Sensitivity of HRPT2 Mutation Screening to Detect Parathyroid Carcinoma and Atypical Parathyroid Adenoma of Thai Patients

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Objective: *Detected HRPT2 mutation in parathyroid carcinoma or atypical parathyroid adenoma in sporadic hyperparathyroidism in Thai patients.*
Material and Method: *Samples of parathyroid carcinoma, typical (atypical) adenoma or hyperplasia were obtained for HRPT2 gene (17 exons) study since September 2001 to August 2010 both somatic and germline by SyBr Green PCR method.*
Results: *Parathyroid carcinomas and atypical paathyroid adenoma from 5 of 26 patients (10 of 32 samples) were tested for HRPT2 mutations. Only somatic HRPT2 mutations were found in tumor from 2 patients but three patients found both somatic and germline HRPT2 mutations. Exon 15 of HRPT 2 gene was the best sensitivity (sensitivity 80.0%; p < 0.001, 95% CI 0.75-1.05) followed by exon 2 and exon11 (sensitivity 60.0%; p = 0.007, 95% CI 0.60-0.99) to detected parathyroid carcinoma or atypical parathyroid adenoma.*
Conclusion: *HRPT2 mutations by SyBr Green PCR sensitive method to detected parathyroid carcinoma or atypical adenoma from benign parathyroid tissues. Exon 15 was the best sensitive to detected parathyroid carcinoma or atypical adenoma. Genotyping of such family members for germline mutation would focus implementation of clinical and biochemical monitoring of carriers of these mutations.*

Keywords*: Parathyroid carcinoma, Atypical parathyroid adenoma, HRPT2*

J Med Assoc Thai 2011; 94 (Suppl. 2): S17-S22
Full text. e-Journal: *http://www.mat.or.th/journal*

The tumor suppressor gene HRPT2 (also called CDC73) was recently identified by positional candidate cloning[1]. Germline mutation of HRPT2 confers susceptibility to the hyperparathyroidism-jaw tumor syndrome (HPT-JT), an autosomal dominant syndrome with high but incomplete penetrance[2].

Hyperparathyroidism (HPT) is one of the most common endocrinopathies, believed to affect approximately three individuals per 1,000 adults[3]. Sporadic HPT may occur as primary, secondary, or tertiary disease. Primary HPT can be attributed to a single adenoma in 80.0-85.0% of cases, multiglandular hyperplasia in 15.0-20.0% of cases and carcinoma in less than 1% of cases[4]. Parathyroid carcinomas are an uncommon and often devastating cause of hyperparathyroidism. These carcinomas characteristically result in more profound clinical presentations of hyperparathyroidism than parathyroid adenoma. A systematic literature review of 22,225 cases of primary hyperparathyroidism reported between 1995 and 2003 revealed that parathyroid carcinoma accounted for 0.7 percent of the cases.

HRPT2 gene encodes a 531 amino acid protein (parafibromin) that contained with 17 exons in transcription processes. Inactivation of the HRPT2 tumor suppressor gene is associated with hyperparathyroidism-jaw tumor (HPT-JT) syndrome and several sporadic parathyroid carcinomas[5,6].

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Because it is frequently clinically and histologically difficult to determine whether an atypical parathyroid tumor is benign or malignant and it is also unclear whether pathology is benign or malignant nature. The subsequent discovery of a parathyroid carcinoma in a normocalcemic carrier and the reoccurrence of atypical adenoma in the normocalcemic index case suggest that adding neck ultrasonography to the surveillance protocol may have a role in early detection of potentially serious parathyroid neoplasms in such families. This result of HRPT2 mutations might occur in malignant screening role from benign parathyroid tumors.

Material and Method

Parathyroid specimens were carefully reviewed, and the diagnosis confirmed according to the World Health Organization guidelines. Frozen and paraffin-embedded specimens from parathyroid tissue were obtained from 26 subjects (12 female and 14 male) who underwent parathyroidectomy during September 2001 to August 2010 for hyperparathyroidism at Department of Otolaryngology-head and neck, Rajavithi Hospital, College of Medicine, Rangsit University, Bangkok, Thailand.

Peripheral blood samples were also available from 4 of these patients. Patients gave informed consent according to protocols approved by ethics committee. Further classification of adenoma, hyperplasia (secondary or tertiary), or carcinoma was established according to detailed WHO guidelines[7]. No case of jaw or renal tumor was found. Thirteen sporadic adenomas (8 adenomas, 1 atypical adenoma and 4 carcinomas) that consisted of 10 in primary, 13 in secondary and 3 in tertiary hyperparathyroidism, as well as four parathyroid carcinomas and one atypical adenoma were also collected.

DNA and RNA preparations

Parathyroid tissue was frozen in liquid nitrogen immediately after surgical removal and stored at -80°C or below. Peripheral blood was collected into EDTA anti-coagulant tubes and stored at -80°C. DNA was extracted from the frozen tissue and peripheral blood leucocytes according to standard procedures. RNA was extracted from frozen tissue using TRI Reagent (Sigma-Aldrich Corporation, St Louis, MO) according to the manufacturer's protocol.

HRPT2 mutation analysis

Real time polymerase chain (20 µl) reaction (RT-PCR) by SyBrGreen®-Roche® used primer specific sequences for HRPT2, each designed to target on the basis of a full search of the GenBank database (www.ncbi.nlm.nih.gov) with sensitivity of 93.0% and specificity 100.0%. The total volume of obtained from patients who underwent parathyroidectomy, was 200 µl and underwent automated DNA extraction (MagNa pure Compact Nucleic and Isolation Kit-Roche®). The DNA was eluted into 50 µl of nuclease-free water and stored at -20°C for repeated RT-PCR analysis.

Primer sequences are listed (Fig. 1) from the 5' to the 3' ends. Primers were constructed on the basis of the alignment of the complementary DNA sequence (GenBank accession number XM-029845) wit the genomic sequences of AL390863 and AL139133. Sequences of primers forward and 17 reverse are located within 5' and 3' untranslated regions, whereas all other primers are derived from intronic sequences[8]. The PCR conditions were as follow for all reactions except those involving exon 12 to 13: a 20 µl reaction mixture with 25 ng of DNA, 20 pmol of each primer, 200 mM of each deoxy-ribonucleoside triphosphate, 1.25 U of Amplitaq Gold DNA Polymerase (Applied Biosystems), 1 x PCR buffer (Applied Biosystems) and 1.0 to 1.5 mM magnesium chloride was subjected to initial denaturation at 95°C for 10 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing as noted above for 30 seconds and elongation at 72°C for 30 seconds, with a final period of extension at 72°C for 10 minutes. The PCR conditions for exons 12 to 13 were as follow: a 50 µl reaction mixture with 25 ng of DNA,

Exon	Forward Primer	Reverse Primer	Size	Temperature
			bp	°C
1	GAGGACGGCTGTTAGTGCT (PCR)† CGAAGGAGGAGGAGGAAGAG (SEQ)†	CCCCTTCTTTCCTTACCCTA	451	55
2	TAAATGAATCCAGCCTGAAG	AGGCCAGACCCTGTCTCT	251	58
3	AAAGTGCTGGGATTCTAGG	TGGACAAAAATGAAGGTAGG	599	58
4–5	AACATGTTTTTGCAGAGCTG (PCR)† ACCTAGAGAAAATCACCATA (SEQ)†	CTCCTCAGGTTACTGCAATC	451	55
6	GGCCTAAAGACACTGATACC	CGAACTTAAGAGCAAAGAGG	351	55
7	GAATGCCTGCTGTGAAAA	TGTGAAGGAGCTTGCATTT	502	55
8	CTCTGGATCAATATCTTAGTAGTGG	GTCTTCAACGTTACTACACTGC	348	55
9	ATGGTCATGCTACTGCACTC	CCAACCCTTACCCTTAAACA	251	55
10	CAGAGATAGTCTTAACCAGCTTC	CTTCAACATGTGCTACTCACAT	384	55
11	AACATGTTCAGTGGAGTAACC	TGCACTGTTACGATCTTTTG	251	55
12–13	TGGTTAACTGAAACTGCAGA	GTATCTCAATATCCTACGTACAGG	713	58
14	ATCTTCCCATTTTCATCACG	CCCCATCTCTTAAAAAGCAA	321	55
15	TGCCTAAGGGATTTATAGTAGC	ACATCATATGCGCAGAACT	281	55
16	GGCGTGTATAAACCCTGAAT	GAAAGAAGGGAATTAGGGAA	401	55
17	GAGGAGTGTTATTTCTAGCTTATTC	GATCAATCTGTGACCTTCTTCA	301	55

Fig. 1 Primer and condition of PCR amplification of HRPT2 gene in parathyroid carcinoma; size was mean about product size in basepairs, temperature was mean annealing temperature. †Different primers was used for PCR and sequence (SEQ) reactions. For all other exons, the same primer was used for both sequencing and PCR[5].

50 pmol of each primer, 200 mM of each deoxyribonucleoside triphosphate, 2 U of Eppendorf HotMaster DNA Polyerase (Brickman) and 1 x HotMaster *Taq* buffer with 2.5 mM magnesium (Brickman) was subjected to initial denaturation at 96°C for 6 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 45 seconds, with a final period of extension at 72°C for 10 minutes.

Statistical analysis

The x^2 contingency test was performed to compare the occurrence of mutations between five cases of sporadic carcinoma or atypical adenoma and 21 cases of sporadic non-carcinomas. Sensitivity and specificity was analysised by SPSS version 17.0 by $p < 0.05$ is considered as significant.

Results

HRPT2 mutation analysis

HRPT2 mutations were detected in the DNA from five of five sporadic parathyroid carcinoma samples, three of five HPT-JT parathyroid tumors (three families) (Table 1). Two mutations were detected in any of the other 21 tumors sequenced (data not shown). Here we found of HRPT2 mutations in 100.0% (five of five) of four sporadic parathyroid carcinomas and atypical adenoma studied. Multiple mutations was detected in patient 2 and patient 3, we can detected 13 of 15 primers in somatic mutation in patient 2 (exon 1, 2, 4-5, 6, 8, 9, 10, 11, 12-13, 14, 15, 16 and 17) and 14 of 15 primers (exon1, 2, 3, 4-5, 6, 8, 9, 10, 11, 12-13, 14, 15, 16 and17) in somatic mutation with 10 of 15 primers (exon 1, 2, 3, 4-5, 7, 8, 11, 12-13, 15 and 16) in germline mutation in patient 3.

Exon 15 mutation was found in 8 of 9 samples that available in detected HRPT2 mutation in parathyroid carcinoma and adenoma followed by exon 2 (6 of 9) and exon 11 (5 of 9). The other exons weren't frequent to found in these study (Table 1). Any other two mutations that found in 21 benign parathyroid tissue (exon 14 and 16 in two patients).

Sensitivity analysis

Exon15 of HRPT2 gene was the best sensitivity (sensitivity 80.0%; $p < 0.001$, 95% CI 0.75-1.05) for both somatic and germline preparations followed by exon 2 and exon 11 (sensitivity 60.0%; $p = 0.007$, 95% CI 0.60-0.99) to detected parathyroid

Table 1. HRPT2 gene analyses in patients with parathyroid carcinoma and atypical adenoma

Patient Exon	Patient1		Patient2		Patient3		Patient4		Patient5	
	soma	germ	soma	germ	soma	germ	soma	germ	soma	germ
1	-	NS	+	-	+	+	-	-	-	+
2	+	NS	+	-	+	+	-	+	+	-
3	-	NS	-	-	+	+	-	-	-	-
4-5	-	NS	+	-	+	+	-	-	-	-
6	-	NS	+	-	+	-	+	+	-	-
7	-	NS	-	-	-	+	-	-	-	-
8	-	NS	+	-	+	+	-	-	-	-
9	-	NS	+	-	+	-	-	-	-	-
10	-	NS	+	-	+	-	-	-	-	-
11	+	NS	+	-	+	+	+	-	-	-
12-13	-	NS	+	-	+	+	-	-	-	-
14	+	NS	+	-	+	-	-	-	-	-
15	+	NS	+	-	+	+	+	+	+	-
16	-	NS	+	-	+	+	-	-	-	-
17	-	NS	+	-	+	-	-	-	-	-

Note: + = mutation HRPT2, - = no mutation HRPT2, germ = germline, soma = somatic (fresh tissue or block tissue), NS = no samples (no sample from germline preparation in patient 1 because she death after 3days admission with severe hypercalcemia and cardiac arrested). The number in the exon column is number of the exon; patient 1 to 5 were parathyroid tissue preparations of carcinomas and atypical adenoma parathyroid tissues□; only two mutation were found in normal 21 parathyroid tissues, one was sample exon16 mutation and another was exon 14 mutation (data not shown).

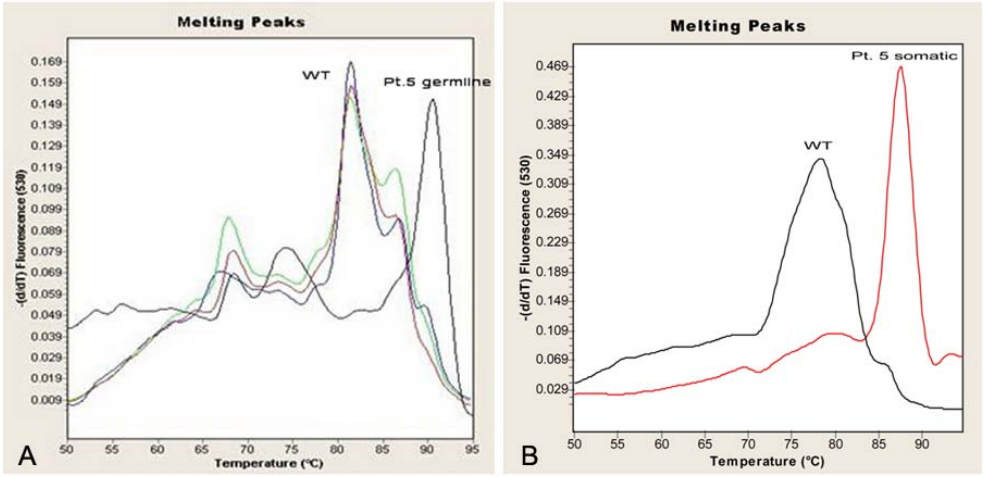

Fig. 2 SyBr Green PCR result A) Exon 1 result from SyBr Green PCR method from patient 5 germline preparation(black line), B) Exon 2 result from SyBr Green PCR method from patient 5 somatic preparation (red line).

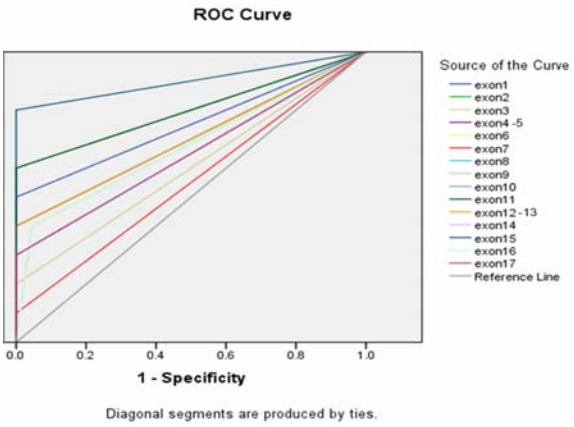

Fig. 3 ROC curves of HRPT mutations from somatic and germline mutation of five parathyroid carcinoma and atypical adenoma.

carcinoma or atypical parathyroid adenoma. But in somatic preparation exon 15 was 100.0% sensitivity to detected patathyroid carcinoma and atypical adenoma as shown in Table 1.

ROC analysis

As shown in Fig. 3, exon 15 was best sensitivity and specificity to detect parathyroid carcinoma and atypical adenoma by significant ($p < 0.001$, 95% CI 0.75-1.05, AUC = 0.90). Exon 2 and 11 (same line in Fig. 2) were followed sensitivity and specificity ($p = 0.007$, 95% CI 0.6-1.0, AUC = 0.80).

Discussion

Primary hyperparathyroidism (PHPT) is the most common cause of hypercalcemia in the general population. The disease has an incidence as high as 1 in 1,000 individuals and occurs more commonly in women than in men (3: 1). In approximately 80.0% of cases of PHPT, a single benign adenoma is the cause of abnormal parathyroid function. In 15.0-20.0% of cases, PHPT is due to hyperplasia of all four parathyroid glands. Rarely, multiple adenomas can be seen. In less than 1.0% of cases, the most scarce and deadly presentation of PHPT occurs, namely that of parathyroid carcinoma[9].

Recently, mutations in a newly identified tumor suppressor gene, HRPT2, have been associated with the development of parathyroid carcinoma in hyperparathyroidism-jaw tumor syndrome (HPT-JT) and in sporadic parathyroid carcinoma[1]. Somatic mutations have been detected in four of four tumors of sporadic parathyroid carcinomas, in five of five cases of HPT-JT and in one case of familial isolated hyperparathyroidism[6] like our result that found five somatic mutated of five tumors.

Here we report the finding of HRPT2 somatic mutations in 100% (five of five) of sporadic parathyroid carcinomas same like study[6]. These results demonstrate a strong association between exon15 mutation of HRPT2 and malignancy in parathyroid tumors ($p < 0.001$). This study was the first that can identify mutation of exon 11 and 15 of HRPT2 mutation. Furthermore, our findings of three germline mutations that first time in Thai population.

About 36.0% (18 of 50) of HRPT2 mutations are located in exons 2, 11, or 15. Exon 2 contains 12.0%

(6 of 50) of all mutations detected. Exon 7 is by far the largest exon, hinting at an important role for this exon and 14.0% (7 of 50) of exon 15 that contains in this study. But we has been shown to harbour only 2.0% (1 of 50) of all HRPT2 mutations in exon 7 was detected in this study contrast to previous report[1] that found 21.0%. Sometime that may have different in ethic that caused of different in genotypic manisfestations.

Conclusion

In the light of the strong association between mutations of HRPT2 and sporadic parathyroid carcinoma demonstrated in this study, it is hypothesised that HRPT2 mutation is an early event that may lead to parathyroid carcinoma and suggest intragenic mutation of HRPT2 as a marker of malignant potential in both familial and sporadic parathyroid tumors. Our finding of germline mutations in three of five HPT-JT families support the conclusions of Carpten et al[1] that HRPT2 is the causative gene in HPT-JT and in a subset of familial isolated hyperparathyroidism (FIHP).

The three germline mutations in the HPT-JT families are, like those found in the sporadic carcinomas, predicted to prematurely truncate the protein, whereas the FIHP mutation is a missense mutation of unknown consequence. Whether there is a phenotype/genotype correlation between mutation type and the presence of parathyroid disease alone, or in conjunction with jaw tumours or renal lesions (that is, FIHP or HPT-JT) will require a larger cohort of FIHP families with HRPT2 mutations to establish. Similarly, it is uncertain whether the severity or number of ''hits'' affecting HRPT2 determines the presentation of familial tumours as benign, cystic, or malignant.

Surprisingly, our study found multiple mutations in two patients, one patient had found both somatic and germline mutations. We needs the further study to investigate these mutations have result to clinical presentation in the humans.

In conclusion, these results also provide further evidence for HRPT2 as the causative gene in parathyroid carcinoma and a subset of atypical adenoma. Efficient detection of HRPT2 mutations should begin with exons 15, 2 and 11, which together harbour 36.0% of mutations found in Thai population. Exon 15 was the best sensitive to detected parathyroid carcinoma or atypical adenoma in Thai patient.

Potential conflicts of interest

None.

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ความไวของการคัดกรองการกลายพันธุ์ HRPT2 เพื่อค้นหามะเร็งต่อมพาราไทรอยด์ และเนื้องอกจากต่อมพาราไทรอยด์ที่มีลักษณะผิดปกติของคนไทย

สถิตย์ นิรมิตรมหาปัญญา, ทองคำ สุนทรเทพวรากุล, ชัยชาญ ดีโรจนวงศ์, วีระศักดิ์ ศรินนภากร, พรเอก อธิพันธ์, ปิยะธิดา หาญสมบูรณ์, สืบสาย คงแสงดาว

วัตถุประสงค์*: ค้นหาการกลายพันธุ์ของ HRPT2 ในมะเร็งต่อมพาราไทรอยด์และเนื้องอกจากต่อมพาราไทรอยด์ที่มีลักษณะผิดปกติในผู้ป่วยไทย*

วัสดุและวิธีการ*: ตัวอย่างของมะเร็งต่อมพาราไทรอยด์ และเนื้องอกจากต่อมพาราไทรอยด์ที่มีลักษณะผิดปกติ (atypical adenoma) ถูกนำมาวิเคราะห์การกลายพันธุ์ของจีน HRPT2 โดยศึกษาตั้งแต่ กันยายน พ.ศ. 2544 ไปถึง สิงหาคม พ.ศ. 2553 จากเนื้องอกต่อมพาราไทรอยด์ และสายเลือดโดยวิธี SyBr Green PCR*

ผลการรักษา*: มะเร็งต่อมพาราไทรอยด์ และเนื้องอกจากต่อมพาราไทรอยด์ที่มีลักษณะผิดปกติ จากผู้ป่วยตัวอย่างมี 5 ราย จากทั้งหมด 26 ราย (10 ตัวอย่างจากทั้งหมด 32 ตัวอย่าง) นำมาตรวจการกลายพันธุ์ของ HRPT2 โดยพบว่ามี 2 ราย ที่มีการกลายพันธุ์ของ HRPT2 เฉพาะในชิ้นเนื้ออีก 3 ราย พบการกลายพันธุ์ของ HRPT2 ทั้งจากชิ้นเนื้อและสายเลือดพบว่า exon 15 ของจีน HRPT2 มีความไวดีที่สุด (ความไว 80.0%; p < 0.001, 95% CI; 0.75-1.05) รองลงมาด้วย exon 2 และ 11 (ความไว 60.0%; p = 0.007, 95% CI; 0.60-0.99) ในการตรวจหามะเร็งต่อมพาราไทรอยด์ และเนื้องอกจากต่อมพาราไทรอยด์ที่มีลักษณะผิดปกติ*

สรุป*: การกลายพันธุ์ของ HRPT2 โดยวิธี SyBr Green PCR เป็นวิธีที่มีความไวในการวิเคราะห์แยกมะเร็งต่อมพาราไทรอยด์ และเนื้องอกจากต่อมพาราไทรอยด์ที่มีลักษณะผิดปกติจากเนื้องอกจากต่อมพาราไทรอยด์ที่มีลักษณะปกติ (benign) โดย exon 15 มีความไวดีที่สุด และการตรวจพบการกลายพันธุ์ในสายเลือดทำให้นำไปใช้ให้เกิดผลของอาการทางคลินิก และชีวเคมีของคนที่เป็นพาหะของการกลายพันธุ์เหล่านี้*