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การสูญเสียภาวะเฮทเทอโรไซโกตบนโครโมโซมที่ 11 ในมะเร็งหลังโพรงจมูกที่มีเอปสไตน์บาร์ เกี่ยวข้องกับไวรัส

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การสูญเสียภาวะเฮทเทอโรไซโกต บนโรคโมโซมที่ 11 เป็นภาวะที่พบบ่อยในการพัฒนาของเนื้อเยื่อมะเร็งหลายชนิด พบว่ามียีนต้านมะเร็งอยู่อย่างน้อย 3 ยีน บนโครโมโซมที่ 11 คือ ยีนต้านมะเร็งของวีมส์ทูเมอร์ 1 เมน 1 และอเท็กเซียทีแลงจิเอกเทเซีย โดยพบอยู่ที่ 11p15, 11q13 และ 11q23 ตามลำดับ ในปัจจุบันความรู้ที่เกี่ยวข้องกับขบวนการพัฒนามะเร็งหลังโพรงจมูกมีน้อยมากการศึกษานี้เราศึกษาถึงการสูญเสียภาวะเฮทเทอโรไซโกทบนโครโมโซมที่ 11 เพื่อพิสูจน์หาและดูว่าบริเวณใดบนโครโมโซมที่ 11 จะพบมีความเกี่ยวข้องกับการพัฒนาของมะเร็งหลังโพรงจมูกที่เกี่ยวข้องกับไวรัสเอปสไตน์บาร์ โดยใช้ซอร์ทแทนเดมรีพีทส์โพลี่มอร์ฟิสึมโดยศึกษาในเนื้อเยื่อมะเร็งเปรียบเทียบกับเนื้อเยื่อปกติ 25 ราย พบว่าการสูญเสียภาวะเฮทเทอโรไซโกตพบที่ 11q23 45.5% และ 11q13 พบ 22% และ 11p พบ 30.4% ดังนั้นยีนอเท็กเซียทีแลงจิเอกเทเซียและเมน 1 น่าจะมีบทบาทในการพัฒนามะเร็งหลังโพรงจมูก

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Microsatellite Instability in Epstein-Barr Virus Associated with Nasopharyngeal Carcinoma

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Nasopharyngeal carcinoma (NPC) is common in Southern China and among Eskimos in Arctic regions with incidences approximately 20-50/100,000 persons per year[1-3]. Intermediate incidences are reported in Saudi Arabia, Caribbean nations, and Southeast Asia with incidences approximately 8-12/100,000 persons per year[2]. From the annual report of cancer at Chulalongkorn Hospital in 1994, NPC was ranked fourth among all cancer in males. Etiologic factors that play an important role in the development of NPC are Epstein-Barr-Virus (EBV), genetic factors, and environmental factors[4-8]. EBV is believed to be closely associated with NPC, in which the EBV genome and specific anti-EBV antibody are regulary found in NPC patients[8]. Several studies, using PCR and *in situ* hybridization exhibited the expression of EBV genome in NPC tumor specimen and suggested the EBV infection was a consistent factor for the evolution of NPC WHO type II, III[8-11].

Carcinogenesis has been described as a result of multiple genetics changes[12,13]. Recently, a distinct type of genetic alteration during tumorigenesis, called microsatellite instability (MSI) has been discovered. MSI was characterised by the expansion or contraction of short tandem repeat sequences, microsatellite. This phenomenon is caused by a defect in DNA mismatch repair gene. At least four of these genes, hMSH2, hMLH1, hPMS1 and hPMS2, have been identified and characterized[14-18]. Thus, the presentation of MSI can be considered as a phenotype of replication error which usually leads to enhance further gene mutations.

MSI was first described in hereditary non-polyposis colorectal cancer[14,15]. Further study demonstrated MSI in several sporadic and hereditary cancers i.e., colorectal, endometrial, prostate, ovarian, head and neck, esophagus, breast, testis and bladder[19-25]. In this study, we examined whether and how often MSI is presented during NPC development.

MATERIAL AND METHOD

Tissues and DNA Extraction

Primary NPC tissues and blood were collected from 23 patients prior to treatment at Chulalongkorn University Hospital. Tissues were

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divided into two parts. The first part was sent for routine histological examination. The second part was immediately stored in liquid nitrogen until used. The biopsied samples were selected for further analysis if they were histologically diagnosed as NPC according to the WHO classification and if they contained neoplastic cells at more than 70 per cent of the total tissue. DNA was extracted from tumor tissue and blood by routine method.

EBV detection by PCR

For the detection of EBV DNA in tumor tissue, a previously described PCR protocol was used with some modifications[26]. Cell line B958, EBV transformed human lymphocytes (American Type Culture Collection) were used as positive controls and the patients' leukocytes as negative controls. Two sets of primers were used. The first was 5'GTAGAAGGCCATTTTTCCAC3' and 5' CTCCATCGTCAAAGCTGCA3', amplifying the non-polymorphic EBV nuclear antigen 1 (EBNA-1) and generating a product of approximately 610 bp. The second was a human b-actin genomic sequence, 5'ATCATGTTTGAGACCTTCAA3' and 5'CATCTCTTGCTCGAAGTCCA3' which generated a product of approximately 318 bp.

The duplex PCR reaction was performed in a total volume of 20 µl using 50 ng of DNA with the following final concentrations: 200 µM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (*p*H 9.0), 0.1% Triton X-100 and 0.5 units of Thermus aquaticus DNA polymerase (Promega). The final concentration for each primer was 0.5 mM: For the PCR reaction, initial denaturation was at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, with an extension at 72°C for 1 min and a final extension at 72°C for 7 min. The PCR products were then analyzed on a 2 per cent agarose gel. The DNA from NPC tissues which presented EBV genomes was used for microsatellite analysis.

Microsatellite analysis

Thirty nine microsatellite markers for PCR analysis are listed in Table 1. The primers were obtained from Research Genetics (U.S.A.). One strand of each primer pair was end-labeled for 1-2 hours at 37°C in a volume of 10 µl containing 10 µM primer, 0.025 mCi (γ-^{32}P) ATP at 3,000 Ci/mmol, 10 mM $MgCl_2$, 70 mM Tris HCl *p*H 7.6, 5 mM DTT and 10 units of T4 polynucleotide kinase. Without further separation of the unincorporated nucleotides, the kinase reaction mixture was directly added to the PCR buffer mix.

The PCR reaction was performed in a total volume of 10 µl using 50 ng of genomic DNA in 200 µM each of deoxynucleotidetriphosphates (dNTPs), 10 mM tris HCl *p*H 8.4, 50 mM KCl, 1.5 mM $MgCl_2$ (for all reactions; excepted D20S470 added 2.0 mM $MgCl_2$) and 2.0 units of Tag polymerase (Perkin Elmer Cetus). Each of the primer pair was performed in optimal concentration listed in Table 1. For PCR reaction 1; the initial denaturation step was 95°C for 4 min then followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min.

Reaction 2 ; the initial denaturation was at 95°C for 4 min followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min.

Reaction 3 ; the initial denaturation was at 95°C for 4 min followed by 25 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min.

Reaction 4 ; the initial denaturation step was 95°C for 4 min, then followed by 5 cycles of step down PCR denaturation at 94°C for 1 min, annealing at 60°C, 59°C, 58°C, 57°C, and 56°C for 1 min respectivly, extension at 72°C for 2 min and 25 cycles f denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min.

Final PCR products (3 µl) of each reaction were mixed with 1.5 µl formamide-loading buffer, heated at 95°C for 2 min, put on ice for 30 sec and then loaded onto the denatured 6 per cent polyacrylamide / 7M urea sequencing gel. The DNA fragments were size-fractionated on a 35x43 cm gel for 3 hour at 80 W until the tracking dye (bromphenol-blue) reached the bottom of the gel. After electrophoresis, the wet gel was wrapped with clean plastic wrap and exposed to Kodak T-mat X-ray film for 6-24 hour at -70°C with an intensifying screens.

RESULTS

All biopsy specimens were histologically proven to be NPC, WHO type II 13 cases and WHO type III 10 cases. MSI was detected in only

Table 1. List of microsatellite markers for microsatellite instability study.

Chromosome arm	Loci	Location	Size	Primer concentration (μM)	PCR reaction
1P	D1S243	1p36.1-36.2	142-170	0.30	3
1Q	D1S103	1q31-q32	82-102	0.10	1
2P	D2S131	2p	229-247	0.40	2
2Q	D2S102	2q33-q37	138-162	0.30	1
3P	D3S1600	3p14	182-198	0.10	3
3Q	GLUT2	3q26.1-q26.3	184-222	0.05	1
4P	D4S174	4p11-p15	175-195	0.50	1
	D4S1599	4q	142-156	0.30	3
4Q	D4S1554	4q11-q35	184-208	0.30	4
5P	D5S392	5p	83-117	0.20	3
5Q	D5S346	5q21-q22	96-122	0.30	3
6P	D6S309	6p	254-272	0.20	4
6Q	IGF2R	6q27	158-166	0.20	1
7P	D7S517	7p	239-257	0.10	1
7Q	D7S486	7q31	133-146	0.20	4
8P	NEFL	8p	137-147	0.30	3
8Q	D8S88	8q22	76-100	0.10	1
9P	D9S169	9p21	259-275	0.10	3
9Q	D9S51	9q	135-159	0.05	1
10P	D10S89	10p11.2-pter	140	0.30	3
10Q	D10S169	10q11.2-qter	99-117	0.10	1
11P	D11S554	11p	234	0.20	4
11Q	D11S976	11q23	130	0.20	3
12P	D12S341	12p	114-130	0.10	4
12Q	MFD133	12q	161-175	0.20	1
13Q	D13S284	13q13RBI	197-227	0.20	4
14Q	D14S118	14q	230	0.20	4
15Q	GABRB3	15q11-13	181-201	0.10	1
16P	D16S287	16p13.11	201-225	0.20	1
16Q	D16S511	16q22-24	182-222	0.20	4
17P	D17S945	17p13	186-208	0.30	3
17Q	KRT9	17q21NMEI	182-198	0.20	4
18P	D18S59	18pter-p11.22	148-164	0.20	3
18Q	DCC	18q21.1	106-160	0.30	1
19P	D19S221	19p	191-211	0.10	4
19Q	D19S412	19q	89-113	0.30	4
20P	D20S470	20p	280	0.20	2
20Q	D20S17	20q12-q13.1	130-140	0.20	1
21Q	D21S258	21q	184-206	0.20	1
22Q	IL2RB	22q	149-163	0.20	1

one (no. 38) of the 23 tumors. The MSIs were evidenced by the presence of allelic fragments not found in the matched normal DNA. Fig. 1 shows the representative of MSI at DCC locus. Of the 39 markers analyzed 37 were dinucleotide repeat sequences, and the remaining 2 were trinucleotide repeat sequences. MSIs were observed in both trinucleotide repeats. For dinucleotide repeats, the frequency of MSI was observed 62 per cent (23/37) (Table 2).

DISCUSSION

Microsatellite instability (MSI) is presented as variations in the length of microsatellite repeats in tumor DNA when compared with matched normal DNA. An abnormalities in size of microsatellite loci has been observed in various types of cancer as well as in hereditary non-polyposis colorectal cancer (HNPCC)[14,27-30]. In HNPCC mutations in a number of DNA mismatch repair genes (hMSH2, hMLH1, hPMS1, and

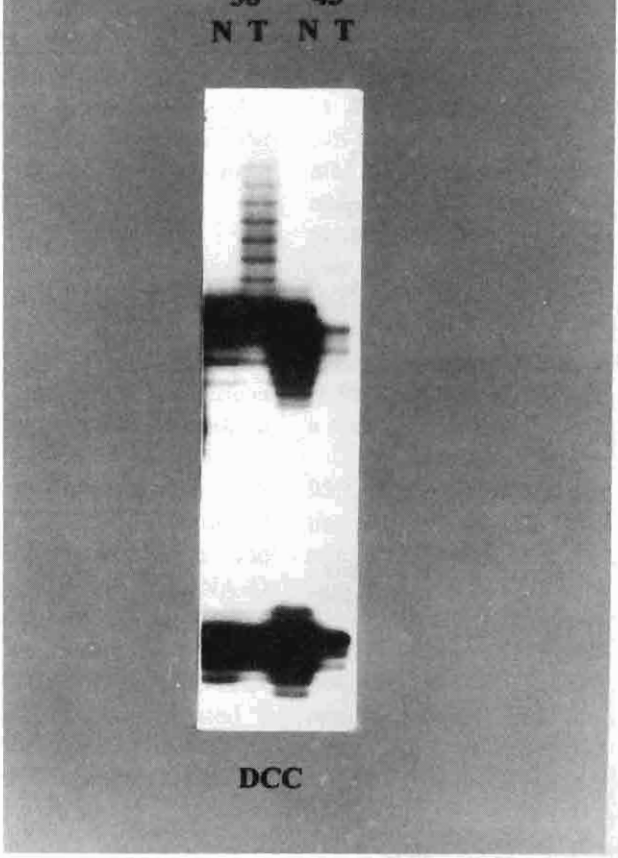

Fig. 1. An autoradiograph showed the microsatellite instability at DCC locus. N, normal DNA ; T, tumor DNA.

Table 2. Microsatellite instability results in NPC patient no. 38

Patient no.	Type of microsatellite	%MSI (no. of MSI/ no. of loci)	Loci name
38	Dinucleotide	62% (23/37)	D1S103, D2S102, D3S1600, GLUT2, D4S174, D4S1599, D4S1554, D5S346, D6S309, IGF2R D8S88, D9S51, D10S169, MFD133, D13S284 GABRB3, D16S511, D17S945, KRT9, DCC, D19S221, D19S412, IL2RB
	Trinucleotide	100% (2/2)	D2S131, D20S470

hPMS2), have been reported. These results in a decreased accuracy of DNA mismatch repair gene system during DNA replication[14,29,30]. Thus MSI was thought to be a phenotype of replication error which facilitate the accumulation of mutations. In addition, MSI has also been reported to occur in various sporadic cancers at various frequencies such as endometrium cancer (17-25%), [31]. Stomach cancer (18-39%),[31] colon cancer (12-28%),[31] head and neck cancer (28%)[21]. This study presented the phenomenon of MSI in only 4 per cent of NPC patients (1/23). The incidence of MSI from this report is similar to that previously observed in breast cancer (4%), liver cancer (3%), prostate cancer (3%), esophagous squamous-cell carcinoma (2%)[31]. This result suggests that the phenomenon of MSI is a relatively rare event during NPC development.

SUMMARY

The result reported here represents the first human genomic screen for MSI in Epstein-Barr-Virus associated NPC. The analysis revealed the incidence of MSI only 1 of 23 cases (4%) which indicates that MSI is less common in NPC development.

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ความไม่คงตัวของไมโครแซทเทลไลท์ในมะเร็งหลังโพรงจมูกชนิดที่สัมพันธ์กับเชื้อเอปสไตน์บาร์รไวรัส

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การศึกษาการเปลี่ยนแปลงของหน่วยพันธุกรรมที่เกิดจากความผันแปรของขนาดความยาวของไมโครแซทเทลไลท์ในจีโนมของเนื้อเยื่อมะเร็ง หรือ ความไม่คงตัวของไมโครแซทเทลไลท์ ถูกพบครั้งแรกในมะเร็งลำไส้ใหญ่ชนิดที่สัมพันธ์กับประวัติทางครอบครัว ซึ่งพบว่ามีความเกี่ยวข้องกับการกลายพันธุ์ของยีนที่ทำหน้าที่ตรวจสอบและซ่อมแซมความผิดพลาดของ ดีเอนเอ ในกระบวนการเรพลิเคชั่น นอกจากนี้ยังสามารถตรวจพบปรากฏการณ์ เอ็มเอสไอ ในมะเร็งชนิด สปอราดิกอื่น ๆ อีกด้วย อาทิเช่น มะเร็งลำไส้ใหญ่, โพรงมดลูก, รังไข่, ศีรษะและคอ, และ หลอดอาหาร แต่ยังไม่มีรายงานการศึกษาความสัมพันธ์ระหว่างปรากฏการณ์เอ็มเอสไอในมะเร็งหลังโพรงจมูกชนิดที่สัมพันธ์กับเชื้อเอปสไตน์บาร์รไวรัส ในการศึกษานี้ผู้วิจัยได้ทำการคัดเลือกไมโครแซทเทลไลท์จำนวน 39 มาร์กเกอร์ นำมาเพิ่มขยายด้วยเทคนิคพีซีอาร์ ทำการเปรียบเทียบระหว่างดีเอนเอของเนื้อเยื่อปกติ (เลือด) กับเนื้อเยื่อมะเร็งจากผู้ป่วยมะเร็งหลังโพรงจมูกจำนวน 23 ราย ผลการทดลองพบอุปติการณ์ของเอ็มเอสไอเกิดขึ้นเพียง 1 ใน 23 ราย (4%) การศึกษาในครั้งนี้แสดงให้เห็นว่าปรากฏการณ์เอ็มเอสไอพบได้ไม่บ่อยในการพัฒนาการของมะเร็งหลังโพรงจมูก

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Reliable Sex Determination of Mouse Preimplantation Embryos by PCR Amplification of Male-specific Genes in Single Blastomeres†

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Prenatal diagnosis (PND) for genetic diseases has traditionally involved the screening after a pregnancy is in progress by biochemical or molecular analysis of fetal materials obtained by chorionic villus sampling (CVS) or amniocentesis[1]. However, couples who undergo PND have to face the results of PND ; once the fetus is confirmed to be defective, the couples have only one of two choices: termination of pregnancy or having a diseased child. Selective termination of pregnancies in the first or second trimesters is a difficult and painful decision faced by the couples and may be unacceptable for some religious and moral reasons.

In recent years, technical development of assisted reproductive technology and polymerase chain reaction (PCR)[2] offer the real possibility of diagnosis of genetic diseases in the earliest stage of human life called preimplantation diagnosis (PID)[3-5]. It has the advantage that the diagnosis is made before implantation of the embryos, so that termination of pregnancy can be avoided. For X-linked recessive diseases, when no specific diagnosis is available, sex determination of preimplantation embryos may be the only way to avoid the births of an affected male child through replacement of only unaffected female embryos into the uterus. However, clinical application requires efficient and reliable assay procedures which avoid false-positive or false-negative results. It was suggested that more basic research should be done before moving on to the routine application of PID in clinical practice[6]. Previous works have utilized the PCR to amplify Y-chromosome-specific repetitive sequence from a single blastomere in order to determine embryonic sex[3,7,8]. However, the disadvantage of this strategy is that the absence of a signal is used to select female embryos for replacement but does not control for loss of the cell or failed amplification by PCR. Recently, accuracy of sexing procedure has been improved by using multiplex PCR where X- and Y- chromosome- specific sequences were co-amplified from a single blastomere[9]. The other functional gene, Sry, has recently been found to be present in a particular sex-determining region of the mouse Y chromosome[10] and is the most potent candidate for testis-determining factor[11]. This study, therefore, investigates the reliability of sex determination through detection of

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DNA Diagnosis for Clinical and Prenatal Diagnosis of Spinal Muscular Atrophy in Thai Patients

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Spinal muscular atrophy (SMA) is among one of the most common autosomal recessive diseases (incidence 1 in 6,000 live births)[1,2]. SMA is characterized by degeneration of the anterior horn cells of the spinal cord leading to symmetrical muscle weakness and wasting of voluntary muscles. Pathological and electrophysiological evidence of muscle denervation is also found in most patients. The SMAs are classified depending on the age of onset, the maximum muscular activity achieved, and age of survival. Type I SMA is the most severe form known as Werdnig-Hoffmann disease. The children can be recognised at birth or within 6 months of age. They are unable to sit unaided and respiratory involvement is usually responsible for death before 2 years of age. In type II SMA, the intermediate form, children are able to sit but unable to stand or walk unaided. If respiratory function is preserved, such patients may be nonambulatory and live beyond 2 years of age. In type III SMA, the mildest form known as Kugelberg-Welander disease, children have an onset after the second year of life and the course of the disease is chronic[3,4].

The locus of the gene responsible for all three forms has been mapped to chromosome 5q 13.3[5-7]. This suggests that they are the result of allelic mutations. Two genes associated with SMA have been identified in this region, the neuronal apoptosis inhibitory protein (NAIP) and the survival motor neuron (SMN) genes[8,9]. While NAIP gene was deleted in some patients, SMN gene was either absent or interupted in the majority of patients (98.6%)[8-10]. The SMA critical region is also presented with many repeated elements. The SMN gene, too, has a highly homologous copy with a more centromeric location. This copy is present in 95.5 per cent homology of the SMN gene. The two genes differ in their exons by only two base pairs, one in exon 7 and one in exon 8[11].

Recently van der Steege et al[11] described a DNA test to confirm clinical diagnosis of SMA. This technique was PCR-based and able to rapidly detect exon 7 and exon 8 deletion of SMN gene and diagnosed 49 SMAs from 51 patients. Thus, this method is useful to confirm the diagnosis and perform prenatal diagnosis of SMA patients. To establish molecular diagnosis for SMA in Thailand we tested the PCR-based method and confirmed the diagnosis on one type I, four type II and one type III SMA patients. In addition, prenatal diagnosis was performed for two families.

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MATERIAL AND METHOD

Subject

Blood samples were collected from parents and patients. The SMA patients were diagnosed using standard clinical diagnosis criteria[4]. Amniocytes were collected from amniocentesis of two requesting families with previous children suffering SMA.

Molecular Analysis

Genomic DNA was extracted from peripheral blood leukocytes or amniocytes by standard procedures. The PCR analyses were performed according to previously described in van der Steege et al with some modification[11]. Exon 7 was amplified with intron 6 primer R111[9] (5'-AGA-CTA-TCA-ACT-TAA-TTT-CTG-ATC-A-3') and mismatch primer X7-Dra (5'-CCT-TCC-TTC-TTT-TTG-ATT-TTG-TTT-3') using 5 ng/μl of genomic DNA in 200 μM each of dNTP, 67 mM Tris-HCl *p*H 8.8, 16.6 mM (NH4)2SO4, 10 mM b-mercaptoethanol, 170 mg/BSA, 6.8 mM EDTA, 1.5 mM $MgCl_2$, 0.25 μM for each primer and 0.7 units of Thermus aquaticus DNA polymerase (Perkin-Elmer) in 20 ml reaction. The PCR was initially denatured for 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C and a final extension of 72°C for 7 min. PCR products were subsequently digested to completeness with DraI. Exon 8 DNA was amplified with primers 541C960 (5'-GTA-ATA-ACC-AAA-TGC-AAT-GTG-AA-3')[9] and 541C1120 (5'-CTA-CAA-CAC-CCT-TCT-CAC-AG-3')[9] with the same PCR conditions. PCR products were subsequently digested with DdeI. Electrophoresis was carried out in 2.5 per cent agarose gel containing 0.5 mg/ml ethidium bromide at 3 to 5 V/cm for 3 hours in 1xTBE.

RESULTS

The goal of this study was to establish the molecular diagnosis of SMA in Thailand for confirmation of clinical finding and prenatal diagnosis. The PCR was tested on five Thai and one Burmese SMA patients, 12 individuals, who were parents of the patients, and two amniocytes of pregnancy with a previous child with SMA.

Deletion Detection

van der Steege et al designed this PCR technique to rapidly detect SMN gene deletion of exon 7 and 8. This PCR products, cut by restriction enzymes and visualised in a gel, give a clear and specific pattern for SMA patients. PCR products of exon 8 of the SMN gene and the copy gene can readily be distinguished since the copy gene contains a recognition site for the restriction enzyme DdeI and this site is absent in exon 8 of SMN. In cases of exon 7, an oligonucleotide hybridizes adjacent to the variant site and introduces a mismatch and created restriction site DraI in the PCR product of exon 7 of the copy gene, but not in the PCR product of the SMN gene.

Fig. 1. demonstrates digested PCR product from SMA patients and their parents. Exon 7 and exon 8 of SMN gene deletion is demonstrated as the absence of the uppermost band of the PCR products. All 6 SMA patients represented three types of SMA, CS was type I, PP, SJ, NV and NN type II, and TS type III. SJ, TS and NV had deletion involving both exon 7 and 8. PP and NN had deletion of exon 7 only and CS had deletion of exon 8 (Table 1). None of the parents detected a total absence of either exon 7 or 8.

Prenatal diagnosis from amniocentesis were performed on two families. For one family an idex case, PP was alive. For the other, the previous child was diagnosed SMA type I and had deceased without any DNA being stored. The first family, PP's sib, deletion of exon 7 was detected therefore the diagnosis was done prenatally as SMA type II. The second family, VM's amniocytes on the otherhand had no deletion of SMN gene detected.

DISCUSSION

Since the incidence of SMA is approximately 1 in 6,000 with little ethnic variation, there should be approximately 200 SMA patients born per year in Thailand. Most patients especially SMA type I are severe and die in early life. This represents a resonable proportion of the population in real need for prenatal diagnosis. We are setting up a standard protocol to improve the diagnosis and management of these patients and families. The PCR-based method provides a rapid and simple method to diagnose SMA patients pre and postnatally[11]. This assay identified more than 98 per cent of SMA patients in Europe[11]. It is less known whether this technique will be able to detect most SMN mutations in the Thai population. We successfully identified deletion of SMN

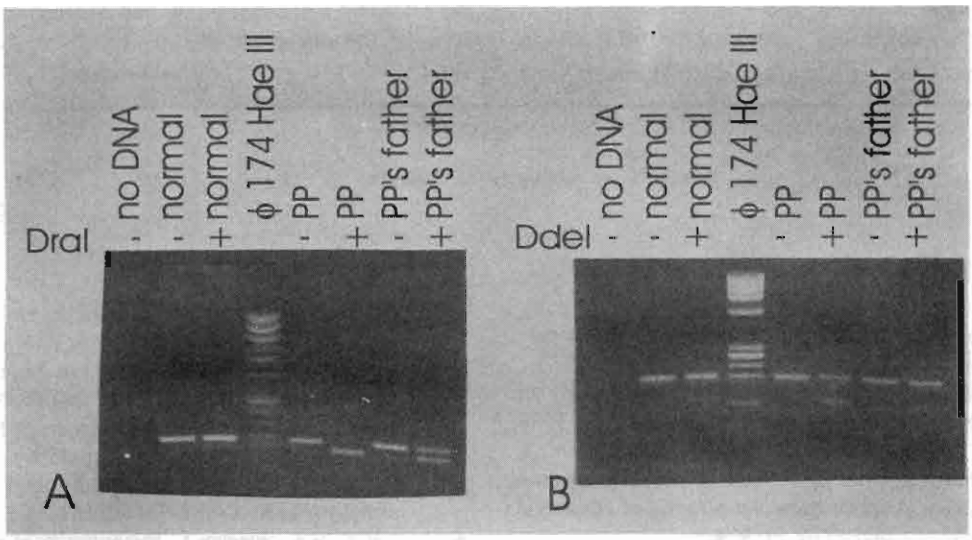

Fig. 1. Demonstrate PCR results of patient PP and her father. + is the PCR products were treated with restriction enzyme DraI or DdeI. - is the PCR products were not treated with restriction enzyme DraI or DdeI. A is PCR products of exon 7. B is PCR products of exon 8. Patient PP had deletion of exon 7 but not exon 8.

Table 1. PCR analysis of exon 7 and 8 deletion of SMA patients.

Name	Type	Exon 7	Exon 8
PP	II	D	**UD**
PP's sib	amniocentesis	D	**UD**
SJ	II	D	D
TS	III	D	D
NV	II	D	D
NN	II	D	UD
CS	I	UD	D
VM's amniocytes	amniocentesis	UD	UD

I, II and III indicate SMA type I, II and III respectively.
D and UD indicate deletion present and absent respectively.

genes in all five Thai and one Burmese patients tested. This may indicate that mutation of the SMN gene in the Asian population is similar to the Caucasian and this technique should apply to the Thai population as well. In addition, this PCR did not detect homozygous deletion in any parents and confirmed the accuracy of the test. This technique is also simple, fast and convenient for prenatal diagnosis. A family who requests the test, will receive the answer within 1 day. Since the test will not detect all SMA cases, DNA of an index case, previous child with SMA, is required to be tested before chorionic villus sampling or amniocentesis. If not, the family must be informed that the negative test result does not always mean the fetus is not affected with SMA. This also emphasizes the need for genetic counselling about recurrence risk and prenatal diagnosis issue while children are diagnosed SMA.

SUMMARY

To improve the mode of clinical and prenatal diagnosis for Thai SMA patients, DNA diagnosis has been set up. We used the published PCR technique from van der Steege et al to detect deletion of the survival motor neuron genes and confirmed the diagnosis of six patients. No false positive was detected from normal individuals. In addition, prenatal diagnosis was performed from amniocytes of two cases with a previously affected child. One of them showed exon 7 deletion and was diagnosed prenatally.

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การตรวจดีเอ็นเอเพื่อวินิจฉัยทางคลินิคและวินิจฉัยก่อนคลอดสำหรับผู้ป่วยโรคสไปนัลมัสคูลาร์อะโทรฟีในคนไทย

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โรคสไปนัลมัสคูลาร์อะโทรฟีอยู่ในกลุ่มโรคที่ถ่ายทอดแบบลักษณะด้อยที่พบบ่อยที่สุดชนิดหนึ่ง เพื่อที่จะพัฒนาการวินิจฉัยทางคลินิคและการวินิจฉัยก่อนคลอดสำหรับผู้ป่วยคนไทย การวินิจฉัยดีเอ็นเอได้ถูกทำขึ้นโดยผู้วิจัยได้ใช้วิธีการปฏิกิริยาลูกโซ่โพลีเมอร์เรสที่ถูกพัฒนาขึ้นโดย van der Steege และคณะ เพื่อตรวจหาการขาดหายของยีนเซอไววัลมอเตอร์นิวรอน ที่ เอกซอน 7 หรือ 8 ซึ่งได้ช่วยยืนยันโรคในผู้ป่วย 6 ราย โดยไม่พบมีการตรวจผิดที่ให้ผลบวกในคนปกติเลย นอกจากนี้ ยังตรวจวินิจฉัยก่อนคลอดโดยใช้เซลล์จากน้ำคร่ำ 2 ราย จากผู้ที่มีประวัติลูกคนก่อนป่วยด้วยโรคนี้ พบว่าใน 1 ราย มีการขาดหายของเอ็กซอนที่ 7 และให้การวินิจฉัย โรคก่อนคลอด

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Diagnosis and Carrier Detection in a Duchenne Muscular Dystrophy Family by Multiplex Polymerase Chain Reaction and Microsatellite Analysis

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Duchenne muscular dystrophy (DMD) is among the most common human genetic diseases, affecting approximately 1 in 3,500 male births[1]. It is an X-linked recessive muscle wasting disorder which leads to unrelenting death by the third decade of life, caused by mutation in the dystrophin gene, located on the short arm of the X chromosome at XP 21.2[2]. Because of the extremely large size of this gene (nearly 2,400 kb) the most molecular defect in DMD patients is intragenic deletion which accounts for more than 60 per cent of mutation[3,4].

Since at present there is no cure or effective treatment for this disorder, much emphasis has been given to prevention. This involves determining the carrier status of at risk women, the ascertainment of having an affected son and genetic counselling for such women. Currently molecular techniques for diagnosis and carrier detection of DMD are now available. Multiplex polymerase chain reaction (PCR) described by Chamberlain et al[5] and Beggs et al[6] can detect about 98 per cent of DMD gene deletion. Although this procedure is very useful for rapid diagnosis of affected individuals, it provides no information on the carrier status of at risk women within families. To access carrier status of at risk women in DMD families, linkage analysis and/or dosage analysis on southern blot, for "deletion", has to be used[7-9]. Since microsatellite or short tandam repeats (STR) are highly repetitive genomic elements of 1-5 nucleotides, it has become widely used as a genetic marker for gene mapping and linkage analysis [10,11]. STR markers covering the entire dystrophin gene have been described by Clemens et al[12] and Taylor et al[13]. As these STR markers fall within a deletion hot spot region of the DMD gene, they are often deleted in affected patients and show loss of heterozygosity in female carriers. The use of microsatellite or STR analysis is thus a relatively new approach for carrier detection of DMD[12-15]. Here, we have used multiplex PCR to detect dystrophin gene deletion and microsatellite or STR analysis to determine the carriers in a family in which two living boys are affected by DMD.

MATERIAL AND METHOD

Subject

A family was referred to the Department of Pediatrics, Chulalongkorn Hospital for further investigation in view of the family history of muscular dystrophy (Fig. 1). Blood samples were collected from all living members within two generations of the family.

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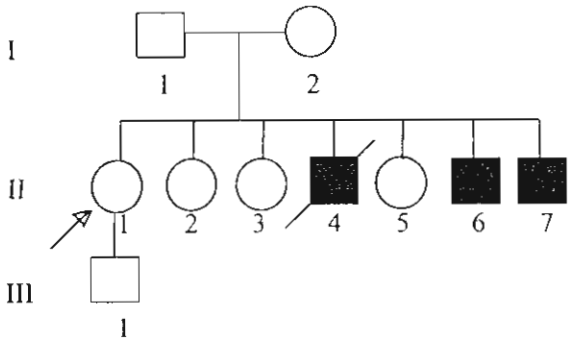

Fig. 1. Pedigree of the DMD family.

Multiplex PCR analysis

Genomic DNA was extracted from peripheral blood leukocytes by standard procedures [16,17]. Multiplex gene amplification systems were performed using the primers described by Chamberlain et al[5]. The multiplex amplification was essentially as described with some modifications. The PCR was carried out in a final volume of 25 µl containing 16.6 mM $(NH4)_2SO_4$, 67 mM Tris-HCl (*p*H 8.8), 6.7 mM $MgCl_2$, 10 mM 6- mercaptoethanol, 170 mg/ml bovine albumin, 6.8 EDTA, 1.5 µM of each dNTP, 250 to 500 ng DNA, 1 unit of Thermus aquaticus DNA polymerase (Pharmacia), primer sets of exon 4(I), 8(a), 12(g), 17(b), 19(c), 4.1 kb Hind III(d) at 0.5 µM and primer 0.5 Kb(e), 1.2/3.8 Kb(f), and 3.1 Kb(h) Hind III at 1 µM (The letters in parentheses are the designated letters from Chamberlain et al, 1990). The simplex PCR reactions were for each set of primer in a total volume of 25 µl using 150 ng of genomic DNA in 200 µM each of the dNTP, 10 mM Tris-HCl *p*H 8.4, 50 mM KCl, 1.2 mM $MgCl_2$, 0.5 mM for each primer and 0.75 units of Thermus aquaticus DNA polymerase (Pharmacia). The PCR was initially denatured at 95°C for 6 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec, extension at 65°C for 4 min and a final extension of 65°C for 7 min. Twelve µl of the reaction products was electrophoresised on 3 per cent nusieve agarose containing 0.5 µg/ml ethidium bromide for 3 hours at 5 V/cm in 1xTBE.

STR analysis

Five STR markers within or linked to the DMD gene, y'5', p44, p45, p49 and p50, which located 5', in intron 44, 45, 49, 50 repectively[13], were selected to test this DMD family. One strand of each primer pair was end-labeled for 1-2 hours at 37°C in a volume of 10 µl containing 10 µM primer, 0.025 mCi [32P] ATP (Amersham) at 3000 Ci/mmol, 10 mM $MgCl_2$, 5 mM DTT, 50 mM Tris-HCl, *p*H 7.6 and 9 units of T4 polynucleotide kinase (New England).

The PCR reactions were performed in a total volume of 10 µl using 25 ng of genomic DNA in 200 µM each of the dNTPs, 10mM Tris-HCl *p*H 8.4, 50mM KCl, 1.2 mM $MgCl_2$ and 0.5 units of Thermus aquaticus DNA polymerase (Promega). Each primer set was added to the PCR mixture with the 0.3 µM final concentration. The PCR reactions were performed as follows; initial denaturation was at 95°C for 4 min followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, extension at 72°C for 2 min and a final extension of 72°C for 7 min. Two microliters of each reaction was mixed with 1 µl formamide-loading buffer, heated at 95°C for 2 min, put on ice for 30 seconds and then loaded onto 6 per cent polyacrylamide/7M urea gel. DNA fragments were size fractionated on a 40x70 cm gel at 70 watts until the tracking dye, Bromphenol blue, almost reached the buttom of the gel. After electrophoresis, the wet gel was wrapped with clean wrap with 3M blotting paper and exposed to Kodak XAR-5 X-ray film for 18 hours at -70°C with an intensifying screen.

RESULTS

The main propose of this study was to establish the molecular diagnosis of DMD in Thailand for antenatal and presymtomatic diagnosis and carrier detection. Here we demonstrate molecular study of a DMD family for diagnosis and carrier testing.

Pedigree Analysis

A family was referred to the Department of Pediatrics, Chulalongkorn University Hospital for further investigation in view of the family history of muscular dystrophy (Fig. 1). There were four healthy daughters and three affected sons. The first sons, II4, was diagnosed clinically of DMD. He was noted to have progressive weakness at the age

of 7, loss of ambulation by the age of 14 years and had died at the age of 18 years. The 2 living sons were diagnosed with DMD by standard clinical diagnostic criteria, including serum creatinine kinase level and histopathological assessment of muscle biopsy specimens. II6 is 14 years old and clinical investigation confirmed the DMD diagnosis. The youngest son (II7) however is 6 years old and is beginning to develop the clinical characteristics of DMD. The mother and all daughters aged 25, 24, 20 and 16 years, II1, II2, II3 and II5 respectively, appeared normal. II1 is married and has a 6 month old son. At first visit II1 brought her youngest brother to be evaluated for the possibility of developing DMD and of her being a carrier.

Detection of the DMD gene deletion

To evaluate the molecular alteration of the dystrophin gene in this family, multiplex PCRs of nine sets of primers were tested. Patient II6 and II7 demonstrated only eight PCR products and missed the fifth band from the top, representing exon 51 (Fig. 2). This indicates that both II6 and II7 had deletion of dystrophin gene cover exon 51 and confirmed the diagnosis of DMD for both patients. I2, II1 and control had nine PCR products. However, the fifth band of the mother's, I2, demonstrated weaker intensity, while II1 had the same intensity as control. This suggested the dosage difference and confirmed the carrier status of the mother and that II1 was not a carrier for the disease.

STR analysis for linkage analysis

In order to confirm the noncarrier status of II1, we tested 5 STRs of this family, these STRs were y'5', p44, p45, p49 and p50, which were distributed over the dystrophin gene and mutations usually located among these markers. Since multiplex PCR showed deletion of exon 51, the p50 should be the most linked STR marker in this study. At the beginning, the STRs were tested for the

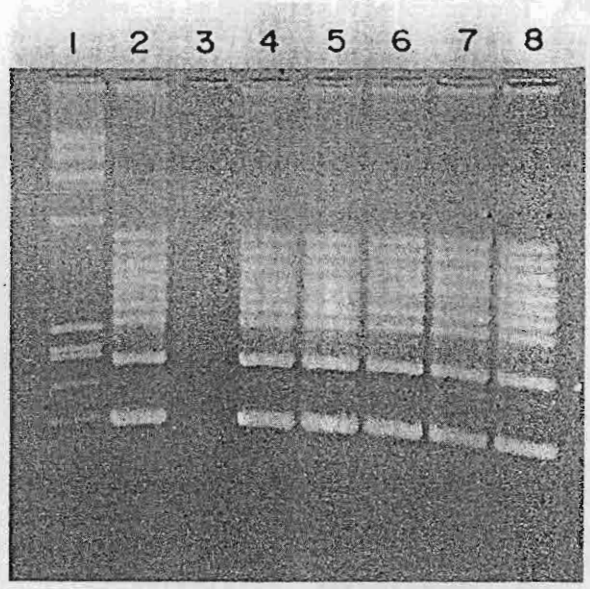

Fig. 2. Detection of the DMD gene deletions of the DMD family by multiplex DNA amplification. Lane #1 from the left was HaeIII digested ϕX174 DNA molecular weight standard. Lane #2 and 3 were the positive and negative controls respectively. Lane #4, 5, 6, 7, 8 were the PCR product of father (I1), mother (I2), the second male patient (II6), the third male patient (II7) and their eldest sister (II1) respectively. The multiplex PCR fragments correspond (top to bottom) to regions e, f, c, b, h, a, g, d and i, respectively. II6 and II7 demonstrate the deletion of the h band and I2 demonstrates the decrease in intensity of the h band.

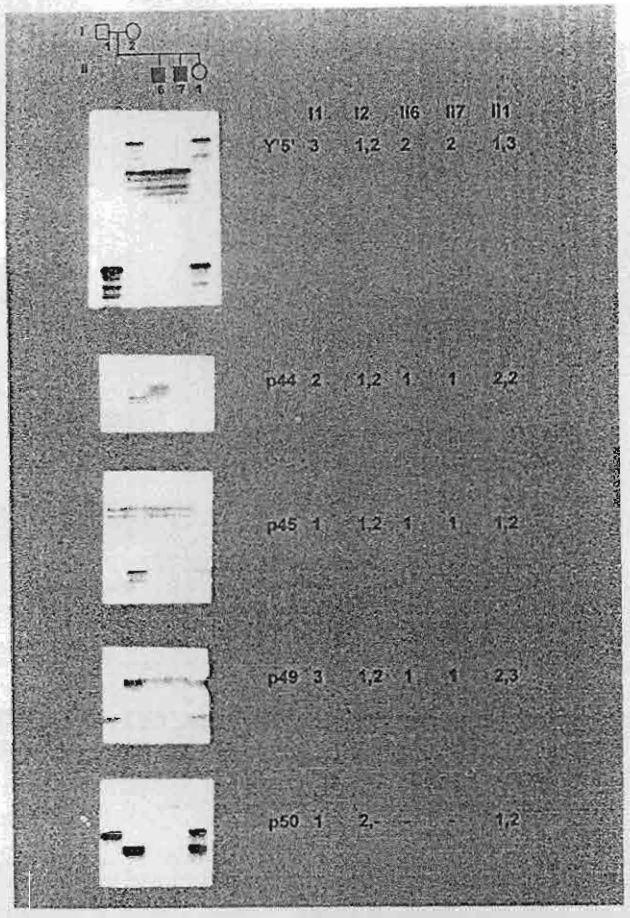

Fig. 3. Demonstrates the STR results of I1, I2, II1, II6 and II7. Y'5', p44, p45, p49 and p50 are the STRs. Y'5', p44, p45 and p49 showed informative linkage analysis. P50 detected gene deletion.

parents, II1, II6 and II7 (Fig. 3). Y'5', p44, p45 and p49 demonstrated two alleles for the mother thus all of these markers were informative for linkage analysis. In addition, the II6 and 7 alleles were the same suggesting the link of the STRs' alleles to the mutations. Since II1 had different STRs' alleles from patients II6 and II7, II1 was not a disease carrier. While all mentioned STRs demonstrated informative linkage datas, p50 detected gene deletion and directly confirmed the deletion of disease allele in the carrier mother but not in II1. Deletion of the dystrophin gene was demonstrated by the absence of PCR products in two affected sons and suggested that the mother was rather hemizygote for this allele. Since II1 p50 STR was heterozygote, she was not carrier.

Further tests have been performed for II2, II3, II5 who were the younger sisters of this family (Fig. 4). P50 demonstrated one allele for II2, II3 and II5. This concluded that they were carriers for this disease. Interestingly the II3's y'5' allele was not linked to the disease. This was a result of genetic recombination between this marker and the deletion of II3 disease chromosome.

DISCUSSION

Since Duchenne Muscular Dystrophy is a serious disorder for which at present there is no

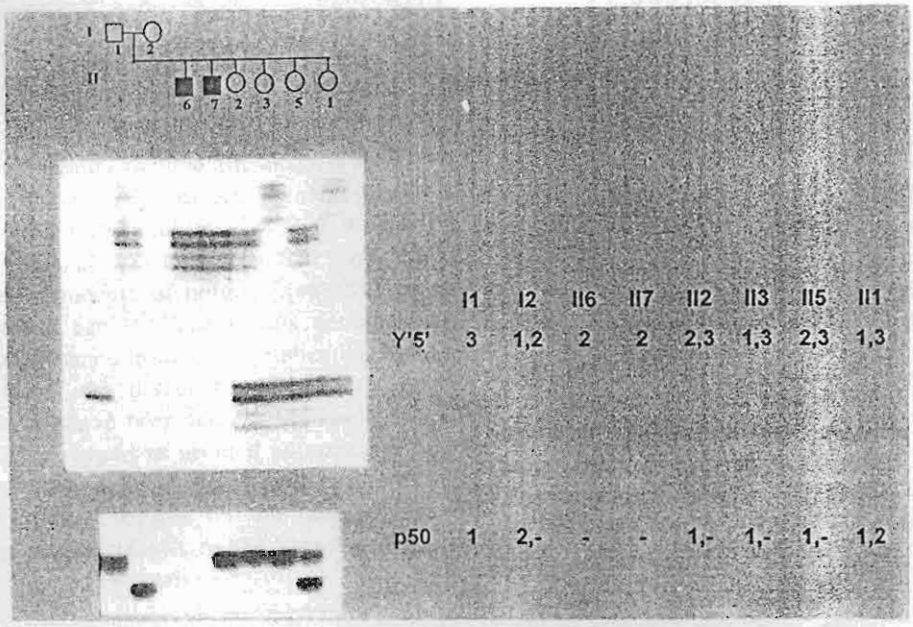

Fig. 4. Demonstrates the STR result of y'5' and p50 for all members in generation I and II. P50 detected the carrier status of II2, II3 and II5. Genetic recombination between y'5' and p50 was demonstrated in II3.

effective treatment, much emphasis has been given to prevention. This involves the ascertainment of women likely to have an affected son, prenatal diagnosis and provision of genetic counselling for such women. In genetic counselling, the most important issues, are to determine the probability of a woman being a carrier and the risks of her having an affected son. Before the development of DNA diagnostic tests, carrier screening was based on mathematical estimates of pedigree risk and phenotype analysis[18]. Conventional screening with clinical examination, creatine kinase assay, muscle biopsy and electromyography had a significant false negative rate owing to the process of random x-chromosome inactivation[19]. Furthermore, no reliable prenatal diagnosis was available. Thus, the women who were carriers of the disorders were faced with the decision of terminating a pregnancy involving a male fetus with a 50 per cent chance of the fetus being unaffected. Because of the advance in DNA technolgy, molecular techniques for diagnosis, carrier detection and prenatal diagnosis of DMD have been described elsewhere[5-10,12]. These allow the affected family the opportunity for reassurrance about carrier status and having unaffected offspring which have dramatically improved the management and prevention of this disorder.

We have used multiplex PCR described by Chamberlain et al to detect dystrophin gene deletion in a DMD family. Two affected boys demonstrated intragenic deletion of exon 51. The father and eldest daughter showed normal results, whereas, the mother showed decrease in intensity of exon 51. Because accurate densitometry on Southern blot analysis[9,20] or PCR amplification[21] can reveal a dosage difference between control and carrier. Thus, there was a possibility for I2 to be a carrier. To confirm this, we performed linkage analysis by microsatellites or STR polymorphic markers to determine carrier status of the mother and all daughters. This confirmed that I2, II2, II3 and II5 were carriers and II1 was not. Since the STR analysis provides direct evidence for carrier detection, it is a more accurate and conclusive method than the dosage analysis.

STR analysis has several advantages for routine laboratory over classical biallelic RFLP marker systems. It is more rapid, requires much less DNA and provides higher informativity than southern analysis. Therefore, the STR analysis using markers covering the entire dystrophin gene may be an essential method for analysis of DMD families. We also plan to perform prenatal diagnosis of this disorder. The multiplex PCR described by Chamberlain et al was tested on four samples of DNA from amniocyte of normal pregnant women with advanced age[22]. The results demonstrated all nine DNA bands in all cases, indicating that no false negative was detected. All carriers in the DMD families have been followed-up and planned for prenatal diagnosis as deemed appropriate. As a result, they can make major decisions regarding their reproductive option on the basis of these diagnostic service results rather than the inaccuracies of phenotype analysis.

SUMMARY

We used multiplex PCR and a microsatellite or STR analysis for diagnosis and carrier detection in a DMD family. Two affected patients both demonstrated deletion of exon 51 by multiplex PCR. The microsatellite or STR analysis showed that the mother and all sisters except the eldest sister of the patients carried the disease allelle. Therefore, all of them except the eldest sister were carriers. We present the need to introduce the molecular techniques to improve a mode of diagnosis and management of DMD patients in the Thai community.

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การวินิจฉัยและตรวจหาพาหะในครอบครัวโรคกล้ามเนื้อเสื่อมดูเชนโดยวิธีปฏิกิริยาลูกโซ่โพลิเมอเรสและวิเคราะห์ไมโครแซทเทลไลท์

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โรคกล้ามเนื้อเสื่อมดูเชนเป็นโรคพันธุกรรมที่พบบ่อยประมาณ 1:3,500 ของทารกเพศชายที่คลอดมีชีวิต เป็นโรคที่ทำให้กล้ามเนื้อฝ่อลีบและอ่อนแรงอย่างรุนแรงจนผู้ป่วยมักเสียชีวิตก่อนอายุ 30 ปี เนื่องจากปัจจุบันยังไม่มีการรักษาที่ได้ผล การวินิจฉัยโรคและพาหะที่ถูกต้องรวดเร็วอาจช่วยป้องกันและลดอัตราการเกิดโรคนี้ได้ คณะผู้วิจัยได้ทำการทดลองใช้วิธีปฏิกิริยาลูกโซ่โพลิเมอเรสและวิเคราะห์ไมโครแซทเทลไลท์เพื่อตรวจวินิจฉัยและหาพาหะโรคในครอบครัวซึ่งมีบุตรชายคนโตเสียชีวิตและบุตรชายที่ยังมีชีวิต 2 คน ป่วยด้วยโรคกล้ามเนื้อเสื่อมดัชเชน ผลการศึกษาพบว่าบุตรชายที่ยังมีชีวิตอยู่ทั้ง 2 คน มีการขาดหายของยีนดีสโตรฟิน มารดาและบุตรสาว 3 คนเป็นพาหะนำโรค ส่วนบุตรสาวคนโตไม่เป็นพาหะนำโรค ดังนั้นวิธีการทางอณูพันธุศาสตร์นี้สามารถใช้ตรวจเพื่อวินิจฉัย และค้นหาพาหะโรคนี้ได้

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