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ORIGINAL ARTICLE

การศึกษาความผิดปกติของโครโมโซมในผู้ป่วยชาวไทย โดยวิธีการตัดชิ้นส่วนโครโมโซม

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บทคัดย่อ

การตัดชิ้นส่วนและการเพิ่มปริมาณดีเอ็นเอของโครโมโซมที่เฉพาะเจาะจง ตามด้วยการติดฉลากเรืองแสง การเตรียมโพรบ เพื่อไฮบริไดซ์กับโครโมโซมระยะเมทาเฟสของคนปกติ หรือวิธี “micro-FISH” สามารถใช้ในการวินิจฉัยทางเซลล์พันธุศาสตร์ก่อนคลอดและหลังคลอดได้ วัตถุประสงค์หลักของการศึกษานี้คือตรวจวิเคราะห์ต้นกำเนิดความผิดปกติของโครโมโซมในผู้ป่วยไทย ด้วยวิธีการตัดชิ้นส่วนโครโมโซม และวัตถุประสงค์รองเพื่อประยุกต์วิธีการนี้มาเสริมวิธีการตรวจวิเคราะห์โครโมโซมในงานประจำ วิธีการศึกษาคือ ตัดโครโมโซมที่สนใจจากโครโมโซมระยะเมทาเฟสของผู้ป่วยที่ย้อมแถบจี จำนวน 5-10 ชิ้น นำมาเพิ่มปริมาณดีเอ็นเอ ด้วยวิธี degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) โดยใช้ digoxigenin-11-dUTP เป็นโมเลกุลรายงาน ตรวจจับโมเลกุลของ digoxigenin-11-dUTP ด้วย mouse anti-digoxigenin และ anti-mouse immunoglobulin ที่ติดด้วยสี Alexa 488 เทคนิค “micro-FISH” สามารถนำมาใช้เสริมกระบวนการวินิจฉัยโครโมโซมทางคลินิกในงานประจำได้ เช่น โครโมโซมผิดปกติที่เกิดจากการสลับที่ของโครโมโซม, โครโมโซมที่มีส่วนที่เกินแทรกระหว่างเนื้อโครโมโซม และโครโมโซมเครื่องหมายที่ไม่ทราบแหล่งที่มาของความผิดปกติของโครโมโซม ที่ด้วยวิธีการศึกษานี้ประสบความสำเร็จ ในการวินิจฉัย การสลับที่ของโครโมโซม X และ 1 (chromosome X;1 translocation) ถึงแม้ว่าในการศึกษานี้จะไม่สามารถบอกความแน่นอนระดับแถบอย่างแม่นยำในกรณีที่โครโมโซมมีส่วนหนึ่งของโครโมโซมเพิ่มเติมขึ้นมาจากสภาพปกติ วิธี “micro-FISH” สามารถนำมาใช้หาที่มาของชิ้นส่วนของโครโมโซมที่เกิดจากการจัดเรียงผิดพลาดแบบไม่สมบูรณ์ได้

คำสำคัญ: Chromosome painting, Micro-FISH, Chromosome microdissection, DOP-PCR, Chromosomal marker

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ORIGINAL ARTICLE

Delineation of chromosome abnormalities in Thai patients by chromosome microdissection

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Abstract

Chromosome microdissection and *in vitro* amplification of dissected chromosomal fragments, followed by labeling and fluorescence *in situ* hybridization (FISH) to normal metaphase chromosomes or "micro-FISH" can be used to identify marker chromosomes for both prenatal and postnatal cytogenetic analysis. The primary objective of this study is to identify the origin of marker chromosomes in three Thai patients with chromosomal disorders. Secondary objective is to apply this method for routine laboratory practice. The method involves the microdissection of five to ten fragments of the related chromosomes from a GTG-banded metaphase spread. Then, the dissected chromosomal fragments were amplified using degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR). The PCR products were labeled by PCR with digoxigenin-11-dUTP. Digoxigenin-labeled probes were detected with mouse anti-digoxigenin and the hybridization signal was detected by anti-mouse immunoglobulin conjugated with Alexa 488. We demonstrated the advantage of this approach in routine clinical cytogenetic testing for the analysis of cases involving a translocation, an insertion or a marker chromosome with an additional material of unknown origin. The micro-FISH probe was used successfully to determine the X and 1 translocation chromosome unidentifiable by conventional cytogenetic (GTG-banded) analysis. This study could not exactly detect the band that resulted from duplication of itself. For conclusion, micro-FISH technique provides a possibility to determine the origin of unbalanced chromosomal rearrangements.

Keywords: Chromosome painting, Micro-FISH, Chromosome microdissection, DOP-PCR, Chromosomal marker

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Introduction

Due to more details map of the human genome, chromosomal rearrangements are more frequently associated with clinical disorders. It is well known that classical banding techniques are difficult to determine the rearrangement of complex chromosomes because of limitation on banding resolution, a skill acquired with experience over time and not easily amenable to automation. For the conformation of chromosome identification and elucidation of the origin of marker chromosomes and complex chromosome rearrangements, it has become routine to utilize chromosome-specific painting probes. Chromosome microdissection and the reverse painting fluorescence *in situ* hybridization (FISH) technique is one of the most useful methods for the identification of structurally abnormal chromosomes.

Marker chromosome (mar) is a structurally abnormal chromosome in which no part can be identified by a routine cytogenetic technique. Whenever any part of an abnormal chromosome can be recognized, it is a derivative chromosome[1]. The incidence of supernumerary marker chromosomes (SMCs) found at prenatal diagnosis varies from 0.4/1000 to 1.5/1000[2-5]. Examples of marker chromosomes include ring, derivative, dicentric, and minute chromosomes. Present literature pointed out that supernumerary marker chromosomes were found in 0.14–0.72/1000 newborns and more common in prenatal testing at 0.65–1.5/1000[6]. The significance of a marker chromosome depends on several factors, including inheritance, mode of ascertainment, origin of chromosome, and morphology as well as structure of the marker. The significance of a marker chromosome depends on several factors, including inheritance, mode of ascertainment, chromosomal origin, and morphology as well as structure of the marker.

In this study, chromosome microdissection, DOP-PCR and FISH based method were applied for rapid analysis of the origin and constitution of cytogenetically visible chromosome rearrangements detected by standard cytogenetic method in Thai patients.

Materials and Methods

Subjects and clinical report

The PHA-stimulated human lymphocyte suspensions from three patients were previously stored at -20°C as fixed cells in fixatives (3:1 methanol:acetic acid). These specimens were remaining samples obtained from routine service of Human Genetics Unit, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok. The patients were previously diagnosed as having chromosomal disorder based on clinical manifestation and abnormal karyotypes. The study was performed following the approval of Ramathibodi Institutional Review Board.

Patient A, a 3 year and 11 month-old Thai girl presented with delayed development. Growth parameters were body weight of 13.7 kg (25th centiles) and height of 92 cm (3rd centiles). Physical examination revealed short stature, slightly blue sclerae, left cup-shaped ear, and low anterior hairline. She also had problems with hyperactivity and aggressive behavior. The patient was born prematurely by caesarean sections at eight months of gestational ages due to ruptured amniotic membrane. Birth weight was 2,350 gram. The initial karyotype analysis was 46,X,der(X),t(X;?1)(q26;?q25). CGG repeat analysis of FMR1 gene for fragile X syndrome reveled normal result. Renal ultrasonography and echocardiography revealed no congenital kidney, urinary, and cardiac defects. The maternal and paternal karyotypes showed 46,X,t(X;1)(q26;q25) and 46,XY, respectively.

Patient B was a 4-year-old boy who presented with globally delayed development and cyclic vomiting. He also was diagnosed with hiatal hernia and gastroesophageal reflex disease (GERD). Physical examination revealed mild hypotonia, otherwise unremarkable. Magnetic resonance imaging (MRI) of brain revealed mildly and globally decreased amount of white matter

and normal myelination. Plasma ammonia level, plasma amino acid and urinary organic acid profiles were normal, ruling out disorders of amino acid and organic acid metabolisms. Chromosome analysis of 12 GTG-banded metaphase cells of the 400-550 BPHS level, and examination of an additional 20 cells revealed a 46 chromosomes with apparently male karyotype that had a material of unknown chromosomal fragment inserted into the long arm of chromosome18 at band 18q21.1, designated as 46,XX,ins(18;?)(q21.1;?). The maternal and paternal karyotypes showed 46,XX,add(18)(q21.1) and 46,XY, respectively.

Patient C, a 23-year-old Thai woman who was 23-week-pregnant with her first pregnancy presented with fetal cardiac anomaly detected by routine prenatal ultrasound. Fetal echocardiogram showed common atrium, single atrioventricular (AV) valve, and single ventricle. Chromosome analysis of 11 GTG-banded metaphase spread of the 400-550 BPHS level, and examination of an additional 24 cells revealed a 46 chromosomes with apparently female karyotype that had a material of unknown chromosomal fragment attached to the long arm of chromosome 7 at band q22, designated as 46,XY,add(7)(q22). The parental karyotypes were not investigated.

Metaphase chromosome preparation

Metaphase spreads were prepared according to routine procedures on a cover slip (22x54 mm.). To avoid contamination, cover slips were previously soaked in 1% HCl and treated with absolute ethanol for at least 30 min prior to use. Immediately, drop the cell suspension on the cover slip and air dry. Then, the cover slip was rinsed with distilled water and stored in absolute ethanol at -20°C[6]. The metaphase chromosomes were GTG-banded before microdissection.

Microdissection and Amplification of dissected chromosomes by DOP-PCR

Microdissection was performed with glass microneedles. The microneedle was controlled by a manual hydraulic micromanipulator (MM-188, Narishige, Japan) on an inverted microscope (Nikon Diaphot, Japan). Five to ten chromosomes were collected in 20 μl collection drop (containing 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3 and 0.1 mg/ml gelatin). The dissected fragment was pre-amplified by DOP-PCR reactions and tested according to slightly modified protocol of Engelen et al.[6]. The DOP-PCR experiments contained a negative control consisting of all PCR components except microdissected DNA and a positive control with 25 pg total healthy human DNA with normal karyotype. DOP-PCR products were smear ranging between 100-1,000 base pair (bp), and the bulk of products are approximately 400 bp on a 2% agarose gel. Subsequently, the PCR was done using universal primer 5'-CCGACTCGAGNNNNNN ATGTGG-3' [7].

Generation of chromosome painting probes, FISH and image processing

The PCR products were precipitated, purified and PCR-labeled using 1 mM digoxigenin (DIG)-11-dUTP (Roche, Germany). For the DOP-labeling-PCR, twenty cycles were performed at 94°C for 1 min, at 56°C for 1 min, and at 72°C for 30 sec, with a 3 min final extension at 72°C.

Metaphase spreads for FISH were prepared from lymphocytes using standard methanol: acetic acid (3:1) fixation[8]. Digoxigenin-labeled probes were detected with mouse anti-digoxigenin (Roche Diagnostics GmbH, Mannheim, Germany). Then, the hybridization signal was detected by anti-mouse immunoglobulin conjugated with Alexa 488 (Invetrogen, UK). Finally, the slides were mounted with antifade solution containing 4', 6'-diamino-2-phenylindole (DAPI II, Roche Diagnostics GmbH, Mannheim, Germany). Results were compared with inverted DAPI images of the same metaphase chromosomes[9].

Determination of hybridization specificity

In a trial study, the normal chromosome 1 probe was accurately hybridized to chromosome 1 in

normal male metaphase cells (**Figure 1**). After the result of this experiment was successful, we applied the micro-FISH to identify the abnormal chromosomes in the studied cases. The specificity of the probe was checked by hybridization with microdissected probe from the patient's marker chromosomes to metaphase cells from the same patient, and resulted in positive signal on only abnormal chromosome in this patient.

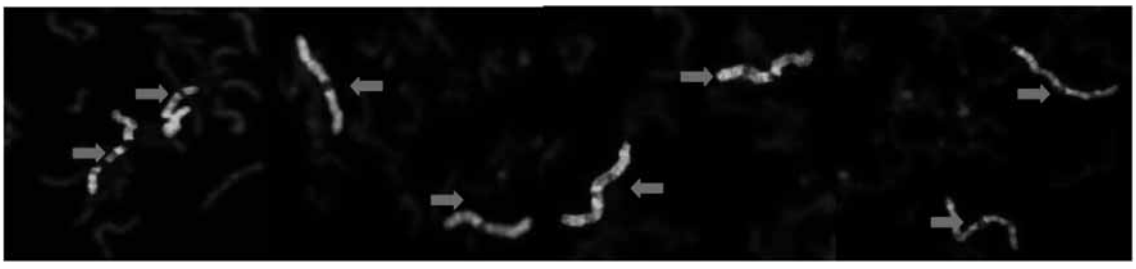

Figure 1 Hybridization specificity of dissected chromosome 1 probe. Four Metaphase cells of a healthy male after hybridization with normal chromosome 1 probe. The microdissected probe showed specific painting signals on the whole length of the chromosome 1 (arrow).

Results

The karyotypes and metaphase FISH results of Patients A, B, and C were shown in **Figures 2-4**. The probe produced from the derivative chromosome X from Patient A was shown to hybridize with signal on whole length of the abnormal X chromosome, the normal X chromosome from pter-q26, and both normal chromosomes 1 from q25-qter on the patients' metaphase chromosomes (Figure 2C and D). Based on this result, The karyotype for Patient A is revised to 46,X,der(X)t(X;?1)(q26; ?q25).rev ish 46,X,t(X;1)(q26;q25). This probe was shown to hybridize to normal X chromosome from pter-q26 and on both normal chromosomes 1 from q25-qter on normal metaphase chromosomes of healthy male control (Figure 2E & F).

The produced probe from the abnormal chromosome 18 [ins(18;?)(q21.1;?)] of patient B showed hybridization signals on the whole length of the abnormal chromosome 18 and the whole length of the normal chromosome 18 of patient B (Figure 3C & D), and the two normal chromosomes 18 of metaphase chromosomes from a normal healthy control (Figure 3E & F). These results cannot elucidate the origin of abnormal chromosome segment that is inserted to chromosome 18 at band 18q21. To verify the accuracy of the microdissected probe, we prepared another microdissected probe from normal chromosome 18 and hybridized this probe onto metaphase cells from normal male control and patient B. Hybridization signals were observed on the normal and abnormal chromosomes 18 from the patient, and on the entire chromosomes 18 of the control (Figure 3G & H). There was no negative signal on the abnormal chromosome 18 from the patient.

The probe derived from abnormal chromosome 7 [add(7)(q22)] was hybridized onto the patient's metaphase cells, which showed signal on the whole length of the normal and abnormal chromosomes 7 (Figure 4C & D). Hybridization between this probe and metaphase cells of normal healthy control showed signal on the whole length of normal chromosomes 7 (Figure 4E & F). Because this result cannot determine the origin of abnormal chromosome 7 segment that attached to 7q22, an additional probe from normal chromosome 7 was produced and hybridized to metaphase cells from a normal male control and the patient. Hybrdization signals were shown on entire chromosomes 7 of the control individual and the patient (Figure 4G & H).

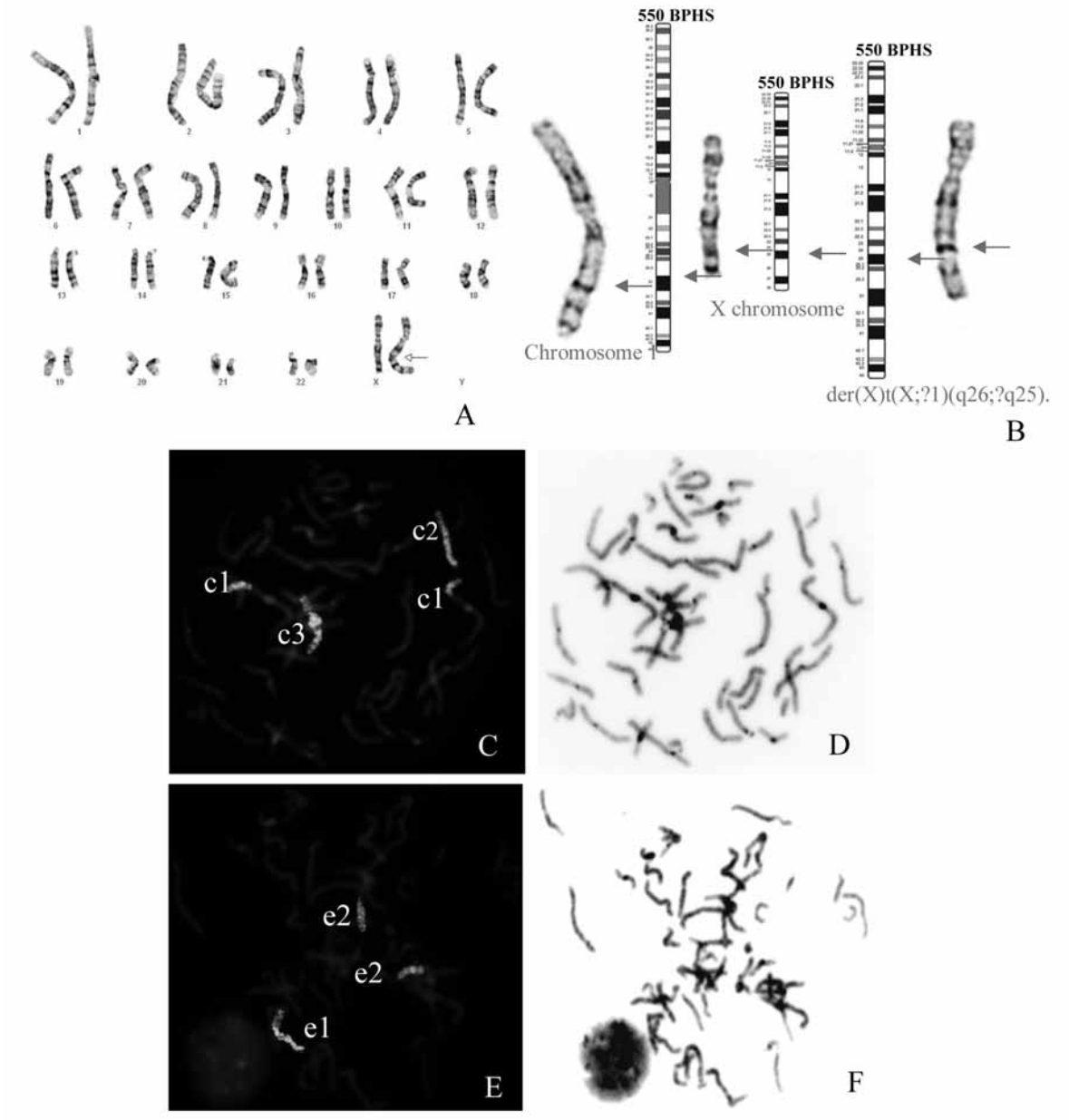

Figure 2 Patient A: karyotype and FISH

A. GTG-banded karyotype of 46,X,der(X)t(X;?1)(q26;?q25)

B. Comparison of GTG-banded chromosomes and ideograms of normal chromosome 1, chromosome X and derivative chromosome X. Arrows indicate the break points.

C. The microdissected probe of abnormal chromosome X was hybridized onto abnormal metaphase from patient A. Hybridization signals were shown on whole length of the abnormal X chromosome (c3), the normal X chromosome from pter-q26 (c2), and both normal chromosomes 1 from q25-qter (c1).

D. Inverted DAPI counterstaining of Figure 2C.

E. Hybridization between the microdissected probe of abnormal chromosome X and normal metaphase chromosomes from a normal male control. Hybridization signal are shown on the normal X chromosome from pter-q26 (e1) and on the normal chromosomes 1 from q25-qter (e2).

F. Inverted DAPI counterstaining of Figure 2E.

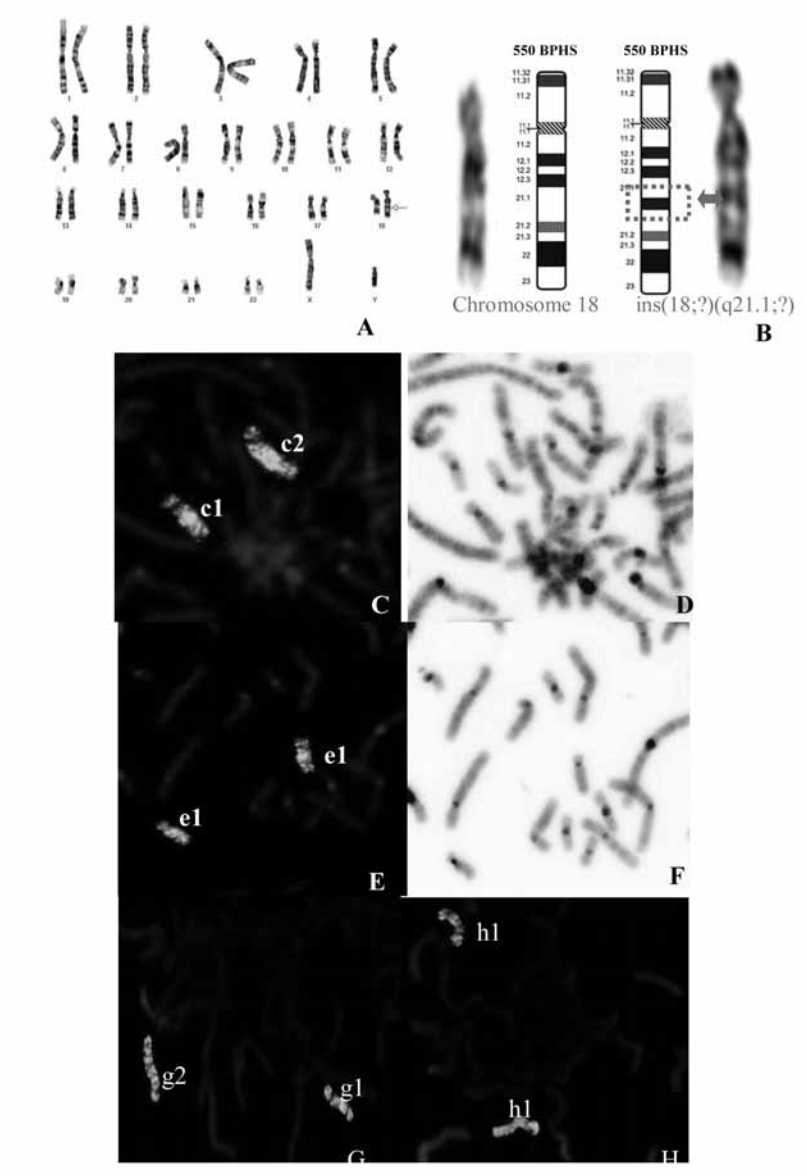

Figure 3 Patient B: karyotype and FISH

A. GTG-banded karyotype of 46,XX,ins(18;?)(q21.1;?)

B. Comparison of GTG-banded chromosomes and ideograms of normal chromosome 18. Arrows indicate the insertion point.

C. The microdissected probe of ins(18;?)(q21.1;?) was hybridized on the metaphase of patient B. The results show painting on the normal (c1) and abnormal (c2) chromosomes 18.

D. Inverted DAPI counterstaining of Figure 3C.

E. The microdissected probe of ins(18;?)(q21.1;?) was hybridized on normal metaphase chromosomes. The hybridization signals reveal painting on both of the entire chromosome 18 (e1).

F. Inverted DAPI counterstaining of Figure 3E.

G. The microdissected probe of normal chromosome 18 was hybridized on metaphases from patient B. The hybridization results show painting on normal (g1) and abnormal chromosomes 18 (g2) of the patient.

H. The microdissected probe of normal chromosome 18 was hybridized on normal metaphase. The hybridization signals are shown on both chromosomes 18 (h1).

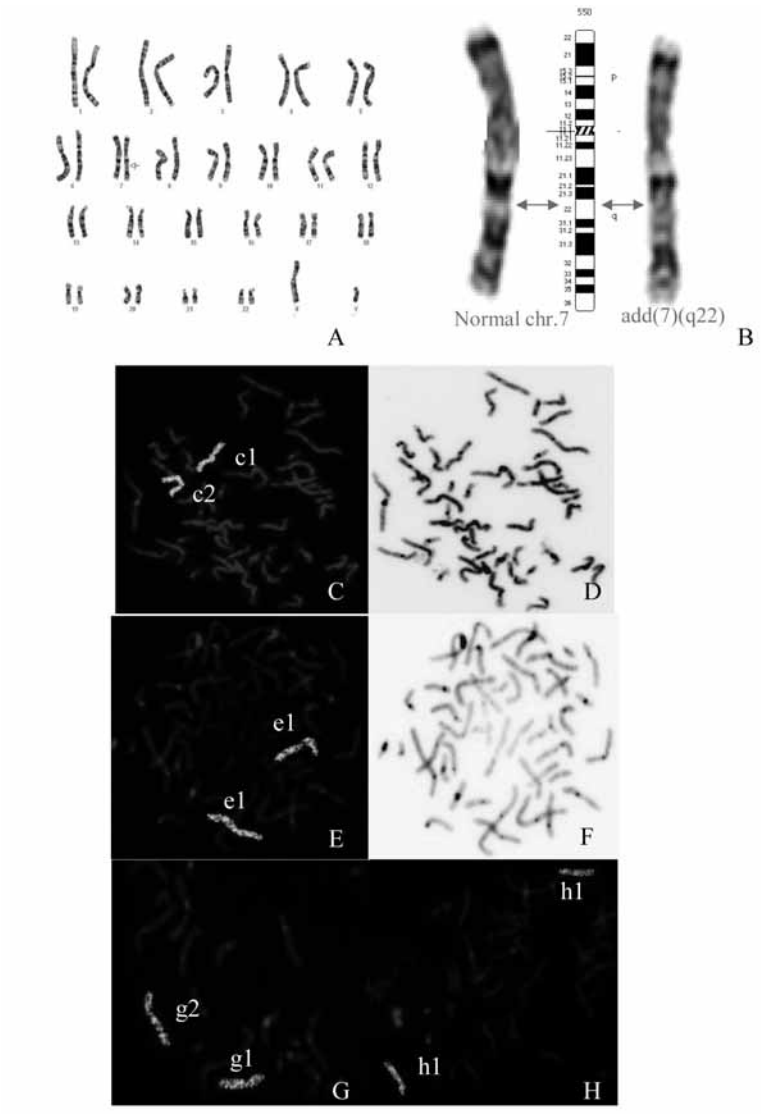

Figure 4 Patient C: karyotype and FISH

A. GTG-banded karyotype of 46,XY,add(7)(q22).

B. Comparison of GTG-banded chromosomes and ideograms of normal chromosome 7. Arrows indicate the addition.

C. The microdissected probe of add(7)(q22) was hybridized on metaphase from patient C. Hybridization signals show painting on the whole length of normal (c1) and abnormal chromosome 7 (c2).

D. Inverted DAPI counterstaining of Figure 4C.

E. The microdissected probe of add(7)(q22) was hybridized on normal metaphase chromosomes. Hybridization signals are shown on the entire chromosomes 7 (e1).

F. Inverted DAPI counterstaining of Figure 4E.

G. The microdissected probe of the normal chromosome 7 was hybridized on abnormal metaphase of patient C. Hybridization signals show painting on the whole length of normal (g1) and abnormal chromosome 7 (g2).

H. The produced probe of the normal chromosome 7 was hybridized on normal metaphase of patient C. Hybridization signals are shown on the entire chromosomes 7 (h1).

Discussion and Conclusion

In this study, we demonstrate the use of combined micro-FISH technique, chromosome microdissection, DOP-PCR, and reverse chromosome painting to determine the origin of chromosomal abnormality in three patients. Patient A involved unbalanced translocation of chromosome X and 1 in which was uncertain by conventional banding techniques. Taken together of the micro-FISH results and G-banding analysis, it was suggested that the revised karyotype of this patient is 46,X,der(X)t(X;?1)(q26;?q25).rev ish 46,X,t(X;1)(q26;q25). Clinical phenotypes of Patient A are consequences of trisomy for 1q25-qter and monosomy for Xq26-qter.

Translocations between X chromosome and autosome or [t(X;A)] is a rare rearrangement with estimated incidence of 1 to 3/10,000 live births[10]. The deletion of Xq25-qter or Xq26-qter often resulted in normal stature and premature menopause/premature ovarian failure[11-13]. Pure trisomy for distal 1q has been rarely reported and often resulted in significant dysmorphic features, congenital malformations of multiple organs such as heart and genitourinary tract, and severe psychomotor retardation[14-16]. Partial trisomy of 1q25-qter is likely a lethal condition unless the additional segment is translocated to an X and thus at least partly inactivated[17]. Generally, there is a preferential X inactivation of the apparently normal X chromosome in individuals with balanced X/autosome translocation[18]. However, for those with unbalanced X/autosome translocation, the derivative X may be preferentially inactivated in order to rescue the severe phenotypes resulted from partial trisomy of the translocated autosomal segment[18].

There is a handful report of translocation between chromosomes distal Xq and distal 1q. Yatsenko et al., described a de novo unbalanced translocation, [46,X,der(X),t(X;1)(q28;q32.1)], in a 9-month-old girl who presented with mild dysmorphic features and developmental delay without malformation of major organs[19]. FISH analysis confirmed monosomy for Xq28-qter and trisomy for 1q32.1-qter in the patient. Late replication of the derivative X was observed in 80% of cells analyzed by replication studies, indicating that the majority of the translocated 1q segment was also inactivated along with the process of X inactivation of the derivative X chromosome. They concluded that preferential inactivation of the extra 1q segment likely resulted in mild phenotypes of their patient[19]. Collins et al., reported *de novo* unbalanced translocation [46,X,der(X),-t(X;1)(q24;q31.1)] in a 5-year-old girl with multiple congenital malformations and developmental delay[20]. The translocated 1q segment remained active in the tissue studied, which may explain the more severe phenotypes in their patient as a result of functional trisomy for distal 1q[20].

The affected female patients, pure trisomy for distal 1q, had minor dysmorphic features including low-set ears, downslanting palpebral fissures, high palate, abnormal dermatoglyphic patterns, and mental retardation without major anomalies[17]. A 3-year-old girl with partial trisomy for 1q25.3-qter and partial monosomy for distal Xp born to a t(X;1) translocation carrier mother, was found have short stature, hypertrichosis, and other signs of de Lange syndrome[21]. Another a 6-year old girl with psychomotor retardation and minor physical abnormalities, had an unbalanced X and 1 translocation which resulted in partial trisomy 1q and partial monosomy for Xp[22].

Patient A has milder phenotypes similar to the cases reported by Yatsenko et al, but in contrast to the case described by Collins et al[19-20]. It is highly probable that the mild phenotypes in patient A is a result of inactivation of the majority of the translocated 1q segment due to preferential X inactivation of the derivative X. This can be answered by replication study and/or X inactivation analysis, of which unfortunately were not performed in the present study. Patient A

is predicted to have premature ovarian failure in the future as those often seen in patients with distal Xq deletion, either pure monosomy for distal Xq or in combination with partial autosomal trisomy[11-13].

In patients B and C, the reverse whole chromosome painting (WCP) using probes derived from the responsible patients and normal control revealed no difference in hybridization signals on the metaphase cells of the patients and the control. This data in conjunction with the absence of negative signal on the abnormal chromosome 18 from patient B, and on the abnormal chromosome 7 from patient C, suggest that the inserted segment on chromosome 18 (of patient B) is potentially the portion of chromosome 18, and the origin of this attached segment (in patient C) is potentially part of the chromosome 7. This suggests the limitation of the reverse WCP using micro-FISH method used in the present study that it may not be sufficient to characterize the unknown origin of the extra chromosomal material. To further identify the origin of the unknown segment, additional methods such as high resolution (prometaphase) banding, micro-FISH with band-specific probe, comparative genomic hybridization (CGH), and/or CGH array may be necessary for investigation of the patients' and/or the parental karyotypes.

We demonstrate that the micro-FISH technique is a rapid method and easy to perform. Only 5-10 copies of each chromosome are needed to be dissected in order to prepare a WCP, and the preparation of specific fluorescence probe can be accomplished in less than 24 hours. Metaphase spread prepared from 2-4 year-stored cell pellet in standard fixative mixture can be successfully used for microdissection. This strategy is a reliable method to establish a relationship between genotype and phenotypes, and more accurate result obtained the more comprehensive genetic counseling can be offered to the patients and their families.

In summary, this study emphasizes the value of using micro-FISH in combination with conventional banding techniques as an alternative method to determine the origin of chromosomal rearrangements in clinical cytogenetic diagnosis. However, there is also limitation in obtaining the precision of chromosomal segment inserted/added in the chromosomal rearrangement. Additional testing may be required to refine the involved chromosomal segment.

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