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ORIGINAL INVESTIGATION

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Timing of the absence of *FMR1* expression in full mutation chorionic villi

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Abstract Fragile X syndrome is caused by the expansion of the CGG repeat in the 5' untranslated region of the *FMR1* gene. This expansion leads to methylation of the *FMR1* promoter region thereby blocking FMR1 protein (FMRP) expression. Prenatal diagnosis can be performed on chorionic villi samples (CVS) by Southern blot analysis. Alternatively, for males, an immunohistochemical method has been introduced for CVS. In this study, we have used this immunocytochemical method for CVS in full mutation male fetuses at different times of gestational age, varying from 10.0–12.5 weeks, and in two cases of full mutation female fetuses (>13 weeks). FMRP expression studies in CVS from full mutation male fetuses (10.0–12.5 weeks) illustrate the timing of the disappearance of FMRP expression in these CVS. Until approximately 10 weeks of gestation, FMRP is expressed normally in full mutation male CVS, whereas FMRP is completely absent at 12.5 weeks of gestation. FMRP expression in full mutation female CVS (>13 weeks) is completely absent in a number of villi, whereas other villi show normal FMRP expression. Unlabelled villi can only be present in the absence of the expression of the full mutation *FMR1* gene on one X-chromosome together with the X-inactivation of the normal X allele. FMRP positive villi can be explained by an active normal X allele. The presence of both positive and negative villi indicates that X-inactivation in human CVS is a random process. No villi are found with a mixture of both FMRP-expressing and non-FMRP-expressing cells. This indicates that X-inactivation occurs very early in development, before the villi start to proliferate, and that X-inactivation in villi is a clonal process. In addition, our results indicate that the timing of both X-inactivation and full mutation *FMR1* allele inactivation is different, i.e. X-inactivation occurs earlier in development than inactivation of the full mutation.

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Introduction

Fragile X syndrome is the most common form of inherited mental retardation affecting 1:4000 males (Turner et al. 1996; for a review, see Kooy et al. 2000). Mental retardation and developmental delay are the most significant clinical features of fragile X syndrome (Hagerman 1996). This X-linked disorder is caused by the absence of the fragile X mental retardation protein (FMRP). The gene defect causing the absence of FMRP is an expansion of the trinucleotide $(CGG)_n$ repeat present in the 5' untranslated region of the fragile X mental retardation gene 1 (*FMR1*; Oberlé et al. 1991; Verkerk et al. 1991; Yu et al. 1991). This trinucleotide repeat is highly polymorphic and alleles can be divided into three groups. The first group contains the alleles ranging between 5 and 50 repeat units. Repeats of this size remain stable upon transmission. The two other groups, called pre- and full mutations (PM and FM, respectively), behave unstably upon transmission to the next generation. Both contractions and expansions are observed, the latter being the most prominent (Rousseau et al. 1991). Alleles between 50 and 200 CGGs are called PM. Although PMs are expanded and behave unstably, they do not block the expression of FMRP (Devys et al. 1993; Verheij et al. 1993); however, recent studies have reported an increase in the *FMR1* mRNA and reduced levels of FMRP in PM male carriers (Tassone et al. 2000, 2001). PMs can expand upon both male and female transmission. Expansion to an FM (>200 CGGs) occurs only upon female transmission. Only females with a PM can transmit an FM to their offspring. FMs coincide with methylation of the promoter region of *FMR1*, thereby blocking the transcription of *FMR1*. This results in the absence of FMRP, which causes

the fragile X disease phenotype (Pieretti et al. 1991; Verheij et al. 1993).

The presence of the FM in patients usually correlates with the methylation of the promoter region of *FMR1*, including the CGG repeat. The methylation status can be determined by Southern blot analysis by using methylation sensitive restriction enzymes (Oostra et al. 1993; for a review, see Oostra and Chiurazzi 2001). The methylation status of the promoter region is always correlated with the expression of FMRP. Why and how this methylation occurs is not known. It has been hypothesized that it is not the repeat expansion itself, but the methylation that is the most important factor causing the disease phenotype (McConkie-Rosell et al. 1993). The methylation of the promoter region, including the CGG repeat, is responsible for the blocking of the transcription, resulting in the absence of FMRP. The observation that FM males, who do not show methylation, do express FMRP supports the idea that an expanded repeat itself is not enough to block the expression completely (De Vries et al. 1996; Hagerman et al. 1994; McConkie-Rosell et al. 1993; Smeets et al. 1995; Taylor et al. 1999; Wohrle et al. 1996, 1998).

In FM females, the situation is more complex, because their cells contain two X-chromosomes. Dosage compensation in somatic cells of normal females is necessary for the expression of equal amounts of X-linked genes compared with males and this is achieved by inactivation of one of the two X-chromosomes. The process of X-inactivation occurs shortly after blastocyst implantation during embryonic development (Tan et al. 1993). Once X-inactivation is established, it is maintained during further cell proliferation and differentiation of the embryo. The choice regarding which X-chromosome is inactivated is a random process. For females with one normal and one FM allele, this implies that, in 50% of the cells, the normal X-chromosome will be inactivated and, in 50% of the cells, the X-chromosome containing the FM allele will be inactivated. If the FM allele is inactivated by X-inactivation, the normal allele will produce normal amounts of FMRP. In theory, this will be the case in 50% of the cells. In the other 50% of the cells, the normal allele will be subject to X-inactivation. As a consequence, the FM allele will be at the active X-chromosome. However, since the FM is inactivated too, this allele will not be active resulting in two inactive *FMR1* loci in these cells. One allele is inactivated because of the FM (If= inactivation of the *FMR1* FM) and the normal allele is inactivated because of X-inactivation (Ix= X-inactivation of the *FMR1* allele). Thus, in these cells, there is no FMRP expression. These two mechanisms of inactivation complicate the disease phenotype in FM females. Depending on the X-inactivation, a certain percentage of cells are normally expressing FMRP, whereas other cells lack FMRP expression.

In the case of prenatal diagnosis, DNA from chorionic villi can be used. In the literature, a number of studies have been described in which prenatal diagnosis has been performed on chorionic villi samples (CVS; Devys et al. 1992; Grasso et al. 1996; Iida et al. 1994; Sutherland et al. 1991; Suzumori et al. 1993). In most described male cases, a methylation-sensitive restriction enzyme has been used. In this way, both the repeat size and the methylation status of CVS have been determined. An alternative prenatal diagnostic method on chorionic villi is based on the absence of FMRP in cytothrophoblasts from FM male fetuses (Losekoot et al. 1997; Willemsen et al. 1996). In the present study, we have used this method to study FMRP expression in extra-embryonic tissue of FM male fetuses at various times of gestational age (10.0–12.5 weeks) and FM female fetuses (>13 weeks). The present study on CVS from FM female fetuses might give more insights into the pattern and timing of X-inactivation in human extra-embryonic tissues.

Materials and methods

Nomenclature

Gene symbols used in this article follow the recommendations of the HUGO Gene Nomenclature Committee (Povey et al. 2001).

Tissue processing and immunohistochemistry

Chorionic villi obtained from pregnancies at risk for fragile X syndrome were tested for FMRP expression. Biopsy material was sent to our laboratory from various Clinical Genetic Centres in The Netherlands. In these centres, the CVS were taken at various times of gestational age, varying from 10.0 weeks to 12.5 weeks. In total, 17 FM male fetuses, varying from 10.0 weeks to 12.5 weeks, were studied. Two cases of FM female fetuses are described in more detail below.

Chorionic villi biopsy from both female fetuses showed an FM allele by Southern blot analysis. The parents decided to terminate the pregnancy. After informed consent by the parents, fetal tissues, including chorionic villi and somatic tissues, were collected and analysed. Immunohistochemical detection of FMRP was performed on chorionic villi (whole-mount and sections) and on brain sections of case 1. Briefly, chorionic villi and fetal tissues were either embedded in Tissue-Tek (Miles, USA) and immediately frozen in liquid nitrogen, or chorionic villi were whole-mount fixed for 10 min in 3% paraformaldehyde and subsequently permeabilized in 100% methanol for 20 min. Immunohistochemistry with monoclonal antibodies against FMRP (Devys et al. 1993), followed by an indirect immunoperoxidase technique, was performed on both cryostat sections (8 μm thick) and whole-mount chorionic villi (Willemsen et al. 1996). Sections were counterstained with haematoxylin, dehydrated and mounted with Entellan. Finally, slides were examined with a Zeiss Axioskop microscope.

Results

Immunohistochemistry

The FMRP expression pattern in cryostat sections of chorionic villi from FM male fetuses at 10.5, 11.5 and 12.5 weeks of gestation are illustrated in Fig. 1. We focussed on the presence or absence of FMRP in cytotrophoblast cells, because cells in the mesodermal stroma were also labelled in sections immuno-incubated with the omission of the first antibody, especially at early stages. At 10.5 weeks, FMRP expression was similar to the expression pattern observed in control male fetuses (data not

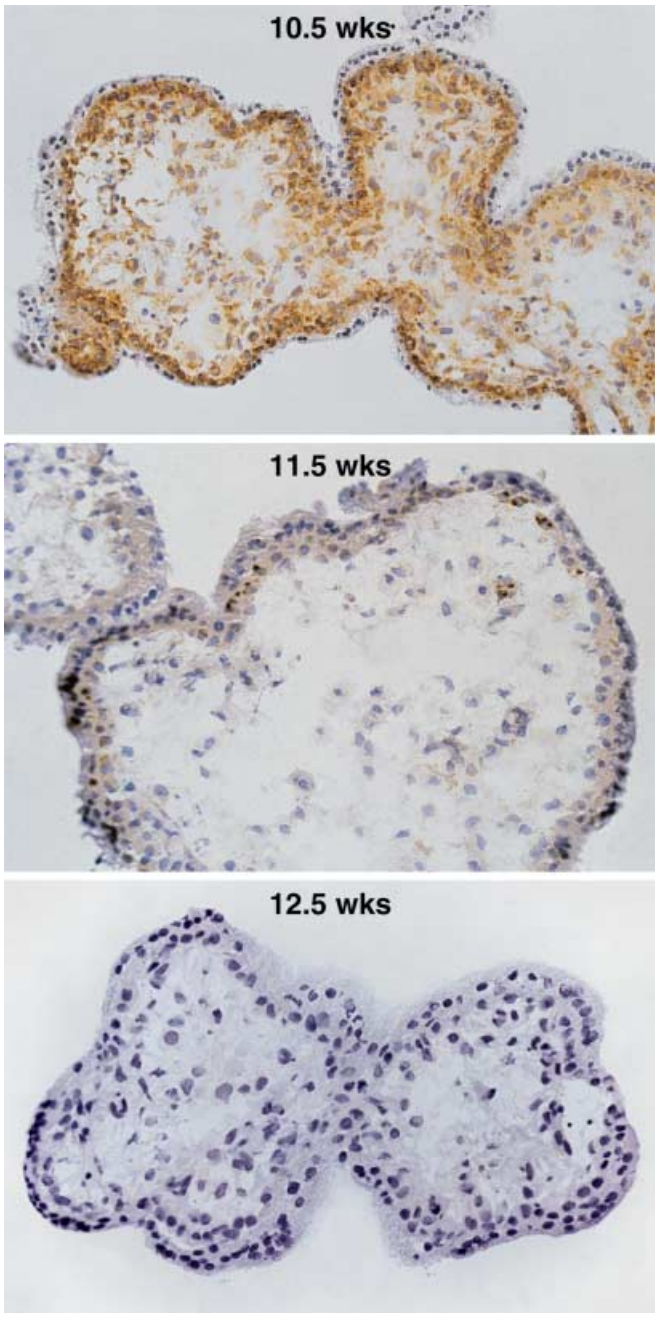

Fig. 1 Expression pattern of FMRP (*brown* precipitate) in chorionic villi samples (CVS) of full mutation males at 10.5, 11.5 and 12.5 weeks of gestational age. At 10.5 weeks, FMRP expression is similar to that in CVS from control fetuses. By 11.5 weeks, an intermediate pattern of FMRP expression is observed. At 12.5 weeks, all cytotrophoblast cells are completely devoid of FMRP

shown), with strong labelling in cytotrophoblast cells. An intermediate pattern was observed at 11.5 weeks, with weak labelling in most of the cytotrophoblast cells and strong labelling only in some cells. In contrast, at 12.5 weeks of gestation, all the cytotrophoblast cells were totally devoid of FMRP.

Chorionic villi from the female fetuses of the first biopsies (10.5 weeks) were not available for FMRP expression studies. Fetal tissues, including chorionic villi and brain, obtained from termination at 13.0 weeks of gestation (case 1) and 13.5 weeks of gestation (case 2) were used for immunohistochemistry. For case 1, two strategies were used to study FMRP expression in these tissues. First, immediately after termination, the chorionic villi were fixed as whole-mounts in order to investigate the overall expression of FMRP in the various villi. This method enabled us to compare the expression pattern between villi (Fig. 2A, B). We observed chorionic villi that were completely devoid of FMRP (Fig. 2A) and villi that were labelled for FMRP (Fig. 2B). This pattern can be explained by assuming that, in the FMRP negative villi (Fig. 2A), the normal allele is subjected to X-inactivation, whereas in the FMRP-positive villi, the normal allele is active.

Second, cryostat sections from sampled chorionic villi (as a mixture) and brain tissue taken from both female fetuses were prepared and immuno-incubated for FMRP. Both fetuses showed the same labelling pattern. For villi (Fig. 2C), high FMRP expression was observed in cytotrophoblast cells, although not all villi showed positive labelling. Both positively and negatively labelled villi were apparently present within the plane of a section. Indeed, within one cross-section of a villus, the cytotrophoblast cells were either positively labelled or unlabelled. A similar labelling pattern was found in brain tissue (Fig. 2D). Some neurons were totally devoid of labelling, whereas other neurons were strongly labelled. Interestingly, the positively or negatively labelled neurons were always located in small groups together, suggesting a clonal origin of neighbouring neurons.

Discussion

A new method for the prenatal diagnosis of the fragile X syndrome was described by Willemsen et al. in 1996. This method is based on the presence of FMRP in the cytotrophoblasts of control male fetuses and the absence of FMRP in the cytotrophoblasts of FM male fetuses. However, gestational age is considered to be important, because of the timing of the inactivation of the FM allele in chorionic villi. Until 10 weeks of gestation, FMRP is expressed in FM male CVS as in control male CVS. This is thought to occur because of the lack of inactivation and methylation of FM alleles at this age. This is in line with the observation that methylation-sensitive enzymes are able to digest DNA in the promoter region of FM CVS taken at week 10 (Devys et al. 1992; Grasso et al. 1996; Iida et al. 1994; Sutherland et al. 1991; Suzumori et al. 1993; unpublished data). The inactivation of *FMR1* is complete at 12.5 weeks of gestation resulting in a total lack of FMRP (Fig. 1). The prenatal diagnosis of the fragile X syndrome with the FMRP protein test on male CVS should thus be performed at 12.5 weeks or later to obtain reliable results. Until now, this immunohistochemical test has been reported only for male CVS. Here, we report the results of this method for the CVS of two female fetuses with a fully expanded CGG repeat in the *FMR1* gene.

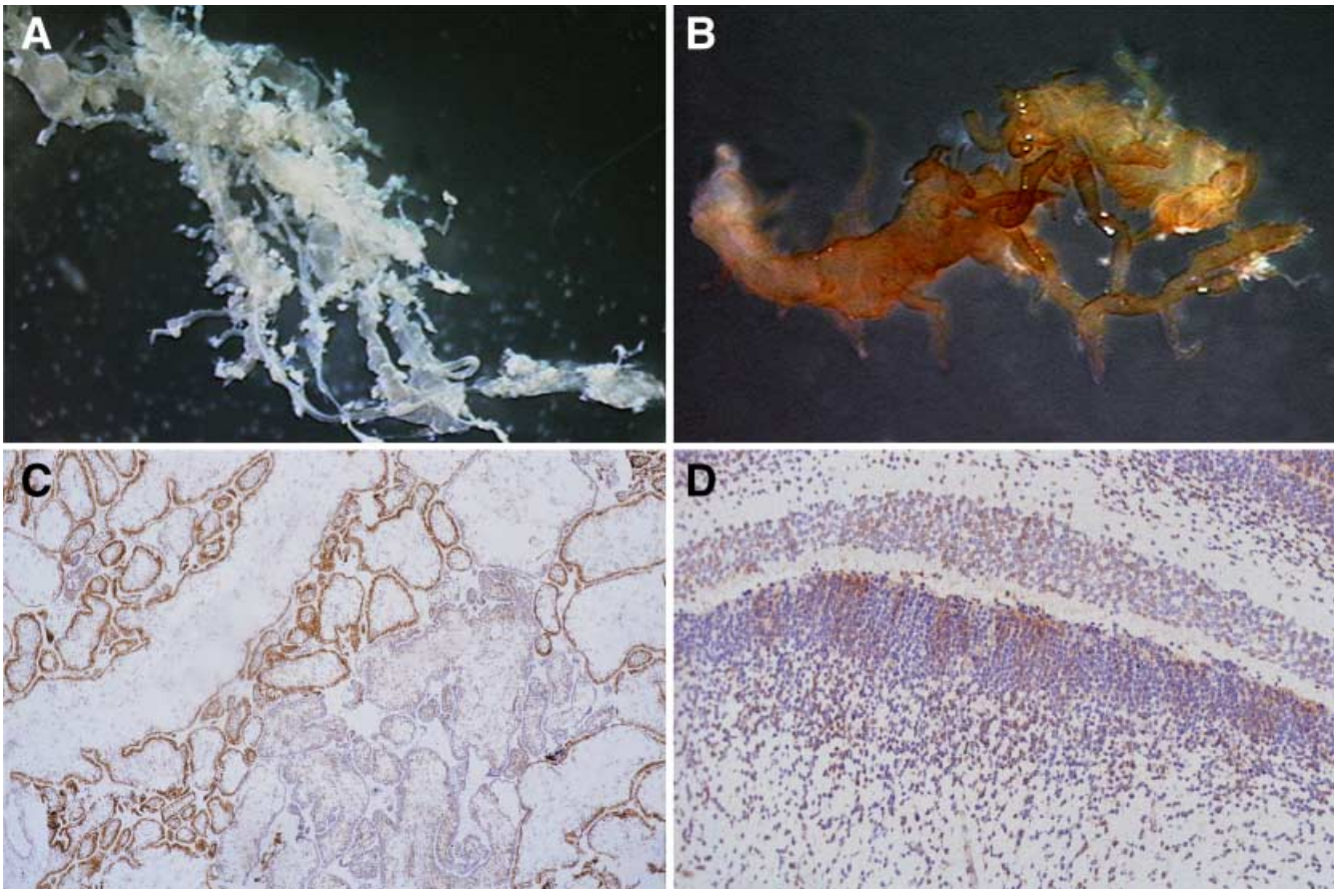

Fig. 2 Chorionic villi from full mutation female fetuses were either completely negative (**A**) or completely positive (**B**) for FMRP (*brown* precipitate). **C** In cryostat sections of the chorionic villi, FMRP was expressed in cytotrophoblast cells of a number of villi. Other villi were completely negative for FMRP. **D** In the brain of the full mutation female fetus, FMRP-positive and FMRP-negative neurons were always located together in small groups

Immunohistochemistry of chorionic villi (gestational age of more than 13.0 weeks) from two FM female fetuses showed that chorionic villi were either completely positively or completely negatively labelled for FMRP (Fig. 2). Since both positive and negative villi were present, this indicates that X-inactivation is a random event in human chorionic villi. In contrast, X-inactivation of extra-embryonic tissues in rodents differs from that in embryonic tissues as random X-inactivation is observed in embryonic tissues, whereas in extra-embryonic tissues, the paternal X-chromosome is inactivated (Takagi and Sasaki 1975). In the human, either of the X-chromosomes of CVS can be subject to X-inactivation: the villi are either completely positive or completely negative for FMRP and no villi have been found in which some cells express the protein and others not. This observation indicates that the development of villi is a clonal process. X-inactivation apparently occurs very early in development, before the chorionic villi start to proliferate, a process that takes place after blastocyst implantation. Because X-inactivation is maintained upon proliferation and differentiation, villi become either completely positive or completely negative upon cell proliferation. All cells in a single villus originate from one progenitor cell in which X-inactivation has occurred.

In conclusion, the lack of FMRP expression in the cytotrophoblasts from FM male fetuses reflects the inactivation of the FM, whereas the lack of FMRP expression in cytotrophoblasts from FM female fetuses reflects both the random inactivation of the X-chromosome and the inactivation of the FM. The timing of both inactivation processes is different, i.e. X-inactivation occurs earlier in development than the inactivation of the FM.

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