

Molecular analysis of PAX8 gene in unrelated patients with congenital hypothyroidism

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Citation

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Abstract

The present study was carried out to screen for potential mutations or polymorphisms in the human PAX8 gene, which may be associated with congenital hypothyroidism (CH). The study cohort consists of 44 unrelated patients with CH. PCR-based analysis of exons 2 to 9 of the PAX8 gene was performed followed by non-radioactive Single Strand Conformation Polymorphism (SSCP) detection. Two novel polymorphisms were detected and confirmed by DNA sequencing: a C>G transversion in intron 5 (Cint51G) and a phenotypically silent polymorphism in exon 9 (C372T). When analysed using Workbench web software, the C>G change creates a new restriction site, BbvCI whereas the C>T change in exon 9 creates a new BstNI restriction site. Both the BbvCI and the BstNI polymorphisms neither create nor destroy a splice site, verified using SpliceView web software at the WEBGENE page. Since both polymorphisms can be screened by restriction endonuclease digestion, normal healthy subjects were screened for the polymorphisms to obtain population frequencies. Restriction analysis performed on 133 healthy individuals revealed that a total of 25 were heterozygous and 1 was homozygous for the BbvCI polymorphism in exon 5 of the PAX 8 gene. On the other hand, BstNI polymorphism in exon 9 of the PAX8 gene was not detected in 105 healthy individuals screened. Our PCR-SSCP results did not detect any novel mutation on the PAX8 gene suggesting that mutation in PAX8 gene is not the cause of CH phenotypes in this cohort of patients.

INTRODUCTION

Primary congenital hypothyroidism (CH) occurs in babies who are born without the ability to produce adequate amount of thyroid hormone, due to disorders of the thyroid gland development [3]. CH is linked to several genetic defects including those in PAX8 gene [2,5]. PAX8 gene encodes a transcription factor, PAX8, which is known to bind to specific DNA sequence via its conserved paired-domain (Prd domain) [1]. PAX8 is expressed in the developing kidney, some areas of the brain and follicular thyroid cells in the adult thyroid [1,6,7,8]. PAX8 recognizes specific DNA sequence at the promoter region of thyroglobulin (TG) [1,7] and thyroperoxidase (TPO) [1,6] genes that are exclusively expressed in thyroid. Human PAX8 gene contains at least 10 exons. Mutations in the Prd domain of the PAX8 gene have been associated with CH with thyroid gland dysgenesis [2,8] and dyshormonogenesis [2,9]. The aim of the study was to screen for potential mutations or polymorphisms in the human PAX8 gene in a cohort of 44 unrelated Malaysian patients with CH.

MATERIALS AND METHODS

SUBJECTS, COLLECTION OF SAMPLES AND DNA EXTRACTION

Blood samples were obtained from 44 unrelated patients with CH, confirmed biochemically with low T4 and high TSH levels in blood, who attended the Paediatric Clinic at the University of Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia. Thyroid scanning and Technetium-99m scintigraphy revealed that 31 patients have thyroid gland of normal size and position whereas 13 patients have either no thyroid gland or thyroid gland of either abnormal in size or position. Genomic DNA was extracted from peripheral white blood cells from each individual using the Qiagen kit (Hilden, Germany) according to the manufacturer's instructions. Samples obtained were with consent and approval granted by the Ethical Committee (Institutional Review Board) of the UMMC in accordance to the ICH GCP guideline and the Declaration of Helsinki. All samples were kept at -20oC and subjected to similar treatment.

GENETIC SCREENING

Mutation screening of the PAX8 gene was performed using PCR-SSCP analysis technique. Samples showing variation in SSCP banding pattern, were then sent for sequencing.

Screening of samples from unaffected individuals representing the general population was also carried out to assess the presence of the mutation/polymorphism in the population.

PCR-SSCP ANALYSIS

Complete coding region of the PAX8 gene (exons 2 to 9) was PCR-amplified in a total of 8 fragments using primer information described by Kozmik et al., 1997 [4]. PCR optimisation and amplification were performed using standard techniques and equipment.

SSCP analysis was carried out using MDETM gels and electrophoresis was performed in the Protean II electrophoresis cell system (BioRad, USA) with a temperature-regulated water circulator. For each PCR fragment, SSCP optimisation was carried out by repeating the electrophoresis using 2 different electrophoresis temperatures (10oC and 20oC) and at 2 different gel concentrations (0.5x and 0.75x).

DNA SEQUENCING ANALYSIS

Following PCR-SSCP screening, PCR fragments showing variations in SSCP banding patterns (mobility shifts) were sent for DNA sequencing. Prior to sequencing, PCR products were purified using commercially available purification kit, QIAquick (Qiagen, Germany). DNA sequencing was carried out using an automated ABI Prism Gene Sequencer (Model 377, version 2.1.1) at AMCAL, University of Malaya, Kuala Lumpur, Malaysia.

BBVC1 AND BSTNI PCR-RFLP ASSAY

The sequence change identified creates a new BbvCI and BstNI restriction sites. The fragments containing the mutated regions were PCR-amplified and then digested with either BbvCI or BstNI restriction enzyme, following the manufacturer's instructions (New England Biolabs, USA). The digested products were separated using 2% agarose mini-gel, pre-stained with ethidium bromide.

RESULTS

PCR-SSCP analysis of PAX8 gene demonstrated shifts in banding patterns in PCR products that contain exons 5 and 9. For exon 5, 6 of the 44 patients (13.6%) showed the mobility shift. Out of the 6 patients, 2 have normal and 4 have abnormal glands. PCR products containing exon 5/intron 5 and exon 9 of PAX8 gene that show mobility shift when analysed using SSCP analysis are shown in Figure 1. Subsequent DNA sequencing analysis showed that the

aberrant SSCP banding patterns in exon 5 was due to a heterozygous C to G transversion at position 51 in intron 5 which was 51bp downstream from the exon 5 (Cint51G) (Figure 2). The C>G change, when analysed using Workbench web software, creates a new restriction site for BbvCI restriction enzyme. BbvCI digestion of the wild type allele yielded uncut fragment of 255 bp in size whereas the allele carrying the Cint51G polymorphism yielded two bands, 197 bp and 58 bp in length, as well as one band of 255 bp in size (Figure 3A) indicating heterozygosity for the polymorphism. When normal healthy individuals were screened for the Cint51G by BbvCI restriction digestion (n=133), 25 were heterozygous and 1 was homozygous for the Cint51G polymorphism. The BbvCI polymorphism neither create nor destroy a splice site, verified using the SpliceView web software at the WEBGENE page.

Figure 1 PCR-SSCP analysis showing mobility shifts for exon 5/intron 5 (A) and exon 9 (B) of PAX8 gene.

Figure 1

Figure 1a

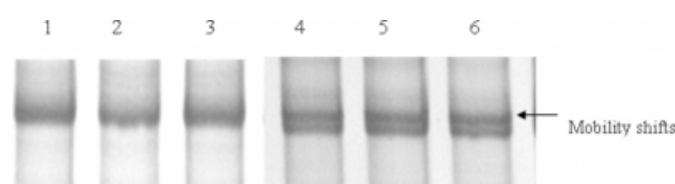


Figure 2

Figure 1 b

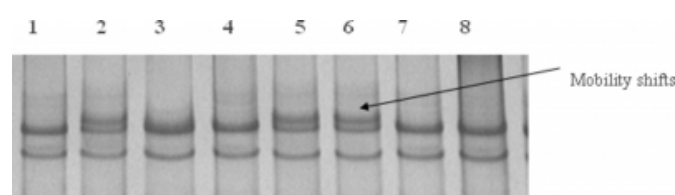


Figure 2 Sequence analysis of PAX8 gene showing;A: a C to G transversion in exon 5/intron 5 and B: a C to T transversion in exon 9.

The position of the substituted nucleotide is indicated by an arrow.

Figure 3

Figure 2a

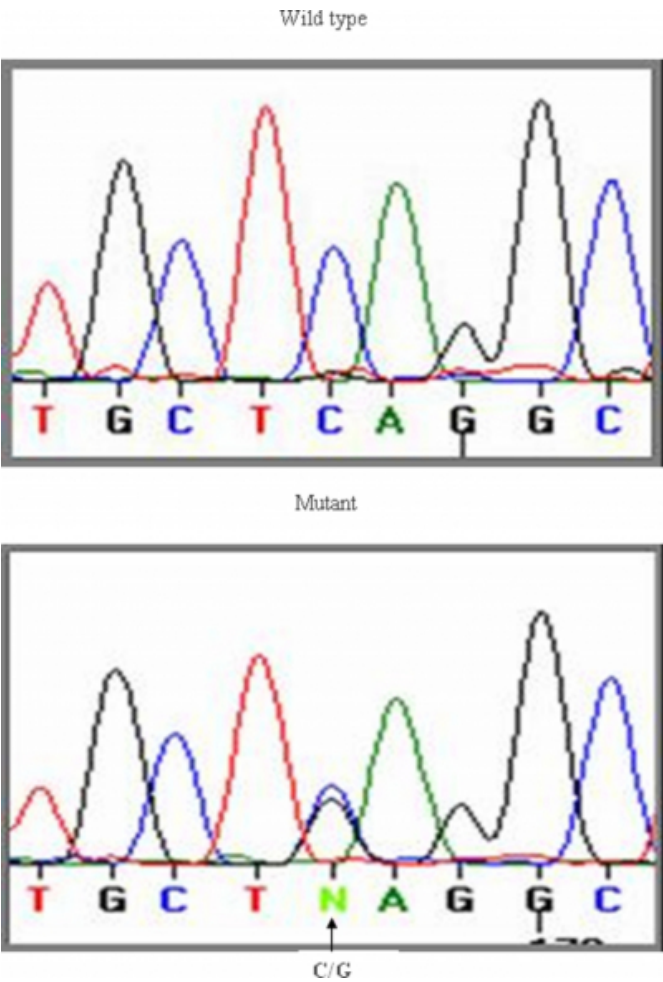


Figure 4

Figure 2b

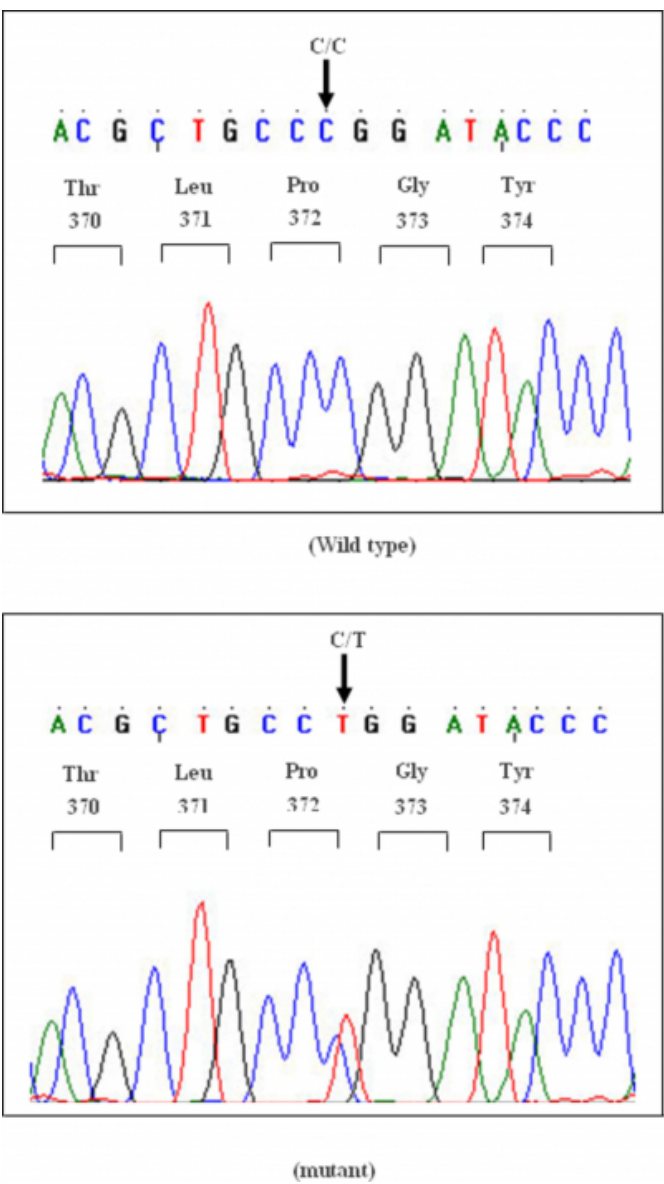


Figure 3. BbvC1 and BstNI restriction enzyme digestion of PCR-amplified exon 5/intron 5 (A) and exon 9 (B) of PAX8 gene. Numbers on the right indicate the length of the restricted products. (+) denotes the presence and (-) denotes its absence of a restriction site.

Figure 5

A: Lane 1: One hundred base pair DNA ladder Lanes 2, 5, 6: DNA from individuals homozygous for the wild-type allele Lane 4: DNA from individual heterozygous for the Cint51G polymorphism Lane 7: DNA from individual homozygous for the Cint51G polymorphism.

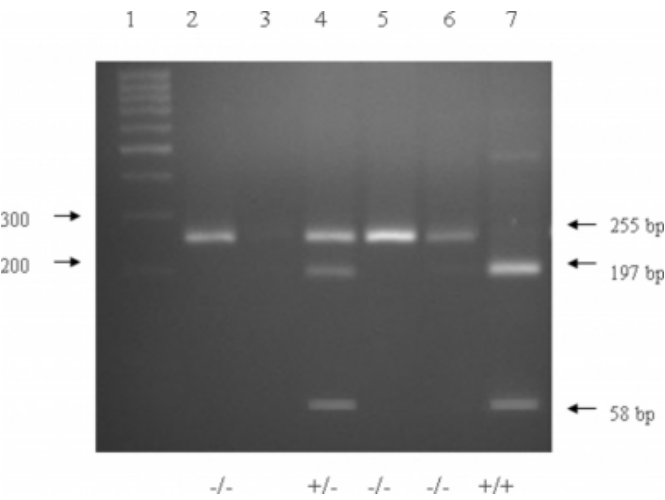
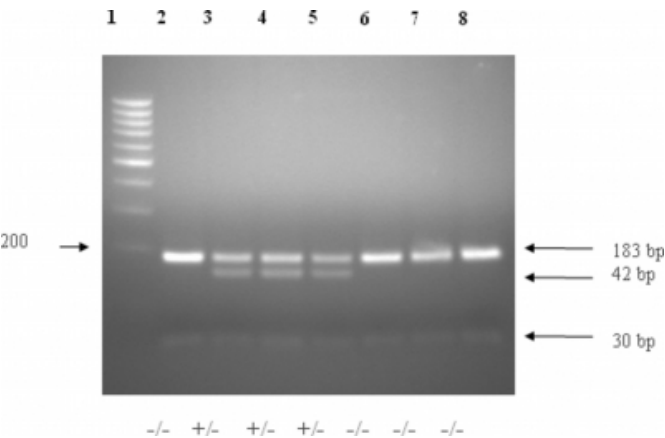


Figure 6

B: Lane 1: One hundred base pair DNA ladder Lanes 2, 6, 7, 8: DNA from individuals homozygous for the wild-type allele Lanes 3, 4, 5: DNA from individual heterozygous for the C372T polymorphism



Subsequent DNA sequencing analysis of exon 9 revealed that the mobility shift was due to a C>T transition at the third base of codon 372 (C372T). The C>T change creates a restriction site for BstN 1. The C372T polymorphism was detected only in 1 out of the 44 patients and it is not detected in 105 healthy individuals screened.

DISCUSSION/CONCLUSION

In humans, heterozygous loss-of-function mutations of PAX8 is responsible for congenital hypothyroidism with varying phenotypes. PAX8 gene mutations had been identified in CH patients with normal as well as abnormal

thyroid gland [9]. A patient who carries a heterozygous novel Q40P PAX8 mutation has overt CH with thyroid hypoplasia. In contrast, her mother who is a heterozygote for the same mutation has a thyroid gland of normal size [9]. The autosomal dominant transmission of a PAX8 gene mutation is in contrast to the findings in knockout mice where heterozygous mutation did not show pathological phenotypes [11].

To date, 6 PAX8 gene mutations have been reported in both sporadic and familial CH cases [2,5,9]. All of these mutations involve single base substitution (Table 1) and are located in the paired domain of the PAX8 gene. Most of the mutations have been shown to affect DNA-binding activity.

Figure 7

Table 1: Mutations and polymorphisms identified in the gene.

Location	Sequence change	Codon	Amino acid	Comments
exon 2	CGC → CAC	31	Arg → His	Published mutation ²
exon 2	CAG → CCG	40	Gln → Pro	Published mutation ⁹
exon 3	CAG → CGG	54	Ser → Gly	Published mutation ¹¹
exon 3	TGC → TAC	57	Cys → Tyr	Published mutation ⁵
exon 2	CTT → CGT	62	Leu → Arg	Published mutation ²
exon 3	CGA → TGA	108	Arg → Ter	Published mutation ²
intron 5	TCA → TGA	At position 51 in intron 5, i.e., 51 bp downstream from exon 5	—	Polymorphism found in this study
exon 8	TTT → CTT	329	Phe → Leu	Published polymorphism ¹⁰
exon 9	CCC → CCT	372	Pro → Pro	Polymorphism found in this study

In addition, 1 silent mutation in PAX8 gene (F329L) had also been reported [10]. In this study, none of the 44 patients carry mutations that can be associated with their CH phenotypes. However, 2 silent mutations that have not been reported previously were detected. The Cint51G polymorphism in intron 5 (BbvC1 polymorphism) is detected in 13.6 % in the unrelated CH patients and 19.5% normal population. The C372G polymorphism in exon 9 (BstN1 polymorphism) is rare and was detected only in the CH patients (2.3%). Unlike the previously reported polymorphism [10], screening of the BbvC1 and BstN1 polymorphisms in a population is made simple due to the presence of the sites for the respective restriction endonuclease. Therefore, these two polymorphic sites in combination with others could be used in genetic linkage studies to determine a unique haplotype associated with defective alleles that cause the CH phenotypes in family

members with CH.

In conclusion, our PCR-SSCP results did not detect any mutation involving point mutation or minor deletion on the PAX8 gene in the CH patients. Mutations in genes other than PAX8 such as TTF-1, TTF-2 and TSH receptor could be responsible for the CH phenotypes. However, since SSCP analysis is limited to detecting point mutation or minor deletion, mutations involving major deletion or rearrangement that cannot be detected through PCR-SSCP technique could not be completely ruled out. Two novel polymorphisms, BbvC1 and BstNI were found in this study. To the best of our knowledge, both polymorphisms have not been documented elsewhere.

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