SEGREGATION ANALYSIS OF RARE AUTOSOMAL FOLATE SENSITIVE FRAGILE SITES

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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TO MY HUSBAND BIBHUTI BHUSAN
AND
MY DAUGHTER URMIMALA

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ABSTRACT

Twelve families with rare autosomal folate sensitive fragile sites were identified. Segregation analysis of fragile sites in these families was undertaken to assess differences in transmission by carrier mothers and fathers. In addition, determination of the sex-ratios of the probands, transmitting parents, fragile site carrier children (excluding probands) and fragile site non-carrier children in the sibships and comparison of the ratio of the fragile site carriers to the non-carriers was undertaken. We also included 20 families with rare autosomal folate sensitive fragile sites from the literature for meta-analysis, 13 of these families were informative for segregation analysis.

The segregation analysis in our study families and in the families from the literature showed paternal fragile site transmission deviates significantly from the expected 50% for a mendelian co-dominant trait. Comparison of the sex-ratios in different groups showed a significant excess of transmitting females in the literature families. Literature review data also confirmed a significant excess of males among fragile site non-carriers. Comparison of the fragile site carriers versus fragile site non-carriers in combined data showed a non significant excess of non-carriers.

A major finding of this study was evidence for a deficiency of offspring expressing fragile sites when transmission is through fathers implying either gametic selection or the phenomenon of parental genomic imprinting.

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1.0 INTRODUCTION

1.1 NATURE AND SCOPE OF THE PROBLEM

Fragile sites (FS) are specific points on chromosomes which tend to break when the cells are cultured and treated in a culture medium with specific chemical agents or exposed to specific conditions of tissue culture. FS are expressed as chromatid breaks or isochromatid gaps or breaks. FS are characterised by a change of chromosome morphology with elongated and thinned chromosomes. Fragility also results in acentric fragments, chromosomal deletions and triradial figures. An important feature of FS is that when they are present in any individual or kindred they are always expressed or present at the same locus.

FS are rarely expressed in over 50% of metaphases. This is likely due to inadequate techniques used to induce and express FS. FS are regions of chromosomes which fail to compact for mitosis and this failure likely lies not in the protein components but in the structure of the DNA itself (Sutherland, 1979a). Laird et al. (1987) suggested that FS in human chromosomes represented regions of delayed or late replicating DNA. Incomplete DNA replication and chromatin condensation caused by late replication may result in observed chromosome gaps and fragility at FS.

The rare folate sensitive FS on the X-chromosome is associated with the commonest inherited form of intellectual disability in males called the fragile X syndrome (Chudley and Hagerman, 1987). Recently, other rare FS have drawn attention for investigations. However, the purpose and significance of the rare autosomal FS remain in question. In general, they are considered to be chromosomal variants or polymorphisms. Some recent

reports clearly indicated an increased frequency of rare autosomal folate sensitive FS among a population of patients referred to diagnostic chromosome studies or mentally retarded with respect to the randomly selected neonates as normal controls (Sutherland, 1985d; Chudley et al., 1990). Fryns et al. (1986) and Kähkönen et al. (1989) did not find any such differences. However, the rate of expression in a group of mentally retarded people compared to a group of mentally normal individuals, was statistically significantly different (Kähkönen et al., 1989).

FS may also coincide with the break points of chromosomal rearrangements in cancer cells which might suggest that at least a portion of the aberrations at FS are truly breaks and that these breaks may occur *in vivo* and have clinically important consequences (Yunis et al., 1984; Hecht and Hecht 1984a).

To date, 18 rare autosomal folate sensitive FS have been confirmed (Hecht, 1988) and the frequencies of this group of FS vary among different studies, 1 in 769 (Quack et al., 1978), 1 in 250 (Petit et al., 1986), 1 in 15 (Fryns et al., 1986), 1 in 90 (Kähkönen et al., 1989), and 1 in 263 (Chudley et al., 1990). These differences may be due to ethnic differences or may be due to differences in study methods.

Sutherland (1982a) reported that whenever an abnormal individual with folate sensitive FS was found, the transmitting parent was almost always the mother. Rare autosomal FS are believed to be inherited in a mendelian co-dominant fashion (Sutherland, 1979a); but, segregation analysis of rare autosomal folate sensitive FS by Sherman and Sutherland (1986) suggested a deviation from expected ratios. The expression of the rare autosomal folate sensitive FS seemed to differ with the sex of the

transmitting parent.

The results of the segregation analysis by Sherman and Sutherland (1986) were very interesting and unique. More data was needed to confirm these unique findings and hence we wanted to analyze the segregation patterns in our study families with rare autosomal folate sensitive FS.

1.2 PURPOSE OF THE STUDY

1.2.1 General Objectives

We planned to examine the families of individuals with rare autosomal folate sensitive FS to determine if any distortions in segregation or sex ratios were evident. Additionally, we planned to review the family histories to determine correlation between the presence of rare autosomal folate sensitive FS, their percentage of expression and health status in the individuals.

1.2.2 Specific Objectives

We planned:

1. to identity probands with rare autosomal folate sensitive FS and to obtain complete family pedigrees and health records from relevant family members.

2. to determine whether a consistent clinical phenotype correlates with existing FS and their percentage of expression in individuals.

3. to determine the sex ratio of the probands with rare autosomal folate sensitive FS.

4. to determine sex ratio of the transmitting parents.

5. to compare the proportion of total number of FS carriers vs non-carriers in the families.

6. to determine the sex ratio of the total number of FS carrier children and of the total number of FS non-carrier children from all the families with rare autosomal folate sensitive FS.

7. to analyze the segregation ratio of rare autosomal folate sensitive FS to determine if differences were present if the FS was transmitted by father or mother.

8. to review recently published data to identify more families with rare autosomal folate sensitive FS to incorporate with our local family data for meta-analysis.

2.0 REVIEW OF LITERATURE

2.1 HISTORY OF FRAGILE SITES

In 1965, A. Dekaban first reported a FS on the long arm of a C-group chromosome. Subsequently, FS on all human chromosomes except chromosome number 21 have been reported. Lejune et al. (1968) first demonstrated that such sites were heritable and Sutherland (1979a) first concluded that such sites were inherited in a mendelian co-dominant fashion. During the study of a family with fragile 16, Magenis et al. (1970) first coined the term "Fragile sites". Prior to this, such sites were referred as breaks in the same general regions on a particular chromosome.

2.2 GENERAL CONSIDERATIONS OF FRAGILE SITES

FS appear as chromosomal breaks in non-random sites when exposed to specific

chemical agents or conditions of tissue culture. Presently, at least 105 FS are known (Hecht, 1988) and they are important as chromosome markers. G.R. Sutherland (1979a) proposed the following definition of FS:

1) Usually present on both chromatids, a non-staining gap with variable width.
2) The site always would express exactly at the same point on the chromosome in cells from any individual or kindred.
3) FS show the mendelian co-dominant mode of inheritance.
4) Fragility would produce acentric fragments, chromosomal deletions, triradial figures and the like.

The gaps at FS could have resulted from extreme despiralisation of DNA due to failure of compact folding in the metaphase chromosome (Chaudhuri, 1972). Furthermore, as the FS are heritable, they are probably the manifestation of information which is coded by DNA (Sutherland, 1979a) or the reason that causes the failure of compactation of DNA lies in the structure of DNA itself at the FS (Sutherland and Hecht, 1985a). With changes in the composition of culture medium, 2 hours prior to harvest and at the time of the addition of colchicine, the frequency of the FS expression can be altered, which indicates that the expression of the FS must be influenced directly at the time of chromatid spiralization in the late G_2 or early prophase. FS could also be the viral DNA modification sites where viruses may be able to modify specific DNA sequences causing specific lesions which superficially resemble the lesions seen at FS (Sutherland, 1979b). The origin of the triradial chromosomes are probably by chromatid breakage which follow mitotic non-disjunction of the distal acentric fragment of the long

arm of a chromatid (Ferguson-Smith, 1977).

2.3 CLASSIFICATION OF THE FRAGILE SITES

Chromosomal FS have been recognized on human chromosomes for about 25 years. They are classified into two major groups depending on their frequency in the general population - a) Rare FS, b) Common FS.

There are many differences between rare and common FS. The rare FS are infrequent in the population and segregate in a simple mendelian fashion whereas common FS are frequent. Common FS may be induced by several environmental factors (Rao et al., 1988) and they also segregate in a mendelian fashion (Sutherland, 1979a). Rare autosomal folate sensitive FS are always present on only one homologue and express as chromatid breaks, deletions and triradials. On the other hand the common FS sometimes are expressed on both homologues and are usually seen as chromatid lesions. Common FS appear to be universally present and a property of the human as well as the animal genome. The terms used to denote these sites are "constitutive" (Daniel et al., 1984; Yunis and Soreng, 1984) and "common" (Glover et al., 1984; Sutherland and Hecht 1985b), as well as "hotpoints" (Zhou et al., 1984) and autosomal "lesions" (Sutherland, 1983).

FS show a very broad range of frequencies from very rare to very common and thus, certain FS with an intermediate frequency cannot be classified either as rare or as common. These are essentially polymorphic variants (Sutherland and Hecht, 1985a). Hecht (1986) suggested three classes of FS; a rare FS might be one with a frequency of

less than 1% in the population, a polymorphic FS would have a frequency of 1%-50% and a common FS would have a frequency of greater than 50% in the population.

At least 105 FS are now recognized comprising 25 (23%) rare and 80 (77%) common FS. Both rare and common FS are subclassified based on their mode of induction (Hecht, 1988) (Table I and Table II).

Eighty common FS are subclassified into 69 Aphidicolin-inducible (66%), 3 5-Azacytidine-inducible (3%), 6 Bromodeoxyuridine-inducible (6%), and 2 unclassified (2%). Twenty-five rare FS are subclassified into 19 folate-sensitive (17%), 3 Distamycin-A-inducible (3%) 2 Bromodeoxyuridine-inducible (2%) and 1 unclassified (Hecht, 1988).

2.4 TISSUE CULTURE CONDITIONS

2.4.1 Culture Media

The key event to the detection of FS was the discovery that this fragility may be expressed only under highly specific culture conditions. Sutherland, 1977, observed that to elicit the expression of several FS, it was necessary to culture lymphocytes in the medium 199. Deficiency in folic acid and thymidine was the essential feature of medium 199. FS expressed under such conditions became known as folate sensitive FS (Table I). Since this finding, a number of other compounds, namely the anti-folate methotrexate and aminopterin, antibiotics trimethoprin and pyrimethamine - inhibitors of folate metabolism; fluorodeoxyuridine (FUdR), fluorodeoxycytidine (FdC) and trifluorothymidine - the inhibitors of thymidylate synthetase, that can also affect the expression of folate sensitive FS have been identified (Sutherland 1979b; Glover 1981; Tommerup et al., 1981; Jacky and Sutherland, 1983).

Table I. Classes of Rare Fragile Sites

cs*	FSFS**	Dist. A***	BrdU****	Uncl +
1	2q11.2, 2q13			
2	2q22.3			
3				
4				
5				
6	6p23			
7	7p11.2			
8	8q22.3	8q24.1		8q13
9	9p21.1, 9q32			
10	10q23.3, 10q24.2		10q25.2	
11	11q13.3, 11q23.3			
12	12q13.1, 12q24.13		12q24.2	
13				
14				
15				
16	16p12.3	16q22.1		
17		17p12		
18				
19	19p13			
20	20p11.23			
21				
22	22q13			
X	Xq27.3			

* Chromosome; ** Folate sensitive fragile site; *** Distamycin-A-induced; **** BrdU-induced; + Unclassified

Table II. Classes of Common Fragile Sites

cs*	Apc**	Aza +	BrdU ++	Uncl
1	1p36, 1p32, 1p31.2, 1p22, 1p21.2, 1q21, 1q25.1, 1q44.1	1q12, 1q42		
2	2p24.2, 2p16.2 2p13, 2q21.3 2q31, 2q32.1 2q33, 2q37.3			
3	3p24.2, 3p14.2 3q27			
4	4p16.1, 4p51 4q31		4q12	4q27
5	5q15, 5q31.1		5p13, 5q15	
6	6p25.1, 6p22.2 6q13, 6q21, 6q26			
7	7p22, 7p14.2 7p13, 7q21.2 7q22, 7q31.2 7q32.3, 7q36			
8	8q22.1, 8q24.1 8q24.3			
9	9q22.1, 10q25.2	9q12	9p21	
10	10q22.1, 10q25.2 10q26.1		10p21	
11	11p15.1, 11p14.2 11p13, 11q13 11q14.2, 11q23.3			
12	12q21.3, 12q24			
13	13q13.2, 13q21.2		13q21	
14	14q23, 14q24.1			
15	15q22			
16	16q22.1, 16q23.2			
17	17q23.1			
18	18q12.2, 18q21.3			
19	19q13			
20	20p12.2			
21				
22	22q12.2			
X	Xp22.31, Xq22.1 Xq27.3			
Y				Yq12

* Chromosome; ** Aphidicolin induced; + 5-Azacytidine induced; ++ BrdU induced; Uncl Unclassified

There are several other FS which are not sensitive to folic acid concentration to the culture medium, but they can be induced in the presence of certain other compounds in the culture medium. Scheres et al. (1980) and Sutherland et al. (1980) found a new class of FS, namely fra (10) (q25), expressed only if the culture medium contained BrdU for at least 8-24 hrs. before harvesting. The fra (16) (q22) and fra (17) (p12) may be expressed spontaneously in some individuals, but they can also be induced by Distamycin-A, netropsin, BrdU, bromodeoxycytidine (BrdC), Hoechst 33258, interferon and caffeine (Schmid et al., 1980; Croci, 1983; Shabtai et al., 1983).

2.4.2. Factors Affecting Expression of Folic Acid Sensitive Fragile Sites

2.4.2.1. Chemical Factors

Sutherland (1979b) observed that expression of FS was almost completely inhibited when medium TC199 was supplemented with folic acid 24 hrs. prior to harvest. Folic acid probably acts late in S or early in G_2 phase.

Fontash (1981) and Mattei et al. (1981) first became successful to induce high levels of expression of fragile X in fibroblasts and lymphocytes by using folic acid antagonists methotrexate and aminopterin. These inhibit dihydrofolate reductase and thus, are effective in the induction of folate sensitive FS in the late S or early G_2 phase.

Sutherland (1979b) discovered that folic acid inhibits the expression of FS. Sutherland and Hecht (1985f) found that thymidine had the same ability to inhibit expression of folate sensitive FS. From these findings, they suggested that the biochemical environment required for folate sensitive FS expression was a relative deficiency of thymidine monophosphate or thymidylate during DNA synthesis. BrdU, an analog of thymidine and BrdC (Bromodeoxycytidine) also inhibit the expression of folate

sensitive FS (Sutherland et al., 1985c). Sutherland et al. (1985c) surprisingly determined that though low levels of thymidine inhibited FS expression; high concentration of thymidine (0.5 - 3.0 mM), but not of its analog BrdU, induced FS. The likely explanation of this unusual finding came from the study of Richard et al. (1961). They showed high levels of thymidine triphosphate inhibited ribonucleotide reductase - the enzyme converted cytidine diphosphate to cytidine triphosphate - a critical requirement for FS expression being in short supply during DNA synthesis (Fig. 1). The high levels of BrdU concentration still resulted in a relative deficiency of deoxycytidine for DNA synthesis requirements. Freese (1959) found that this was overcome by the incorporation of enol form of BrdU in the newly synthesised DNA strand in place of deoxycytidine (Fig. 1).

Howard-Peebles et al. (1980, 1981) reported methionine as an essential compound for expression of fragile X even under folic acid and thymidine deprivation. Other attempts to confirm this report were partly successful. Some individuals with FS at 10q23, 11q13, 12q13 and Xq27 showed reduced frequencies of expression without methionine.

Actinomycin D, Ethidium bromide and Hoechst 33258 showed no effect on folate sensitive FS although they may act as inducing agents for some of the folate sensitive FS (Jacky and Dill, 1983).

Foetal bovine serum is most commonly used in the culture medium. For good cell growth Sutherland (1979b) used a 5% concentration of it, though there was no detailed study of serum concentration versus frequency of FS expression. Howard-Peebles et al. (1981) claimed that high serum concentrations inhibits fragile X expression.

Aphidicolin is known to induce chromosomal aberrations in a highly non-random manner. It is a specific inhibitor of DNA polymerase α and inhibits DNA synthesis by blocking progression of the replication fork and interferes with the joining of adjacent

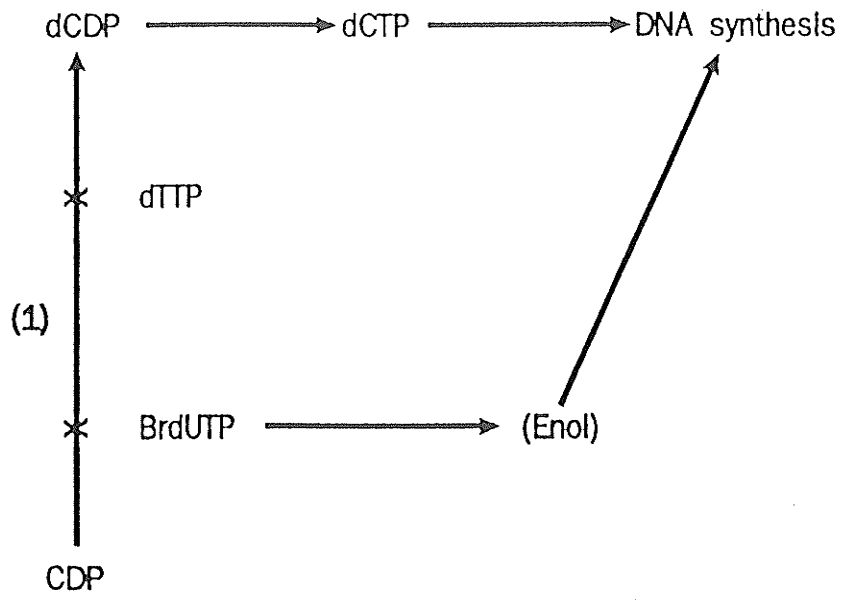

Figure 1. Pathways of nucleotide metabolism affected by high thymidine and BrdU concentration.

Key:

CDP=cytidine diphosphate; BrdUTP=Bromodeoxyuridine triphosphate; dTTP=deoxythymidine triphosphate; dCDP=deoxycytidine diphosphate; dCTP=deoxycytidine triphosphate, "x"=inhibition of enzyme activity; (1)=Ribonucleotide reductase

DNA intermediates, preferentially at 2q31, 3p14, 6q26, 7q32, 16q23 and Xp22 sites. Recent evidence suggested that an additional polymerase was also inhibited by aphidicolin (Dresler et al., 1986). The sites most sensitive to aphidicolin damage also show increased expression by thymidylate stress which may partially inhibit polymerase α and uridine plays a role for this increased expression under thymidylate stress (Reidy, 1987). The fragile X, which can also be induced by thymidylate stress, can not be induced by aphidicolin. These aphidicolin induced FS are also expressed in low frequencies of metaphases spontaneously and are termed "hot spots" and belong to a new class of FS called the common FS (Glover et al., 1984) (Table II).

2.4.2.2. Physical Factors

pH: The addition of folic acid to the medium TC 199 inhibited FS expression (Sutherland, 1979b). When this folic acid was dissolved in a bicarbonate solution this extra bicarbonate, along with folic acid, resulted in a rise of pH in the medium. A control experiment was performed to ensure that the addition of bicarbonate alone, i.e. the rise of the pH of the medium was not responsible for inhibition of FS expression. This increase of pH caused the frequency of lesions at the FS to increase rather than decrease. This increasing effect was highly significant for sites at 2q13, 20p11, Xq27, but not for those 10q25, 11q13, 16p12 and 12q13 (Sutherland et al., 1981). The rate of folate uptake by cells was inversely proportion to pH and higher intracellular levels of folate in a steady state situation have been found at lower pH of the culture medium (Branda et al., 1982).

Duration of Culture: Two-day cultures are not satisfactory for FS expression. After approximately four days of culturing the frequency of expression reaches a maximum for

fragile X (Jacobs et al., 1980; Jennings et al., 1980; Howard-Peebles et al., 1981) and 12q13 (Sutherland and Hinton, 1981). After four days the expression decreases along with the quality of chromosome preparation. Depletion of media components that inhibit expression may account for this temporal effect.

Age of the Blood: Older blood when cultured under condition of folate depravation, yielded lower frequencies of fragile X expression than fresh blood, but higher expression resulted by using FUdR induction (Brookweel et al., 1982). Yet Jacky and Sutherland (1983) could not find any difference in frequencies of expression either on storage of blood at 4°C for a period up to seven days or between folate-free culture medium and FUdR induction. Mattei et al. (1981) and Fontasch et al. (1983) found a decrease in frequency of expression with storage time.

Effects of Harvesting and Microscopy: Higher frequency of fragile X in fibroblasts have been reported by Jacky et al. (1980) when sodium citrate was used as the hypotonic agent instead of KCl. Howard-Peebles et al. (1981) found higher frequency of fragile X expression when the slides were air dried, not flame dried. Frequency of expression also increased with the length of the metaphase chromosome (Jacky and Sutherland, 1983). Zankl et al. (1982) found that Giemsa staining of chromosome and normal bright field microscopy was not satisfactory to recognize the fragile X, but the frequency could be doubled by the use of phase contrast and increased even further when orcein stain was used.

Effects of Cocultivation: Eberle et al. (1982) were the only ones who studied the co-cultivation of fragile X lymphocytes with normal lymphocytes and their study showed a decreased frequency of fragile X expression which suggested that normal cells in culture produce a soluble factor which inhibits FS expression.

2.5 EXPRESSION OF FRAGILE SITES IN OTHER CELL TYPES

The first attempt to elicit FS expression in cells other than lymphocytes made by Magenis et al. (1970) was unsuccessful. They used fibroblast cell culture. In 1973, Ferguson-Smith reported expression of fra (2) (q13) in fibroblast cells but the frequency was lower than the frequency in lymphocytes. Sutherland (1979b) studied fibroblasts from carriers of FS at 2q13, 10q23, 11q13, 16q22, 20p11 and Xq27 under folate deprived condition. Some of these FS were expressed up to 4% of metaphases, but no fragile X have been expressed. Jacky and Dill (1980) first reported fragile X in fibroblasts using rigorous folate restriction but their approach was not reproducible.

A simple reliable method for the induction of folate sensitive FS including the fragile X in fibroblasts was described by Sutherland and Baker (1986). This method involved addition of 600 mg/l thymidine to the cultures 24 hrs. before harvest. Sutherland et al. (1984) reported fra (10) (q25) from several individuals and fra (16) (q22) from one individual were expressed with BrdU in fibroblasts. Common FS have been reported to appear in fibroblasts when fragile X induction was attempted, but no studies on their induction with aphidicolin have been reported.

Sutherland (1979b) studied lymphoblastoid cell lines (LCL) from individuals with fra (2) (q13) and fra (X) (q27) under conditions of folic acid and thymidine deprivation and found no expression of FS. Jacobs et al. (1982) showed that fragile X in LCL can be expressed by FUdR.

Sutherland (1979b) also studied the bone-marrow from a male with fra (X) (q27) and from a carrier of fra (16) (p12) and found no expression of any of these FS. However, lymphocytes from these persons expressed fragile X in 10% of metaphases and 16p12 in 46% of metaphases respectively.

Numerous groups have been reporting expression of fragile X in amniotic fluid cells after exposing them to various conditions designated to induce FS. All these studies have been done for prenatal diagnosis.

Very few data have been published on the expression of FS in hybrid cells. When fragile X fibroblasts were fused with normal fibroblasts and aminopterin was used as an inducer of fragile X, the expression depressed to 4%-7% from 6%-12% (Bryant et al., 1983). Wegner et al. (1982) cultured human/mouse hybrid cells with methotrexate, aminopterin or FUdR to express fragile X and found no expression. Nussbaum et al. (1983) cultured human/hamster hybrid cells where fragile X was the only human chromosome and used FUdR and methotrexate. They found the induction of the FS on X chromosome. Warren et al. (1984, 1987) also found fragile X in human/hamster hybrid cells.

2.6 CLINICAL SIGNIFICANCE OF RARE FRAGILE SITES

2.6.1 Significance of Fragile X

FS on Xq27 were first described by Lubs (1969), when he reported a family with several mentally retarded males. Eight years following this report, Harvey et al. (1977) reported 8 additional families with this marker X chromosome and mental retardation. Since then, it has been established that this is the only FS known to have definite clinical significance and is associated with the X-linked Martin-Bell form of mental retardation in males. The FS at Xq27.3 is folate sensitive. Sutherland (1983) estimated that 19-55 in every 10,000 males are afflicted. Between 2% and 6% of the institutionalised males with severe mental retardation may have fragile X (Blomquist et al., 1982; Jacobs et al., 1983). The degree of retardation varies from severe to borderline. In addition, several

males with fragile X chromosomes have shown normal intelligence. The affected males have enlarged testes, large and protruding ears, a slightly enlarged forehead with prominent supra orbital ridges, mild short stature and prominent mandible. Occasionally, cleft palate and hypospadias are present. Autism has been reported in many cases, otherwise most affected males are cheerful and cooperative. The pathogenesis of the clinical abnormalities of fragile X are unknown. Many of these physical features may be related to a connective tissue dysplasia (Opitz et al., 1984; Hagerman et al., 1984). Further studies of connective tissue abnormalities may reveal a specific biochemical abnormality. The physical features associated with connective tissue disorders can help in clinical diagnosis, but the relationship to mental retardation in the fragile X syndrome is still unknown. This fragility on Xq27 may only be an *in vitro* marker associated with but not responsible for the clinical phenotype. Possibly both the fragility and clinical abnormalities may be the reflection of a single gene mutation or the fragility on Xq27 may be directly responsible for the pathology by predisposing to gaps or breaks *in vivo* (Michels, 1985). Hecht et al. (1982) suggested that any male patient with unexplained mental retardation should have chromosome analysis to determine whether a fragile X chromosome is present or not. Four percent to 56% of metaphases express fragile X in affected males (Howard-Peebles, 1983). In one study, Chudley et al. (1983) found the expression of fragile X in affected males varied between 5% and 50% (average ~20%). Soudek et al. (1984) found that the proportion of positive cells was usually consistent in an individual over time, and they hypothesised that the proportion may be related to other familial or genetic factors rather than directly to the degree of retardation. Furthermore, 1/3 of the carrier females suffer from some degree of mental retardation and approximately 50% of heterozygous females have either mental retardation or educational

difficulties (Fishburn et al., 1983; Chudley and Hagerman, 1987).

2.6.2 Significance of Rare Autosomal Fragile Sites

The autosomal fragile sites (FS) are generally considered as chromosomal variants and their meaning is more controversial. No consistent pathologic role for these FS has been confirmed. However, Glover et al. (1988) reported that FS predisposed to deletions and interchromosomal recombination. These findings led them to speculate that cells with such deletions would have greatly reduced fitness and probably die and thus, only rarely be seen in cytogenetic preparations. Maltby et al. (1987) reported a patient with fra (10) (q23) which was expressed in 12% of metaphases and in 66% of the expression was as deletion at 10q23. Voullaire et al. (1987) reported a chromosome deletion of 11q23-11qter in a child from a family with fragility at 11q23. Jayakar et al. (1986) unexpectedly found fra (2) (q13) in two cases of infantile autism out of their 20 study patients. Several other reports have documented a variety of neurodevelopmental abnormalities and MR in individuals with rare FS (William et al., 1976; Annerén et al., 1981; Gruichaoua et al., 1982; Chodirker et al., 1987). Chudley et al. (1990) found five rare FS in a mentally retarded (MR) population study. Fifty-five percent of cases in this MR population were classified as idiopathic with no identifiable etiology. Four of the five rare FS identified in that study were found in this idiopathic MR group. From this finding, they concluded that rare FS were over represented in the idiopathic MR group and might be etiologically related to the MR. Garcia-Sagredo et al. (1983) reported a boy with multiple congenital anomalies and mental retardation and a *de novo* balanced translocation involving 16q22, and his father and sister with fra (16) (q22). Hecht and Hecht (1984a, 1984b) presented evidence for a significant excess of chromosome breaks

and rearrangements in bands containing FS in amniocenteses, spontaneous abortions, stillbirths and livebirths. This findings suggested that certain FS may be fragile in meiosis and thus, predispose to chromosome breakage in meiosis and so would tend to produce chromosome rearrangements in gametes and in conceptuses. They also suggested that certain FS are meaningful with regard to *in vivo* chromosome breakage while others are innocent. The FS also can mediate breakage and non-random chromosome rearrangements involving the FS in dividing somatic cells (Warren et al., 1987; Beek et al., 1983). Venkatraj et al. (1987) found a significant relationship between break points and FS in aborters who have had at least two consecutive fetal losses. This might be the result of meiotic chromosomal rearrangement predisposed by FS, leading to production of a fetus with an aberrant genotype. Stetten et al. (1988) described a woman with multiple miscarriages who had a rare FS at 12q13. Smith et al. (1985) reported a male with two FS at 9p21 and 12q13, who had a cytogenetically abnormal offspring, some of whose cells had an extra chromosome fragment. Côté et al. (1978) found a woman with fra (16) (q22) whose son and grandson had an extra portion of chromosome 16 distal to this FS. Dunner et al. (1983) reported a family with fra (16) (q22) ascertained through a newborn infant with multiple congenital anomalies and fra (16) (q22). Moric-Petrovic et al. (1984), Donti et al. (1979), Sutherland (1979b) and Kubien et al. (1977) identified many of these autosomal FS in patients and families with birth defects or mental retardation or both. However, chromosome analysis is most often performed in such patients and thus, there might have been considerable bias in ascertainment. However, William and Howell (1976) suggested hypothetically that *in vivo* breakage at FS might result in a variety of aneuploid cell lines and this breakage "at critical stages of development could initiate a variety of defects, depending upon the presumptive tissues

in which that breakage occurred." Yunis et al. (1987) found that diverse mutagens and carcinogens induced a large number of breakages at FS. From this finding they suggested that the FS might be the general targets of mutagenic action and a class of FS involved in such mutation which disrupt the active DNA sequences and thus, promote genetic defects as well as cancer.

A possible causal relationship between FS and cancer break points (CBP) has been suggested. This suggestion came from the finding of patients with rare or common FS expressed in their peripheral blood lymphocytes and who had a malignant lesion in which some cells had a chromosomal break apparently at the same site as the FS (Yunis et al., 1984; De Braekeleen et al., 1985). Yunis (1983) found a highly significant correlation between FS and break points for specific structural chromosome defects known so far in leukemias, lymphomas and malignant solid tumours. Le Beau (1988) found remarkable concordance between the chromosomal locations of FS and break points associated with leukemia and lymphoma. Hecht and Sutherland (1984c) showed statistically significant association between FS and cancer specific break points. According to Berger et al. (1985) the evidence for the relationship between FS and CBP is largely circumstantial and came from a significant correlation between break point location and the chromosome bands in which the FS are present. The evidence also came from the anecdotal reports of individuals with FS and cancer. The break point in their malignant cells corresponds with the location of their FS as reported by Pathak et al. (1982), Sessarego et al. (1983), and Yunis (1984), but it has not been established that they truly coincide. There is no evidence that a rare FS predisposes its carrier to malignancy. Sutherland (1988) reasoned that other than fra (16) (q22) association with M4E0, there has been no series of patients with rare FS and malignant disease or CBP close to the FS. No discernible increase in

malignant diseases has been found in FS families. Also if rare FS have in any way predisposed their carrier to neoplastic disease then such disease would be expected to be familial. Again, all individuals are probably carrier for the common FS, sometimes even in homozygous condition, they are unlikely to be important to any individual in terms of increased risk of cancer. This would also suggest that no individual is predisposed to neoplastic disease because of a FS, rare or common (Berger et al., 1985). Therefore, further studies are required to specify the role of autosomal FS in cancer biology, other birth defects and mental retardation.

2.7 POPULATION DATA

2.7.1 Frequency of Fragile Sites

The first population survey to detect a FS was carried out by Gerald et al. (1970) in 3,543 newborns in the prebanding era and only one baby with a FS on a C-group. chromosome was detected. Sutherland (1985d) conducted a population cytogenetics study for folate sensitive FS on 2,439 randomly selected neonates, on 1,936 referred patients, on 502 special school students and on 128 sheltered workshop employees. The carrier frequency of folate sensitive FS was 1 in 100 individuals in the retarded group and about 1 in 700 individuals in the neonates. In the patient group it was intermediate at about 1 in 260. The incidence of folate sensitive FS in patients in this study was about twice that in other reported series. Fra (17) (p12) was seen in 1 of 368 babies but no fra (16) (q22) was detected in this study. The frequency of folate sensitive fragile X in institutionalised retardates was 2%-6% in males (Blomquist et al., 1982; Jacobs et al., 1983). In a survey, Turner et al. (1980) found that 7% of the girls from schools for the mildly retarded carried the fra (X) (q27).

Sanfilippo et al. (1983) found fra (16) (q22) in 4 of 155 institutionalised retardates and 14 of 1,444 patients in Italy. In another study in 1984, Sutherland found fra (16) (q22) in 8 of 491 patients. The combined Australian and Italian frequency of fra (16) (q22) was about 1 in 90 individuals. The fra (10) (q25) is present in 1 in 40 individuals in the population (Sutherland, 1982b).

In another study, Petit et al. (1986) detected 13 folate sensitive FS carriers among 405 mental retardates, and they derived a combined frequency of folate sensitive FS of 1 in 250 persons (0.26%) from all other studies, irrespective of the nature of study groups. Chudley et al. (1990) found the incidence of rare autosomal FS to be 1 in 65 in as MR population (1 in 45 in an idiopathic MR subgroup and 1 in 147 in those with MR of known etiology) and 1 in 263 in neonates. In 1989, Kähkönen et al. gave an overall frequency of folate sensitive FS of 1 in 90 while Takahashi et al. (1988) found this FS frequency to be 1 in 204 in healthy subjects.

Thus, the frequency of folate sensitive FS has differed greatly among different studies from 1 in 15 (Fryns et al., 1986) to 1 in 769 (Quack et al., 1978). This variation in frequencies might be due to ethnic differences between study groups or differences in study methods (Sutherland, 1985d; Takahashi, 1988). Considering all the population studies that have been done, and when grouped according to mental status, the total frequency of rare autosomal folate sensitive FS appears greater in mental retardates (1/51) than in mentally normal individuals (1/194). This difference in total frequency is mostly due to a low frequency of these FS in neonates and might be due to difficulties in detecting FS in cord blood obtained from neonates (Sutherland, 1985c).

2.7.2. Ethnic Distribution of Fragile Sites

The presence of fragile X has been reported in all racial groups including Europeans, Japanese, Filipinos, Polynesians and Australian aboriginals reported by Turner and Jacobs (1983), in South Africans Zulus by Venter et al. (1981) and in American blacks by Howard-Peebles et al. (1980). However, there is very little information about the presence of other FS in non-European populations. In other racial groups, the most studied FS is 10q25 which shows a polymorphism in frequency in Australian white populations. This would be followed by 16q22 which has also been found in Australian white populations with a frequency of approximately 1 in 64 persons (Sutherland, 1985d). Studies by Takahashi et al. (1988) in Japan found four folate sensitive FS, 2q11, 11q13, 11q23 and 17p12 (in which 17p12 was new, and showed a frequency of 1 in 204 persons). He also discovered three Distamycine-A inducible FS 8q24, 16q22 and 17p12 (in which 8q24 was a new one with a frequency of 1 in 70 persons). Furthermore, he found one BrdU requiring FS, 10q25 with a frequency of 1 in 340 persons.

2.7.3 Segregation Patterns of Fragile Sites

2.7.3.1 Fragile X

Sherman et al., (1984, 1985) showed that the segregation ratio of fra (X) (q27) in males was 0.406 which was significantly different ($P<0.028$) from the expected ratio of 0.5 for a fully penetrant X-linked gene. From this finding it could be concluded that if FS followed a mendelian dominant fashion of inheritance, then 20% of males with the fragile X "gene" could not be identified in the families with fragile X "gene". The reason for this might be that some males with fragile X were not classified as mentally retarded due to mild expression of the "gene". However, in some males where the fragile X gene

was present, but not expressed either at phenotypic or cytogenetic level, they would transmit this fragile X gene to their daughters and could have grandsons with the fragile X syndrome.

On the other hand, accurate segregation studies of fragile X in the females were not possible because many obligate fragile X carrier females do not show the expression of this fragility on their X chromosome cytogenetically. Only in one third of these obligate carrier females has the phenotypic expression of the fragile X syndrome been found.

2.7.3.2 Autosomal Fragile Sites

The autosomal FS are assumed to follow a mendelian co-dominant pattern of inheritance. Segregation analysis of rare autosomal folate sensitive FS was first assessed by Sherman and Sutherland (1986) in view of the unusual findings of the segregation ratio (0.406) of fragile X in males which was significantly different from the expected ratio of 0.5 for a fully penetrant X-linked gene (Sutherland and Hecht, 1985e). They concluded that the segregation of rare autosomal FS was not straightforward. They noted expression of the folate sensitive FS differed with the sex of the transmitting parent. They split their data by sex of the carrier parent to examine if lack of expression of the gene specific to a defined group of carrier. The test was found to be highly significant ($P<0.005$). The gene responsible for FS expression was presumably only 50% penetrant in the offspring of carrier father as only 25% of the offspring of carrier fathers expressed the FS. On the other hand, the gene was fully penetrant when transmitted by carrier mother, i.e. 50% of the offspring of carrier mothers expressed the FS. They could not find any differences of penetrance between sons and daughters of carrier fathers. They also found the same pattern of segregation differences for BrdU sensitive FS, 10q25,

though the trend was not statistically significant. The Distamycin-A induced FS, 16q22 appeared to be fully penetrant with co-dominant segregation.

2.8 PRENATAL DIAGNOSIS FOR FRAGILE SITES

The association of fra (X) (q27) and the Martin-Bell form of mental retardation could provide a strong reason for prenatal diagnosis (Sutherland, 1977). Jenkins et al. (1986) concluded that positive results in amniocytes are reliable for prenatal detection for the presence of fragile X. Negative or low frequency expression of fragile (X) should require further studies. There is a concept that certain autosomal rare FS might predispose to chromosome breakage and rearrangement in meiosis, but still it remains only a possibility and thus premature to justify prenatal diagnosis or counseling for the carriers of autosomal FS (Hecht and Hecht, 1984a; Hecht and Hecht, 1984d). However, Garcia-Sagredo et al. (1983) justified prenatal diagnosis for a FS carrier for other reasons. A general approach to genetic counseling for autosomal FS carriers would be to reassure and inform them that to the best of our current knowledge, FS are normal chromosome variations (Hecht and Hecht, 1984d; Chudley et al., 1990).

2.9 MECHANISM AND BIOCHEMISTRY OF FRAGILE SITE EXPRESSIONS

Chaudhuri et al. (1972) first gave an explanation for the origin and nature of general achromatic lesions. These chromatid gaps resulted from extreme despiralisation of the DNA due to the failure of compact folding in the metaphase chromosome. He considered four factors to be involved in spiralization of chromosomes: the DNA itself, histones, non-histone proteins and divalent cations. Sutherland (1979b) first proposed a possible explanation for the structural nature of FS. As the frequency of expression of FS can be

altered by changes to the composition of the culture medium 2 hrs. prior to harvest, then the sites must be influenced directly at the time of spiralization of the chromatids in late G_2 or early prophase and as the FS are heritable, they are probably a manifestation of the DNA coded information.

An alternative explanation came from the nature of inhibitors and inducers of folate sensitive FS. The roles of these chemicals indicate that the process of expression is more likely to be operating during DNA synthesis affected by pyrimidine biosynthesis, specifically the production of thymidine monophosphate (dTMP). The steps of this pyrimidine metabolism are believed to be (1) the conversion of uridine monophosphate (dUMP) to dTMP (by methylation of dUMP) and (2) 5, 10-methylene tetrahydrofolate (5, 10-meTHF) to dihydrofolate (DHF). Both reactions are catalysed by thymidiylate synthetase. Folic acid results in an increase in 5, 10-meTHF, which in turn leads to an increase in dTMP production, whereas thymidine is directly phosphorylated to dTMP (Sutherland, 1979b). Thus, they inhibit FS expression providing dTMP for incorporation into DNA during DNA synthesis. (Fig. 2).

Inducers of FS, such as MTX, aminopterin, FUdR, trifluorothymidine, FCdR, trimethoprin, pyrimethamine, folic acid and thymidine deficiency all lead to limitation of the dTMP pool available for DNA synthesis. MTX, aminopterin and trimethoprin block the conversion of DHF to THF by inhibiting dihydrofolate reductase (Sutherland, 1979b). FUdR, trifluorothymidine and FCdR all are inhibitors of thymidylate synthetase which converts dUMP to dTMP (Glover, 1981; Tommerup, et al., 1981) (Fig. 2). These explanations imply that the FS is a section of thymidine rich DNA which cannot complete synthesis with limited dTTP in the nucleotide pool. Goulian et al. (1980) found that indirect inhibition of thymidylate synthetase by MTX in human lymphoid cell lines

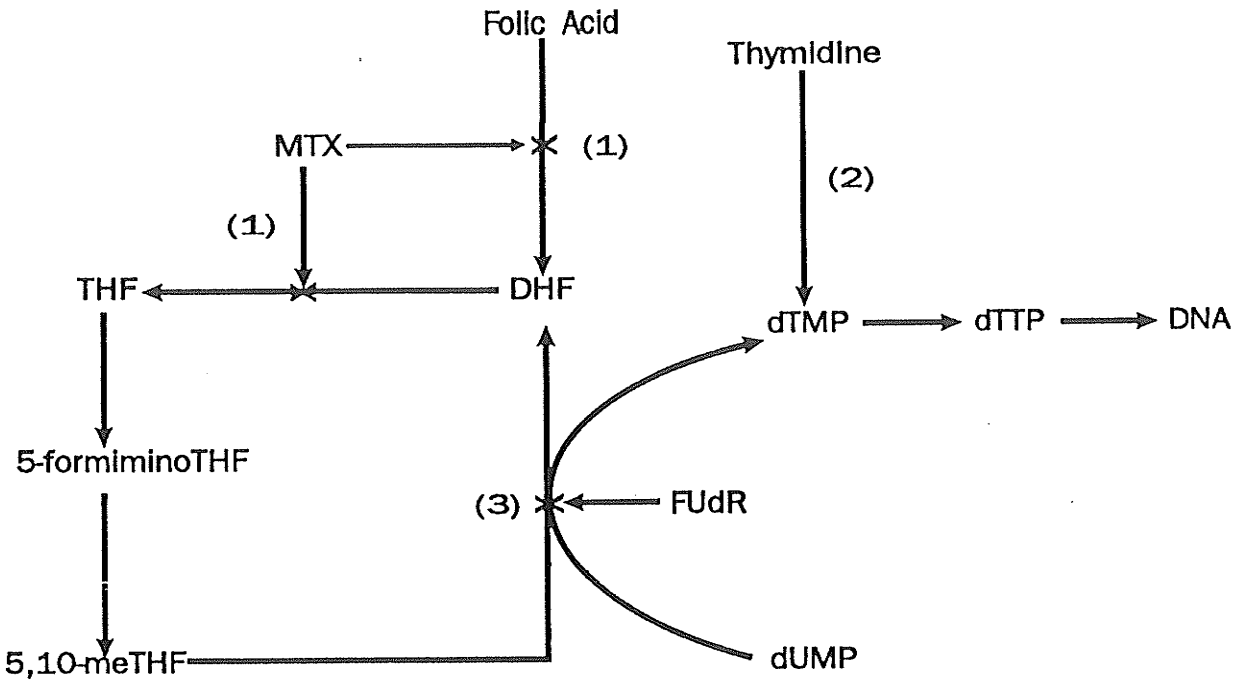

Figure 2. Pathways of nucleotide metabolism affected by restriction of folic acid and thymidine or induction by MTX or FUdR.

Key:

MTX=Methotrexate; DHF=dihydrofolate; THF=Trihydrofolate; (1)=dihydrofolate reductase; dUMP=deoxyuridine monophosphate; FUdR=Fluorodeoxyuridine; (3)=thymidine synthetase; dTMP=deoxythymidine monophosphate; dTTP=deoxythymidine triphosphate; (2)=thymidine kinase; "x"=inhibition of enzyme activity.

resulted in an increase in misincorporation of uracil into the DNA, possibly producing double-strand breaks due to futile DNA repair when dTTP was limited. Any inhibition of dTMP synthesis resulted in a pronounced change in the relative pool sizes of increased intracellular deoxyuridine triphosphate (dUTP) and decreased dTTP. Deoxyuridine triphosphate usually occurs within the cell at very low concentrations. During DNA replication the enzyme cannot efficiently distinguish between dUTP and dTTP (Reidy, 1988). Since dUTP is rare in the nucleotide pool, under normal circumstances there is very little chance of dUTP being incorporated in place of dTTP during DNA synthesis and thus, does not cause any problems (Goulian et al., 1980). The ratio of dUTP to dTTP increases over 1000-fold when folate metabolism is inhibited. Under these circumstances the dUTP appears as a DNA component (Goulian et al., 1980; Sedwick et al., 1981; Luzzatto et al., 1981). Krumdieck et al. (1983) suggested that misincorporation of uracil in place of thymine resulted in undermethylated dTMP-poor regions of DNA was the molecular event immediately responsible for expression of folate sensitive FS. This replacement of thymine by uracil results in the loss of the methyl group, which normally appears in an exposed position in the major groove of the DNA double helix (Goulian et al., 1980). The loss of the methyl group in this key region, be it by conversion of 5-methylcytosine to cytosine or thymine to uracil, interferes with the binding of proteins to DNA which provides a mechanism for chromosome folding or condensation (Comings and Riggs, 1971; Razin and Riggs, 1980). Simpson et al. (1979) showed that chromatin assembly can take place with the synthetic copolymer poly $d(G\text{-}C)_2$, which contains no 5-methyl substituents. From this finding, Hagerman (1984) proposed a different model for FS expression where the 5-methyl moiety did not play a major role. The mechanism was the rapid repair of misincorporated dUMP residues, both through action of uracil-N-

glycosylases and through possible proof reading functions of the eukaryotic DNA replication machinery. With these two actions, such abnormal residues were almost quantitatively removed under normal circumstances followed by new DNA synthesis for repair purpose. If such repair processes were carried out under dTTP depletion conditions, the resulting DNA may end up with extensive single-strand nicks or gaps. Thus, the FS expression was the consequence of an abortive repair and/or replication process, not the mere presence of altered bases without methyl groups. Lin et al. (1987) noticed that at 2 hrs. before harvesting a significant pattern of uridine-induced FS repairing with supplementation of thymidine. This repairing increased with the amount and time thymidine was supplied to the culture. At 12 hrs. this repairing mechanism reached a maximum. They observed that this repairing mechanism was very efficient, with a level of 90% rescue achievable though they were not able to fully repair all uridine-induced chromosome breaks. They concluded that this might be due to heterogeneity in the mechanism of FS induction. Expressions of some FS may be inhibited by addition of thymidine, whereas in others expression was not affected. Sutherland et al. (1985c) found that high concentrations of thymidine induced folate sensitive FS instead of inhibiting them. They also found that high levels of 5-bromodeoxyuridine (BrdU), an analogue of thymidine, did not induce folate sensitive FS. An explanation for this finding was that high levels of thymidine elevated the level of dTTP which inhibited ribonucleotide reductase-catalysed reduction of cytidine diphosphate to deoxycytidine diphosphate (dCDP) (Reichard et al., 1961). This latter nucleotide appeared to be a critical requirement for DNA synthesis at FS (Fig. 3), and hence, folate sensitive FS were expressed when either dCTP or dTTP was in short supply during DNA synthesis. Meuth et al. (1979) found an elevated level of dCTP with low levels of

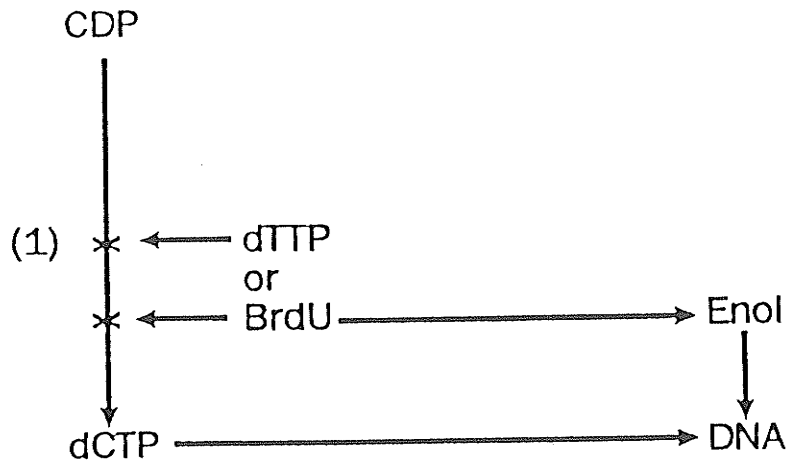

Figure 3. Inhibition of ribonucleotide reductase activity by elevated level of dTTP but not by BrdU.

Key:

CDP=cytidine diphosphate; dTTP=deoxythymidine triphosphate; BrdU=Bromodeoxyuridine; (1)=ribonucleotide reductase; dCTP=deoxycytidine triphosphate; "x"=inhibition of enzyme activity.

thymidine. This implies that the dCTP/dTTP ratio must be maintained within a certain range to inhibit FS expression (Sutherland et al., 1985c). The reason for elevated levels of dCTP with low dTTP is that a certain level of dTTP controls the activity of ribonucleotide reductase which converts cytidine diphosphate to dCDP. This dCDP eventually converts into dCTP. With low levels of dTTP the activity of ribonucleotide reductase is not under control and thus the level of dCTP becomes elevated (Meuth et al., 1979). The dCTP/dTTP ratio is important because with a low level of dTTP the dCDP can be converted to dUTP as well as dCTP. When the level of dCDP is higher there is more chance of production of dUTP to elevate the level of uracil in the nucleotide pool. This higher uracil level increases the misincorporation of uracil in newly synthesized DNA to induce FS (Sutherland, 1988).

Like thymidine, high concentration of BrdU also inhibits ribonucleotide reductase, although it does not induce FS. A possible explanation for this finding is that higher concentrations of BrdU resulted in a relative deficiency of dCDP for DNA synthesis. This can be overcome by the incorporation of the "enol" form of BrdU into the newly synthesized DNA strand in place of dCTP (Freese et al., 1959) (Fig. 3).

Distamycin-A induced FS can also be induced by the closely related oligopeptide antibiotic netropsin and Hoechst 33258 (Schmid et al., 1980; Sutherland et al., 1984). All these compounds are direct DNA binding ligands with high affinity for A-T rich regions. Binding is accompanied by subtle changes in DNA conformation and block the activity of DNA-dependent enzymes such as DNA and RNA polymerases, restriction endonucleases and DNAse (Wartell et al., 1974; Hahn, 1975; Zimmer, 1975; Simmer et al., 1980). They also inhibit chromosome contraction upon binding, especially in A-T

rich heterochromatin, producing uncoiled chromosome regions and non-staining gaps that resemble FS (Schmid et al., 1981; Hayman et al., 1981). The gaps may result from altered DNA conformation or possibly from the ability to inhibit DNA-dependent enzymes which may also influence induction of this group of FS.

The effects of BrdU for the BrdU-inducing FS include its incorporation into DNA in place of thymidine possibly driven by altered nucleotide pools (Roy-Burman, 1970; Reichard et al., 1961). On the chromosomal level BrdU affects chromosome spiralization and staining characteristics. It also causes breaks in mammalian chromosomes (Hsu et al., 1961).

The common FS are weakly induced by conditions or agents that also induce the folate sensitive FS and strongly induced by the DNA polymerase α-inhibiter aphidicolin (Glover et al., 1984). DNA polymerase α is responsible for chromosomal DNA replication. The common FS are specially sensitive to DNA polymerase α-inhibition. Aphidicolin is competitive with dCTP for binding sites on the polymerase molecule (Oguro et al., 1979). It blocks progression of the replication fork (Lonn et al., 1983) and interferes with the joining of adjacent DNA intermediates (Yagura et al., 1982). Such blocks of progression of replication forks would leave single stranded gaps preferentially at the FS helping to express them.

As the conditions inducing folate sensitive FS also induce some common FS, Glover et al. (1984) suggested that these conditions share with aphidicolin the partial inhibition of polymerase α. They also found that aphidicolin was not able to induce the folate sensitive FS, the fragile X, suggesting that there might be separate but closely related underlying mechanisms for induction of these two classes of FS.

The expression of folate sensitive FS in cultured cells can be suppressed by

maintaining normal levels of thymidine or folate and the part of the cell cycle during which the expression of the FS may be suppressed by supplementing thymidine or folate is late S or early G_2 phase. From this finding, Laird et al. (1987) concluded that (1) the DNA at FS is late replicating, (2) DNA that replicates unusually late may miss the normal condensation during G_2 phase and thus creates a visible chromosome gap. Alteration in DNA which could lead to its late replication might be due to mutations that directly affect DNA sequences involved in the timing of replication. Other important genetic alterations might be greatly expanding the distance between two replication origins by unequal recombination (Ledbetter et al., 1986) as well as by insertion of DNA resulting in delayed replication for the region. Some methylation events, which are involved in X chromosome inactivation (Mohandas et al., 1981) might lead to or maintain this late replication. Spontaneous methylation of DNA may occur and be propagated by a "maintenance methylase", an enzyme that can methylate the non-methylated strand of half methylated DNA after semiconservative DNA synthesis.

2.10 PROPOSED MODEL FOR THE DNA SEQUENCE AT THE FOLATE SENSITIVE FRAGILE SITES

Sutherland et al. (1985c) found that both low and high concentrations of thymidine levels induced the expression of folate sensitive FS but BrdU, an analogue of thymidine, did not induce them. Based on these findings they proposed a molecular model for the folate sensitive FS.

Low levels of thymidine decreased the level of dTTP and high levels of thymidine decreased the dCTP levels in the nucleotide pool. This finding implies that the folate sensitive FS are expressed when either dCTP or dTTP is depleted. Again, with the high

concentration of thymidine, the levels of deoxyadenosine triphosphate (dATP) become elevated (Fox et al., 1980). Therefore, it is unlikely that the folate sensitive FS are due to DNA regions which are AT-rich, but high levels of BrdU which also causes the decrease in dCTP levels, does not induce the folate sensitive FS because the enol form of BrdU can pair with guanosine (Freese, 1959) in place of cytosine. This allows DNA synthesis to proceed without leaving the guanosine residues unpaired as happens when excess thymidine inhibits dCTP synthesis or low levels of thymidine causes dTTP depletion. Thus, the proposed model is that the DNA which expresses as a folate sensitive FS is a repeating alternating sequences of polypurine/polypyrimidine rich DNA (Fig. 4). The simple repeating sequence of poly d(AG).poly d(TC) would produce single stranded gaps under dCTP or dTTP depletion since replication could not proceed by primer extension, but with the presence of BrdU when dCTP is limited, such gaps would not result since BrdU can pair not only with guanosine but also with adenosine.

Throughout the human genome, there exists Z-DNA, which is composed of short stretches of alternating pyrimidines and purines - the repeating sequence d(CA).d(GT) (Rich et al., 1984; Hamada et al., 1982).

Sutherland et al. (1985c) proposed that a folate sensitive FS resulted from the amplification (Smith, 1976) of a naturally occurring polypurine/polypyrimidine sequence. The degree of amplification could account for the ease of FS induction and familial differences.

3.0 MATERIALS AND METHODS

The present study has been carried out on 12 families with rare autosomal folate sensitive FS. These family pedigrees are shown in Fig. 5.1-5.12. Of these 12 families,

9 were informative for segregation analysis. Among these family pedigrees, families AG,

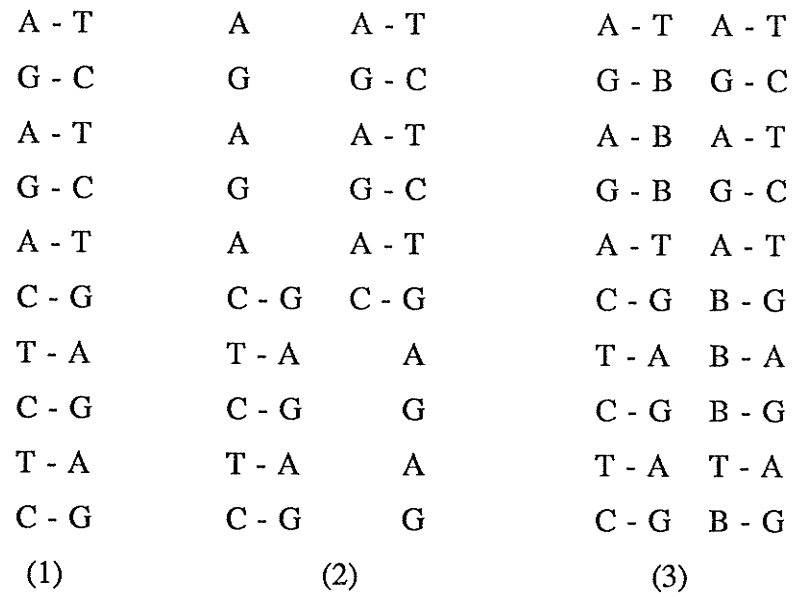

Figure 4. Model proposed after Sutherland et al. (1985c) for a folate sensitive FS.
(1) Possible DNA sequence at the FS
(2) Daughter strands with gaps after replication in low dTTP or low dCTP pool.
(3) Daughter strands without gaps after replication in low dCTP pool but in high BrdUTP pool. Adenosine residues will pair with either thymidine or BrdU.

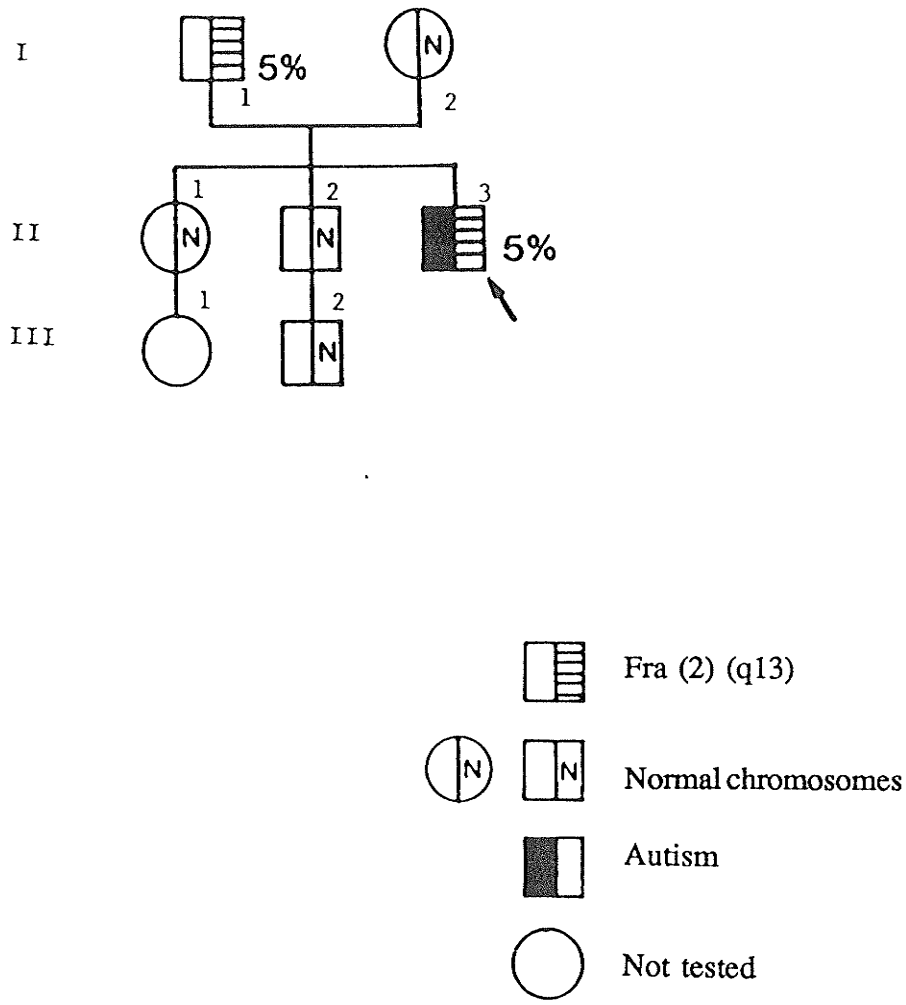

Figure 5.1 **Pedigree of Family LSh**

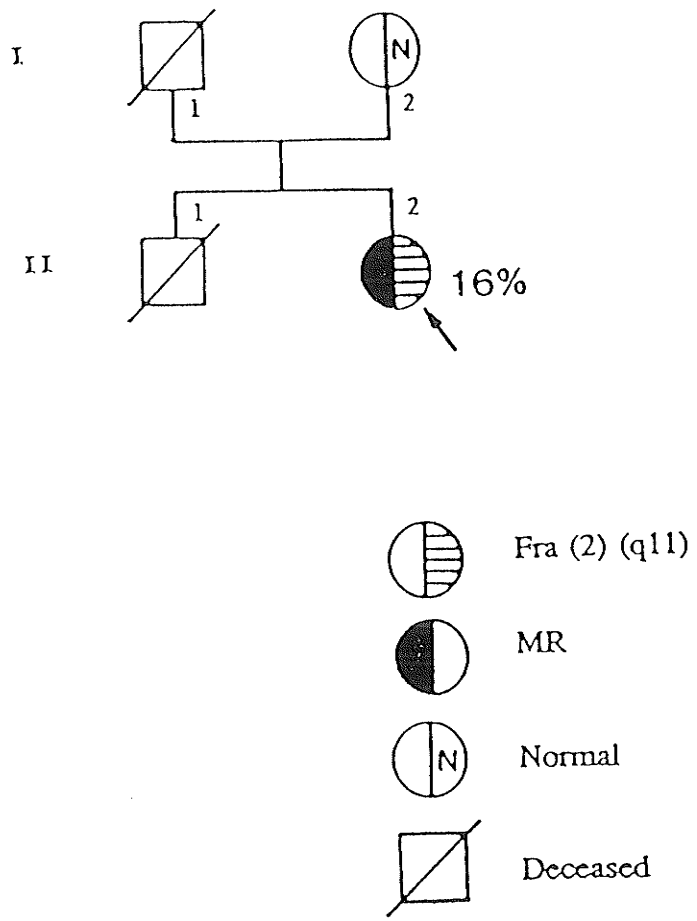

Figure 5.2 **Pedigree of Family CMᶜD**

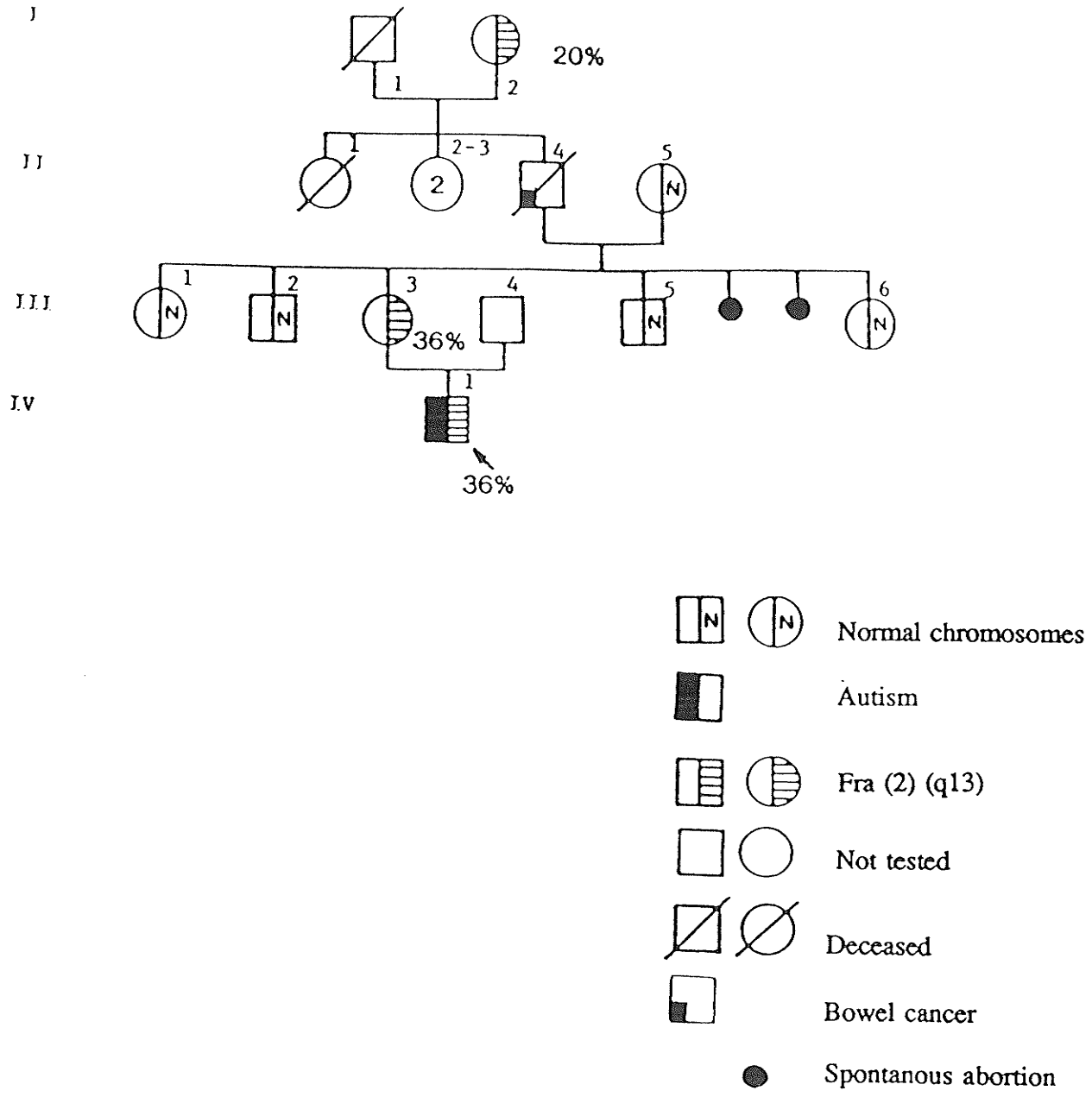

Figure 5.3 **Pedigree of Family AG**

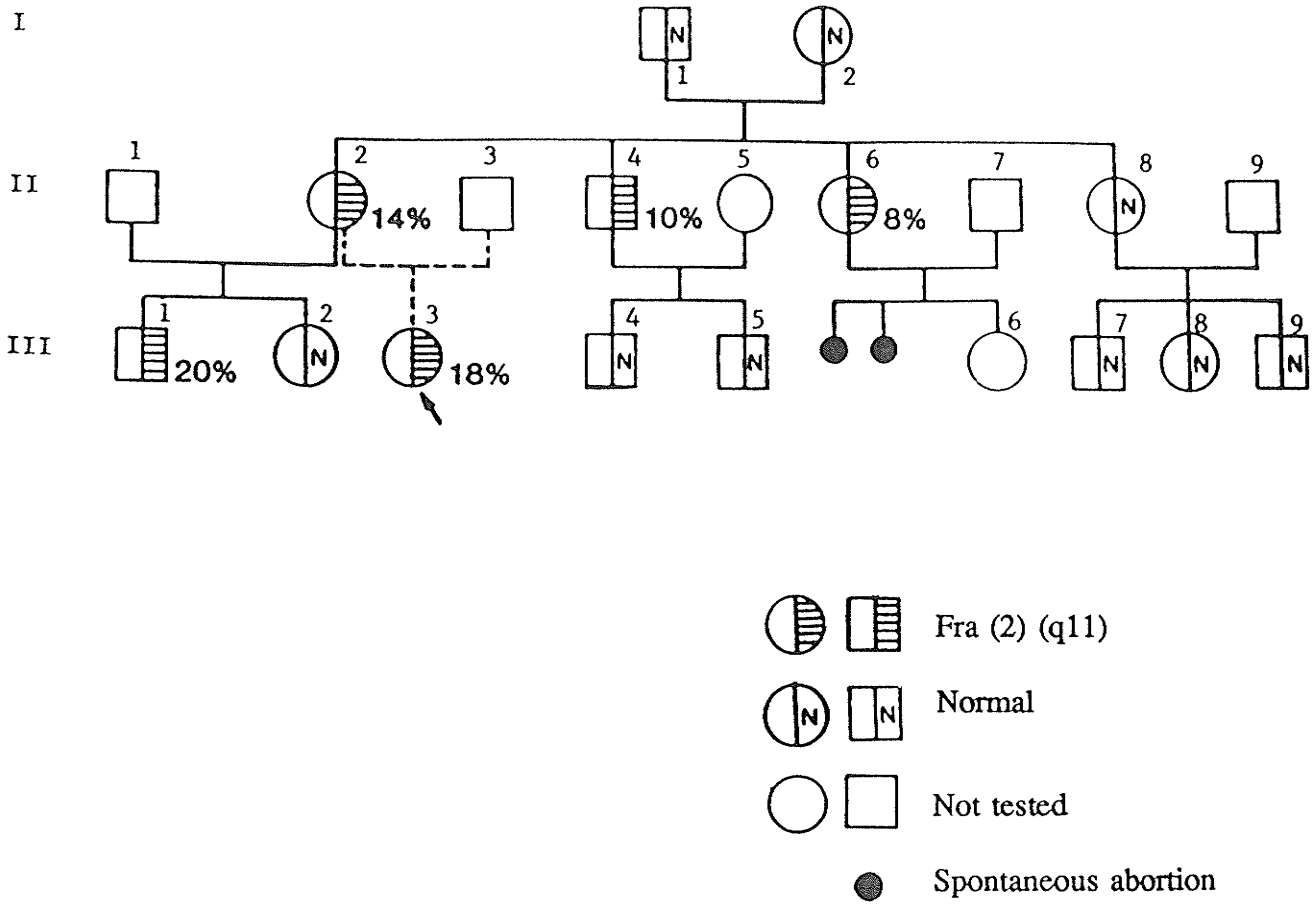

Figure 5.4 **Pedigree of Family SR**

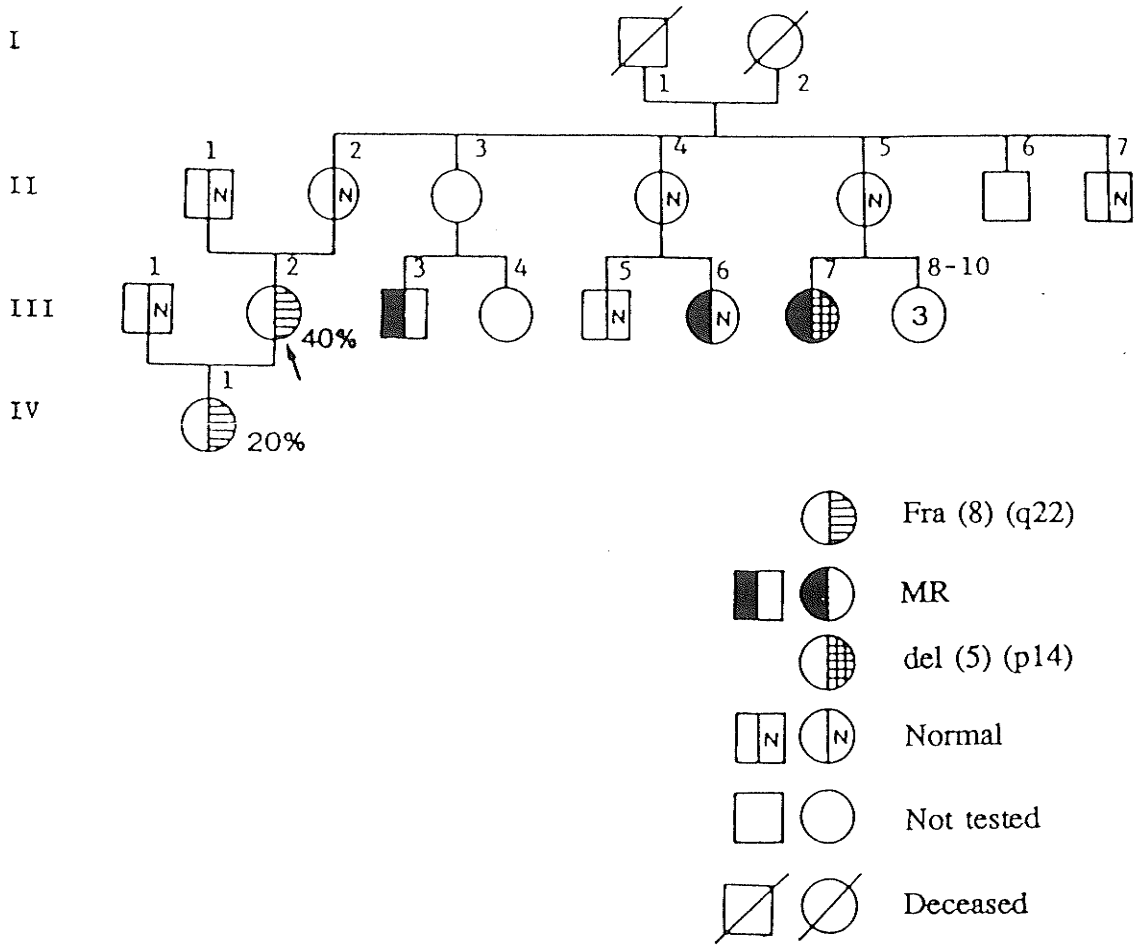

Figure 5.5 **Pedigree of Family WM[c]M**

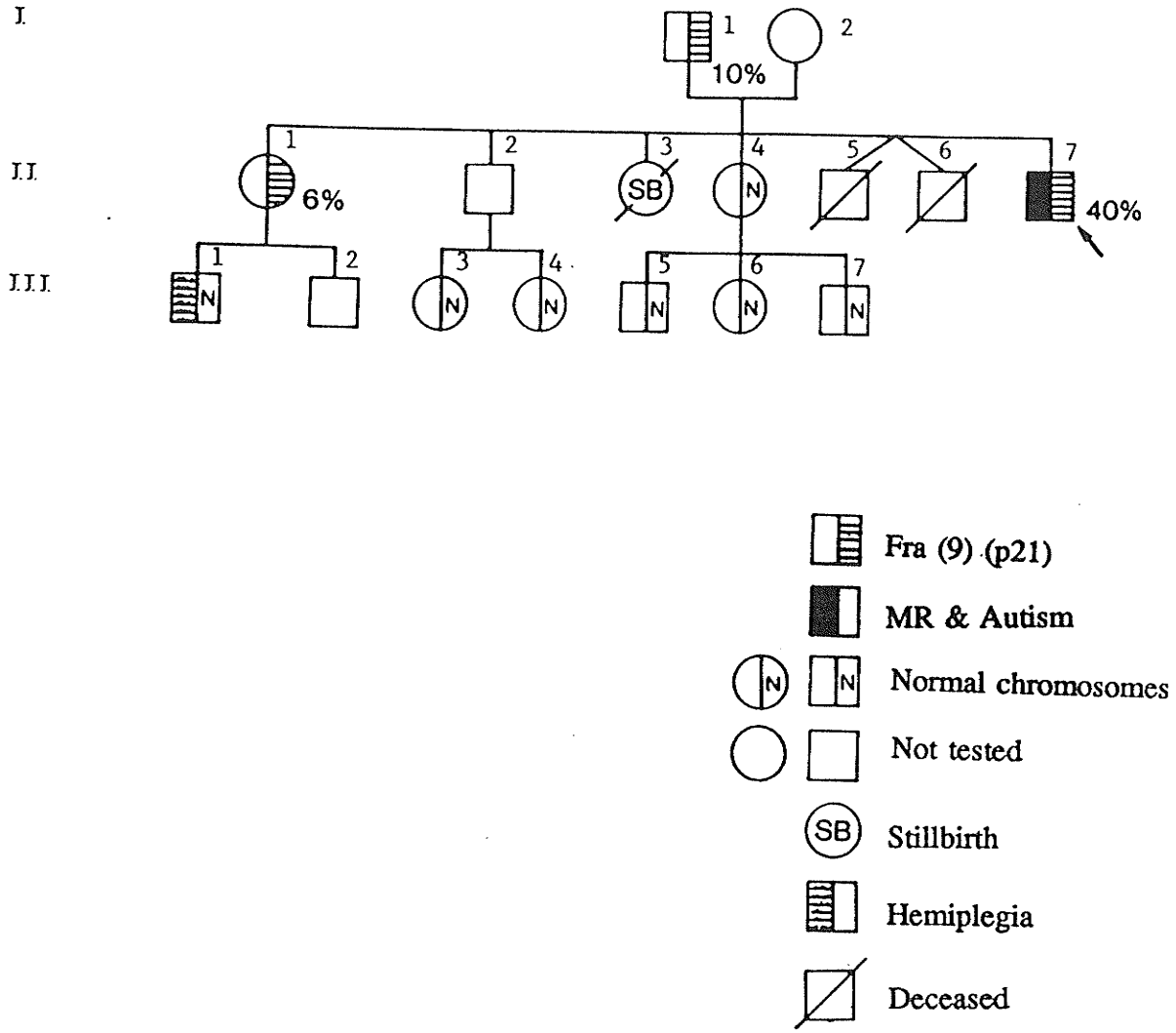

Figure 5.6 **Pedigree of Family BG**

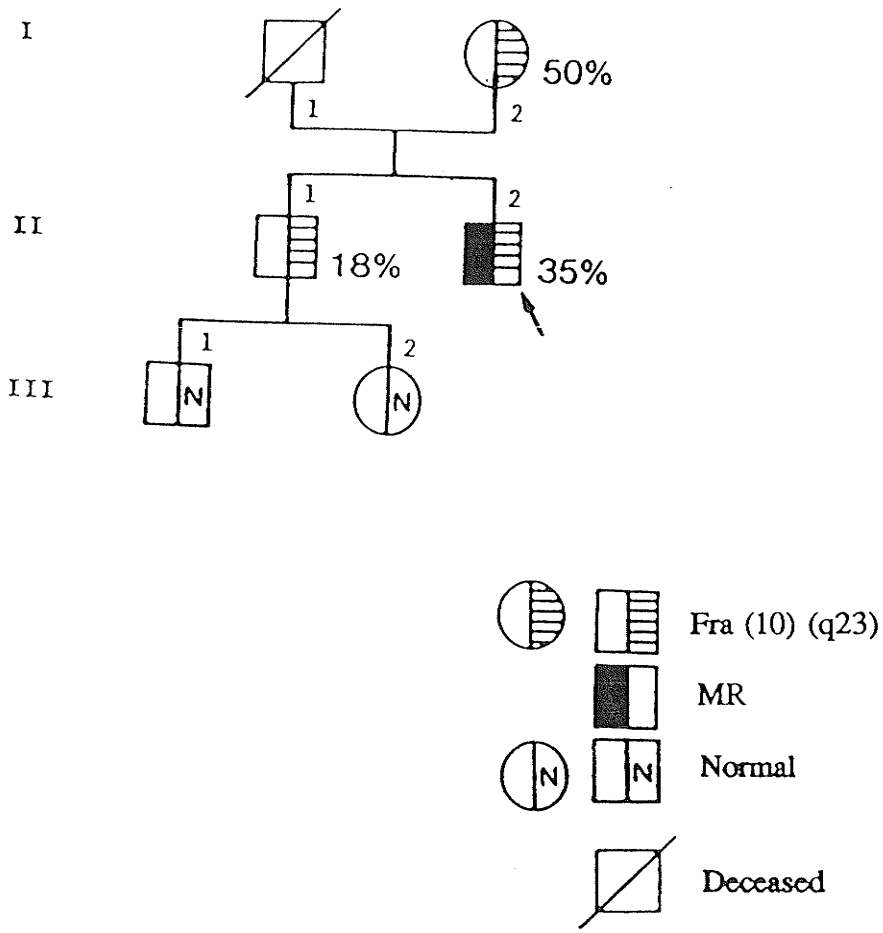

Figure 5.7 **Pedigree of Family LN**

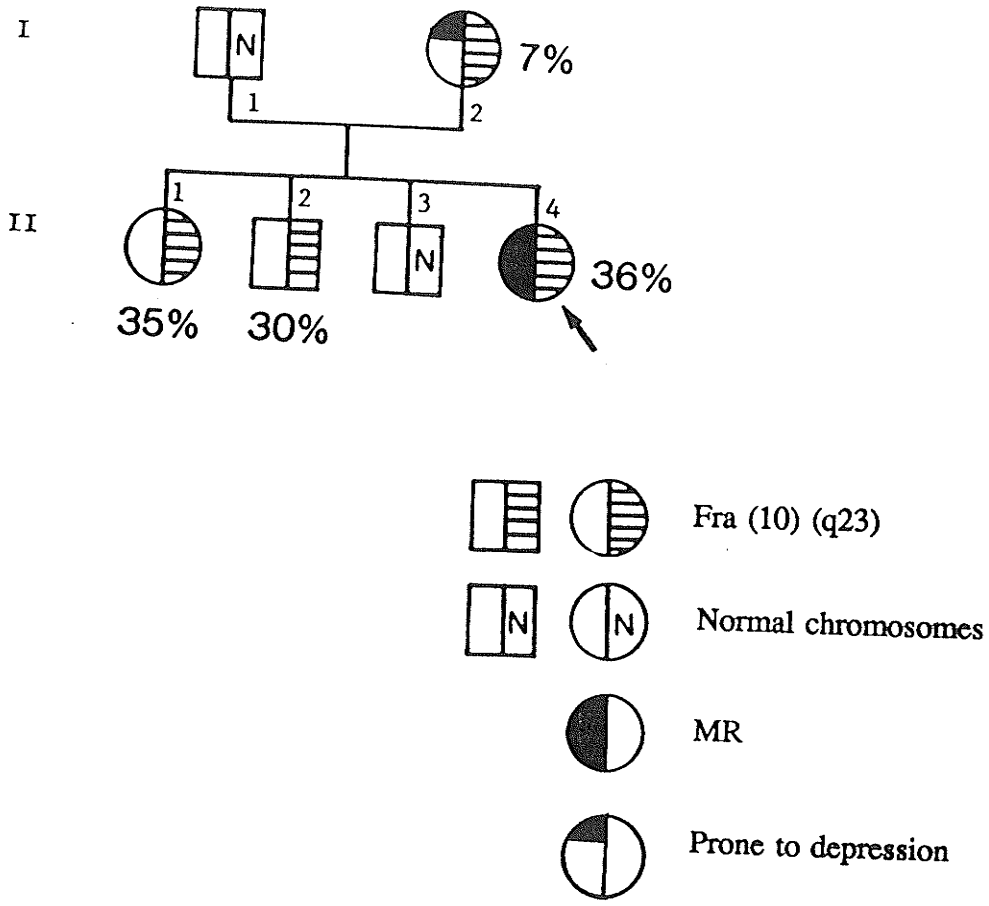

Figure 5.8 **Pedigree of Family SL**

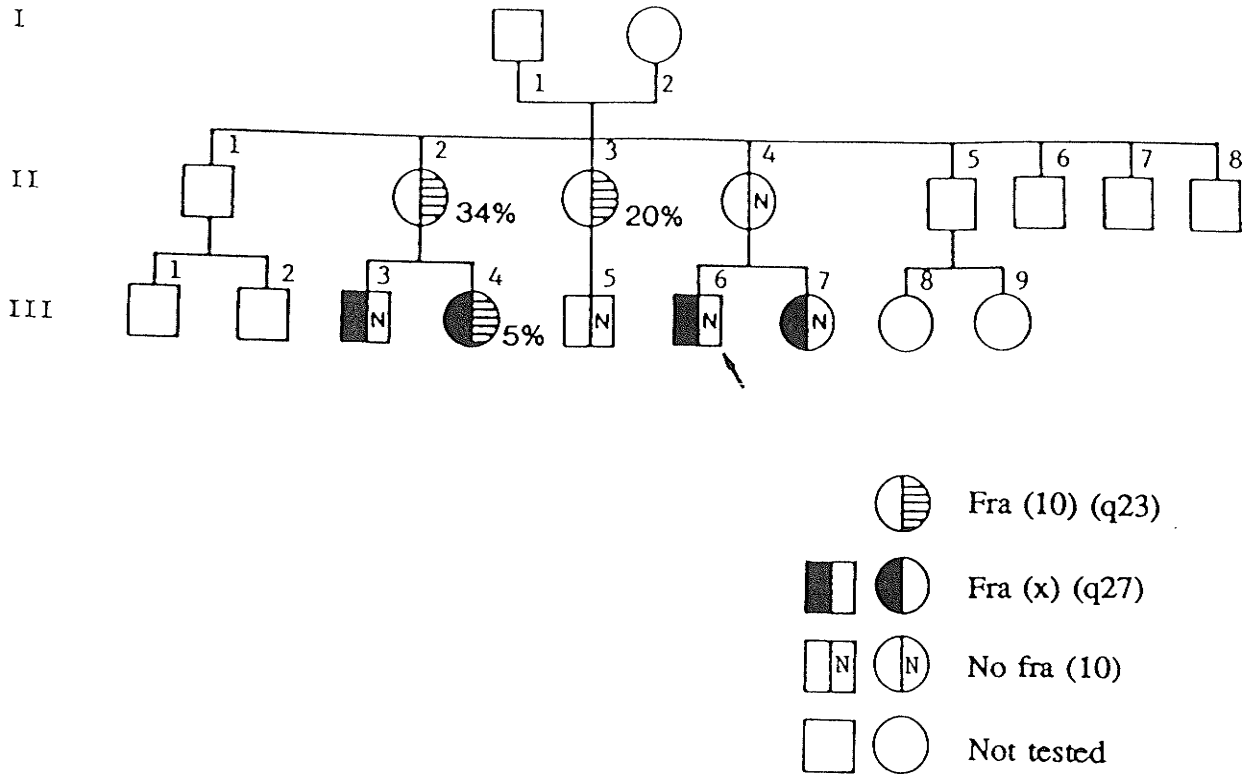

Figure 5.9 **Pedigree of Family JC**

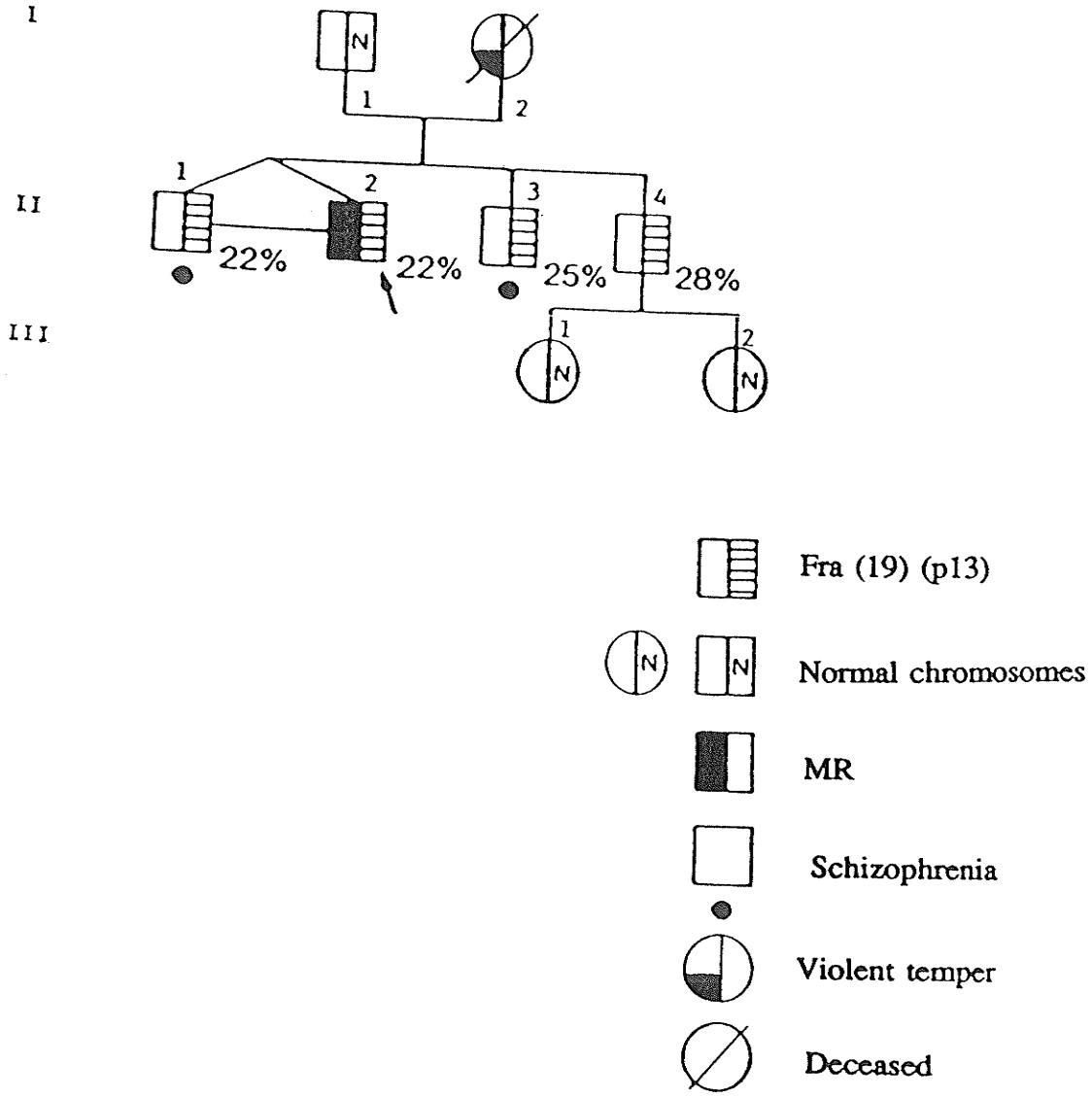

Figure 5.10 Pedigree of Family TM

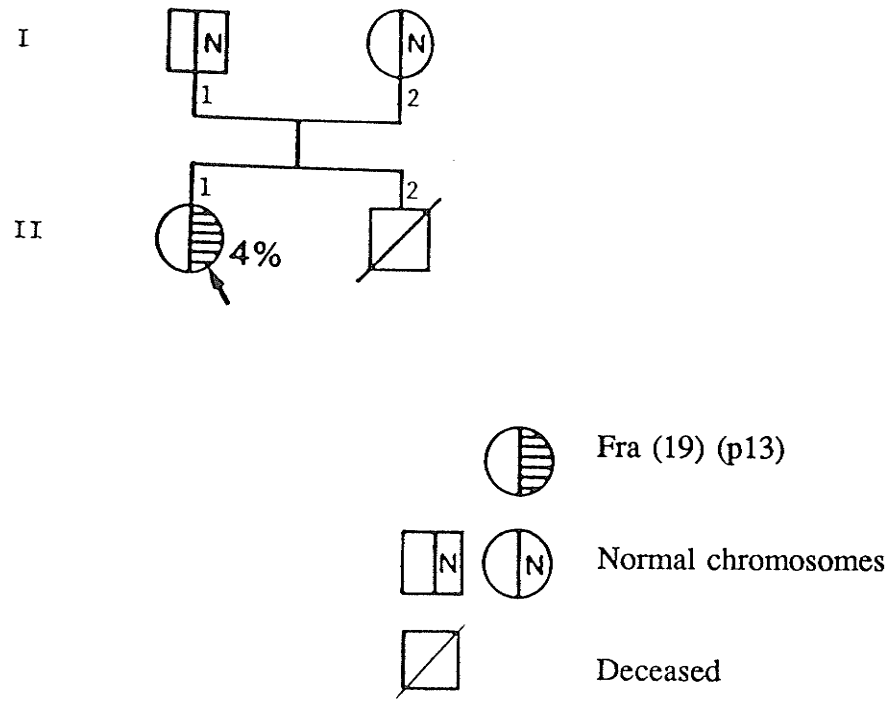

Figure 5.11 **Pedigree of Family TP**

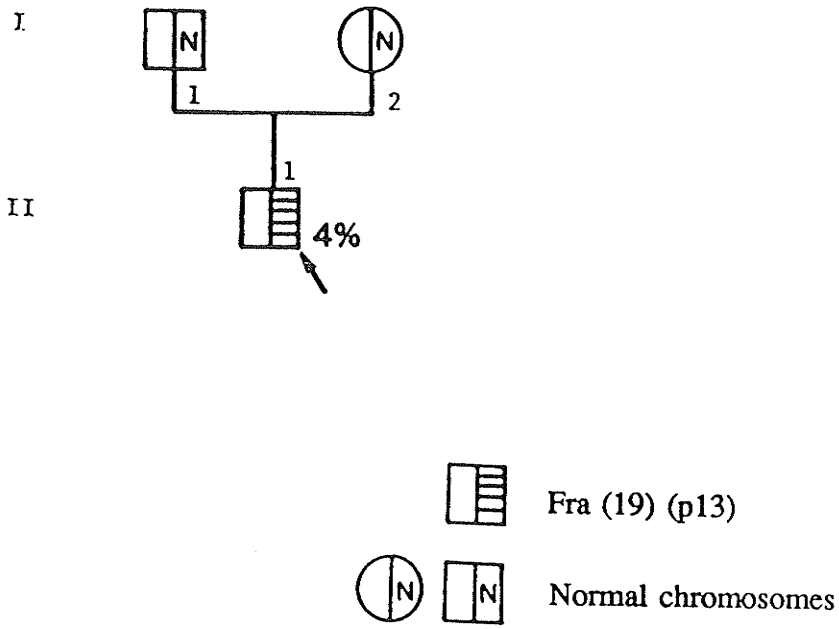

Figure 5.12 **Pedigree of Family SS**

LSh, and TM have been previously published (Jayakar et al., 1986; Chodirker et al., 1987). In this study chromosome analysis has been done by myself on LSh II.1; CM°D I.2; SR I.1, I.2, II.2, II.4, II.6, II.8, III.1, III.2, III.4, III.5, III.7, III.8, III.9; WM°M II.4, II.5, III.5, III.6, III.7, III.8; BG I.1, II.1, II.4, III.1, III.3, III.4, III.5, III.6, III.7; LN II.1, III.1, III.2. The information about the chromosomal status of other participants was provided by cytogenetics service laboratory. In order to expand the number of families for meta-analysis (Esdaile et al., 1989) other families with rare autosomal folate sensitive FS from recently published papers were included (Tommerup et al., 1985; Smeets et al., 1985; Romain et al., 1986; Kähkönen et al., 1989) (Fig. 6). There were 20 families with this group of FS ascertained from the literature; 13 were informative for segregation analysis.

3.1 MATERIALS

3.1.1 Study Families Ascertainment

This study was approved by Faculty of Medicine, University of Manitoba, Research Committee on Human Subjects.

After the ascertainment of the index cases (Fig. 5.1-5.12, shown by arrows), segregation analysis of the FS was carried out in these families after the completion of carrier detection.

There were three different sources used to ascertain probands. The first source was a blind controlled study on an autistic population which included the two probands in the families AG and LSh respectively who were diagnosed as carriers of folate sensitive FS 2q13 (Jayakar et al., 1986).

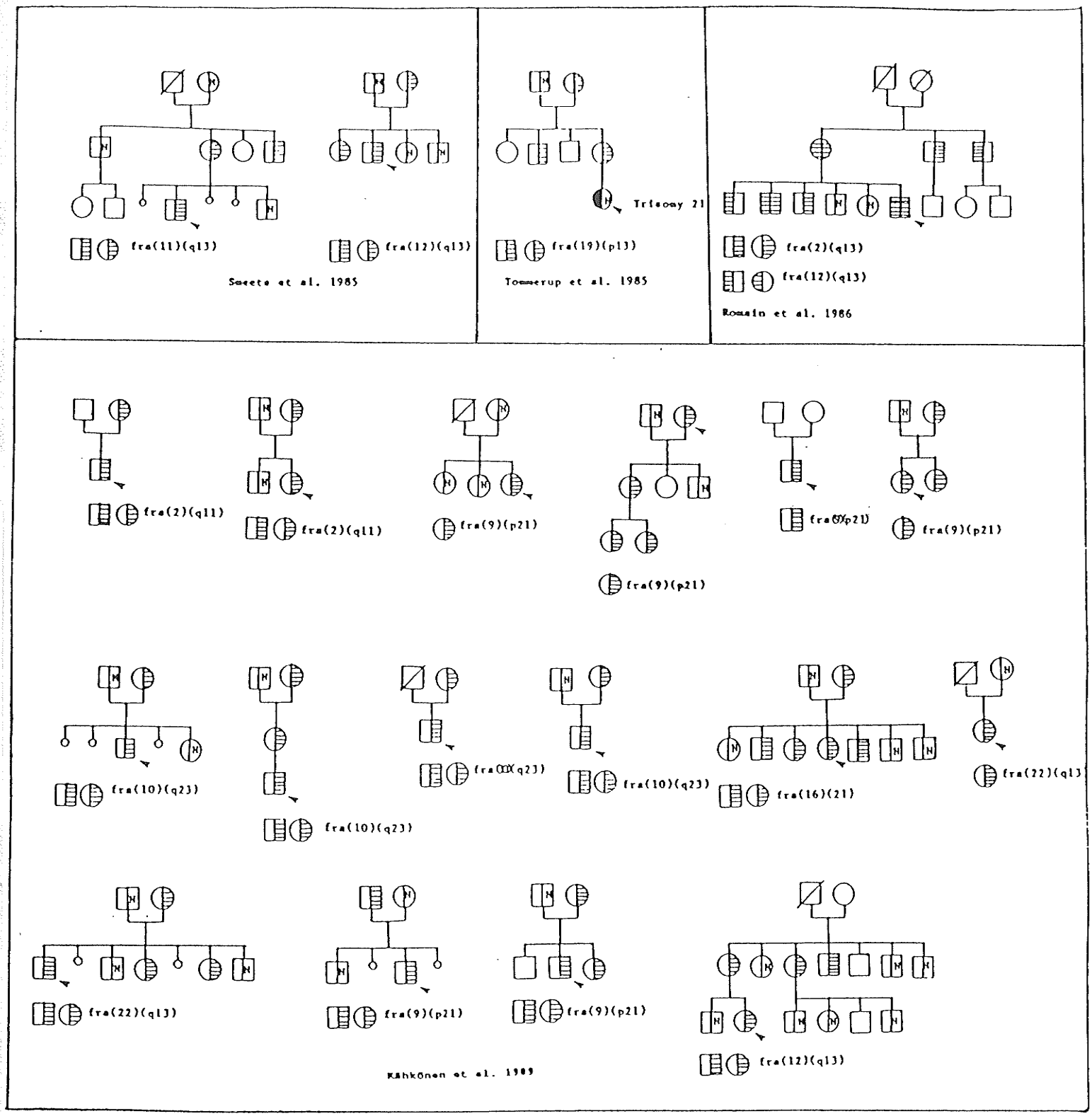

Figure 6. **Literature Review Family Pedigrees**

Key: = **Normal chromosomes;** = **Not tested;** = **Deceased;** = **Proband**

The second source of ascertainment of probands was a large blind controlled study to determine the frequency of FS in newborn (NB) and mentally retarded (MR) populations which included five probands (Chudley et al., 1990). The chromosome analysis on the proband of the family TM was part of this population study (Chodirker et al., 1987) and he was diagnosed as a carrier of folate sensitive FS, 19p13. The probands of the families CM^{c}D, SR, TP and SS were confirmed as the carriers of folate sensitive FS, 2q11 (Fig. 5.2), FS, 2q11 (Fig. 5.4), 19p13 (Fig. 5.11) and 19p13 (Fig. 5.12) respectively during the study to determine the frequency of FS in newborns (NB) populations (Chudley et al., 1990).

The final source of proband ascertainment was through the clinical genetics and cytogenetics service laboratory which provided five probands. The proband in the family WM^{c}M underwent chromosome analysis when she was pregnant for possible prenatal diagnosis for X-linked mental retardation because of her family history of mental retardation. She was diagnosed as a carrier of the folate sensitive FS 8q22 (Fig. 5.5). The proband in the family JC is a fragile X carrier. When the chromosome analysis was done on other family members to detect other fragile X carriers, it was found that this family also has a segregating folate sensitive FS, 10q23 (Fig. 5.9). The chromosome analysis on the probands in the families BG, LN and SL were performed because of mental retardation. They were carriers of folate sensitive FS, 9p21 (Fig. 5.6), 10q23 (Fig. 5.7) and 10q23 (Fig. 5.8) respectively.

3.1.2 Control Subjects Selection

Sex matched controls for the healthy individuals were also included in the study. Control subjects were mostly the members of the Department of Human Genetics,

University of Manitoba. Verbal consent was taken from each control subject and only blood specimens were obtained from them. The control specimens were used to monitor the effect of the modified culture medium on the blood cultures.

3.2 METHODS

3.2.1 Clinical evaluation

A careful and detailed family history for conditions including mental retardation, congenital anomalies, cancer and spontaneous fetal loss were taken from the proband or first degree relatives of the proband. In addition, other carriers identified in the families were interviewed.

3.2.2 Cytogenetic Methods

The first step of the study involved the culture of blood lymphocytes. Before collecting blood samples written informed consent was obtained from each adult study patient. For the patients who were under 18 years of age consent was obtained from their parents. Five ml of peripheral blood was obtained by venipuncture from the study patients and from control patients. Blood samples were collected in heparinised vacutainers. Patients who lived far from the city and who were willing to donate blood samples for the study, were requested to contact their family physician. We requested the physician to send the blood sample to us through a courier service. Blood samples from the patients and controls were usually cultured simultaneously. Samples were coded before culture and the culture were established within 24 hrs. of blood collection.

The lymphocytes were cultured in a medium deficient in thymidine and folic acid. We also used an inducing agent, 5-fluorodeoxyuridine (FUdR) (Glover, 1981). Under a

sterile tissue culture hood, 6-8 drops of whole blood were added to 5 ml of medium 199, previously prepared under aseptic conditions, in tissue culture tubes. The composition of the medium was Earle's salts with glutamine -500 ml; fetal calf serum - 25 ml; phytohaemagglutinin - 5 ml; penicillin G and Streptomycine sulphate (premade) - 5 ml; 7.5% $NaHCO_3$ - 5 ml. The pH of the medium was 7.6. A sterility check had been done before using the prepared medium 199.

After addition of the blood to the medium, the culture tubes were shaken carefully to mix the blood well into the medium. The specimens were then incubated at 37°C in 5% CO_2. To enhance lymphocytes' growth in the culture, the specimens were shaken every day under the sterile tissue culture hood. At 72 hrs. (24 hrs. prior to harvest), 0.1 ml of 5μM solution of FUdR was added to each tube. At this time, the specimen was vortexed to mix the FUdR thoroughly into the culture medium. Two hours prior to harvest 0.1 ml of 2.5 μg/ml solution of colcemid was added to each tube and again the specimen was thoroughly vortexed. To harvest the lymphocytes, the culture tubes were placed in a centrifuge hood and spun at 700-1000 RPM for 7-10 minutes. The supernatant was then poured off quickly and carefully. Five-10 ml of prewarmed (37°C) hypotonic solution (0.075M KCl) was added to each culture tube while the specimen was vortexing. Specimens were then incubated at 37°C for 20 minutes. After 7-10 minutes of spinning in the centrifuge at 700-1000 RPM, the supernatant was again discarded. The cells in the pellet were then fixed with 10 ml of 3:1, methanol to glacial acetic acid, fixative. First, the fixative was added to the cells drop by drop while the specimen was vortexing. When the colour of the specimen turned dark brown then the rest of the 10 ml of fixative was added to the specimen. Tubes were then placed in the cold room for at least 2 hrs., then the tubes were again spun at 700-1000 RPM to recover the pellet.

The pellet was then washed in a fixative 3-4 times spinning after each washing until the supernatant was clear. The cell suspension was dropped on the slides, cleaned with 95% ethanol and kept in the moist chamber at 30°C temperature until dry. Slides were stained conventionally with 4% Giemsa solution and 50 good metaphases were analysed from each individual for evidence of fragile sites, breaks and gaps and other anomalies. If, in a single individual 2 or more breaks appearing in different cells were considered at the same site on the same chromosome, sequential G or Q banding was done to confirm the site, location and the number of the chromosome on which the breaks appeared (Hecht and Sutherland, 1984e).

3.2.2.1 G-Banding with Trypsin (modified method of Seabright, 1971)

Preparation of trypsin stalk solution

10 ml of 0.85% saline (freshly made) was added to the stock vial. Then 2 ml of this trypsin stalk solution was added to 48 ml of 0.85% saline to make it a working solution.

Preparation of Giemsa working solution

2 ml of Giemsa was added to 48 ml of Gurr's buffer, pH 6.8.

Procedure of chromosome banding

Seven day old slides were prewashed in 0.85% of saline for 20-30 seconds. Then the slides were dipped in the trypsin working solution for 5-6 seconds. The temperature of trypsin solution was maintained at 17°C. While the slides were dipped in the trypsin solution, they were continuously shaken in the solution while held with forceps. The slides were then rinsed into two changes of 0.85% saline. They were then stained with

Giemsa working solution for 5-6 minutes. Finally, they were washed in 2 changes of distilled water and air dried. The slides were analyzed under a light microscope and the FS were photographed.

3.2.2.2 Q-Banding (modified from Casperson et al., 1971)

Pre-staining procedures included the treatment of the slides starting with 100% ethanol, then in 70%, 50% and 20% ethanol, for 2 minutes in each. Following this, the slides were dipped in McIlvaine's buffer (pH 4.5) for 5 minutes. The stain used was 0.5% Atebrin (Quinacrine dihydrochloride) in McIlvaine's buffer (pH 4.5). The slides were placed in the staining solution for 20-25 minutes. The differentiation was done using McIlvaine's buffer of the same pH 3 times for 1 minute, 1 minute and 8 minutes respectively. The slides were removed from the buffer and allowed to drain but not to dry. Several drops of distilled water was placed on the slide surface. A coverslip was placed on top avoiding air bubbles carefully. Excess water was removed by blotting with paper towel. The edges of the coverslip were then sealed with nail polish and allowed to dry. The slides were analyzed under the fluorescent microscope and the pictures of the FS were taken on the same day as the staining.

3.2.3 Statistical Methods

3.2.3.1 Comparison of Segregation and Sex Ratios

The chi-square test of significance is used to give a measure of the significance of an observed deviation from the expected value.

This chi-square (X^2) test is designed to assess the significance of deviation from the expected in relation to the number of observation made. It has the virtue of reducing

many different samples of different sizes and with different numerical deviations to a common scale for comparison.

The formula is used as follows:

$$X^2 = \Sigma(O-E)^2/E$$

where X^2 = chi-square

Σ = sum of

O = observed value

E = Expected value

3.2.3.2 Comparison of Percentage of Fragile Site Expression

The paired Wilcoxon rank sum test was used to measure possible differences between the percentages of expression of FS in the carriers with clinical abnormalities and carriers with normal clinical findings. To determine whether any significant differences exist between the family groups with at least one clinically abnormal FS carrier and family groups with only clinically normal FS carriers, Mann-Whitney two tailed test was utilized (Wilcoxon, 1945).

4.0 RESULTS

4.1 CLINICAL FINDINGS

Among our study families (n=12), FS carriers (other than probands) in five families showed to some extent clinical abnormalities. In the family AG II.4, who was an obligate carrier of FS, died of bowel cancer and his wife had two spontaneous abortions. In the family SR II.6, who is a FS carrier, also had two spontaneous abortions. In the family BG, I.1 is a FS carrier whose wife had a stillbirth. In the family SL I.2 is a FS carrier

and is prone to depression. In the family TM I.2, who was a presumed FS carrier and had moodswings with occasional violent outbursts and TM II.1 and II.3 are also FS carriers and both have schizophrenia. The total number of FS carriers in these five families including TM I.2 and AG II.4, (who were obligate carriers) and probands is 21, 7 of which have some clinical abnormalities or fetal loss problem as described above. Furthermore, 5 of these 21 FS carriers are probands. Four of these 5 probands are mentally handicapped and the other one is clinically normal. The other 9 of these 21 FS carriers are clinically normal.

In the other seven study families, namely LSh, CM^{c}D, WM^{c}M, LN, JC, TP and SS the total number of FS carriers is 13, including 6 probands. Only 3 of these 6 probands showed clinical abnormalities. One has a pervasive developmental disorder and 2 are mentally retarded. All the other 7 FS carriers are clinically normal and no obvious correlations of cancer or fetal loss were found with these FS carriers or in the families with FS (Table III).

In the family WM^{c}M, three of the first cousins of the proband are mentally retarded. None of them are FS carriers, one of them has del(5)(p14).

There are 25 FS non-carriers in our study families including 1 proband (fam. JC). This proband and 1 of his cousin (JC III.3) are not autosomal FS carriers but are fra (X) (q27) carriers. Another 1 of these FS non-carriers has hemiplegia (BG III.1)

4.2 CYTOGENETIC FINDINGS

4.2.1 Autosomal Aberrations

Families SR and CM^{c}D have the fra (2) (q11) (Fig. 7) and the rate of expression of this FS in the carriers, ranges from 8%-20%. No other significant chromosome anomalies

were noted in any of the family members tested in these two families. Family SR provided an unusual finding. Neither of the parents of 3 FS carriers (II.2, II.4, II.6) was FS positive.

Families LSh and AG have the fra (2) (q13). The rate of expression of this FS in family LSh is 5% and in family AG is 20%-36%. No other significant chromosome anomalies were found in these two families.

Family WM^{c}M has the fra (8) (q22). The rate of expression of this FS in this family is 20%-40%. One of the first cousins (WM^{c}M III.7) of the proband has del(5)(p14) which was found in 100% of her cells. No other chromosome abnormalities were found either in the FS carriers or in the other family members who were tested.

Families TM, TP and SS have the fra (19) (p13) and the rate of expression of this FS ranges from 4%-28%. Other than this FS expression, no other chromosome abnormalities were found in these families. In the families TP and SS, none of the parents were FS positive.

Family BG has the fra (9) (p21) (Fig. 8) which is the only chromosome abnormality found in this family. The range of expression of this FS is 6%-40%.

Families LN, SL and JC have the fra (10) (q23) (Fig. 9) expression of which ranges from 5%-50% among the FS carriers. No other chromosome anomaly was found in the families LN and SL. Family JC has fra (X) (q27) along with fra (10) (q23).

Individuals with rare autosomal folate sensitive FS, their sexes, the percentage of expression of FS in them and the main clinical findings are shown in Table IV.

No rare FS were identified in the control subjects.

Table III. Findings in clinically abnormal FS carriers

Clinical Findings	**Individuals affected**
Pervasive developmental disorder (Autism)	LShII.3, AGIV.1, BGII.7
Mental retardation with atypical psychosis	CM^{c}DII.2, LNII.2, SLII.4, TMII.2
Schizophrenia	TMII.1, TMII.3
Violent temper	TMI.2
Bowel cancer (wife had 2 miscarriages)	AGII.4
Reproductive losses	SRII.6, BGI.1
Depressive disorder	SLI.2

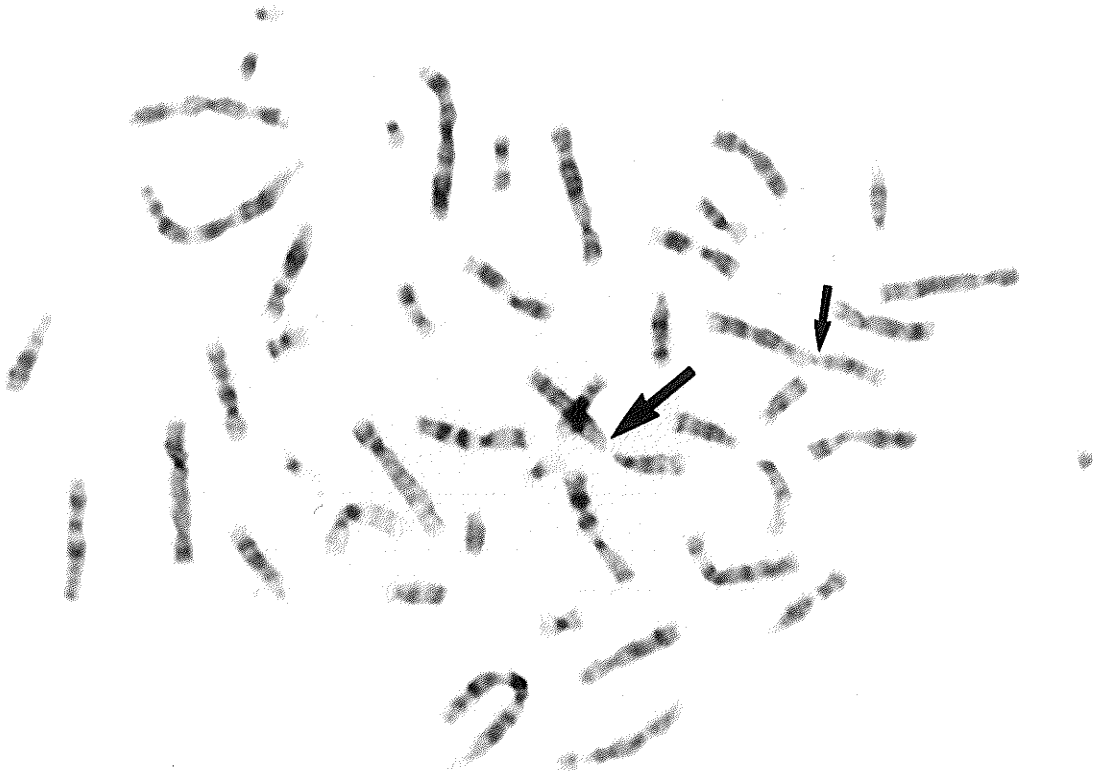

Figure 7. **G-banded metaphase spread from brother of proband of family SR showing fragile site 2q11 (large arrow) and normal chromosome 2 (smaller arrow).**

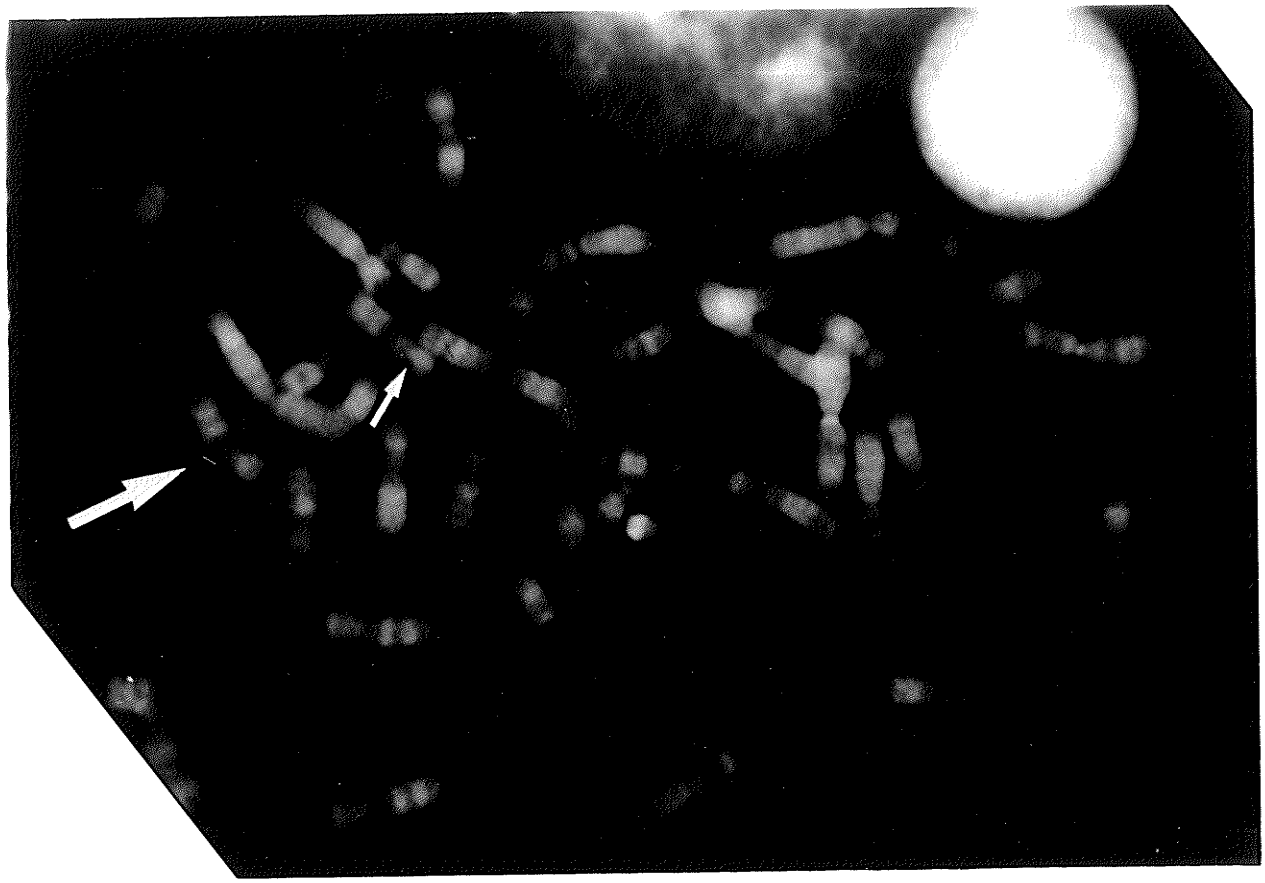

Figure 8. Q-banded metaphase spread from proband of family BG showing fragile site 9p21 (small arrow) and normal chromosome 9 (larger arrow). (Courtesy of Cytogenetics Laboratory, Health Sciences Centre.)

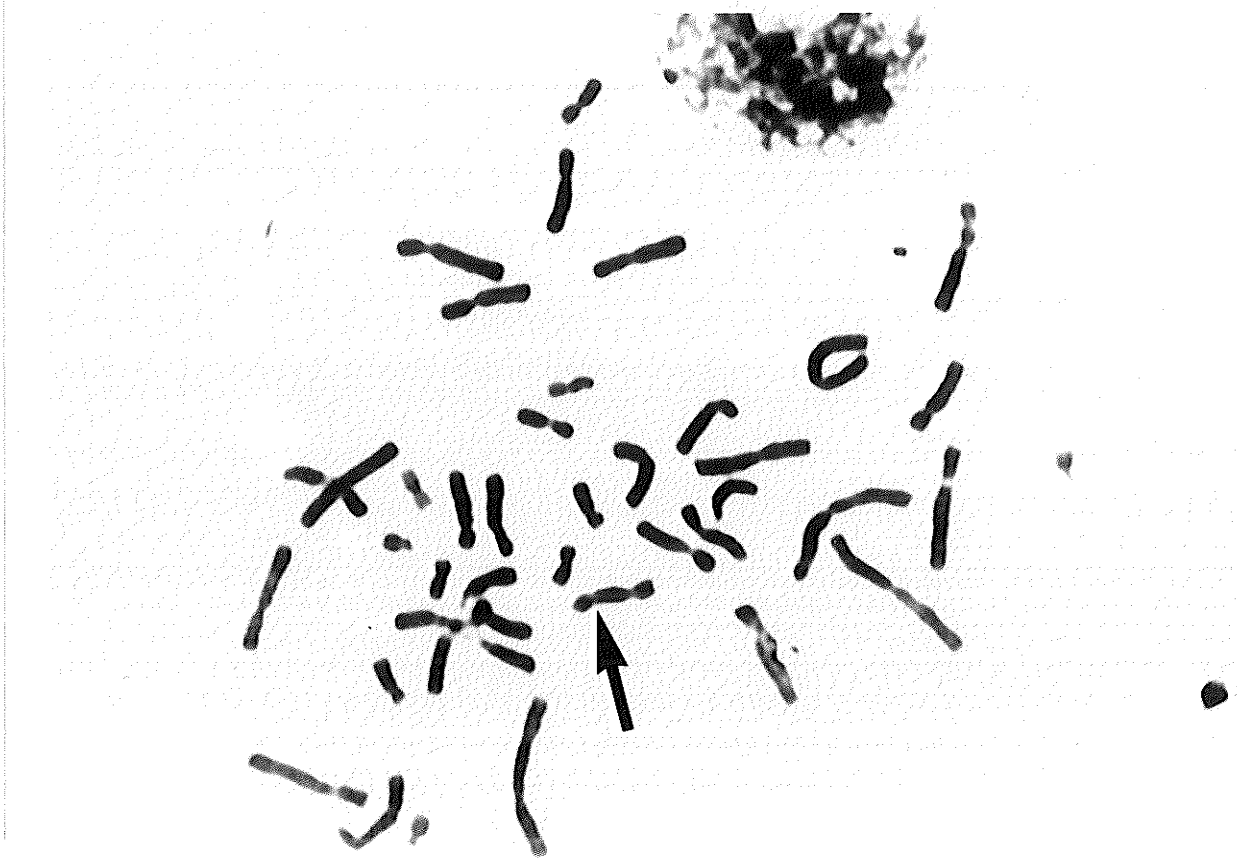

Figure 9. **Conventionally stained partial metaphase spread from brother of proband of family LN showing fragile site 10q23 (arrow).**

Table IV. Individuals with ARFS*, their sexes, percentage of FS expression and phenotypic findings

ARFS*	Family/Individual	Sex	Expression of ARFS (%)	Main phenotype findings
Fra(2)(q11)	CM°D/II.2	F	4/25 (16)	P;MR
	SR/II.2	F	7/50 (14)	Normal
	SR/II.4	M	5/50 (10)	Normal
	SR/II.6	F	4/50 (8)	Normal
	SR/III.1	M	10/50 (20)	Normal
	SR/III.3	F	9/50 (18)	P;Normal
Fra(2)(q13)	LSh/I.1	M	3/60 (5)	Normal
	LSh/II.3	M	5/100 (5)	P;Pervasive developmental disorder
	AG/I.2	F	10/50 (20)	Normal
	AG/II.4 (obligate FS carrier)	M	Deceased	Died of prostate cancer
	AG/III.3	F	9/25 (36)	Normal
	AG/IV.1	M	18/50 (36)	P;Autistic
Fra(8)(q22)	WM°M/III.2	F	18/45 (40)	P;Normal
	WM°M/IV.1	F	5/24 (20)	Normal
2Fra(9)(p21)	BG/I.1	M	5/50 (10)	Normal
	BG/II.1	F	3/50 (6)	Normal
	BG/II.7	M	20/50 (40)	P;MR
Fra(10)(q23)	LN/I.2	F	10/20 (50)	Normal
	LN/II.1	M	9/50 (18)	Normal
	LN/II.2	M	12/35 (35)	P;MR
	SL/I.2	F	4/57 (70	Prone to depression
	SL/II.1	F	10/30 (35)	Normal
	SL/II.2	M	8/28 (30)	Normal
	SL/II.4	F	20/57 (36)	P;MR
	JC/II.2	F	8/24 (34)	Normal P.T.O
	JC/II.3	F	5/25 (20)	Normal
	JC/III.4	F	3/60 (5)	fra(X)(q27) carrier
Fra(19)(p13)	TM/I.2 (obligate FS carrier)	F	Deceased	Violent temper
	TM/II.1	M	11/50 (22)	Schizophrenic
	TM/II.2	M	11/50 (22)	P;Schizo-phrenic, MR
	TM/II.3	M	5/20 (25)	Schizophrenic
	TM/II.4	M	14/50 (28)	Normal
	TP/II.1	F	2/50 (4)	Normal
	SS/II.1	M	2/50 (4)	Normal

Note: P = Proband
*ARFS=Autosomal rare fragile site

4.2.2 Sex chromosome aberrations

In family JC, the proband has the Martin-Bell form of mental retardation and is a carrier of fra (X) (q27). When other family members were tested to find other fra (X) (q27) carriers, it was discovered that this family also has the rare folate sensitive autosomal FS, 10q23.II.2 has fra (10) (q23). She is also an obligate carrier for fra (X) (q27) but this fra (X) (q27) was not expressed in her lymphocytes. Her son (III.3) is a fra (X) (q27) carrier and her daughter (III.4) is the carrier for both fra (X) (q27) and fra (10) (q23).

II.3 is a fra (10) (q23) carrier.

II.4 has neither fra (10) (q23) nor fra (X) (q27). She is, however, an obligate carrier for the fra (X) (q27), though this fragile site did not express in her lymphocytes, because both her son (III.6) and daughter (III.7) are fra (X) (q27) carriers.

4.3 SEGREGATION ANALYSIS OF RARE AUTOSOMAL FOLATE SENSITIVE FRAGILE SITES

To undertake segregation analysis, we used the direct sib method or Weinberg proband method of complete selection of ascertainment. In this method the proportion of affected sibs of proband(s) is obtained by counting each sibship once for each time it has been independently ascertained, omitting the proband each time (Weinberg, 1912).

Our study pedigrees segregating for the FS, 2q11, 2q13, 8q22, 9p21, 10q23, 19p13 and the pedigrees from literature segregating for the FS, 2q11, 9p21, 10q23, 12q13, 16p12 and 22q13 were analyzed together as numbers were too small for a separate analysis of any of the individual FS. The study families (n=9) were partitioned into 16 sibships of which 14 had known carrier parents. The pedigrees from the literature (n=13) were

partitioned into 21 sibships of which 17 had known carrier parents. The segregation of FS in the study sibships with known carrier parents was 33.3% (9/27) which was not significantly lower than the expected 50% for a co-dominant trait (P>0.2). Combined with the sibships from literature, segregation of the FS was 38.5% (27/70) which was not significantly lower than the expected (P>0.1). (Table V)

When the sibships were split by the sex of the transmitting parent, it was found that in our study families the mothers (n=8) transmitted the FS to 61.5% (8/13) of their children, which is not significantly higher than the expected 50% (P>0.8). The fathers, however, (n=6) transmitted the FS to 7.2% (1/14) of their children which is significantly lower than the expected 50% (P=0.05-0.02). The literature data showed maternal FS transmission (n=15) of 42.5% (17/40) which is not significantly lower than expected (P=0.5). Paternal FS transmission (n=2) of 33.3% (1/3) is lower than expected, but obvious the numbers here are very small. Combined data analysis showed maternal transmission of 47.2% (P>0.7). Paternal FS transmission of 11.7% (P=0.05-0.02) deviated significantly from the expected 50%.

4.3.1 Comparison of the Sex Ratios of the Probands

In the study families the sex ratio of the probands is 7M:5F (P>0.7) which did not significantly differ from the expected 1:1 ratio for any co-dominant trait which has equal expression in males and females. The sex ratio of the probands from the literature is 12M:8F (P=0.5). For the combined data, the ratio is 19M:13F (P>0.3). Neither of these two ratios deviated significantly from the expected.

4.3.2 Comparison of the sex ratios of the FS carrier children in the sibships

As we did not include the probands in the segregation analysis, we did not include them in the sex ratio comparison of the FS carriers in the sibships. Sex ratio of the FS carriers in our study families was 6M:5F ($P>0.8$) which was not significantly different from the 1:1 ratio for a co-dominant trait. The FS carriers from literature showed a ratio of 13M:9F ($P>0.5$) which is not significantly different from the expected. Combined data showed a ratio of 19M:14F ($P>0.7$) which is also not significantly deviated from the expected.

4.3.3 Comparison of the Sex Ratios of the FS non-carrier children in the sibships

The sex ratio of the FS non-carriers in our study families was 15M:10F ($P>0.5$) which was not significantly deviated from the expected 1:1 ratio. FS non-carriers from the literature showed a ratio of 23M:7F with significant deviation from the expected ($P<0.05$). Combined data also showed a significant deviation from the expected with a ratio of 38M:17F ($P<0.05$).

4.3.4 Comparison of the Sex Ratios of the Transmitting Parents

FS carrier parents with at least one FS-carrier child (including probands) were incorporated in this comparison study. The sex ratio of transmitting parents in our study families was 6M:8F ($P>0.5$). Data from the literature showed a ratio of 2M:19F ($P<0.01$). Weighted by this excess of transmitting females from the literature, combined data showed a significant excess of transmitting females (8M:27F, $P<0.05$).

4.3.5 Comparison of the Ratios of FS carrier versus FS non-carrier Children in the Sibships

Comparison of the FS carriers (C) versus FS non-carriers (NC) in our study families showed a non-significant difference from expected 1:1 ratio for a co-dominant trait (11C vs 25NC, $P<0.1$). There was noted a non-significant excess of FS non-carriers when the FS carriers were compared with FS non-carriers from the literature (22C vs 30NC, $P<0.4$). Combined data showed a non-significant excess of FS non-carriers (33C vs 55NC, $P<0.1$).

All the results are shown in tabular form in Table V.

Table V. Results

Analysis	Study Families	Literature Families	Combined Data (Study & Literature)	Deviation from Expected & Comment
1. Segregation ratio				
a) Maternal	61.5% (8/13) (P>0.8)	42.5% (17/40) (P=0.5)	47.17% (25/53) (P>0.7)	Not Significant
b) Paternal	7.2% (1/14) (P=0.05-0.02)	33.3% (1/3)	11.7% (2/17) (P=0.05-0.02)	Significant
2. Comparison of sex ratio of probands	7M:5F (P>0.7)	12M:8F (P=0.5)	19M:13F (P>0.3)	Not Significant
3. Comparison of the sex ratio of transmitting parents	6M:8F (P>0.5)	2M:19F (P<0.01)	8M:27F (P<0.05)	Deviation is significant for literature families and combined data but not for study families.
4. Sex ratio of the FS carrier offsprings (excluding probands)	6M:5F (P>0.8)	13M:9F (P>0.5)	19M:14F (P>0.7)	Not significant
5. Comparison of the sex ratio of FS non-carriers in the sibships	15M:10F (P>0.5)	23M:7F (P<0.05)	38M:17F (P<0.05)	Literature review showed a significant excess of males among FS non-carrier children
6. Comparison of the FS carriers (C) vs non-carriers (NC)	11C:25NC (P<0.1)	22C:30NC (P<0.4)	33C:55NC (P<0.1)	Not Significant

4.4 COMPARISON OF PERCENTAGE OF FRAGILE SITE EXPRESSIONS

4.4.1 Comparison of Percentages of FS Expression in Affected as Compared to Non-affected FS Carriers

The paired Wilcoxon rank sum test was used to determine the differences of the percentage of expression of FS among FS carriers with clinical abnormalities and clinically normal FS carriers. The data was tested in two ways: i) we paired the affected FS carriers with the non-affected FS carriers in each family individually irrespective of their sexes. The mean percentages of FS expression for clinically normal vs clinically abnormal FS carriers were 23.0%±13.9 and 27.3%±12.1 respectively. This difference was not significantly different (P=0.33). ii) we then paired sex matched clinically normal FS carriers with clinically abnormal FS carriers in each family individually. The mean percentages of FS expression were 23.4%±11.2 and 24.0%±13.0 respectively and the difference was not significant (P=0.87).

4.4.2 Comparison of Percentages of FS Expression in Affected as Compared to Non-affected FS Carrier Families

We used the Mann-Whitney two tailed test to compare the mean expression of FS in the family groups with no clinically abnormal FS carriers (15.3%±11.2) and the mean expression of FS in the family groups with at least one clinically abnormal FS carrier(22.9%±13.7). No significant difference was noted (P=0.15) but a trend of higher percentage of FS expressions in the families with at least one clinically abnormal FS carrier was suggested.

5.0 DISCUSSION

The significance of the rare autosomal FS is not yet known. Presently, they are considered to be chromosomal variants or polymorphisms. Laird et al. (1987) hypothesised that the molecular basis of the FS expression might be that the DNA at FS is late replicating and so misses the normal condensation during G_2 phase. This alteration for late replication at fragile sites might result from an altered DNA sequences which involves timing of DNA replication. Alternatively, there may be an alteration which expands the distance between two replication origins by unequal recombination, in addition to insertion of DNA could result in delayed replication in the region. The frequencies of rare autosomal folate sensitive FS vary greatly between studies. In a recent review of several surveys, the total frequency of rare autosomal folate sensitive FS appears to be greater in MR populations than in mentally normal populations (Kähkönen et al., 1989). However, an apparently unique feature about this group of FS lies in their pattern of segregation, in that the probability of expression varies with the sex of the transmitting parent (Sherman and Sutherland, 1986).

In this study we analyzed the segregation ratio of rare autosomal folate sensitive FS separately for cases transmitted through father or mother and compared the sex ratios of the probands, transmitting parents, FS carriers and non-carriers and compared the ratio of FS carriers to non-carriers.

5.1 CLINICAL FINDINGS

In our study families, there were 34 FS carriers; 14 of these carriers (including probands) exhibited variable clinical abnormalities. Nine of these clinically abnormal FS carriers are mentally handicapped (Table III). Therefore, the question is raised "Do rare

autosomal folate sensitive FS occur in a higher frequency in MR populations?" Since chromosome analysis is most often performed in such MR patients, FS carrier families with MR patients have a higher chance of being ascertained. Thus there might be considerable bias in concluding that a higher incidence of MR exists among rare autosomal folate sensitive FS carriers.

If FS predispose to chromosome breakage and rearrangement during meiosis and produce chromosomally unbalanced gametes which may result in non-viable conceptuses, or if *in vivo* breakage at FS in dividing somatic cells occur at critical stages of development, this could initiate a variety of defects and might lead to reproductive losses. In our 12 study families, the incidence of spontaneous abortions in the pregnancies of carriers was 8.7% (4/46) vs 0% (0/14) in the non-carrier sibs. Thus, these families do not appear to exhibit excess fetal loss when compared to the general population (risk of miscarriage ~15%). Some of our families were ascertained because of MR or autism. It is not surprising that we found a disproportionately higher incidence of these disorders in our sample as it was biased. We cannot, therefore, comment on phenotypic abnormalities in this group and suggest that they are due to the FS.

5.2 CYTOGENETIC FINDINGS

The percentage of expression of rare autosomal folate sensitive FS in the carriers in a given family is variable. The comparison of the percentage of FS expression between affected and non-affected FS carriers and between affected and non-affected FS carrier families did not show any significant difference. However, Kähkönen et al. (1989) found a statistically significant difference in the rate of expression of FS between mentally retarded and mentally normal groups.

Chudley et al. (1990) did not find any influence of age in common FS expression. On a superficial assessment of the FS data, there was no clear cut evidence for age influence on the percentage of FS expression in individuals in our study families.

The occurrence of fra (X) (q27) together with rare autosomal folate sensitive FS, 10q23 has been found in one of our study families (fam. JC). Amarose et al. (1987) reported a family with rare autosomal folate sensitive FS, 12q24 and Xq27. Smith et al. (1985) also reported another family with two rare autosomal folate sensitive FS, 9p21 and 12q13. These findings make it clear that in some families there can be segregation of more than one heritable rare folate sensitive FS.

5.3 SEX RATIO ANALYSIS

The sex ratios of the probands and FS carriers do not significantly deviate from the expected 1:1 ratio for a mendelian co-dominant trait. Sex ratio of the transmitting parents in our study families does not significantly deviate from the expected ratio. However, our sample size might be too small to determine true differences. The literature review identified rare FS families which were used to enlarge our sample. The combined data provided numbers large enough to more reliability assess the sex ratio of transmitting parents. The combined data sets showed a significant deviation from the expected 1:1 ratio with a significant excess number of transmitting females. This might be due to the fact that when the rare autosomal folate sensitive FS are transmitted through females, the penetrance of expression of FS is 100% and thus 50% of the offspring would be FS carriers; when transmitted through fathers the penetrance of expression is 50% in which case 25% of the offspring would be FS carriers (Sherman and Sutherland, 1986). And thus, with the higher number of FS carriers, maternally transmitted sibships have a higher

chance of being ascertained.

The sex ratio of the non-carriers in our study families did not show any significant deviation from expected ratio. The literature review data showed a statistically significant deviation of the sex ratio from the expected with an excess number of male offsprings. There is no apparent explanation for this finding except this may be due to chance.

Comparison of the FS carriers vs non-carriers showed a non-significant excess number of non-carriers in our study families and in the literature review families when analyzed separately. When both these data sets were pooled, no statistically significant deviation from the expected 1:1 ratio was found.

5.4 SEGREGATION ANALYSIS

5.4.1 Maternal Transmission

When the segregation ratio of rare autosomal folate sensitive FS was analyzed, it was found that in our study families the mothers (n=8) transmitted this FS to 61.5% (8/13) of their children. This percentage of FS transmission was a little higher than the expected 50%, but this deviation is not statistically significant (P>0.8). The literature data showed maternal transmission (n=15) of 42.5% (17/40) (P=0.5) and combined data analysis showed maternal transmission of 47.2% (P>0.7). For both the literature and combined data sets, the maternal FS transmission were little lower than the expected, however, statistical analysis confirmed that these deviations were not significant.

5.4.2 Paternal Transmission

When rare autosomal folate sensitive FS are transmitted through the father, the segregation ratio of FS according to our data showed a significant deviation from

expected. This was also seen in the literature review data and also in combined data and thus did not indicate a simple mendelian pattern of inheritance. One explanation for conditions or traits that do not "mendelize", is genomic "imprinting" (Hall, 1990). Imprinting may provide an explanation for a remarkably diverse set of observations on conditions whose genetic transmission and expression do not conform to the prediction of single gene inheritance. Thus in any trait or disorder that lacks a clear pattern of inheritance, the pedigree should be examined for evidence of imprinting. Examples of genetic diseases where imprinting has been suggested to play a role include myotonic dystrophy, Huntington disease, Prader-Willi syndrome, fragile X etc. (Hall, 1990). The term genomic "imprinting" has been used to refer to the differential expression of genetic material, at either a chromosomal or allelic level, depending on whether the genetic material has come from the male or female parent (Surani, 1986; Monk, 1988; Marx, 1988). This imprinting must involve somatic cell nuclear DNA modification in order to produce these phenotypic differences. It also implies that something happens during germ cells' formation when genetic information is "tagged", temporarily (reversibly) changing the genetic information to permit differential expression. Thus, this appears to be a form of regulation allowing another level of flexibility within the control and expression of the mammalian genome and may explain why mutations in some parts of mammalian genome function differently depending on whether they come from the father or mother. Sapienza (1989) has suggested that the best description of imprinting is that it is a form of dominance modification in which different manifestations of an epigenetic allele-inactivation process occur depending on the parental origin of gametes. An "imprintable" allele will be transmitted in a mendelian fashion but its expression will be determined by the sex of the parent transmitting the gene.

It has been suggested that DNA methylation may play a role in regulating the expression of genes involved in imprinting (Hall, 1990). These heritable changes in gene activity due to DNA modification, not due to DNA sequence change should be referred as "epimutations" to distinguish them form classical gene mutations (Jeggo et al., 1986).

Krumdieck et al. (1983) suggested that the misincorporation of uracil in place of thymine in undermethylated TMP-poor regions of DNA is the molecular event immediately responsible for expression of the folate-sensitive FS, because it is now well-established that loss of methyl groups in this key region interferes with the binding of proteins to DNA. The role of these protein-DNA interactions in establishing the high degree of coiling and folding necessary to condense the extremely long DNA molecules to the small dimensions of metaphase chromosome is well-recognized (Razin and Riggs, 1980; Comings and Riggs, 1971).

Recently, evidence has been obtained for transmission of altered methylation patterns through the germ-line (Schwartz et al., 1986). A specific enzyme might add a methyl group to a non-methylated gene, which would soon become heritably methylated, that is, would be transmitted intact from generation to generation of cells (Holliday, 1989). Now it can be hypothesized that during spermatogenesis *de novo* DNA methylation occurs in the undermethylated FS region which is the key factor for non-expression of the FS in the offspring. *De novo* methylation of non-methylated DNA occurs at a lower rate (Holliday, 1987), which might explain why the expression of the paternally transmitted FS is lower but not totally inhibited. Explanation for this lower segregation ratio can be given in another way. There is evidence that DNA methylation can suppress transcription as well as gene expression (Bird, 1984). If it is assumed that folate sensitive FS is an *in vitro* marker associated with a gene mutation (Michels, 1985), then it can be hypothesized

that the *de novo* methylation or imprinting of that gene during spermatogenesis might be the reason for lower segregation ratio because of the non-expression of that imprinted gene responsible for FS expression.

Hecht and Hecht (1984a) suggested that certain FS may be fragile in meiosis and thus predispose to chromosome breakage as well as rearrangements and deletions in meiosis and tend to produce chromosomally unbalanced gametes. These unbalanced gametes may be selected against fertilization through gametic selection process, they may fail to effect fertilization or they may lead to non-viable conceptuses.

5.4.3 Parental "Non-penetrance"

The negative cytogenetic finding in the parents with more than one FS carrier children (eg. fam. SR) might be the result of nonpenetrance of the gene or genes responsible for FS expression in the carrier parent, or this finding can be explained by the theory of premutation, (Auerbach, 1956), i.e. the mendelian inheritance of a genetic change (itself harmless) that predisposes to a specific mutation in the next generation and which then follow the mendelian pattern of inheritance in the successive generations. This finding could also possibly be explained by the hypothesis of amplification of pyrimidine-rich sequence (PRS) (Nussbaum et al., 1986). This PRS, a site with high frequency of dUMP misincorporation, is present as a normal sequence or simple PRS in the FS and may undergo amplification through unequal crossing-over (with its homologue with simple PRS) to produce the initial lesion of the FS. Individuals with this initial lesion will be "unaffected" but transmitting, because chromosomes with such initial lesions might show a higher rate of unequal crossing-over with its homologue with simple PRS during gametogenesis and result in progression to a longer stretch of pyrimidine rich

DNA in the FS region in the gametes. This increased length of PRS would make this region too long to be repaired by excising misincorporated dUMP during G_2 of the somatic cell division and thus allow this region to be seen as a FS in the next and the successive generations. When more than one child expresses the FS, then it can be assumed that the cause of this FS expression is not a new mutation and one of the parents must carry the FS predisposing factor. Genomic imprinting might be another explanation for this non-expression of FS in a parent which is re-established in next generation (Hall, 1990). Gonadal mosaicism may be an alternate explanation. In the families where neither parent exhibit FS but has a FS carrier child, (eg. fam. TP & SS), this might be explained on the basis of a new mutation. Paternity tests were not performed in any of these 3 families.

6.0 CONCLUSION

In this study no consistent clinical abnormality was identified with any of the rare autosomal folate sensitive FS. This study has confirmed that there is a statistically significant deficiency of offspring expressing FS when transmission is from a FS carrier father. Maternal transmission of FS conforms to that expected of a co-dominant trait. These findings may be due to the phenomenon of parental genomic imprinting, gametic selection, or chromosomal aberrations at meiosis or at a critical stage of development predisposed by FS. The actual basis of these findings remains in question, and further studies will be required to delineate the biological determinants of this apparent segregation distortion.

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8.0 APPENDIX

8.1 Statement to participants and consent form.

FRAGILE SITE

SEGREGATION STUDY

We wish to do a study assessing the frequency and significance of chromosome (carriers of genetic material) abnormalities in individuals who are mentally disabled. We also wish to assess the frequency and significance of a certain type of chromosome abnormality called a "fragile site" or "marker". (Fragile sites refer to a tendency of chromosomes to break at a specific place and does not mean individuals with this finding are fragile). Fragile sites on human chromosomes have only recently been discovered. They are usually passed on in families from one generation to the next, and if a child or older individual has a fragile site, it is likely one of his parents is a carrier for the same fragile site. Only 2 of the over 13 fragile sites is known to be associated with mental disability, the fragile X (a fragile site on the X chromosome). The other fragile sites are on other chromosomes (autosomes). At present we do not know the significane of autosomal fragile sites in regards to an individual's physical or mental health, but it appears most individuals with autosomal fragile sites are healthy and no different from individuals who do not have fragile sites.

I, _____, brother/sister/mother/father/aunt/uncle/grandfather/grandmother/grandchild to _____, agree to have Dr. Chudley or his associates obtain a blood specimen (5 ml - 1 teaspoonful) from myself. The nature and purpose of the study have been fully discussed with me. I acknowledge that my consent for the study is given voluntarily and that I may withdraw from the study at any time without prejudice. The information obtained in this study will remain confidential.

_____ _____

(Witness) (Signed)

_____ _____

(Date) (Relationship)

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