1. Introduction

Introduction

Fragile X syndrome (FXS) is an X-linked disorder caused by an expanded CGG repeat in the *fragile X mental retardation* 1 *(FMR*1*)* gene. It is the most common cause of inherited mental retardation. This syndrome is characterized by mental retardation, abnormal physical features (i.e. elongated face, large and protruding ears and macroorchidism) and behavioral problems (i.e. attention deficit/ hyperactivity and autistic-like behaviors). All affected individuals (full mutation) almost always have a CGG expansion greater than 200 repeats, and the condition is also associated with methylation of the promoter, resulting in gene inactivation and lack of fragile X mental retardation protein (FMRP).

Currently available methods for diagnosis of FXS include cytogenetics, Southern blot analysis, polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR) and immunohistochemical analysis of the FMRP. Of these, PCR and Southern blot analysis are commonly used as the standard diagnostic methods. Since methylation is a fundamental defect of this syndrome, identification of methylation status is crucial for the FXS diagnosis.

Conventional PCR reveals the number of CGG repeats, while methylation status is tested by Southern blot analysis. However, Southern blot analysis has some disadvantages, mainly the cost and time consumed and the large amount of DNA required. In an attempt to find a better methylation detection method, a PCR-based methylation detection has been developed, called methylation specific PCR (MS-PCR).

This study was undertaken to determine the accuracy of MS-PCR as a diagnostic screening method of FXS.

Review of literatures

Fragile X syndrome and DNA methylation

Fragile X syndrome (FXS) is now known to be the most frequent inherited form of neurodevelopmental disability, with an estimated prevalence of 1:4,000-6,000 in males and 1:6,000-8,000 in females (Murray, et al., 1996; de Varies, et al., 1997; Turner, et al., 1996; Morton, et al. 1997). The clinical characteristics of FXS include both physical and neuropsychological features. The main features are mental retardation, macroorchidism (enlarged testes) and typical facial appearances including elongated face, large and protruding ears and prominent jaw. Of these, macroorchidism, elongated face and prominent jaw are easily observed in adult. Some behaviors are frequently found in children including attention deficit/ hyperactivity and autistic-like behaviors. Other physical features (i.e. flat feet, excessive joint laxity, mitral valve prolapse, seizure and hypotonia) and behavioral features (i.e. social difficulty, aggressive outbursts and obsessive-compulsive behavior) are also found but less frequent. A carrier, known as a premutation, is generally normal, but an association has been shown in some individuals, such as prominent ears, mild social phobia and anxiety disorders (reviewed in Hagerman RJ, et al., 1996). Recently, there has been some evidence that carriers may have some clinical features, including premature ovarian failure (a cessation of menses before age 40 years) among female carriers (Schwartz, et al., 1994.; Partington, et al., 1996; Uzielli, et al., 1999) and

fragile X tremor/ ataxia syndrome among older male carriers (Hagerman, et al., 2001; Brunberg, et al., 2003; Leehey, et al., 2003; Jacquemont, et al., 2003).

The FXS was originally described by Martin and Bell, who reported the first pedigree of sex-linked form of mental retardation (Martin, et al., 1943). Later, Lubs (1969) demonstrated its cosegregation with cytogenetic abnormality of the X chromosome as a constriction or isochromatid gap, close to the distal end of the long arm of the X chromosome. Sutherland (1977a, 1997b, 1999) finally defined the nature of this anomaly as a fragile site which can be induced under folate and/ or thymidine depletion culture medium. This fragile site was designated as FRAXA (Fragile site, X chromosome, A site) at Xq27.3. The molecular mechanism of FXS is associated with a massive trinucleotide, Cytosine-Guanine-Guanine (CGG) repeat expansion within the *fragile X mental retardation*-1 *(FMR*1*)* gene. The *FMR*1 gene was identified and sequenced in 1991 by an international collaborative effort (Verkerk, et al., 1991; Yu, et al., 1991; Oberle, et al., 1991; Bell, et al., 1991). *FMR1* spans ~38 kb and consists of 17 exons. The CGG repeats polymorphism is located at the 5'-untranslated region (5′UTR) of exon. The transcription size is about 4.4 kb (Verkerk, et al., 1991; Fu, et al., 1991; Kremer, et al., 1991; Ashley, et al., 1993; Eichler, et al., 1993). The *FMR*1 gene encodes fragile X mental retardation protein (FMRP). FMRP is widely expressed in all tissues and organs. Analysis of the amino sequence of FMRP suggested that FMRP is an RNA binding protein. It binds to messenger RNA (mRNA) and forms a complex called a mRNP (messenger ribonucleoprotein) particle. The FMRP has been shown to associate with polyribosomes and shuttle between the nucleus and cytoplasm. These findings suggested that FMRP may modulate mRNA translation and it exports mRNA from the nucleus to the cytoplasm. In central nervous system, the

FMRP is expressed in the dendritic spine. The regulation of protein synthesis within the dendritic spine is important for synaptic development and brain plasticity. The absence of FMRP during synaptic development may lead to mental retardation. Consistently, abnormal dendritic spines have been observed in brains of FXS patients and in *FMR*1 knock-out mice.

In normal population, the CGG repeat is polymorphic in length and content, usually punctuated by AGG interruption (Eichler, et al., 1994). The normal CGG repeats size ranges from 6-60, with 29 and 30 repeats found on the most common allele (Murray, et al., 1996; Fu, et al., 1991; Kunst, et al., 1996; Brown, et al., 1996). Alleles with between 60 and 200 repeats are called premutation (PM), which is unstable (may expand to a full mutation or a different sizes of premutation) during transmission to the next generation (Heitz, et al., 1992; Turner, et al., 1994). The terms "intermediate" or "borderline" are used to describe individuals with 41-60 repeats. Most intermediate alleles are stable on transmission similar to normal alleles, but 10-30% of these alleles may change in the repeat size similar to premutation alleles (Nolin, et al., 2003). Most affected individuals (full mutation, FM) have a massive CGG repeat expansion over 200 repeats. This *FMR1* fully mutated correlates with abnormal CpG island hypermethylation, which results in the transcriptional silencing and the loss of FMRP (Oberle, et al., 1991; Bell, et al., 1991; Pieretti, et al., 1991; Heitz, et al., 1991) (Fig.1). Of affected individuals, 15-20% have mosaicism, that can be categorized into (1) repeat size mosaicism, in which both full mutation and premutation or normal size alleles are present (Schmucker, et al., 1999; Orrico, et al., 1998), and (2) methylation mosaicism, in which both methylated and unmethylated full mutation are presented (Nolin, et al., 1994). Individuals with mosaicism may have

a higher intellectual level than individuals with a completely methylated full mutation (McConkie-Rosell, et al., 1993; Hagerman, et al., 1994; Smeets, et al., 1995). Presumably, these individuals produce some FMRP from their unmethylated alleles. Rare mutations of the *FMR1* gene are deletions (reviewed in Hammond, et al., 1997) and a point mutation (De Boulle, et al., 1993).

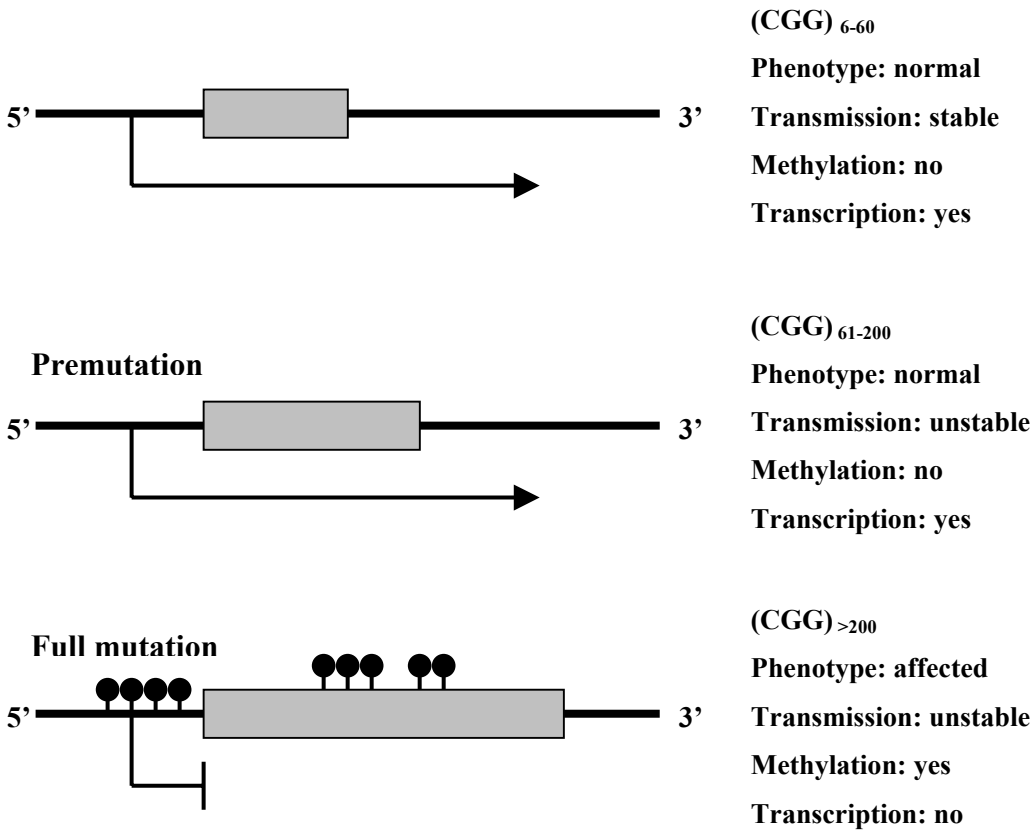

Fig. 1 Schematic presentation of the repeat expansion in *FMR*1 gene seen in premutation and full mutation patients. Expansion over 200 repeats leads to methylation of the promoter, resulting in transcriptional silencing. The *gray bar* represents the CGG repeats; the *black dot* represents methylation of cytosines (adapted from O'Donnell and Warren 2002).

DNA methylation is a covalent modification of post-replicative DNA obtained by adding a methyl group to the carbon-5 position of a cytosine ring to form a methyl cytosine. This modification is present in all organisms from bacteria to human beings. In bacteria, methylation is part of defense mechanism to reduce the amount of gene transfer between species. In human and other mammals, methylaiton is almost exclusively found in a cytosine preceding a guanosine (CpG dinucleotide) in the gene. It has an important regulatory effect on gene expression, especially when involving CpG islands that are located in the promoters or exons of many genes. Most genes on autosomal chromosomes are protected from methylation, while extensive methylation has been associated with transcriptional inactivation of selected imprinting genes and genes on the inactive female X chromosome of females (reviewed in Costello and Plass 2001).

The finding that the FXS has an abnormal methylation in affected males was predicted by Laird (1987). He suggested that the FXS mutation produced an impediment to reactivate (demethylate) of the inactive X chromosome that normally expressed in female germ cells. Failure to demethylate a gene on the inactive X chromosome during oogenesis may produce abnormal phenotypes. Later, Vincent and colleagues (1991) and Bell and colleagues (1991) found the alteration of restriction-fragment patterns in DNA of fragile X patients digested with the methylation-sensitive enzymes. They suggested that the different patterns resulted from the blocking by methylation. These abnormal patterns were similar to the pattern found on the inactive female X chromosome. After that, several studies showed that lack of expression of the *FMR*1 gene was associated with abnormal expansion and methylation of the CGG repeats (Obelé, et al., 1991, Sutcliffe, et al. 1992).

Cytosine residues of all CpG dinucleotides within the CGG repeats and both 5'and 3'-end of the *FMR*1 gene are unmethylated in the DNA of normal and premutation males, whereas these cytosines are methylated in full mutation males and normal inactive X chromosomes (Hornstra, et al., 1993; Hansen, et al. 1992). Methylation of non-CpG cytosines may occur but it is rare (Stöger, et al., 1997). The *FMR*1 does not express from the inactive X chromosome (Kirchgessner, et al., 1995) and the methylation pattern of the *FMR*1 5'CpG island in affected males is similar to the 5'CpG island of other genes on the inactive X chromosome (Hornstra, et al., 1993). These findings suggested that transcriptional suppression of the *FMR*1 gene may occur by the mechanism similar to other genes on the inactive X chromosome. Stöger and colleagues (1997) also found that methylation mosaicism in FXS males was similar to normal alleles with active X or inactive X chromosomes in normal females, suggesting that the methylation mosaicism of some FXS males was similar to on and off states of the *FMR*1 expression that exists in normal females.

The mechanism by which DNA methylation modulates transcription of the *FMR*1 gene is unclear. Adding methyl group does not affect the base pairing, but methyl group protruding into the major groove may affect DNA-protein interaction. DNA methylation may inhibit transcription factor binding. This hypothesis is supported by the study of Schwemmle and colleagues (1997). They studied the interaction of transcription factors with the *FMR*1 promoter and evaluated the DNA methylation of the corresponding region. Footprinting analysis of the methylated promoter revealed no footprints in the methylated promoter region. The absence of the transcription factor binding in this region might be associated with DNA methylation.

Another way of transcriptional silencing is the formation of heterochromatin, which is less accessible to the transcriptional machinery than the euchromatin. Heterochromatin is formed in concert with various epigenetic phenomena. It is comprised of dense DNA methylation and hypoacetylated histones. Several studies on histone deacetylation and DNA methylation have shown that DNA methyltransferases and histone deacetylases are able to interact either directly or indirectly through several pathways (reviewed in Robertson and Wolffe 2000). In addition, it has been found that the 5' end of the *FMR*1 gene of the patients with FXS is associated with deacetylated histones H3 and H4. The treatment of fragile X cells with 5-azadeoxycytidine results in the re-association of acetylated histones with the *FMR*1 promoter and transcriptional activation. These findings suggest that both methylation and histone deacetylation are linked to transcriptional inactivity (Chiurazzi, et al., 1998, 1999; Coffee, et al., 1999; Pietrobono, et al., 2002, 2005).

It is unknown why the expanded CGG repeat becomes *de novo* methylation. Some researchers have proposed that the repeat expansion might induce changes in the chromatin structure and promote methylation in this region. Alternatively, the absence of relevant transcriptional factors might expose the CpG island to *de novo* methylation, which in turn maintain the gene to switch off (reviewed in Migeon 1993). Some have suggested that it might result from the potential of repeats to form a hairpin structure that was a good substrate for DNMT1[1]. Because large enough

[1] DNMT1 is a maintenance methyltransferase enzyme, which is essential for maintaining methylation after each round of replication. It introduces methyl groups on the newly synthesizes DNA strand to convert cytosine to 5-methyl cytosine when the corresponding sequence of the template strand is methylated (Reik, et al., 1999).

expansion was required to form hairpin structure, so methylation occurred with large expansion (Chen, et al., 1995; Burman, et al., 1999). Some have suggested that the *de novo* methylation might be related to the genome defense/ repetitive element methylation system in that the expanded repeat. This might be similar to parasitic elements recognized as foreign DNA. As a result, the expanded repeat became a substrate for *de novo* methyltransferases[2]. (Burman, et al., 1999).

The absence or deficit of FMRP resulting from methylation is the fundamental defect in FXS. Although the repeat is expanded in full mutation range, the expanded repeat does not suppress transcription directly. One study showed that unmethylated full mutation males had higher *FMR*1 mRNA level than normal controls. This finding showed that up-regulation of the *FMR*1 gene occurred in cell with unmethylated full mutation alleles (Tassone, et al., 2000).

Several studies have suggested that the proportion of cells with methylation was associated with cognitive ability in FXS individuals. Also, phenotypic expression of the syndrome was depended on the degree of methylation of the *FMR*1 gene (McConkie-Rosell, et al., 1993; Taylor, et al., 1994; Rousseau, et al., 1994; Steyaert, et al., 1996). For example, in a study of 20 males with a partially methylated full mutation, individuals with < 10% methylation were not retarded (Taylor, et al., 1994). However, Rousseau and colleagues (1994) found no mental retardation in a full

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[2] DNMT3A and DNMT3B are *de novo* methyltransferase enzymes, which methylate the genome after the wave of global demethylation that occurs during early embryonic development. DNA methylation is performed by DNA methyltranferases (Reik, et al., 1999).

Besides these DNA methyltransferases, other genes that affect genomic methylation pattern were reviewed in Bestor (2000).

mutation male with 40% methylation. The study of postmortem tissues from high functioning full mutation male revealed unmethylation in a wide span of CGG repeats (ranges from premutation to full mutation) in blood and most regions of the brain, while methylated full mutation was found in most non-brain tissues and some regions of the brain (Taylor, et al.1999). These findings suggest that lack of mental retardation might be due to sufficient FMRP production in most areas of the brain. Since strand-specific DNA mismatch repair is methyl-directed, in the absence of methylation, erroneous correction of slipped strand misalignment during DNA replication may allow to gain or loss of CGG repeats (Wöhrle, et al., 1998).

A study by Burman and colleagues (1999) suggested methylation of the *FMR*1 was not associated with global methylation defect. They described why some full mutation males are protected from methylation. First, they might fail to maintain methylation at the *FMR*1 region. Second they escaped from *de novo* methylation during early embryogenesis. Third, they had CGG repeats not enough to become methylated.

The methylation pattern at the *FMR*1 locus is established in early embryonic development (Devys, et al. 1992; Burman, et al., 1999). However, embryonic and extra-embryonic methylation patterns are different (Sutherland, et al., 1991; Sutcliffe, et al., 1992; Suzumori, et al., 1992; Yamauchi, et al., 1993; Iida, et al., 1994). The timing of methylation and its heterogeneity has an impact on prenatal diagnosis. A study of methylation in full mutation male fetus was performed by Sutherland and colleagues (1991). They reported the lack of methylation in chorionic villi at 10 weeks of gestation and the presence of methylation in embryonic tissues; however, they did not state the timing of methylation occur in the embryonic tissues. Sutcliffe and

colleagues (1992) found the chorionic villi of a full mutation male at 11 weeks of gestation had mosaic methylation patterns, while tissues from the same fetus at 13 weeks of gestation had completely methylated. Yamauchi and colleagues (1993) studied methylation in a full mutation female fetus and reported no methylation of chorionic villi, but total methylation had occurred in the brain and other tissues. Iida and colleagues (1994) studied the methylation status of the *FMR*1 in normal male and female fetuses. They examined fetuses at 8 weeks of gestation including brain, cord blood, chorionic villi and placenta. The male fetuses had no methylation in both embryonic (brain and cord blood) and extra-embryonic (chorionic villi and placenta) tissues, while fifty percent of cells in the female fetuses were methylated in embryonic tissues, according to the X-inactivation, but no methylation was observed in extra-embyonic tissues. They suggested that the CpG islands of the *FMR*1 gene escaped lyonization in extra-embryonic tissues. Later, Willemsen and colleagues (2002) found that in the chorionic villi of full mutation males, the FMRP was expressed normally at 10 weeks of gestation, while it was absent in 12.5 weeks of gestation. Because the methylation status of the *FMR*1 promoter region always correlated with the expression of FMRP, this finding might reflect the timing of methylation in chorionic villi of full mutation males.

In general, *FMR*1 methylation is restricted for full mutations; however some premutations may exhibit methylation. Smiths and colleagues (1994) reported a mentally retarded male with methylated premutation in a small percentage of his cells. Hagerman and colleagues (1996) reported a male with methylated premutation in blood and buccal cells who presented with attention deficit and mild physical features of FXS. Steyaert and colleagues (1996) also described a boy with methylated

premutation in a small percentage of his cells. This boy presented with mild physical and behavioral characteristics of FXS. Tassone and colleagues (1999) reported that premutation individuals with the identical *FMR*1-CGG repeat mutation had different methylation patterns that was unmethylted in skin fibroblasts and methylated in a small percentage of lymphocytes. These findings suggest that different methylation patterns can exist in different tissues of premutation carriers. However, when methylation is present in only a small percentage of cells, it may not affect FMRP expression. Especially, if the absence of methylation in the brain results in sufficient FMRP production, mental retardation is unlikely to occur.

Methylation-specific PCR for diagnosis of fragile X syndrome

A definite diagnosis for FXS is very important in clinical practice, and necessary for informative genetic counseling, proper clinical management and further investigation. It is, however, difficult to diagnose by clinical examination alone because some of the clinical characteristics are commonly found in other syndromes (Lachiewicz, et al., 2000; Stoll, et al., 2001). FXS diagnosis has been based on chromosome analysis using modified culture techniques to induce fragile site. However, this method is difficult and unreliable because it may exhibit false positive results caused by other fragile sites, such as FRAXD (Xq27.2), FRAXE (Xq28) and FRAXF (Xq28) (Sutherland, et al., 1990, 1992; Hirst, et al., 1993). Also, false negative results occasionally occur, especially in FXS carriers.

DNA analysis, based on the detection of an alteration in the *FMR1* gene, is providing increasingly reliable. PCR is suitable for the diagnosis of FXS as a rapid and accurate estimation of the CGG repeats. Several PCR-based diagnostic and screening methods have been reported with variable results. The major problem in the

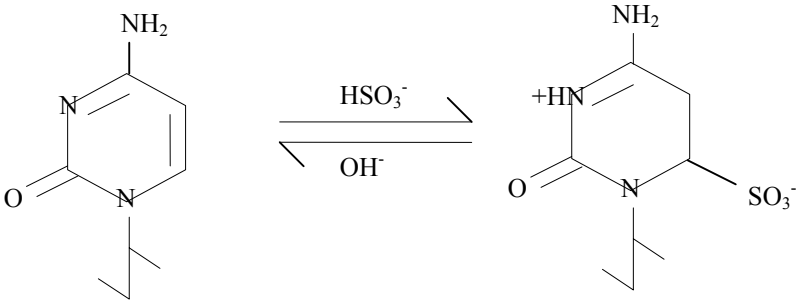
use of conventional PCR techniques is the almost 100% GC content of the fragment containing the repeats and the very high GC content of the flanking regions. The published 1-kb *Pst*I fragment (Fu, et al., 1991) containing the CpG island and the CGG repeats has about 70% GC composition. This very high GC content in the target DNA, resulting in high denaturing temperatures and the formation of stable secondary structures that make the amplification failure. In order to overcome these obstacles, PCR protocols usually add chemical additives, such as dimethylsulfoxide (DMSO), polyethylene glycol, and formamide, together with the ultilization of various thermostable polymerases such as Pfu-, or expand long template-polymerase, and partial or complete substitution of 7-deaza dGTP for dGTP (Erster, et al., 1992; Pergolizzi, et al., 1992; Brown, et al., 1993; Chong, et al., 1994; Levinson, et al., 1994; Condorelli et al., 1996; Haddad, et al., 1996; Hecimovic, et al., 1997; Larsen, et al., 1997). The PCR reaction provides a successful amplification in normal and small premutation alleles, but full mutation alleles are usually not amplified. In addition, mosaic premutation/ full mutation patients may have PCR product in the premutation ranges only, leading to a false negative diagnosis. Southern blot hybridization is a further test used for all suspicious cases. This procedure includes the digestion of genomic DNA using a pair of restriction enzymes (methylation sensitive and methylation insensitive enzymes), followed by the hybridization of the digested DNA with a specific probe (Rousseau, et al., 1991). The advantage of Southern blot analysis is that the methylation status is obtained as well as the approximate number of CGG repeats. However, the main disadvantages of this technique are that it is tedious and time consuming, requires a large amount of DNA, and also low sensitivity in its ability to distinguish a low ratio of mosaicism. Other techniques, such as protein

expression detection and mRNA level analysis are available (Willemsen, et al., 1995, 2000; Pai, et al., 1994) but they are generally not appropriate for this type of screening test due to complicated techniques.

Because methylation is a prognostic indicator of phenotypic expression in FXS and the difficulties in amplifying DNA fragments due to GC rich template. A methylation specific PCR (MS-PCR) method for screening of FXS has been developed (Weinhäusel, et al, 2001; Zhoe, et al., 2004). MS-PCR is based on the methylation sensitive conversion of cytosine (C) residues to uracil (U) by bisulphite on single-stranded DNA and subsequent amplification of the modified anti-sense strand with specific primers. Moreover, the bisulphite treatment dramatically reduces the GC component of the region and thus melting temperature (Tm) and secondary structures are no longer obstacles for PCR amplification. The method has many advantages compared with the previous PCR-based diagnostic tests for FXS in which 7-deaza dGTP was used. Since there is no need for the partial or complete substitution of 7-deaza dGTP for dGTP in PCR, therefore, the PCR products are directly visualized by ethidium bromide staining (Latimer, et al., 1991).

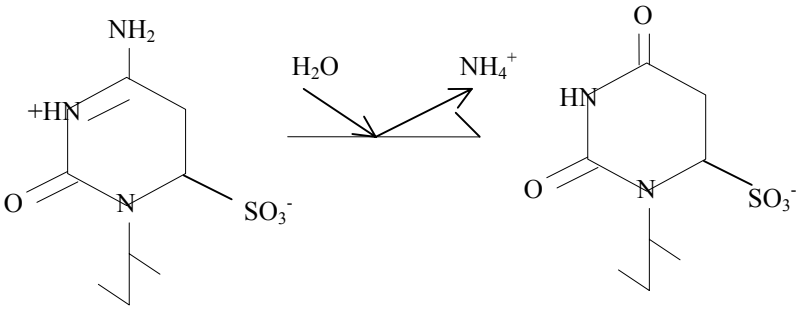
The method relies on the ability of sodium bisulphite to convert cytosine residues to uracil on single-stranded DNA (Frommer, et al., 1992; Clark, et al., 1994). In double-stranded DNA, the rate of cytosine deamination is less than 0.1% of the rate of single-stranded DNA (Feil, et al. 1994). 5-methyl cytosine can also react with bisulphite, but the reaction is extremely slow and the equilibrium favors 5-methyl cytosine rather than the deaminated product, thymine. The deamination of cytosine by sodium bisulphite involves the following steps:

Step1. Sulphonation: The formation of the cytosine sulphonate by addition of sulphonate group to the 5-6 double bonds of cytosine. This step is controlled by pH, bisulphite concentration and temperature, and can be reversed. The forward reaction is favored by low pH and the reverse reaction by high pH.



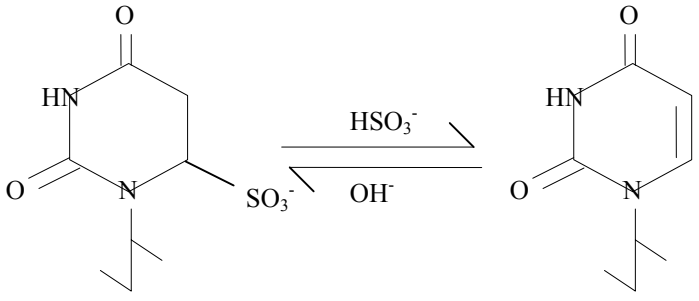
Cytosine **Cytosine sulphonate**

Step2. Hydrolytic deamination: The cytosine sulphonate undergoes hydrolytic deamination to give a uracil sulphonate. This step is catalyzed by basic substances, such as sulphite, bisulphite and acetate anions.



Cytosine sulphonate **Uracil sulphonate**

Step3. Desulphonation: The removal of the sulphonate group by a subsequent alkali treatment, to give uracil.



Uracil sulphonate **Uracil**

MS-PCR is suitable for rapid screening when it is compared to the Southern blot analysis. MS-PCR is simpler, less time consuming and eliminates possible false positives due to partial digestion by the methylation-sensitive enzymes used in the Southern blot analysis. Moreover, the method does not require radioactivity, biotin or digoxigenin, thus reducing the expense.

Besides FXS, MS-PCR can be applied in diagnosis of other methylation-related diseases including Prader Willi syndrome, Angelman syndrome and some types of cancer (Herman, et al., 1996; Kubota, et al., 1997; Das, et al., 1997/98). To date, there is no guideline for FXS diagnosis using MS-PCR since the method has only been reported in recent years.

Objectives

1. To establish a multiplex methylation specific PCR as a screening method for diagnosis of fragile X syndrome
2. To compare a multiplex methylation specific PCR with gold standard methods