mmol/L added IMP (n = 6), respectively. The lower recoveries reflect coprecipitation of IMP with proteins, which agrees with previously published findings (1). Imprecision values (as CV, n = 10) were 2.1%, 1.2%, and 1.0% (within-day CV) and 4.2%, 3.2%, and 2.4% (between-day CV) for 0.06, 0.54, and 3.00 mmol/L additions of IMP, respectively. The reproducibility values (CV) of migration times for 10 samples from healthy volunteers were 0.92%, 2.8%, and 2.5% for run-to-run, sample-to-sample, and between-day measurements (n = 10), respectively. Because of the use of acidic separation medium, we observed no interference during the analysis of samples from 80 healthy blood donors and 20 patients undergoing azathioprine therapy.

With this simple capillary electrophoresis method, we estimated a reference interval (n = 80, 38 males and 42 females) for healthy white individuals of 13.3–112.2 nkat/g Hb (5%–95%), with a median of 57.8 nkat/g Hb, which agrees with the previously published data (3).

Availability of alternative methods is important because available analytical equipment varies among laboratories. Measurements of ITPase are important because ITPase deficiency may alter 6-mercaptopurine (azathioprine) metabolism, leading to adverse reactions (3), and the deficiency affects >10% of the population (4).

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References

Novel Mutation (c.G1124A) in Exon 9 of the APOB Gene Causes Aberrant Splicing and Familial Hypobetalipoproteinemia

To the Editor:
Familial hypobetalipoproteinemia (FHBL) is commonly caused by mutations in the apolipoprotein B gene (APOB). The APOB gene encodes 2 proteins, apolipoprotein (apo) B-48 and apo B-100. Apo B-48 is formed in the intestine and is essential for the formation and recognition of dietary derived chylomicrons, and apo B-100 is found in VLDLs and LDLs of hepatic origin and is involved in the endogenous transport of triglycerides, cholesterol, and fat-soluble vitamins. A number of abnormally truncated apo B proteins have been described, and by convention are referred to by a centile system reflect-
ing their apparent $M_r$ in relation to apo B-100 \((1)\).

Truncations shorter than apo B-27 are not expressed in lipoproteins, and those shorter than apo B-75 are underrepresented in LDL \((2-4)\). Consequently homozygous mutations in the N-terminal third of APOB result in the virtual absence of both apo B-48 and apo B-100 and their corresponding lipoproteins, and thus very low concentrations of plasma triglycerides, cholesterol, and the fat-soluble vitamins. This condition is known as FHBL and is characterized clinically by failure to thrive, steatorrhea, and eventually both central and peripheral neurological abnormalities \((1)\). Heterozygotes usually experience a milder phenotype or are asymptomatic.

We report a novel APOB mutation, identified in a family with low total cholesterol and apo B concentrations in plasma. The proband, a 64-year-old man, had an LDL cholesterol concentration of 1.4 mmol/L and an apo B concentration of 0.39 g/L, and his 2 daughters both had LDL cholesterol concentrations $\approx$0.5 mmol/L, and apo B concentrations $<0.35$ g/L. In the mother, the concentrations of these analytes were within reference intervals. Western blotting of plasma from all 4 individuals showed no apo B truncations. DNA sequencing of the exons and exon/intron boundaries of the APOB gene revealed a novel heterozygous c.G1124A mutation in the proband and his 2 daughters, which was not present in the mother. No other APOB gene mutations were identified.

The c.G1124A mutation predicts a p.Ser348Asn substitution in the $\beta\alpha_1$ domain, which is essential for lipoprotein assembly. The p.Ser348Asn substitution may affect the structure or function of this domain but is predicted to be benign, according to Polyphen (http://www.polyphen.com), with a position-specific independent counts difference score of 0.675. Alternatively the mutation at the ultimate nucleotide of exon 9 could affect splicing at the adjacent intron 9 donor splice site, with various potential splicing outcomes (Fig. 1A).

Indeed, the programs SpliceView (http://l25.itba.mi.cnr.it/~webgene/wwwspliceview.html) and NNSplice (http://www.fruitfly.org/seq_tools/splice.html) predicted that the G$\rightarrow$A mutation would abolish splicing at the normal donor splice site of intron 9, and activate a cryptic donor site 40 bp into the intron. Gene Splicer (http://www.tigr.org/tdb/GeneSplicer/gene_spl.html) also predicted abolishment of the normal donor site but did not predict the usage of a cryptic splice site.

To confirm these in silico predictions we performed minigene expression studies. A minigene construct spanning exons 8–11 and the intervening sequences was cloned into the pcDNA3.1/V5-His TOPO TA vector and then transfected into COS-7 cells. After 48 h the mRNA was isolated and reverse transcription PCR was performed. The cDNA was amplified using primers within exon 8 and exon 11. The expected 650-bp product was visualized in the wild-type, and a larger product of 690 bp was observed in the mutant (Fig. 1B). DNA sequencing of the 690-bp and 650-bp bands revealed that the increase in size of the mutant product reflected the inclusion of the first 40 bp of intron 9. A cryptic donor splice site between c.1124 + 40 and c.1124 + 41 was activated in the mutant construct, and the normal intron 9 acceptor site was used. Predictably, this message results in a frame shift in the translated protein, a substitution of serine 348 to lysine, and the insertion of 92 new amino acids before a premature stop is encountered at residue 440 (Ser348LysfsX93). The resulting mutant protein, a truncated apo B-9.7, would not be viable for lipoprotein formation. In vivo the majority of the transcripts would be expected to use the cryptic splice site in intron 9, creating the truncated apo B-9.7 and causing the observed FHBL.

![Fig. 1. Pre-mRNA splicing of the G1124A minigene construct.](image-url)

(A), diagram of the minigene construct and the potential splicing outcomes from the G1124A mutation. (B), 2% agarose gel showing the wild-type (WT) and mutant (MUT) cDNA products arising from splicing of the minigene construct in COS-7 cells. Two different-sized bands are visible at 650 and 690 bp, respectively.
From this analysis, we have shown that the novel c.G1124A mutation causes FHBL by disrupting splicing. We identified 3 family members who were heterozygous for this mutation but were largely asymptomatic because each still had 1 normal APOB allele.

This case highlights the difficulty of interpreting novel mutations identified in diagnostic laboratories and the need for a clear strategy to determine their significance. If sufficient family members are not available, linkage analysis may be uninformative and functional analysis is essential.

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Circulating Cell-Free Placental mRNA in the Maternal Plasma as a Predictive Marker for Twin-Twin Transfusion Syndrome

To the Editor:

Twin-twin transfusion syndrome (TTTS), which is a serious complication in monochorionic diamniotic twins (MCDA-T), involves unequal blood flow via the placental vascular anastomoses from the donor to the recipient twin. Although the placental anastomoses are present in all MCDA-T and both fetuses are genetically identical, TTTS occurs in only 15% of MCDA-T, and much of the pathophysiological basis of TTTS remains poorly understood. Clinically, a staging system based on the ultrasound features of TTTS is widely used for the management (1) but not for the prediction of TTTS. In addition, the known predictive findings observable by ultrasonographic examination are detectable only in a small portion of TTTS cases (2). New predictive markers are therefore desirable for the early detection and prevention of TTTS. Recently, placental mRNAs, such as human placental lactogen (PL) and some other hormones were detected in maternal plasma, and concentrations of each marker were measured with quantitative real-time reverse transcription (RT)-PCR (3, 4). Thus, circulating cell-free mRNA (cf-mRNA) in maternal plasma has become an attractive target for the noninvasive monitoring of pregnancy disorders (3, 5).

The purpose of the present study was to investigate the use of cf-mRNA concentration in maternal plasma as a predictive marker of later TTTS. The study participants included 17 pregnant women who visited the Obstetrics Clinic of Nagasaki University Hospital at 12–21 weeks of gestation for management of their pregnancy with MCDA-T. Included as a control group were 135 singleton pregnant women without medical complications at similar gestational age. All of the participants gave written informed consent, and the study was approved by the Research Ethics Committee of Nagasaki University. Although none of the 17 cases of MCDA-T were complicated by TTTS at the time of blood sampling, TTTS subsequently developed in 5 cases (TTTS group), but not in the remaining 12 cases (no-TTTS group). Gestational ages at diagnosis of TTTS were 15–25 weeks. The 3 groups had no significant differences in population characteristics, including the maternal age, the number of nulliparous women, and the gestational age at the time of sampling (data not shown).

The blood samples (8 mL) from each woman were collected into an EDTA tube, and the plasma sample was stored at −20°C until use. After cf-mRNA was extracted from maternal plasma, a quantitative 1-step real-time RT-PCR assay was performed using an ABI 7900T Sequence Detector (Perkin-Elmer) as described previously (4). Primer sets and TaqMan probes for each gene and single-strand, and synthetic DNA oligonucleotides from each amplicon used for a calibration curve were prepared as described previously (4). Then, plasma concentrations of cf-mRNA for human PL and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured and converted into multiples of the median (MoM) of the controls adjusted for gestational age, as described previously (5). The differences between the TTTS and the no-TTTS groups were evaluated with the Mann–Whitney U-test. Significant difference was defined as a P value <0.05.

The median (minimum–maximum) cf-PL mRNA MoM values were 1.80 (0.89–3.81) in the TTTS group, 1.14 (0.77–1.35) in the no-TTTS group, and 1.00 (0.82–2.05) in the control group, respectively. At adjusted gestational age the cf-PL mRNA concentration was significantly higher in the TTTS group than in the no-TTTS group (Mann–Whitney U-test, P = 0.035), whereas there was no significant difference of cf-PL mRNA concentration between the no-TTTS group and the control group (P = 0.41; Fig. 1). In addition, the median cf-GAPDH mRNA MoM value in the maternal plasma was significantly higher in the TTTS