembranous VSD and 10 patients (33.3%) were diagnosed with outlet VSD based on echocardiography report. However, the information of the defect for one of the patients (3.4%) was not available.

SNPs identification and genotyping

A total of 10 common SNPs were identified in the cases group including one in 5' upstream promoter region (rs7236774), six in exonic regions (rs368427729, rs145686330, rs3764482, rs3809922, rs780863704 and rs3809923) one in intronic region (rs3736242) and two in 3' untranslated regions (rs375444823 and rs16950113). No significant difference was observed in the genotype frequency bet-

ween both groups for all SNPs identified. The distributions and list of SNPs identified in the study are shown in **Figure 1** and **Table 1** respectively. The genotypes of allelic frequencies of each SNP are shown in **Table 2**.

Linkage disequilibrium and haplotype analysis

Based on the identified SNPs genotype data, we identified 10 SNPs that were found to be in Hardy-Weinberg equilibrium in both cases and controls. The 10 SNPs were classified into three haplotype blocks derived by solid spline method in Haploview in 30 case-control data with MAF>0.05. The first block, spanning 1 kb of LD encompassed three SNPs in exon 7 and one SNP in 3'UTR region namely rs16950113, rs375444823, rs3809923, rs3809922 and rs-

SMAD7 in VSD

Table 2. Genotypes and alleles frequency of identified SNPs of *SMAD7*

SNPs	Genotypes	Patients n=30 (%)	Controls n=30 (%)	P-value	Alleles	Patients n=30 (%)	Controls n=30 (%)	P-value
rs145686330	CC	29 (96.67)	30 (100.00)	1.000 ^a	C	59 (98.33)	60 (100.00)	1.000 ^a
	CA	1 (3.33)	0 (0.00)					
	AA	0 (0.00)	0 (0.00)					
rs3736242	GG	16 (53.33)	19 (63.33)	0.432	G	45 (75.00)	48 (80.00)	0.512
	GA	13 (43.33)	10 (33.33)					
	AA	1 (3.33)	1 (3.33)					
rs3764482	CC	23 (76.67)	25 (83.33)	0.519	C	53 (88.33)	55 (91.67)	0.543
	CT	7 (23.33)	5 (16.67)					
	TT	0 (0.00)	0 (0.00)					
rs3809922	CC	24 (80.00)	24 (80.00)	1.000	C	54 (90.00)	54 (90.00)	1.000
	CT	6 (20.00)	6 (20.00)					
	TT	0 (0.00)	0 (0.00)					
rs780863704	GG	29 (96.67)	30 (100.00)	1.000 ^a	G	59 (98.33)	60 (100.00)	1.000 ^a
	GA	1 (3.33)	0 (0.00)					
	AA	0 (0.00)	0 (0.00)					
rs3809923	CC	24 (80.00)	24 (80.00)	1.000	C	54 (90.00)	54 (90.00)	1.000
	CG	6 (20.00)	6 (20.00)					
	GG	0 (0.00)	0 (0.00)					
rs375444823	GG	29 (96.67)	29 (96.67)	1.000 ^a	G	59 (98.33)	59 (98.33)	1.000 ^a
	GA	1 (3.33)	1 (3.33)					
	AA	0 (0.00)	0 (0.00)					
rs16950113	AA	23 (76.67)	18 (60.00)	0.165	A	53 (88.33)	48 (80.00)	0.211
	AG	7 (23.33)	12 (40.00)					
	GG	0 (0.00)	0 (0.00)					
rs7236774	GG	28 (93.33)	22 (73.33)	0.080 ^a	G	58 (96.67)	52 (86.67)	0.095 ^a
	GC	2 (6.67)	8 (26.67)					
	CC	0 (0.00)	0 (0.00)					
rs368427729	GG	29 (96.67)	28 (93.33)	1.000 ^a	G	59 (98.33)	58 (96.67)	1.000 ^a
	GA	1 (3.33)	2 (6.67)					
	AA	0 (0.00)	0 (0.00)					

^a: Fisher exact test applied.

780863704 of *SMAD7*. The second block, spanning 5 kb encompassing the SNPs in exon 3, intron 3 and exon 4 which were rs145686-330, rs3736242 and rs3764482 whereas the third block consisted 2 SNPs each, located at the 5'upstream promoter region and at exon 1; rs7236774 and rs368427729. Two SNPs in exon 7, rs3809922 and rs3809923 showed strong LD. The other SNPs were in low linkage disequilibrium ($r^2 < 0.08$) with each other.

Haplotypes for each group were established whereby the GG haplotype of rs368427729 and rs7236774 in block 3 was statistically significant between both groups ($p=0.0398$). The major alleles of both SNPs conferred protection

with significant association. By changing the minor allele (C) of rs7236774, the haplotypes conferred towards susceptibility of VSD. On the contrary, the frequency of three haplotypes in block 1 and two haplotypes in block 2 were not significance for both groups ($p>0.05$) as shown in **Table 3**. Result of pairwise LD for 10 SNPs is illustrated in **Figure 2**.

Discussion

Outlet VSD, also known as subarterial or subpulmonic VSD is a defect which has completely muscular rims that is situated within the outlet septum of the heart (Corno 2003). Outlet VSD is relatively common among the Japanese and

SMAD7 in VSD

Table 3. Haplotype association analysis of 10 SNPs for cases and controls groups according to haplotype blocks generated using case-control data from Haploview

Haplotype	Case frequency (%)	Control frequency (%)	P-value
Block 1			
rs16950113-rs375444823-rs3809922-rs3809923-rs780863704			
A/G/C/C/G	75.0	69.9	0.505
G/G/C/C/G	11.7	18.8	0.278
A/G/G/T/G	10.0	10.0	1.000
A/A/C/C/G	1.70	0.40	0.512
Block 2			
rs3764482-rs3736242-rs145686330			
C/G/C	70.5	71.7	0.884
C/A/C	16.2	20.0	0.589
T/G/C	9.50	8.30	0.817
T/A/C	2.10	0.00	0.255
Block 3			
rs368427729-rs7236774			
G/G	83.3	95.0	*0.039
G/C	13.3	3.30	*0.047
A/G	3.30	1.70	0.558

*Statistically significant; $P < 0.05$.

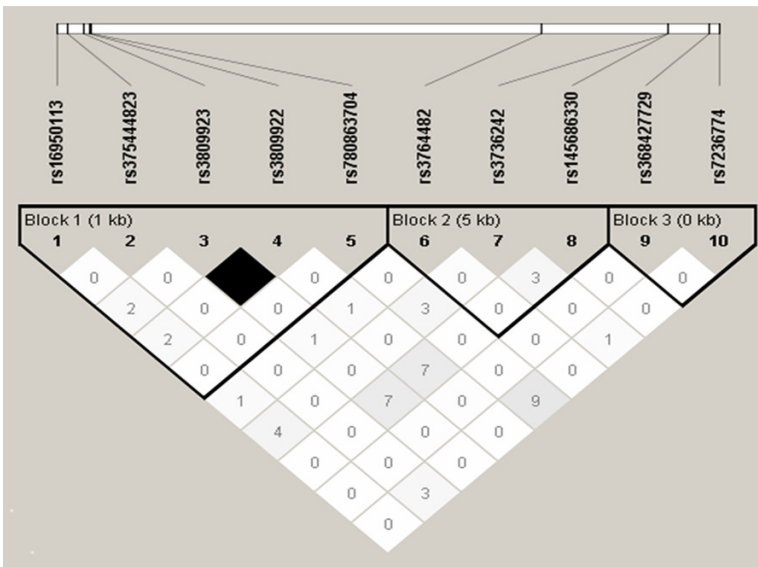


Figure 2. Pairwise linkage disequilibrium (LD) structure of *SMAD7* gene within 48920219 to 48950736 kb. The LD blocks were derived by solid spline method. The numbers in the box represents r^2 value. Dark shading (rs3809922 and rs3809923) indicates strong LD.

Chinese populations, accounted for 30% and 22.6% of their studies subjects respectively [14, 15]. However, the incidence of this type of VSD in the United States is much lower with approximately 8% of occurrence when compared to the Asian countries [16, 17]. Present

study reported 33.3% of outlet VSD, which is comparable but slightly higher than the Asian populations at large. The high incidence rate of outlet VSD in Kelantan, when compared to other states in Malaysia posed the question of interstate variations that still need to be elucidated. The big difference between age of VSD group (mean age 13 ± 8.39 years old) and healthy group (mean age 23 ± 3.69 years old) is mainly due to the difficulty in obtaining informed consent from parents of normal healthy newborns and children. As CHD is an anomaly that is present since birth, the age of the normal healthy individuals is insignificant for this study.

The TGF- β signaling from the cell membrane to the nucleus is mediated by the SMAD proteins family. *SMAD6* and *SMAD7* function as intracellular antagonist in the TGF- β signaling pathway [18]. Both reported to have critical function in the cardiogenesis. The *in vitro* study of *SMAD6* suggested that the variants in its MH2 domain contribute to the increased risk of CHD [19]. The *in vivo* study of *SMAD7* also, reported the cardiovascular malformations, particularly VSD due to the significant loss of *SMAD7* function [10]. *SMAD7* is highly expressed in the endothelial cells of developing cardiovascular system of the mouse embryo suggesting the significant role of *SMAD7* in the embryonic development [9].

This is the first study screening of *SMAD7* conducted in Malay population. In this report, 30 case-control subjects were screened on the exonic regions of *SMAD7*. A total of 10 SNPs were identified; in the upstream, exonic, intronic and untranslated (UTR) regions. Of these

SNPs, rs780863704 was found in only one VSD patient but not observed in the controls. This is in accordance with the study performed by Wang *et al.*, where rs780863704 was also observed in a Han Chinese CHD patient but was not seen in 1000 controls (Wang *et al.*, 2013). The absence of this SNP in 1000 Genomes Project further highlighted the susceptibility for CHD in Asian population. Even though this variant did not affect amino acid changes and thus did not affect the protein regulation of SMAD7, it might influence the transcriptional efficiency and stability at the mRNA level. Mutations of the four basic amino acid residues in the basic surface of the SMAD7 in MH2 domain are able to abolish the inhibitory effect of SMAD7 in TGF- β signaling [8]. Chen *et al.* reported deletion of the entire MH2 domain of SMAD7 in mice model with the profound defects in cardiac development [10]. Conversely, the deletion of exon 1, corresponding to MH1 domain of SMAD7 in mice model did not show any cardiovascular anomalies [20].

The intronic variant, rs3736242 is located on the splicing site. The roles of splicing in human disease have been widely reported [21]. However the effect of polymorphisms of splice site variant on SMAD7 is still unclear. Several studies identified many alternatively spliced SMAD protein variants affecting the regulation of signaling pathway. For instance; splice transcript with the removal of the SSXS motif in the MH domain of receptor-regulated SMAD, SMAD8 frequently acts as dominant inhibitors in the subsequent signaling [22]. Three SNPs namely rs3809922, rs780863704 and rs3809923 that was identified in the MH2 domain of SMAD7 suggested that MH2 domain contains inhibitory functions that is important in TGF- β signaling [23].

The association between SMAD7 and VSD was analyzed using a haplotype-based case-control analysis. Two synonymous variant (rs3809922 and rs3809923) were found in complete linkage disequilibrium ($r^2=1.0$) and was in strong correlation with each other. This is consistent with a study by Wang and colleagues that discovered strong linkage disequilibrium ($r^2=0.93$) between these two SNPs (Wang *et al.*, 2013). Complete LD happened when the SNPs have the same allele frequency because they have

not been separated during recombination [24]. In this case, one marker can provide complete information about the other marker suggesting it can be served as representative SNP and can be defined as tag SNP. Several factors can contribute for the complete LD such as the loss of existing haplotype(s) by selection or random drift [25]. However, the measure of LD is strongly affected with the sample size and is strongly biased when the sample size is small ($n < 100$) [26]. Small sample sizes can increase the chance on an individual to inherit two copies of the same alleles from the same ancestor. Therefore, large sample size is needed to compensate for weak LD between the markers.

A better understanding in the genetic and molecular events of SMAD7 signaling will improve the screening and clinical diagnosis of VSD towards the more accurate genetic counseling as well as identification of possible targets of gene therapy in future generations. Future directions involving the whole gene of SMAD7 utilizing high throughput technologies such as GWAS and gene expression studies might result in the discovery of more genetic variants to be studied.

This is the first report on screening of the exonic region of SMAD7 in Malay population. All the identified SNPs were not statistically significant between cases and controls groups. Furthermore, we have identified two SNPs, rs3809922 and rs3809923 which are in complete disequilibrium with each other, suggesting minimal historical recombination. The present study was limited by a relatively small sample size. Therefore, further study with a larger sample size will have to be conducted to elucidate the role of SMAD7 and the association of VSD. The polymorphisms in coding and regulatory sequences should be investigated and taken into consideration as it might be useful in association studies.

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SMAD7 in VSD

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