ORIGINAL ARTICLE

Identification of Y Chromosomal Material in Turner Syndrome by Fluorescence *In Situ* Hybridisation (*FISH*)

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ABSTRAK

Sindrom Turner adalah salah satu keabnormalan kromosom yang kerap berlaku pada bayi-bayi perempuan yang baru lahir. Lebih dari separuh pesakit yang menghidap sindrom Turner mempunyai kariotip 45X. Pesakit yang selebihnya menunjukkan keabnormalan struktur kromosom seks ataupun menunjukkan mozek dengan kromosom seks normal atau abnormal. Pesakit-pesakit mozek sindrom Turner yang mempunyai kromosom seks X yang kedua, kebiasaannya tidak menunjukkan sebarang tanda-tanda klinikal yang penting. Walaubagaimanapun, pesakit sindrom Turner yang mempunyai kromosom Y yang kedua atau bahan kromosom Y, berisiko menghadapi penyakit gonadoblastoma di kemudian hari. Tujuan kajian ini ialah untuk membandingkan teknik sitogenetik konvensional (kariotip) dan pendaflor *in situ* hibridisasi (*FISH*) di dalam mengesan bahan kromosom Y pada pesakit sindrom Turner. Kami juga membuat perbandingan darjah mozek yang dikesan diantara teknik kariotip sitogenetik dan penghibridan pendaflor (*FISH*). Kariotip dan *FISH* telah dijalankan ke atas lapan sampel darah dari pesakit sindrom Turner yang dikumpulkan diantara tahun 2004 dan 2006. Dua pesakit sindrom Turner dikesan mempunyai kawasan penentuan seks pada gen kromosom Y (SRY) dengan menggunakan teknik *FISH*. Kadar pengesanan kes-kes mozek sindrom Turner juga bertambah sebanyak 88% setelah menggunakan teknik *FISH*. Hasil kajian ini menunjukkan bahawa teknik *FISH* lebih efektif dari teknik kariotip di dalam pengesanan bahan kromosom serta penentuan darjah mozek Y pada pesakit sindrom Turner. Teknik *FISH* juga adalah cepat dan lebih kos efektif di dalam diagnosis sindrom Turner dalam penentuan darjah mozekisme.

Kata kunci: sindrom Turner, *FISH*, sitogenetik, gen SRY

ABSTRACT

Turner syndrome is one of the most common chromosomal abnormalities affecting newborn females. More than half of patients with Turner syndrome have a 45X karyotype. The rest of the patients may have structurally abnormal sex chromosomes or are mosaics with normal or abnormal sex chromosomes. Mosaicism with a second X sex chromosome is not usually of clinical significance. However, Turner syndrome patients having a second Y chromosome or Y chromosomal material are at risk of developing gonadoblastoma later

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in life. The aim of this study is to compare the results of conventional (karyotyping) and molecular cytogenetics (*FISH*), and discuss the advantages and limitations in the diagnosis of Turner syndrome. We also aim to compare the degree of mosaicism identified using conventional cytogenetics and *FISH* techniques. Conventional cytogenetics and *FISH* analyses were performed on eight peripheral blood samples of patients with Turner syndrome collected between 2004 and 2006. From this study, two out of eight patients with Turner syndrome were found to have the **s**ex determining **r**egion on the **Y** chromosome (SRY) gene by *FISH* analysis. Our results showed that the rate of detection of mosaic cases in Turner syndrome was also increased to 88% after using the FISH technique. We concluded that *FISH* is more superior to conventional cytogenetics in the detection of the Y chromosomal material. *FISH* is also a quick and cost effective method in diagnosing Turner syndrome and assessing the degree of mosaicism.

Key Words: Turner syndrome, fluorescence *in situ* hybridisation (*FISH*), Y chromosomal material, SRY gene

INTRODUCTION

Turner syndrome is one of the most common chromosomal abnormalities affecting 1 in 2500 newborn females (Rosenfeld 1994). It is characterised by short stature, gonadal dysgenesis, congenital heart disease and renal anomalies. The syndrome is also characterised by a variety of somatic features including neck webbing, cubitus valgus, short neck and widely set nipples. The cytogenetic abnormality associated with Turner syndrome was first described by Ford and co authors in 1959 (Ford 1959). Since then, a variety of other karyotypic findings associated with Turner syndrome have been determined. The classical 45, X is identified in about half of the patients. The remaining half have either structurally abnormal sex chromosomes (for example 46,X,i(Xq)) or are mosaics with other cell lines with normal (46, XX) or abnormal sex chromosomes (Jacobs 1997). In addition, a cell line containing the Y chromosome is present in 5% of patients and a further 3% of cases have an unidentifiable marker sex chromosome, presumably derived from a Y chromosome (Magenis 1980).

The Y chromosome specific gene SRY is one of the key genes involved in human sex determination. The SRY gene encodes a testis specific transcription factor that plays a key role in sexual differentiation and development in males and is located on the distal region of the short arm of the Y chromosome (Sinclair 1990). SRY expression initiates a network of gene activity that transforms the undifferentiated gonad, genital ridge into testis. Studies have confirmed that Turner syndrome patients with a Y chromosome – derived material in their genome, may go on to develop gonadoblastoma later in life (Manuel 1976, Gravholt 2000).

It has been proposed that all female patients with Turner syndrome with a 45, X karyotype carry a cell line containing two sex chromosomes at a low level of mosaicism (Held 1992). However, the low level of mosaicism is undetectable by standard conventional cytogenetic analysis. Previous studies have indicated that it was important to identify the type and degree of mosaicism since it appears to affect the prognosis and occurrence of stigmata and morbidity (Barrenas 2000, Landin 2001).

The laboratory diagnosis of Turner syndrome involves the identification of chromosomal abnormality using genetic methods. Conventional cytogenetics has been the gold standard in the identification of this chromosomal abnormality. Recently,

molecular cytogenetic studies such as *FISH* are rapidly becoming part of clinical practice in diagnosing Turner syndrome and the presence of Y chromosomal material.

Conventional cytogenetic analyses are capable of identifying Y chromosomal material in 4%-20% of patients with Turner syndrome (Jacobs 1997). However, Y chromosomal material may be present in only a few cells, and therefore, routine conventional analyses may miss the Y chromosome. The use of molecular techniques in detecting the presence of Y chromosome material is becoming increasingly important in determining those at risk of developing gonadoblastoma. Many studies have reported that molecular studies such as *FISH* are far superior to conventional cytogenetic analysis in the identification of the Y chromosomal material and the detection of mosaicism (Gravholt 2000, Quilter 1998, Robinson 1995, Hanson 2001, Alvarez 2003).

The main aim of this study is to compare the results of conventional (karyotyping) and molecular cytogenetics (*FISH*), and discuss the advantages and limitations in the diagnosis of Turner syndrome.

MATERIALS AND METHODS

A total of eight patients with clinical features of Turner syndrome were seen in the endocrine outpatient clinic in Hospital Universiti Kebangsaan Malaysia (HUKM) between 2004 and 2006. Five millilitres of peripheral blood was drawn from patients and collected in ethylene diamine tetracetic acid (EDTA) tubes. The blood samples were then sent to the Cytogenetics Unit, HUKM for conventional cytogenetic and *FISH* analyses.

Conventional cytogenetic analysis

Metaphase cells were obtained from PHA stimulated blood lymphocytes following standard protocols. Slides were stained by conventional Giemsa banding method.

Fluorescence in situ hybridisation (FISH) analysis

FISH analysis was performed on the same peripheral blood samples harvested for cytogenetic analysis. Ten micro litres of dual-colour probe cocktail consisting of SRY (SpectrumOrange-labelled, orange) and CEP X (SpectrumGreen-labelled, green) probes (Vysis, USA) was applied to the sample and contained with coverslips sealed with rubber cement. The sample and probe were co-denatured and hybridised using the Vysis HYBrite Denaturation/Hybridisation System. The HYBrite unit was programmed to allow 5 minutes of denaturation at 73°C, followed by overnight hybridisation at 37°C. Post-hybridisation wash was performed in 0.4X SSC/0.1% NP-40 (72°C, 2 minutes) followed by a wash in 2X SSC/0.1% NP-40 (room temperature, 1 minute). The slides were air dried in the dark, then counterstained with 10 micro litres of DAPI (4,6-diamidino-2-phenylindole). The *FISH* signals were visualised using Vysis filter sets and an Olympus BX51 epi-fluorescence microscope attached to a *FISH*View image acquisition and analysis system for FISH (Applied Spectral Imaging, Germany).

SRY probe

The SRY gene is located within 10kb of the pseudoautosomal region of Yp. The LSI SRY probe is used for detecting deletions of SRY or presence of the gene in rearrangements involving the X chromosome, autosomes and marker chromosomes.

RESULTS

Karyotyping

Five out of eight cases were found to have a mosaic karyotype with conventional cytogenetic analysis (Table 1). All of the mosaics involve a monosomy 45,X cell line

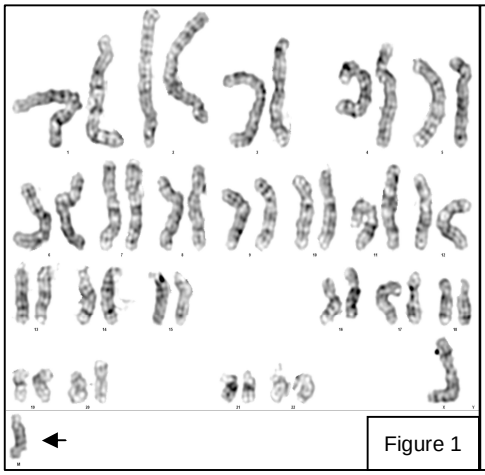
in combination with at least one other cell line; one case with a normal cell line, two cases with a cell line with at least one structurally abnormal X chromosome, and two cases with a cell line containing a possible Y material (Table 1, Figure 1). The remaining three cases were non - mosaics; two cases were 45,X (Figure 2) and one case showed a karyotype of 46,X,i(X)(q10) (Table 1).

FISH

FISH was used to identify the presence of the SRY gene and to determine the origin of the marker chromosome.

Table 1: Karyotypes of 8 patients with Turner syndrome using conventional and molecular cytogenetics

Patient	Age	Conventional Cytogenetics	Molecular cytogenetics (FISH)
TS1	Unknown	45X,46X,i(Xq)	45X,46XX
TS2	12	45X, 46X + mar	45X, 46X+Y (50%)
TS3	Unknown	45X,46XX	45X,46XX
TS4	29	45X,46X+mar	45X,46XX
TS5	49	45X	45X
TS6	32	45X	45X, 45X +SRY
TS7	Unknown	46Xi(X)(q10)	45X,46XX
TS8	Unknown	45X,46X,i(X)(q)	45X,46XX



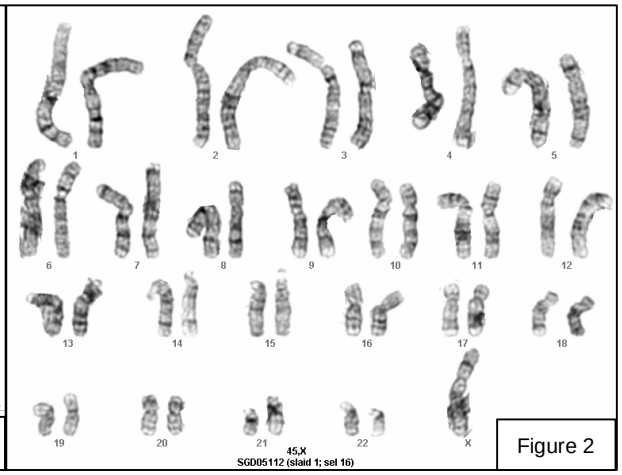
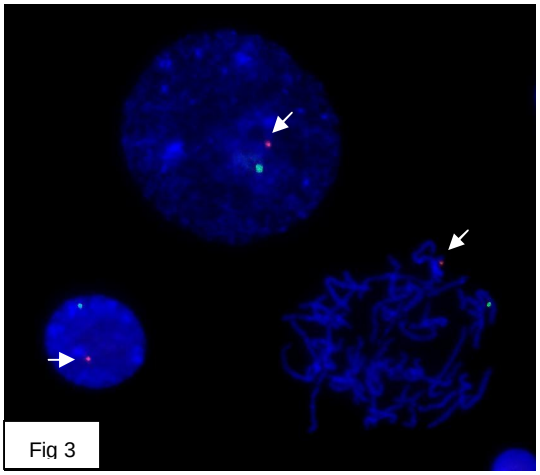
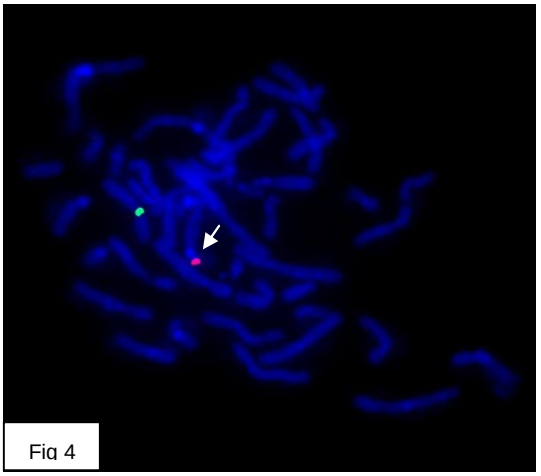


Figure 1: Karyotype of patient TS2 showing 46, X with a marker chromosome (arrow)
Figure 2: Karyotype of patient TS5 showing 45, X

Two out of eight cases analysed were found to have the SRY gene (Figure 3). One case (TS2) showed presence of Y chromosomal material in 50% of the cells. This confirms the finding of the karyotypic analysis that the marker identified was a Y chromosome. In the second positive case (TS6), 88% of the cells showed Y chromosomal material. However, it was observed that the SRY gene in this second case (TS6) was translocated onto one of the autosomes (Figure 4). The other six cases were negative for the SRY gene.

Analysis with CEP X probe confirmed that all cases were consistent with the karyotypic findings. However, in one non-mosaic case identified by conventional karyotyping, (TS7), the *FISH* analysis showed an additional normal cell line (Table 1, Figure 5).





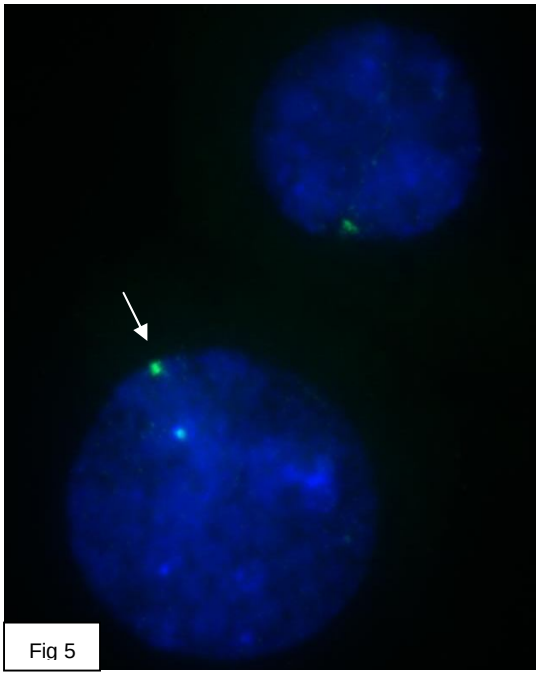


Figure 3: *FISH* analysis of patient TS6 showing interphase cells and metaphase spread with SRY gene (red signals, white arrows)

Figure 4: *FISH* result in patient TS6 showing translocation of the SRY gene onto one of the autosomes (red signal, white arrow)

Figure 5: *FISH* result of patient TS7 showing two interphase cells with one cell displaying 45, X only (one green signal) and another cell with an extra and intense green signal, consistent with an additional normal cell line 45XX (green signal, white arrow)

DISCUSSION

Turner syndrome is characterised by a range of clinical stigmata and cytogenetic analysis is the definitive investigation for patients with this syndrome. In Turner syndrome, there is an increased risk of developing gonadal neoplasms if Y chromosome material is present (Gravholt 2000). Therefore, the detection of Y chromosome material in Turner syndrome is of diagnostic importance.

Conventional cytogenetic analysis (karyotyping) detects Y chromosome mosaicism in about 5% of patients with Turner syndrome (Alvarez 2003). However, if Y chromosome material is present in only a few cells, it may be missed by the conventional method which routinely analyse 30 metaphases only.

Some authors have recommended the PCR method in the investigation of Y chromosome material in all patients with Turner syndrome, or in cases when a marker of undetermined origin is found (Rosenfeld 1994, Iezzoni 1997). Most of these studies had utilised PCR method as a screening procedure in identifying Y chromosome material, and *FISH* to confirm the findings of the PCR. Although PCR is a highly sensitive method in identifying Y chromosomal fragments, it is difficult to quantify the proportion of Y positive cells using the PCR method. *FISH* analysis, on the other hand, can easily quantify the proportion of cells containing Y chromosome material. Studies have indicated that the higher the proportion of cells bearing the Y chromosomal material, the more likely it is for the patient to develop gonadoblastoma (Rocío 2005, Prandstraller 1990). Therefore, the risk assessment of developing gonadoblastoma in Turner syndrome can be made possible by using *FISH* analysis.

FISH can also quantify the level of X chromosomal mosaicism. The purpose of quantifying the level of X chromosomal mosaicism is important because the type and degree of mosaicism appears to affect prognosis, and the occurrence of stigmata and morbidity (Barrenas 2000).

In this study, *FISH* was used to identify and confirm the presence of Y chromosome material or the SRY gene in patients with Turner syndrome. It was also used to analyse the level of mosaicism identified. The results of *FISH* were then compared with the karyotypic findings (Table 1).

In our study, two (TS2 and TS6, Table 1) of the eight patients with Turner syndrome were found to have the SRY gene by *FISH* analysis. Of these two, one patient (TS6) was originally thought to have a non mosaic 45, X karyotype by conventional method karyotype. However, *FISH* analysis confirmed the presence of the SRY gene in her peripheral blood. The *FISH* analysis also revealed that the SRY gene has translocated onto one of the autosomes (Figure 4). The findings of this particular case indicated that although conventional cytogenetic method (karyotyping) is the definitive diagnosis of Turner syndrome, karyotyping alone cannot be used to identify the autosome for which the SRY gene has translocated onto. Further molecular cytogenetic analyses such as spectral karyotyping (SKY) is required to confirm the origin of this autosome. SKY is a molecular cytogenetic technique which combines *FISH* and direct labelling technology for identification and analyses of complex chromosomal translocations.

The Y chromosome has an indistinct banding pattern and it can sometimes be difficult to determine the structure of the Y material from G banded preparations alone. In our study, one patient (TS2) was found to have a mosaic karyotype with a marker chromosome by conventional cytogenetics. Subsequent *FISH* analysis confirmed that the marker chromosome was a Y chromosome bearing an SRY gene. This shows that determination of the origin of the marker chromosome can be made possible with *FISH* studies.

Previous studies have found that the majority of patients with Turner syndrome, who either had a 45,X karyotype or seen to

have a Y chromosome or a marker cytogenetically, tend to be positive for Y chromosome material or SRY gene. The results of our small study have suggested that mosaic patients with cell lines containing two X chromosomes are less likely to be positive for the SRY gene or Y chromosomal material. However, a larger study is required to validate this finding.

Several studies have reported a correlation between phenotype and genotype in Turner syndrome. In general, 45,X monosomics display more stigmata and higher morbidity than mosaics (Gotzsche 1994, Verp 1987). Furthermore, mosaics are known to have less cardiac anomalies and a lower prevalence of cardiovascular disease and hypertension than monosomics (Landin 2001). It is therefore essentially important that the degree of mosaicism is accurately analysed in each patient with Turner syndrome.

In this study, the identification of mosaic cases was increased to 88% by using *FISH* compared to 63% by karyotyping only. The increase in the proportion of mosaic cases was attributed to the detection of cells with the SRY gene in patient (TS6) previously karyotyped as 45,X and also to the presence of 45,X cells in patient (TS7) with 46,X,der(X) karyotype. In this study, the mosaicism identified by *FISH* indicates a more favourable prognosis for these two patients. Therefore, we strongly recommend *FISH* as a quick and cost effective tool in diagnosing Turner syndrome and assessing the degree of mosaicism.

The risk of developing gonadoblastoma increases with age in Turner syndrome. In young patients with 45,X/46,XY or 45,X/46,X,+mar(Y), the risk is essentially zero (Verp 1987). The risk is dramatically increased from 15% to 20% by the age of 30 years. However, a more recent data suggested a lower risk of only 7% to 10 % (Gravholt 2000). At the time of analysis, patient TS6 was 32 years old and the age of patient TS7 was unknown. None of our patients positive for the SRY gene or Y chromosome were known to have had gonadectomies or gonadoblastoma. It is essentially important for these patients to be followed up since they are at risk of developing gonadoblastomas.

In conclusion, *FISH* technique is more superior to conventional cytogenetic analysis (karyotyping) in the identification of Y chromosomal material in Turner syndrome. We strongly recommend *FISH* as a quick and cost effective tool in the diagnosis of Turner syndrome and assessing the level of mosaicism.

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