LETTERS TO THE EDITOR

FMR1 fully expanded mutation with minimal methylation in a high functioning fragile X male

I refer to the paper by Wang *et al* entitled "FMR1 fully expanded mutation with minimal methylation in a high functioning fragile X male" published in *J Med Genet* 1996;33:376-8, in which the authors state that the case described "represents one of fewer than 10 documented which show this mutation pattern". I would like to draw your and the authors' attention to the fact that in 1993 (*Am J Hum Genet* 1993;53:1064-73) we presented four such cases which were not referred to in the above paper. If these had been added to the list, and the possibility of general under-representation of such males in the total sample of fragile X males ascertained through affected probands were considered, this might have somewhat changed the view expressed in the paper that the discrepancy between the size of CGG repeat and methylation in fragile X is "an extremely rare molecular genetic pattern".

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This letter was shown to Dr Taylor and colleagues, who reply as follows.

We thank Dr Loesch for her comments on our paper by Wang *et al* and apologise for our oversight in omitting reference to her article. The incidence of unmethylated full mutations among fragile X males is not known, but this molecular finding may be somewhat less rare than initially supposed. At the time of submitting the article by Wang *et al*, there were few cases published. Since then there have been several additional reports (for example, by Kambouris *et al*, *Am J Med Genet* 1996;64:404-7 and by Lachiewicz *et al* and de Vries *et al* presented at the 1996 Fragile X Conference in Portland Oregon in August). It is of particular interest that all cases have been males with mild manifestations of fragile X syndrome and IQs in the normal range. It is likely that with increasing awareness of the existence of high functioning, non-retarded fragile X males, and of the importance of considering fragile X testing in borderline retarded or learning disabled males, more cases will be identified with minimally methylated or unmethylated fully expanded mutations.

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Raising the sensitivity of fetal RhD typing and sex determination from maternal blood

The fact that all currently available methods for tissue sampling carry procedure related risks has been a motivation for many years to

develop less invasive techniques for prenatal diagnosis, ideally by studying fetal cells in the maternal circulation.¹ Methods directly to PCR amplify sequences specific for the fetal Y chromosome (important in the case of X chromosomal recessive diseases) or for fetal RhD status (to manage rhesus sensitisation) without previous enrichment of fetal cells from maternal blood have successfully been established.^{2,3} However, these methods have so far not reached the precision required for routine application, since rates of false results, about 20%, are too high.^{4,5} On testing PCR based sex determination and RhD typing in over 100 samples of amniotic cells, chorionic villi, cord blood, or peripheral blood, we obtained no false results.

Y chromosome specific PCR was performed by a dual amplification procedure using the primers Y1.5 and Y1.6 in a first round PCR. One microlitre of the first round product was reamplified by nested PCR with Y1.7 and Y1.8. Each round consisted of 30 cycles of 94°C for one minute, 60°C for 30 seconds, and 72°C for two minutes. For determination of fetal RhD status, the primers RD-A3 and RD-2 were used in a first round PCR consisting of 30 cycles of 94°C for one minute, 55°C for two minutes, and 72°C for three minutes. The resulting product was reamplified using primers RD-5 and RD-2 by 30 cycles of heminested PCR with the same cycling parameters. We used two primers specific for sequences of the HER-3 oncogene as internal control (5' GACAAACACTGGTGTGATC 3' and 5' GCCCTTTTATTCTGAATCCG 3'); the other primers have previously been described^{2,3} as have details of the PCR protocol⁶). Using these approaches to determine the fetal sex and RhD status from maternal blood samples, all between 20 and 24 weeks of pregnancy, we obtained a rate of false results of about 26% (over 40 samples were analysed). Whereas we could not at present solve the issue of the false positive cases, we attempted to decrease the false negative rate by increasing the sensitivity of the sequence detection experiment. We separated the PCR products on an agarose gel and transferred them to nylon membranes by Southern blotting.⁷ After UV fixation these blots were hybridised with the relevant radioactively labelled sense primers, washed, and exposed to x ray films at -70°C using intensifying screens.⁸ Artificial dilutions of fetal DNA in adult DNA showed that by this method we were able to increase the sensitivity of detection from one fetal cell in a negative background of 100 000 cells to 1 in 10 000 000 cells. Accordingly, using this approach to analyse maternal blood samples the rate of false negative results decreased from about 20% to lower than 3% for RhD as well as for Y chromosome sequences. Our data indicate that false negative PCR results from maternal blood are more likely based on detection limits rather than on more physiological reasons and our methods increase the potency of PCR to perform less invasive prenatal diagnosis.

However, a major drawback for PCR based prenatal diagnosis from whole maternal blood is the fact that a PCR setting which is highly sensitive, such as the one described here, is also very likely to give false positive results. Such false positive results on fetal sex and RhD status determination from maternal blood have also been obtained by other groups.^{3,9,10} In the case of RhD status determination, general concerns about discrepancies between serological typing and

PCR derived RhD type have been raised.¹¹ Recently, a large scale evaluation of the accuracy of PCR to determine RhD blood type using DNA derived from adult blood samples was performed. Two of 632 RhD positive subjects were characterised as RhD negative and seven of the 133 RhD negative subjects were characterised as RhD positive.¹² These false positive results might also lead to the interpretation that not all RhD negative subjects exhibit total loss of the RhD gene. Our data do not support this interpretation. Taking exhaustive steps to avoid sample contamination and by including positive and negative controls, we did not obtain any false results doing PCR analysis of over 100 samples of amniotic cells, chorionic villi, cord blood, or peripheral blood. However, when we analysed fetal sex and RhD status by PCR from maternal blood samples, we obtained a relatively high rate of false positive results. The assumption that these false positive results are not the result of inaccuracy in our PCR technique is further supported by the observation that analysing artificial DNA dilutions from genomic DNA positive for Y chromosome DNA or the RhD gene with excess of negative DNA did not lead to these rates of false positives. An alternative interpretation might be that these results represent a fundamental biological phenomenon because of fetal cells in the maternal circulation from previous pregnancies or a previous maternal blood transfusion.^{10,13} In the cases presented here we could not explain the observed high rate of false positive results this way.

In summary, the data presented here describe a successful approach to increasing the sensitivity of fetal PCR diagnosis from maternal blood by reducing the rate of false negative results. However, as long as the PCR setting is not optimised to diminish the observed positive results, one might wonder whether this diagnostic approach can routinely be used without previous enrichment of fetal cells.

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