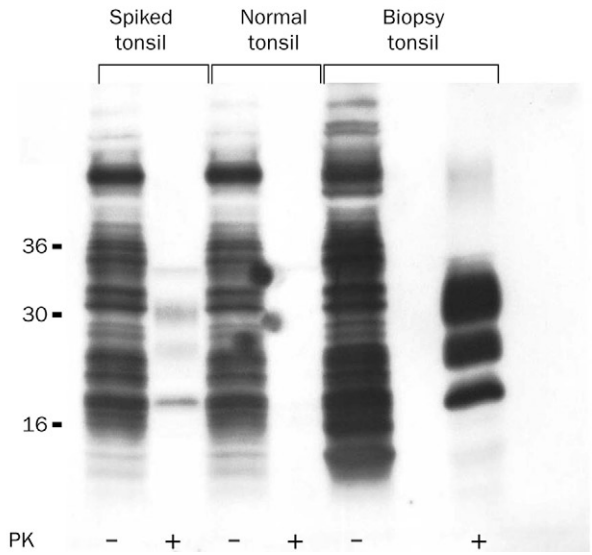

Figure 2: **Diagnostic *PrP*^{Sc} analysis of tonsil biopsy tissue by western blot**

We precipitated 0·16 mL aliquots of 5% homogenates from healthy tonsil and tonsil with suspected vCJD with sodium phosphotungstic acid (Sigma, Poole, UK). We added a spike of 0·05 μL 10% vCJD brain to one aliquot of normal tonsil (spiked tonsil) as a positive control. We compared 20 μL aliquots of samples isolated before centrifugation that were not digested by proteinase K (PK–) with products of proteinase K digestion (PK+) derived from the entire sodium phosphotungstic acid pellets. The tonsil biopsy homogenate has bands indicative of *PrP*^{Sc} after digestion with proteinase K.

This woman is the first case of vCJD in Italy, a country where, as of May, 2002, only 64 indigenous and two British-imported cases of BSE have been identified. Thus, continuous support for surveillance programmes of TSEs in human beings and animals is needed in Italy.

Contributors

V La Bella and F Piccoli diagnosed and cared for the patient during the diagnostic procedures. J Collinge analysed the tonsil biopsy sample. M Pocchiari did the genetic testing. V La Bella prepared the report with help from all other investigators.

Conflict of interest statement

None declared.

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Prenatal exclusion of β thalassaemia major by examination of maternal plasma

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The discovery of the presence of fetal DNA in maternal plasma has provided a new approach for non-invasive prenatal diagnosis. At present, the prenatal diagnosis of β thalassaemia relies on invasive methods. We designed allele-specific primers and a fluorescent probe for detection of the codon 41/42 (–CTTT) mutation in the β globin gene from maternal plasma by real-time PCR. The specificity and sensitivity of the allele-specific assay was confirmed by subjecting plasma, buffy coat, and amniotic fluid samples from 100 pregnancies to screening for the mutation. Subsequently, the assay was applied to the prenatal testing of eight fetuses at risk of β thalassaemia major, the aim being to exclude fetal inheritance of paternally transmitted codon 41/42 mutation. The fetal genotype was completely concordant with conventional analysis and β thalassaemia major was excluded in two of the pregnancies non-invasively.

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β thalassaemia is one of the most common autosomal recessive single-gene disorders in the world.[1] Current established prenatal diagnostic techniques for this disease, such as chorionic villus sampling, are invasive and have an inherent risk of fetal loss. The recent discovery of circulating fetal DNA in maternal plasma has provided an alternative and non-invasive approach for analysing fetal genetic heredity.[2] Thus far, this approach has been useful for the prenatal diagnosis of sex-linked diseases[3] and fetal rhesus D status.[4] We investigated the feasibility and reliability of prenatal exclusion of β thalassaemia major through the analysis of fetal DNA in maternal plasma.

In Southern China, about 90% of all β thalassaemia alleles are caused by four mutations in the β globin gene.[5] We focused our study on the most common of these, namely the deletion of four nucleotides (–CTTT) at codons 41/42. This mutation accounts for 40% of all β thalassaemia alleles in Hong Kong.[5] We designed allele-specific primers and a fluorescent probe (panel) for the specific detection of the codon 41/42 (–CTTT) mutation, using real-time PCR. In this system, the PCR process was monitored through the increase in fluorescent signal released by enzymatic cleavage of the fluorescent probe.[4] The PCR conditions and allele-specificity of the primers were optimised and confirmed with the use of buffy-coat DNA from individuals known to be homozygous for the mutation, and normal volunteers. Such DNA samples also served as the positive and negative controls in the subsequent analyses. Additionally, the presence of

Primer and probe sequences used in real-time PCR for detection of codon 41/42 (–CTTT) β thalassaemia mutation

Allele-specific primers	
CD4142F	5′-ACTCTCTCTGCCTATTGGTCTATTTT-3′
CD4142R	5′-GATCCCCAAAGGACTCA*ACC-3′
Fluorescent TaqMan probe	
CD4142	5′-FAM-CCACCCTTAGGCTGCTGGTGGTCTACCCT-TAMRA-3′

*Position of deleted (CTTT) sequence in normal β globin gene.

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amplifiable DNA from all of the samples used in this study was confirmed by the positive amplification of the normal β globin gene through a previously described real-time PCR assay.[4] All of the analyses were done by operators who were unaware of the identity of the samples being tested.

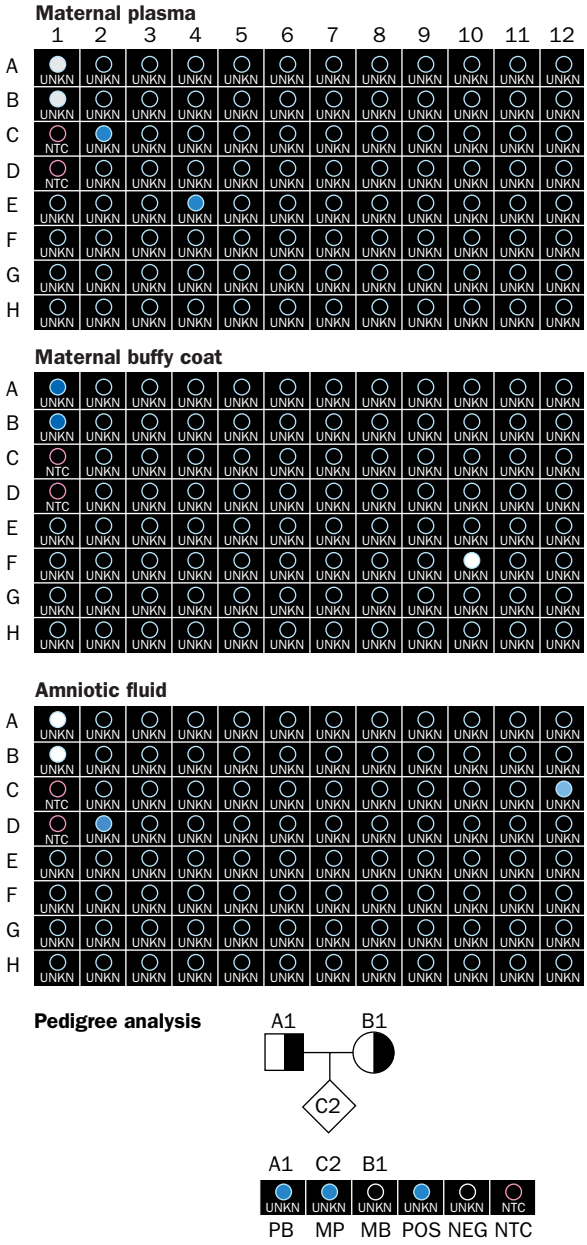
To assess whether the codon 41/42 (–CTTT) mutation-specific assay was applicable to the detection of fetal DNA in maternal plasma, 100 pregnant women (median gestational age 17·4 weeks; range 12–21) attending the antenatal clinic at the Prince of Wales Hospital, Hong Kong, and requiring amniocentesis predominantly for aneuploidy diagnosis were recruited with informed consent and institutional ethics approval. Maternal blood (3 mL) was collected before amniocentesis into edetic acid tubes, and the buffy coat and plasma fractions were stored separately. Amniotic fluid samples (5 mL) were collected into plain tubes. DNA from the buffy coat, plasma, and amniotic fluid was extracted with the QIAamp Blood kit (Qiagen, Hilden, Germany). On analysis of the 100 maternal plasma samples by the mutation-specific assay, two samples showed positive amplification for the mutation (#2921 in well C2, and #2998 in well E4; figure, top panel) and were subsequently confirmed by DNA sequencing.

The positive amplification of a maternal plasma sample can be seen in two situations: (1) if the pregnant woman is a carrier of the mutation, and (2) if a fetal-specific paternally derived mutation is present. To distinguish between these possibilities, the DNA from the amniotic fluid and maternal buffy coat from these 100 pregnancies were analysed by mutation-specific PCR analysis. Only one of the two corresponding maternal buffy coat samples was positive for the mutation (#2998 in well F10; figure, middle panel), whereas both of the corresponding amniotic fluid samples were positive (#2921 in well C12, and #2998 in well D2; figure, lower panel).

These data indicated that, among the 100 pregnancies, one of the pregnant women (#2998) was a carrier for the mutation, consistent with published findings that the carrier frequency of the codon 41/42 mutation in the general population in Hong Kong was 1·2%.[5] This woman had also transmitted the mutation to her offspring; hence the positive amplification in the amniotic fluid sample. For the second case (#2921), the male partner of the pregnant woman was a carrier for the mutation and had transmitted the mutation to his offspring. This case showed that the mutation-specific assay was able to detect a fetal-derived codon 41/42 (–CTTT) mutation in maternal plasma. Overall, these results indicated that this assay was highly sensitive and specific for the tested mutation (Fisher's exact test, $p<0·0001$).

We next investigated the clinical application of this assay by studying couples who presented for genetic counselling and prenatal diagnosis of β thalassaemia. Maternal and paternal blood was collected and subjected to mutation-specific PCR by the prenatal diagnostic service (Queen Mary Hospital, Hong Kong). Among 16 couples who were counselled and investigated, eight couples were informative for our approach, with the male partner carrying the codon 41/42 (–CTTT) mutation, and the pregnant woman carrying another β thalassaemia mutation. Maternal plasma was collected before amniocentesis or chorionic villus sampling at a median gestational age of 15·5 weeks (range 12–19). The maternal plasma DNA samples were analysed by mutation-specific PCR and six cases were positive for the mutation. The results suggested that, among the eight pregnancies, the paternally derived codon 41/42 (–CTTT) mutation was inherited by six of the fetuses. The fetal genotype was subsequently confirmed by conventional prenatal diagnosis, and found to be completely concordant with that predicted by maternal plasma analysis (Fisher's exact test, $p=0·036$).

The absence of inheritance of the paternal mutation in the two negative cases indicated that the fetus was not a compound heterozygote for β thalassaemia. This finding



Real-time PCR detection of codon 41/42 (–CTTT) mutation in screening samples and in a couple referred for prenatal diagnosis of β thalassaemia

Three upper panels show amplification results after 40 cycles of real-time PCR. Filled (blue and white) and unfilled (black) circles represent samples with and without positive amplification, respectively. A1 and B1 are positive controls; C1 and D1 are no template controls; E1 and F1 are negative controls. Lower images show pedigree and amplification results in one of the recruited families. PB=paternal buffy coat; MP=maternal plasma; MB=maternal buffy coat; POS=positive control; NEG=negative control; NTC=no template control. The father (PB) was positive for the mutation, but the mother did not carry the mutation, as evidenced by an unfilled circle in MB. The filled circle for MP indicates that the fetus had inherited the mutation from the father.

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effectively excluded the diagnosis of β thalassaemia major and therefore invasive prenatal diagnostic procedures could have been spared in these pregnancies. The fetuses were subsequently found to be heterozygous carriers of the maternal mutations and therefore had β thalassaemia minor. Among the six positive cases, four of the fetuses were found to be compound heterozygotes and therefore have β thalassaemia major. The other two fetuses had β thalassaemia minor, being heterozygous carriers of the paternal mutation. The figure illustrates the pedigree of one of the positive cases and the corresponding mutation-specific PCR analysis of each family member.

The limitation of the testing strategy as outlined here is that it cannot determine whether a fetus positive for a paternal mutation has β thalassaemia major or β thalassaemia minor—ie, whether or not the fetus has also inherited the maternal mutation. In this series of pregnancies, the non-invasive maternal plasma-based approach was completely accurate for the detection and exclusion of the fetal codon 41/42 (–CTTT) mutation. Additional large-scale prospective collaborative studies will be necessary to confirm these findings.

Similar assays can be set up for the other three common β thalassaemia mutations found in Southern China.[5] When available, these assays can be applied to 30–40% of all at-risk pregnancies. Through the effective exclusion of inheritance of the paternal mutation, the need for invasive prenatal diagnostic procedures could be eliminated in half of these pregnancies. This approach can be adapted to mutations prevalent in other regions, such as southeast Asia and the Mediterranean region.

Contributors

Y M D Lo and D H K Chui conceived of the study. Y M D Lo designed the study and supervised the laboratory work. R W K Chiu designed the mutation detection system, wrote the first draft of the manuscript, and, in conjunction with K C K Chow, did the molecular analysis of the clinical samples. D H K Chui proposed the use of the codon 41/42 mutation. T K Lau and T N Leung recruited the patients and did the clinical correlation.

Conflict of interest statement

Y M D Lo is a co-inventor of the patented technology of fetal DNA detection from maternal plasma. This technology has been licensed to BTG and Plasmagene. Y M D Lo is a consultant and shareholder of Plasmagene.

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