行政院國家科學委員會專題研究計畫 成果報告

著絲點 DNA 之基因組構造，演化及功能的研究
研究成果報告(精簡版)

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Introduction:

The centromere mediates normal mitotic and meiotic process including kinetochore assembly, spindle attachment, sister chromatid cohesion and subsequently proper separation of sister chromatids during mitotic anaphase or segregation of homologous chromosomes during meiosis I (Choo 1997). Centromere malfunction is the leading cause of aneuploidy, classically defined as a deviation of chromosome number from the normal chromosome complement (Hook 1985). Aneuploidy has catastrophic consequences for chromosomal disorders. The epidemiological study has shown that, in human, 45% of spontaneous abortions or 7% of all conceptions are associated with aneuploidy (Jacobs and Hassold 1995), and aneuploidy causes common birth defects (e.g. Down, Klinefelter and Turner syndromes). Aneuploidy is also tightly correlated with almost all types of cancer: there are >84,000 documented cases of abnormal karyotypes associated with human neoplastic disorders (Vig et al. 1989; Mitelman 1994). Detail studies in the structure and molecular building blocks of a centromere would be essential for understanding its crucial role in maintaining the exact chromosome complement of daughter cells or offsprings.

However, centromeric regions of eukaryotic chromosomes still present an enigma that a conserved centromere function is in the face of rapidly evolving centromeric DNA sequence (Sullivan 2001). Each species studies to date, from yeast to worms to flies to humans, the DNA sequences found in the centromere region are composed of quite diverse satellite DNAs. One still could speculate that a specific conserved DNA sequence, like the centromeric DNA of the budding yeast, exits to assemble kinetochore proteins and then to form a functional centromere in higher eukaryotypes. In order to unveil a specific conserved centromeric DNA fundamental for a functional centromere, comparative sequence analysis of the centromere regions in various species is urgently necessary. The full DNA sequences in centromere region in most mammalian species remain unknown. Only the centromeric sequence of human is almost completed (Schueler 2001) by the time the human genome project had been finished (Venter 2001). Never the less, the competent centromeric sequences analysis from human X chromosome revealed as a functional centromere DNA. This may serve as a model for the comparative sequence analysis of centromere loci in other mammalian species for the search of functional centromeric DNA in that species. Consequently, it will provide better insights into the evolution of cenromeric DNA that leads to the formation of currently active centromere.

Almost as a rule, the repetitive satellite DNAs is the main composition of centromere and centromeric DNA sequences diverse among species. Generally, most similar satellite DNAs are restricted to closely related species. In other words, the related (or congeneric) species share a "library" of similar related satellite sequences, some of which could be amplified onto a major satellite DNA in some particular species (Salser et al. 1976; Meštrović et al. 1998). The

evolutionary dynamics of satellite DNAs could be a driving force for the speciation process, thus forming a species-specific profile of satellite DNAs (Ugarković and Plohl 2002). The species-specific profile of centromeric satellite DNA is possible to be demonstracted by means of comparative genomic studies that showed a large fraction of centromeric sequences were repositioned either by independent chromosomal rearrangements or by *de novo* centromere emergence in the independent lineage to become unique to a species (Murphy et al. 2005). Therefore, mapping and sequencing of centromeric DNAs from phylogenetically closely or divergent species will provide a better understanding on the mechanism for centromeric satellite DNA evolution. Furthermore, it could also unravel the puzzle of a functional conserved centromere in the face of rapidly evolving centromeric DNA sequence.

BAC library has tremendous potential to facilitate such studies to shed light on the temporal relationships of extant species by comparative sequence analysis and cytogenetic mapping of genome rearrangement, expansion, and loss (Gomyo et al. 1999; Srinivasan et al. 2002). In addition to decipher genome evolution, BAC resources could be used to address specific questions pertaining to centromeric and pericentromeric evolution, and the mechanisms of karyotypic change between closely related species. The Indian muntjac (*Muntiacus muntjac vaginalis*) has the lowest chromosome number in mammalian species with 6 chromosomes in female and 7 in male. Recent molecular cytogenetic studies landed support to the hypothesis that the karyotype of the Indian muntjac could evolve from an ancient deer species with a karyotype 2n=70 through extensive tandem fusions and several centric fusions (Li et al. 2000). Moreover, the centromere of X+3 chromosome of the species is compound and unique. Therefore, it is an ideal species for studying chromosome rearrangement, comparative genomic and karyotype evolution. Thanks to the constructed BAC library of Indian muntjac in our lab; therefore, it is availability to explore the following studies: the centromeric evolution and function, the underlying mechanism of tandem fusion of Indian muntjac chromosomes, the chromosomal rearrangement, and the karyotype evolution.

Specific Aim:

Studying the genomic organization of Indian muntjac centromere

Systematically sequencing and mapping the genomic organization of Indian muntjac centromeric BAC clones would make an effort in identifying candidate functional centromeric DNAs of Indian muntjac.

Method:

Systematically sequencing:

The hierarchical sequencing strategy was used to sequence the centromeric BAC clone. First, the centromeric BAC DNA was digested into smaller fragments by two different restriction enzymes separately. All restriction fragments then were subcloned. All subclones were sequenced. Second, Paired-end sequencing was used to connect each sequences of subclone.

Computational analysis and comparative sequences analysis:

Sequences of centromeric DNA will be aligned with GenBank database using the BLAST programs for searching the identified satellite DNAs elements. Tandem repeats will be determined by single-base-shift self-comparison (Pluciennicza k et al. 1982). Palindromic and mirror repeats will be identified by eye. The interspersed repeats and low complexity DNA sequences will be screened using RepeatMasker program from http://www.repeatmasker.org/. The composition of sequences will be determined using nucleic acid statistics programs from Biological Workbench (http://workbench.sdsc.edu/).

Comparative sequence analysis will be performed in order to understand the evolution of centromere sequences. This will be achieved by comparing the above identified muntjac centromeric sequences with cenromeric sequences of different species that had been deposited in the GenBank database.

Physical mapping the subclones of BAC:

All subclones, with exception of those containing the known satellite DNA elements, from BAC clones of interest will be mapped onto Indian muntjac chromosomes by FISH experiment for identifying the chromosome localization. BAC-fiber FISH will be also conducted using every subclones as FISH probes for confirming the order of assemble based on the sequences. Two continuous subclones will be labeled with different fluorochromes as FISH probes to hybridize the chromatin fiber for identifying the high resolution genomic organization. .

For BAC-fiber FISH, the freshly isolated BAC DNA is fixed onto the poly-L-lysine-coated slide by heating at 65^{o}C for 10 min. For chromatin-fiber FISH, the cells are fixed onto the slide and then soak in lysis buffer to release the chromatin fiber. The FISH will be performed using digoxigenin labeled BAC vector, biotin-labeled subclones, and FITC labeled the other subclones as probes. The digoxigenin labeled probe is detected with cy5-conjugated anti-DIG antibodies, while the biotin labeled probe is detected with AF568-conjugated avidin. Finally, samples are mounted in antifade mounting medium with DAPI (vector). Fluorescent signals are captured under an Olympus BX51

fluorescence microscope equipped with appropriate filter sets and a cooled CCD camera (Photometrics, Sensys). The FISH image will be normalized and enhanced using the IPlab software. The detailed FISH protocol had been established in our laboratory (Li et al. 2002).

Results and discussions:

I. Construction of an Indian muntjac BAC library and production of the most highly density FISH map of the species

The frequency of clones with inserts was 88% and thus this library corresponds to approximately 4X coverage of Indian muntjac genome. The individual chromosomal location of 2,242 BAC clones on the Indian muntjac metaphase chromosomes were identified by fluorescence in situ hybridization (FISH). Among these clones, 2,107 BAC clones were mapped onto specific loci and 135 BAC clones were mapped onto the centromeric region. This provided the most high density FISH BAC clone map for the species. This dense ordered map could be used as a blueprint for comparative FISH mapping studies of other deer species in order to understand the mechanism of genomic rearrangement and karyotypic evolution. Moreover, the centromeric BAC clones will provide an excellent resource for studying the structure and function of mammalian centromeres. **The result had been published in Zoological Studies 47:282-292 (2008).**

II. Studying the genomic organization of Indian muntjac centromere

In our preliminary BAC mapping study, four BAC clones with the parallel signals on the kinetochore position by FISH experiment were observed (Fig 1). This special parallel signal of BAC clones probably interprets that these BAC clones contain a DNA sequence likely to be responsible for centromere function by associating with kinetochore proteins. Therefore, it is very meaningful to further characterize the genomic organization of these BAC clones by full sequencing. A hierarchical sequencing was performed. First, 17 *Eco*RI digesting fragments were subcloned into pBSK vector and then were sequenced. Second, all sequenced subclones were aligned (Fig 2). The sequence result showed that the BAC clone contained not only cervid satellite II and IV DNAs but also other sequences, such as long interspersed nucleotide elements (LINEs), short interspersed nucleotide elements (SINEs), long terminal repeats (LTRs), other DNA elements and unidentified sequence. It suggested that the centromeric DNA organization is complex than it was expected. Furthermore, EMSA and the artificial chromosome assay will be performed for identifying the critical sequence for centromeric function.

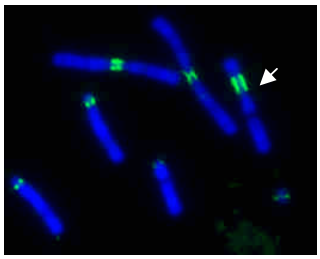

Fig.1: 1296A1 BAC clone DNA was hybridized onto Indian muntjac chromosomes. The FISH signals show parallel pattern on the lateral sides of the primary constriction where kinetochore located.

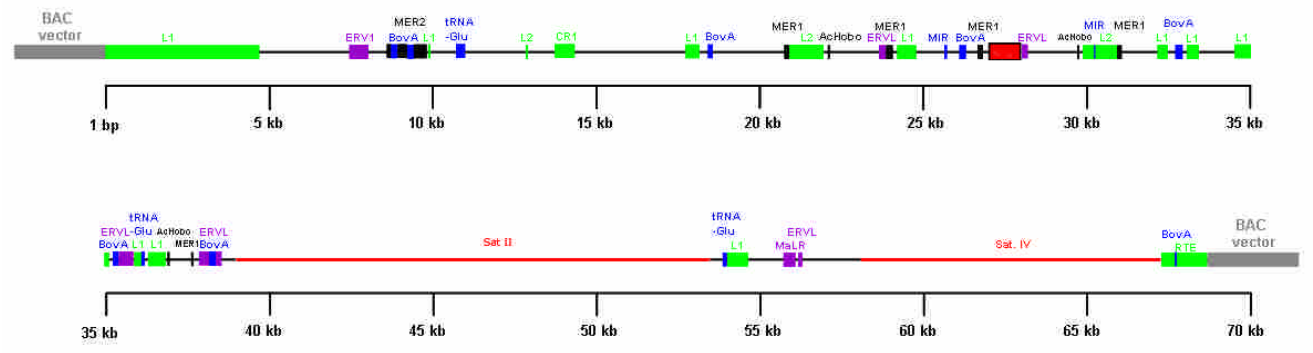

Fig 2. Sequence organization of the Indian muntjac centromeric BAC DNA clone. The sequences contains satellite tracks (red arrows), LINEs (green), SINEs (blue), LTR elements (purple), and DNA elements (black).

Self-evaluation:

In this year's project, our major aim is to studying the genomic organization of Indian muntjac centromere. We had constructed 4X coverage of Indian muntjac BAC library and mapped 2242BAC clones onto the Indian muntjac metaphase chromosomes by fluorescence in situ hybridization (FISH) at the previous project. This part of result had been published in **Zoological Studies 47:282-292 (2008).** In this study, we further characterized the centromeric BAC clone to understand genomic organization of centromere. This result would shed light on the genomic organization of centromere. It is not easy to be identified the centromeric sequence, because of highly repeatitive DNA sequence. Therefore, it was taken much time to identify the centromeric sequence. All together, we should have achieved the progress of the project.

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出席國際學術會議心得報告

計畫編號	NSC96-2311-B040-004
計畫名稱	著絲點 DNA 之基因組構造，演化及功能的研究
出國人員姓名 服務機關及職稱	李月君 中山醫學大學 生物醫學科學系教授
會議時間地點	October 23-27, 2007; 美國加州聖地牙哥
會議名稱	第 57 屆美國人類遺傳學年會
發表論文題目	Identification of marker chromosomes using FISH-based technology and DNA polymorphic marker

一、參加會議經過

第一天下午辦理報到

第二天早上張貼海報及觀賞其他海報內容，下午參觀並聽取數十家廠商的新產品簡介及應用。

第三天早上聽了兩場有關 array CGH 及 CNV 的演講，下午展示海報。

第四天早上聽了一系列有關染色體異常的臨床表徵的演講，下午觀賞海報內容。

第五天早上觀賞海報內容。

二、與會心得

This meeting mainly presents research in human genetics, genomics and molecular biology to make participants be able to :1) discuss the research underway and /or the current topics relevant to their areas of interest in human genetics, 2) demonstrate a gained level of insight into the methods being used by researchers and practitioners in this field, and 3) describe a personal exposure to several stimulating areas of inquiry with speakers in related areas.

I listened to several speech regarding the chromosomal phenotype and laboratory's techniques in the diagnosis of genetic disorders. In the session of chromosomal phenotype, I gain an understanding of the suspected critical regions for spectrum autism. I also realized how a difference in breakpoints in these syndromes will lead to different phenotypes and what suspected mechanism may result in these deletions. Additionally, I learned more new etiology and the pathogenesis of genetic disorders that I never learned before. I truly gain a lot of knowledge in clinics. It is helpful for my research in future. Moreover, I listened to two literatures regarding to the CNV studies.

I also met and discussed with several directors of cytogenetics in US. This discussion also let me realize that array CGH technique had been applied to diagnose the genetic disorders in the some cytogenetic labs in state.

Appendix： 壁報論文

Identification of marker chromosomes using FISH-based technology and DNA polymorphic marker

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Abstract:

A marker chromosome (mar) is a structurally abnormal chromosome in which no part can be identified with conventional cytogenetic techniques. Because of its unclear origin, characterization of marker can be labour intensive and time consuming. It may cause anxiety on the patients and became a challenge for prenatal genetic counseling. We performed SKY and /or FISH to identify a total of 33 cases (20 cases are prenatal) of marker chromosomes. In some of the cases, STR markers were also used to identify the parental origin of the marker chromosomes. All but 3 cases the origin of markers can be unequivocally identify (success rate: 90.9%). Among 30 cases identified; 10 cases were derived from chromosome 15 (33%), 5 cases from chromosome 22 (16.7%), 3 cases from chromosome 18 (10%), 3 cases from X chromosome (10%), 2 case from chromosome 9 (6.7%), 2 case from Y chromosome (6.7%) and 1 case each from chromosomes; 2, 3, 10, 14 and 21 and 14/22 (3.3%).

Three of cases studied had been reported (Chen et al. 2004; Lin et al. 2006; Chen et al. 2006). The study demonstrated the importance of clinical cytogenetic and molecular analysis for diagnosis of marker chromosome and showed how crucial it was for prenatal counseling and management of the pregnancies.

Introduction:

A marker chromosomes (mar) is a structurally abnormal chromosome in which no part can be identified with conventional cytogenetic techniques (ISCN, 1995). Marker chromosome account for about 1:1,100 cases at amniocentesis, 1:4,000 in live born and approximately 1:400 in mentally retarded patients. The chromosomal origin and composition of the marker chromosome are directly associated with the clinical manifestations of the carrier. However, prenatal identification of the marker has been hampered since conventional cytogenetic techniques often fail to determine the marker origin. Because of its unclear origin, characterization of marker can be labour intensive and time consuming. It may cause anxiety on the patients and became a challenge for prenatal genetic counseling. Recent advances of fluorescent in situ hybridization (FISH) base technique have provided a powerful approach for the identification of marker chromosomes.

Here we report 33 cases of marker chromosomes detected during a 4 years period in our

laboratory. Twenty of those cases (60.6%) are detected at amniocentesis. Using combined approaches of G-banding/FISH, SKY, SKY/ FISH and short tandem repeat marker (STR), we successfully characterized 30 of them (success rate: 91%). We reported 3 of those prenatal cases identified which shed light on the genotype-phenotype correlation and facilitated subsequent genetic counseling and pregnancy management.

Materials and Methods:

Clinical cases: The 33 cases with marker chromosome were initially detected from 10 clinical cytogenetic laboratories nationwide (including our own laboratory) and referred to our laboratory for further characterization. Twenty of the cases were diagnostic amniocentesis samples.

Chromosome Identification: G-banding technique of chromosome analysis was performed following the standard protocol. For further characterization of the marker chromosome, spectra karyotyping analysis (SKY) and FISH study were carried out on slides of chromosome preparation from PHA-stimulated lymphocyte cultures or directly on "*in-situ* coverslip" of chromosome preparation of amniotic cell culture. Human SkyPaint kit containing 24 color chromosome specific painting probes (Applied Spectral Imaging Ltd., Migdal Haemek, Israel) and chromosome region specific DNA probes (e.g. subtelomeric probes and centromeric probes from Vysis, Downers Grrove, IL) were used to identify the origin of chromosome. Procedures used for FISH and SKY analysis were described elsewhere (Lin et al., 2006).

Molecular genetic analysis: The genomic DNA of fetus was isolated from blood samples or from amniotic cell culture. Parental DNAs were isolated from peripheral blood samples. Quantitative fluorescent polymerase chain reaction (QF-PCR) using polymorphic short tandem repeat (STR) markers located on specific chromosome region was carried out to determine the parental origin of the marker chromosome.

Results:

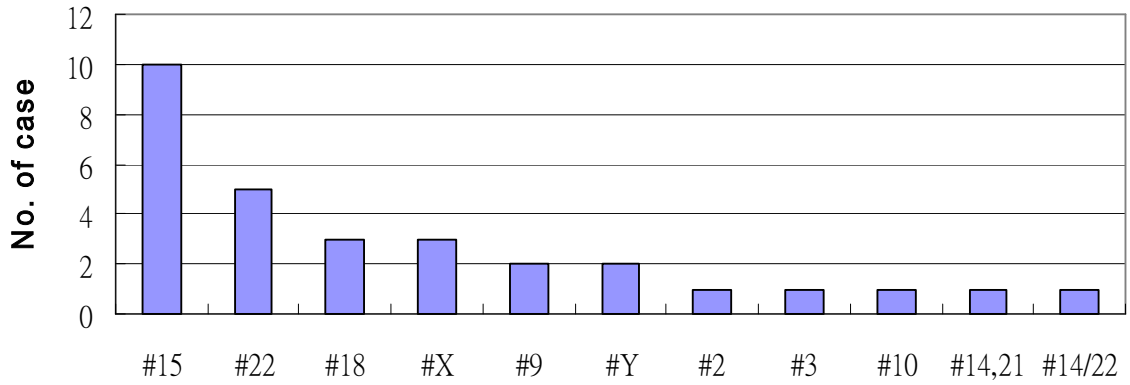
From 2003 - 2006, we performed SKY and /or FISH to identify a total of 33 cases (20 cases are prenatal) of marker chromosomes. In some of the cases, STR marker analysis also used to identify the parental origin of the marker. All but 3 cases of the origin of marker can be unequivocally identified (success rate: 90.9%).

1. Chromosome origin of 30 marker chromosomes identified (Table 1; Fig. 1).

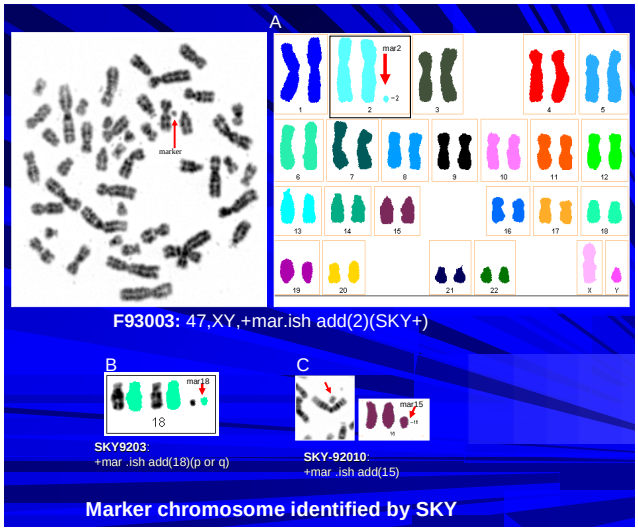
Table 1. Chromosome origin of 30 marker chromosomes

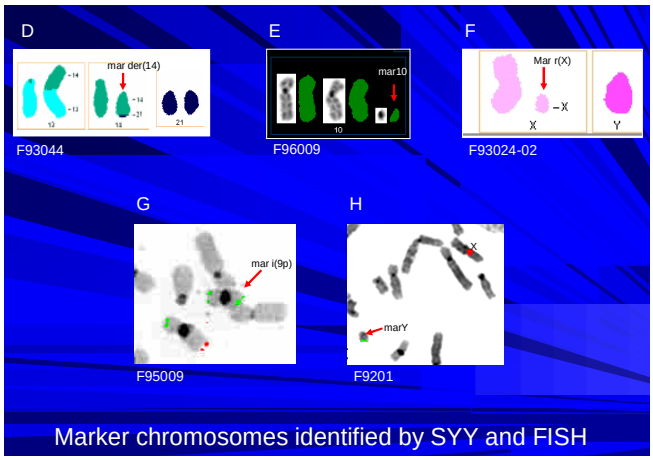
Chromosome origin	Cases	%	Chromosome origin	Cases	%
15	10	33.3	2	1	3.3
22	5	16.7	3	1	3.3
18	3	10.0	10	1	3.3
X	3	10.0	13/21	1	3.3
9	2	6.7	14/22	1	3.3
Y	2	6.7			

Fig. 1. Chromosome origin of 30 marker chromosomes



2. Examples of marker chromosomes identified (Fig. 2).



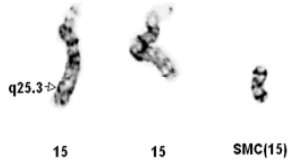


3. Karyotype of patient or fetus with marker chromosome identified (Table 2).

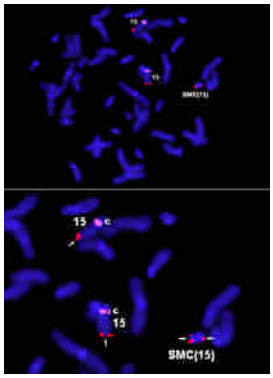
Case No	Lab No	Result
1	**F9201**	46,X,mar.ish der(Y)(DXZ1-,DYZ3+)
2	**F9205**	47,XX,+mar.ish der(15)idic(15)(q11.2)(SKY,D15Z1++,SNRPN-)
3	**SKY9203**	47,XX,+mar ish der(18)(p or q)(SKY+)
4	SKY9207	47,XX,+mar.ish der(15) inv dup(15)(q12) (SKY+,D15Z1++,SNRPN++)
5	**SKY9209***	47,XX,+mar.ish der(22)(SKY+,D22Z4+,CECR7-,CECR2-,BID-,D22S75-)
6	**SKY9210***	47,XY,+mar.ish der(15)(SKY+)
7	**F93003**	47,XY,+mar.ish der(2)(SKY+)
8	F93004	47,XY,+mar.ish del(9)(q13)(SKY+,9pTEL+,9qTEL-)
9	**F93017**	47,XY,+mar . ish der(22)(SKY+, CEP22+)
10	**F93024***	47,XY,+mar.ish r(X)(SKY+, DXZ1+)
11	**F93024-02**	47,XY,+mar.ish der(X)(SKY+, DXZ1+)
12	**F93032**	47,XX, +der(3)t(2;3)(q24;q13)/47,XX,+21 nus ish. 21q22(D21S259x3)[190]/ 21q22(D21S259x2)[10]
13	**F93035**	46,XY,der(13;14)(q10;q10),+mar.ish der(14 or 22)(CEP14/22+)
14	F93040	46,X,mar.ish dic(Y)(q?)(DYZ3++,YpTEL++)
15	F93041	46,X,+r.ish r(X)(DXZ1+)
16	**F93044**	46,XY,der(13;14)(q10;q10),+mar.ish der(14)t(14;21)(q?;q?)(SKY+, 14qTEL-, 21qTEL+)

17	F93051	47,XX,+mar.ish der(22)(SKY+, D22Z1+, BID-)
18	**F93056**	47,XX,+mar.ish i(15)(p10)(SKY+, D15Z4+, D15Z1++, SNRPN-, PML-)
19	**F9305**	47,XX,+mar.ish der(15)(D15Z4+)[1]/46,XX[7]
20	F94004	47XX,+mar.ish der(22)(P109L3-,77H2-)
21	**F94010**	47,XY,+mar.ish der(15)(D15Z4+, D15Z1+, SNRPN-, PML-)
22	**F94013**	47,XX,+mar.ish der(15)(D15Z4+)
23	**F94018**	47,XY,+mar.ish.inv dup(15)(pter→q11::q11→pter) (D15Z4+,D15Z1++, SNRPN-, PML-)
24	F94028	47,XX,+mar.ish der(15)(D15Z4+,D15Z1++,SNRPN-, PML-)
25	F95004	47,XX,+mar.ish der(15) inv dup(15)(q12) (D15Z1++,SNRPN++)
26	F95009	47,XX,+mar.ish i(9)(p10)(9pTEL++)
27	F96002	47,XX,+mar.ish der(22?)(SKY22+, D14Z1/D22Z1, p190.22++)
28	**F96003**	47,XX,t(17;18),+mar.ish der(17;18)(q11.1;q11.2) (17pTEL+, D17Z1+;1 8pTEL+, D18Z1 +, D17Z1 +),+der(18)(D18Z1+)
29	F96004	47,XX,t(17;18),+mar.ish der(17;18)(q11.1;q11.2) (17pTEL+, D17Z1+;1 8pTEL+, D18Z1 +, D17Z1 +), +der(18)(D18Z1+)
30	**F96009**	47,XX,+mar.ish der(10)(SKY10+,CEP10+)

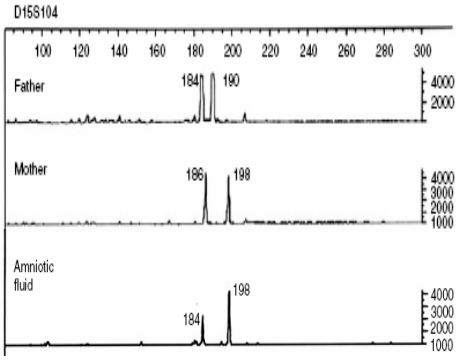
4. Examples of QF-PCR using STR marker for the identification parental origin of marker chromosome (case:SKY9210)



A. G-band analysis detected SMC marker in the fetus.



B. FISH study showed SMC was originated from two copies of distal 15q



C. Representative electrophoretograms of QF-PCR assays at STR markers for distal chromosome 15q using the parental blood and amniocytes showing that the two peaks (184 bp:198 bp) with equal ratio of 1 : 1 in the parents and with a ratio of 1:2.5 in amniocytes. This indicated that the SMC(15) was maternal origin.

5. Reported prenatal marker chromosome cases :

(1) Chen CP, Lin CC, Li YC, Chern SR, Lee CC, Chen WL, Lee MS, Wang W, Tzen CY. Clinical, cytogenetic, and molecular analyses of prenatal diagnosed mosaic tetrasomy for distal chromosome 15q and review of literature. *Prenat Diagn* 2004; 24:767-773.

(2) Lin CC, Hsieh YY, Wang CH, Li YC, Hsieh LJ, Lee CC, Tsai CH, Tsai FJ. Prenatal detection and characterization of a small supernumerary marker chromosome (sSMC) derived

chromosome 22 with apparently normal phenotype. *Prenat Diagn* 2006; 26: 898-902.
(3) Chen CP, Lin SP, Lin CC, Li YC, Hsieh LJ. Chern SC, LeeCC, Chen Lf, Hua HM, Wang W. Prenatal diagnosis of low-level mosaicism for a small XIST-negative supernumerary ring chromosome in a nondysmorphic male fetus. Prenat Diagn 2006; 26:387-391.

Conclusion

1. Using a combination of G-banding and SKY, SKY and FISH we were able to achieve a rate of over 90% for identification the origin of marker chromosome.
2. The parental origin of marker chromosome can be further determinate using quantitative PCR with STR markers.
3. Marker chromosomes are most frequently originated from chromosome 15 and secondly from chromosome 22. It is recommended that chromosome 15 and 22 probes should be used first when a satellite marker was detected.
4. *De novo* marker chromosomes are associated with an increased risk of mental retardation/or physical anomalies (e.g. our reported cases 1) However, some *de novo* sSMC can be benign (e.g. our report case 2 and 3).

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