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Variations of the *Amnionless* gene in recurrent spontaneous abortions

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Recurrent spontaneous abortions (RSA) are estimated to affect 0.5–1% of couples trying to have a child. The causes of RSA are unknown in the majority of cases. This study aimed to determine whether homozygous mutations in the *AMN* gene in a fetus cause spontaneous abortions in humans, as they are known to cause spontaneous abortions in mice. The study was conducted by screening 40 couples and 5 women with three or more unexplained spontaneous abortions for heterozygous mutations in the *AMN* gene using denaturing high-performance liquid chromatography. Altogether, 3 exonic and 11 intronic sequence variations were found. There were no significant differences in the frequencies of the variations between the patients and a control group. One of the exonic variations was non-synonymous, and three of the variations may affect gene splicing. None of the putative phenotype-affecting variations were found in both partners in any couple. These results indicate that RSA in the couples studied cannot be explained by homozygous *AMN* mutations in the fetus. However, two couples had different, potentially deleterious variations in both partners. If these variations have a phenotypic effect, the RSA experienced by these couples may be caused by mutations in the *AMN* gene. In addition, birthplaces of the patients' ancestors revealed some clustering, suggesting that some patients may carry a founder mutation in another gene which may contribute to RSA.

Key words: *Amnionless*/DHPLC/miscarriage/polymorphism/recurrent spontaneous abortion

Introduction

Spontaneous abortions are the most common complication of pregnancy, affecting approximately 15% of all pregnancies. Recurrent spontaneous abortions (RSA), defined as the occurrence of three or more consecutive, clinically detectable pregnancy failures, are estimated to occur in 0.5–1% of all couples (Tulppala *et al*., 1993; Katz and Kuller, 1994). After excluding known causes of spontaneous abortion including genetic, metabolic, endocrine, environmental and anatomic factors, the cause remains unknown in approximately 50% of women who experience RSA (Plouffe *et al*., 1992; Tulppala *et al*., 1993; Clifford *et al*., 1994). Even though genetic factors such as chromosome abnormalities (Stephenson *et al*., 2002) are one of the major and best defined causes of spontaneous abortions, new genetic factors associated with recurrent abortions remain to be found.

One way to identify new genes associated with RSA is to study candidate genes required for normal development in mice. One gene known to cause miscarriage in mice when mutated is *Amnionless* (*Amn*). The *Amn* mutation is a transgene-induced insertion mutation in the distal region of mouse chromosome 12. This mutation is prenatally lethal, and homozygous mutant fetuses die at an early stage of development (Wang *et al*., 1996). Gastrulation begins normally, but despite the apparently normal development of extra-embryonic structures, the embryonic ectoderm of the mutant mice remain small and undifferentiated, and no amnion is generated (Wang *et al*., 1996). Mutant embryos that survive to the tenth day of gestation have headfolds, a beating heart and abundant posterior mesoderm, but they have none of the mesoderms that produces the limb buds, dermis, muscle, vertebrae and other organs of the trunk (Tomihara-Newberger *et al*., 1998). At the onset of gastrulation, the proximal and distal portions of the primitive streak are normally organized, but the middle region is absent in the mutant mouse. The Amn protein has been shown to be required in the visceral endoderm (VE) for normal middle streak formation (Tomihara-Newberger *et al*., 1998), but its exact role in mouse development has not yet been determined. The extracellular region of the Amn protein contains a 70 amino acid cysteine-rich (CR) domain similar to CR domains present in bone morphogenetic protein (BMP)-binding proteins. Therefore, it has been proposed that Amn functions as a part of a BMP-signalling pathway within the VE to direct the expression of a set of genes that control the formation of the middle streak, which is proposed to be directed by a genetic pathway different from the pathways controlling the formation of the proximal and distal parts of the primitive streak (Kalantry *et al*., 2001).

In both mouse and human, *Amn* is expressed in the intestine and kidney (Tanner *et al*., 2003; Strope *et al*., 2004). Mouse Amn is also expressed during gastrulation in the VE (Tomihara-Newberger *et al*., 1998; Kalantry *et al*., 2001). In these tissues, Amn forms a complex with the cubilin (Cubn) protein, a multi-ligand scavenger receptor. The function of Amn in the complex is to ensure the proper localization and endocytosis of Cubn and its ligand (Fyfe *et al*., 2004; Strope *et al*., 2004). An alternative role suggested for Amn in mouse development is that the Cubn/Amn complex may be required for endocytosis of several ligands in the VE, and the complex would thereby facilitate a signalling pathway during gastrulation to coordinate primitive streak assembly (Strope *et al*., 2004).

In humans, the CUBN/AMN complex is required for uptake of cobalamin (vitamin B12), and to date all known homozygous mutations in either gene cause Imerslund–Gräsbeck syndrome (IGS). Although AMN is needed for absorption of cobalamin in humans, it may be a moonlighting protein with more than one function. High similarity between mouse and human *Amnionless* genes suggests that the genes would have the same functions. That mutations in *Amn* cause fetal loss in mice (Wang *et al*., 1996) makes human *AMN* a candidate gene for RSA. The role of *AMN* in the early development of humans has not yet been investigated, and the aim of this study was to determine whether homozygous mutations in the *AMN* gene of a fetus could offer a new explanation for RSA in humans. We hypothesized that parents with recurrent unexplained fetal losses are healthy heterozygous carriers of mutations in *AMN*, and the aborted fetuses would have the mutations in a homozygous state.

Materials and methods

Subjects

Patients with RSA treated at the Department of Gynaecology and Obstetrics of the Helsinki University Hospital during 2001–2004 were chosen for the study. The inclusion criteria for the study included women aged 18–40 years with previous history of recurrent abortion, defined as three or more consecutive abortions. In 44 women, all the abortions had taken place during the first trimester (<13 weeks). In addition to first trimester losses, two women had experienced second trimester (from 13 weeks to 23 weeks) abortions, and one women a third trimester (≥24 weeks) intrauterine fetal death.

Uterine anomalies were checked by ultrasonography or hysterosonogram. Maternal and paternal karyotypes were tested from peripheral blood lymphocyte culture. No patients included in this study had chromosomal or uterine abnormalities. A total of 85 (40 couples and 5 women) patients with unexplained RSA were chosen for this study.

The control group used in the study consisted of 95 women who had at least one normal pregnancy and no history of spontaneous abortion. The ethics committee of the Department of Obstetrics and Gyneacology, Helsinki University Central Hospital, approved this study.

Polymerase chain reaction

DNA was extracted from whole blood collected from patients and controls using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). The 12 exons and 3′-UTR of *AMN* were amplified via PCR, performed in a 25 µl reaction mix containing the following reagents: 50–100 ng of genomic DNA, ×1 Optimized Detergent-free EXT buffer, 2 nmol of each dNTP, 10 pmol of forward primer, 10 pmol of reverse primer and 0.5 units of DyNAzyme EXT DNA-polymerase (Finnzymes, Espoo, Finland). Additionally, DMSO (final concentration 5% [v/v]) was added to some of the amplicons. Thermocycling was perfomed in a PTC-225 DNA Engine Tetrad thermocycler (MJ Research, Waltham, MA, USA). Initial denaturation at 95°C for 2 min was followed by 33–40 cycles of denaturation at 95°C, annealing at 51–66°C depending on the amplicon and extension at 72°C. The lengths of the denaturation, annealing and extension steps varied depending on the amplicon. A final extension was performed at 72°C for 10 min. Information concerning the exact PCR conditions and the primers used is available on request. Amplification of appropriately sized PCR products was confirmed by agarose gel electrophoresis before further analysis.

DHPLC sample analysis

Denaturing high-performance liquid chromatography (DHPLC) analysis was carried out using a Transgenomic WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Omaha, NE, USA) and the associated Navigator software. Before analysing the samples with DHPLC, the PCR products were denatured for 3 min at 95°C and then gradually reannealed by decreasing the temperature from 95 to 50°C over a period of 50 min to enable the formation of heteroduplexes. To obtain optimal resolution of homoduplex and heteroduplex DNA fragments, the temperature was set for partially denaturing conditions. The melting profile of the amplicons was predicted by the Navigator software, but the exact temperature was determined empirically by injecting one PCR product for each amplicon at 0.5 and 1°C under, and 1 and 2°C over the optimal temperature suggested by the Navigator program. Conditions used for DHPLC analysis for each amplicon are available on request.

Sequencing

Following DHPLC screening, samples showing heterozygous peaks were sequenced in order to determine the nature of the sequence change. Additionally, for each amplicon at least 10 samples showing only a homoduplex peak were sequenced to confirm that no variation went undetected. The PCR products were purified using Exonuclease I and shrimp alkaline phosphatase (USB Corporation, Cleveland, OH, USA), and the purified products were sequenced using BigDye version 3.1 sequencing chemistry and an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Genotype determination

By sequencing, four variations were detected in a homozygous state. Even though this study focused on heterozygous variations, we determined the exact genotype of the samples by restriction enzymes in the cases where the variations either created or deleted a restriction site. All the samples were genotyped for variations c.-27T>C, c.1169+42C>G and c.1362+38G>C using restriction enzymes HpaII, BsaJI and AciI (New England Biolabs, Ipswich, MA, USA), respectively. Variation c.-23G>C does not create or delete any restriction site, and therefore the samples were sequenced to determine the exact genotype for this variation.

After the digestion, the restriction pattern was detected by agarose gel electrophoresis.

Predicting the effects of variations

Exonic splicing enhancers (ESEs) are common in both alternative and constitutive exons, where they act as binding sites for Ser/Arg-rich proteins (SR proteins), splicing factors needed in multiple steps of the splicing pathway. ESEfinder (http://exon.cshl.edu/ESE/) is a Web-based resource which scans nucleotide sequences to identify putative ESEs. The ESEfinder was used to predict whether the exonic or intronic sequence variations disrupt the sequence of known ESE-elements.

The variations predicted to change an amino acid were analysed by the SIFT (sorting intolerant from tolerant) program (http://blocks.fhcrc.org/sift/SIFT.html) which predicts whether an amino acid substitution in a protein will be tolerated.

Statistical analysis

Fisher's exact tests were used for statistical analysis of the data. The allele frequencies of the patients and controls were compared to determine if any of the variations were more frequent in either group. Differences were considered as statistically significant for P-values <0.05.

Genealogic analysis

All patients were given questionnaires enquiring as to the full names, dates and places of birth of their parents and grandparents. Answers were received from 82 patients. To investigate the geographic distribution of ancestral birthplaces, the birthplaces of the parents and grandparents were placed onto a map of Finland, including the formerly Finnish areas of Russian Karelia. The maps were compared with the maps of the distribution of the Finnish population at the time the parents/grandparents of the couples were born to determine if these birthplaces were clustered to some specific region.

Results

We screened 85 patients (40 couples and 5 women) with a history of unexplained spontaneous recurrent abortions and 95 controls with DHPLC for mutations in the 12 exons and the 3′-UTR region of the *AMN* gene. The nature of the variations detected was confirmed by sequencing.

In total, 14 sequence variations were found (Table I). Of these, one was found only in patients. Three were found only in controls, while

Table I. *AMN* sequence variations

Location	DNA variation* (predicted amino acid change)	Heterozygous patients (homozygous patients) (n = 85)	Heterozygous controls (homozygous controls) (n = 95)
Exonic			
5	c.363G>A†	4	0
8	c.829A>G (T276A)†	1	1
12	c.1339_1344dup GCCGGG†	2	2
Intronic			
Upstream of SS‡	c.-87C>G	13	15
Upstream of SS‡	c.-74C>T	10	11
Upstream of SS‡	c.-27T>C	48 (15)	50 (19)
Upstream of SS‡	c.-23G>C	46 (9)	44 (9)
4	c.296-75_-66dup GCGTGGCGTG†	0	1
8	c.843+11C>T†	5	1
10	c.1169+42C>G	43 (12)	47 (14)
10	c.1170-6C>T†	2	2
3′-UTR	c.1362+38G>C	34 (11)	40 (14)
Downstream of 3′-UTR	c.1362+518C>T†	0	1
Downstream of 3′-UTR	c.1362+523G>A†	0	1

*Numbering relative to adenine in the first ATG startcodon of *AMN* (UCSC Genome Browser, http://genome.ucsc.edu/).
†Novel variations (variations which have according to our knowledge not been previously reported).
‡SS, start site.

the remaining ten variations were detected in both patients and controls. There were no significant differences in the frequencies of heterozygous (or homozygous) patients and controls for any of the sequence variations. Two of the variations were single-nucleotide substitutions within exons. Sequence variation c.363G>A is a single-nucleotide substitution in exon 5, which changes the last nucleotide of codon 121 in the *AMN* gene (GGG→GGA). This synonymous variation, which was detected only in patient samples, does not predict a change of the amino acid (glycine) and is therefore predicted to be a silent polymorphism. Sequence variation c.829A>G is a non-synonymous single-nucleotide substitution in exon 8, which changes the first nucleotide of codon 276 (ACC→GCC). The variation is predicted to change the amino acid from threonine to alanine. According to the SIFT program, this amino acid change would be tolerated even though the hydroxyl group of threonine makes it much more hydrophilic and reactive than alanine. Ten variations were single-nucleotide substitutions within introns or the 3′-UTR region, and two were duplications. One of the duplications was a 10 bp (GCGTG GCGTG) duplication in intron 4 and the other was a 6 bp duplication (GCCGGG) of codons 447 and 448 in exon 12. This variation is predicted to make the final protein product two amino acids (Ala+Gly) longer.

All 14 sequence variations were analysed with the ESEfinder to predict whether any affect the splicing of the gene by altering the binding sites of the SR proteins needed for splicing. ESEfinder searches for putative ESE motifs (six to eight nucleotides long) and calculates a score for all sequences with a motif match. A score is considered significant and the sequence potentially needed for splicing when the score is greater than the threshold value defined by the program. The results indicate that the single-nucleotide substitutions in exon 8 (c.829A>G), intron 8 (c.843+11C>T) and intron 10 (c.1170-6C>T) may affect the splicing by deleting one or two binding sites for the known SR proteins (Table II).

The 40 couples included in this study were analysed to determine whether some variations existed in both partners of a couple. The only variations detected in both partners were the four most common variations. In no couples were the same exonic or putative splice-site affecting variations found in both partners. In two of the couples, both partners had a different exonic or putative splice site affecting variation. One couple had variations c.363G>A and c.1170-6C>T, and another couple had variations c.843+11C>T and c.1339_1344dup.

The birthplaces of the parents and grandparents (data concerning grandparents not shown) of the patients were mapped to determine whether these were clustered. Compared to the reference map, the geographical distribution of the birthplaces is somewhat uneven (Figure 1), and the results suggest that there may be an eastern enrichment (in the province of Kuopio) of the ancestral birthplaces.

Table II. Predictive score matrices obtained with the ESEfinder software

Sequence variation	SF2/ASF (Thr = 1.956)	SC35 (Thr = 2.383)	SRp55 (Thr = 2.267)
AMN c.829A>G			
Normal allele	2.080 (GAC**A**CCT)		
Variant allele	<1.956 (GAC**G**CCT)		
AMN c.843+11C>T			
Normal allele	2.506 (**C**GCGCGG)	2.584 (GGGC**C**GCG)	
Variant allele	<1.956 (**T**GCGCGG)	<2.383 (GGGC**T**GCG)	
AMN c.1170-6C>T			
Normal allele		2.633 (GC**C**CTCAG)	2.922 (TACGC**C**)
Variant allele		<2.383 (GC**T**CTCAG)	<2.267 (TACGC**T**)

Threshold (Thr) values defined by the ESEfinder and values obtained for normal and mutant alleles for sequence variations predicted to affect the binding sites of SR proteins.

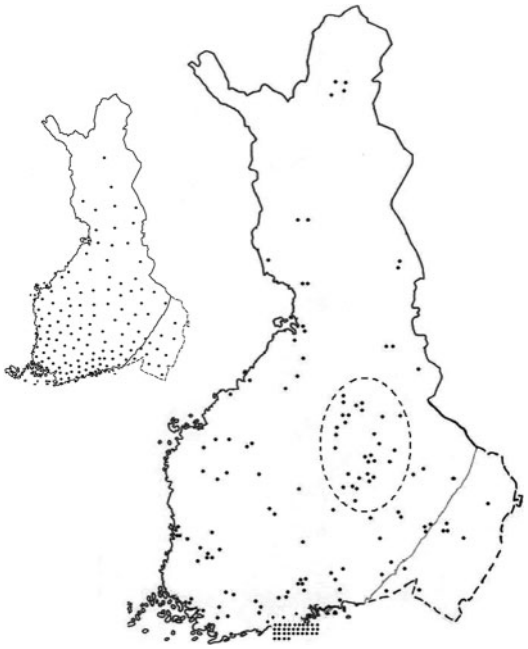

Figure 1. Birthplaces of the parents of 82 patients with RSA. 34 of the parents are born in Helsinki. The region of geographical enrichment of ancestral birthplaces is marked with a circle. The small reference map shows the population density of Finland at the time the parents were born.

Discussion

The high similarity between human *AMN* and mouse *Amn* (Kalantry *et al*., 2001), and the fact that homozygous mutations in *Amn* are known to cause fetal death in the mouse (Wang *et al*., 1996), makes *AMN* a candidate gene for spontaneous abortions in humans. In this study, we have examined whether there is a connection between RSA and mutations in the *AMN* gene. To our knowledge, there are no published data concerning the role of *AMN* in human development. As a result of screening 85 patients using DHPLC, 3 exonic and 11 intronic sequence variations were detected. All the detected variations in *AMN* can be defined as polymorphisms, and there were no significant differences in the frequencies of the variations between patients and controls. Three variations were predicted to affect exon splicing. In two of the 40 couples screened, both partners had different exonic or potential splice site-disrupting variations, but in no couple was the same putative protein-altering variation detected in both partners. These results suggest that RSA in the couples studied is not explained by homozygous mutations in *AMN*. However, the genealogic studies showed clustering in the birthplaces of the parents and grandparents, indicating that the patients may carry a founder mutation in another gene which may contribute to RSA.

The polymorphisms detected in *AMN* may not directly affect protein function but may have effects on the protein indirectly by changing the function of regulatory sequences that control gene expression, by altering the stability of the mRNA or by disrupting the splicing mechanisms of the gene. Splice site mutations may result in exon skipping, activation of cryptic splice sites, creation of a pseudo-exon within an intron or intron retention (Krawczak *et al*., 1992; Nakai and Sakamoto, 1994). We attempted to predict whether the variations detected in *AMN* would disrupt the splicing of the gene using ESEfinder, a Web-based splicing motif recognition program. The program predicted that three sequence variations, c.829A>G in exon 8, c.843+11C>T in intron 8 and c.1170-6C>T in intron 10, may affect splicing. These variations are predicted to abolish one or more of the binding sites for known SR proteins and may therefore result in exon skipping or intron retention. The ESE finder-software, however, only makes predictions about the effect of a variant on the splicing of the mRNA. The presence of a high-score motif in a sequence does not necessarily make the sequence an ESE, and the sequence with maximum score is not necessarily the most effective ESE (Cartegni *et al*., 2003). Further studies are required to determine whether the variations predicted to affect splicing actually disrupt the splicing mechanisms.

Two couples in this study had different exonic or putative splice site variations in both partners. Variations c.363G>A in exon 5 and c.1170-6C>T in intron 10 were detected in one couple and the other couple had variations c.843+11C>T in intron 8 and c.1339_1344dup in exon 12. The variation detected in exon 5 is a synonymous (Gly121Gly) change and is unlikely to affect the protein function. Variations c.1170-6C>T and c.843+11C>T are predicted to disrupt the splicing mechanisms of the gene. The exonic duplication in exon 12 is predicted to make the protein two amino acids longer (Ala+Gly). This variation may affect the structure of the cytoplasmic region of the protein. The other *AMN* variations (c.-27T>C, c.-23G>C, c.1169+42C>G and c.1362+38G>C) found in both partners of a couple are previously reported common variations, also found in homozygous states in both patients and controls, indicating that these variations are neutral polymorphisms. In the two aforementioned couples, there is a 25% chance for every conceptus to be a compound heterozygote with different potentially deleterious *AMN* mutations in the two alleles. Unfortunately, there are no fetal samples available to study whether these variations may be the cause for spontaneous abortions experienced by these couples. If these variations had a phenotypic effect, the RSA in these couples could be explained by mutations in the *AMN* gene. However, at present there is no evidence for a phenotypic effect. Further studies are needed to determine whether the function of *AMN* in human and mouse differs significantly or whether *AMN* is needed both for the uptake of vitamin B12 and normal embryonic development in humans. When considering the data from the Human Genome Project, the human genome sequence appears to encode far fewer proteins than predicted, indicating that some genes may encode a protein with more than one function (Jeffery, 2003, 2004).

Even though we found no clear-cut mutations in the *AMN* gene explaining the RSA in the couples studied, the result of the genealogic studies are interesting and suggest the possibility of an unknown gene causing the spontaneous abortions in some of the patients. The birthplaces of the parents and grandparents of a subset of the patients showed clustering to a region previously known as the province of Kuopio. That the region around Helsinki is overrepresented compared with the reference map is explained by the fact that all patients were treated in Helsinki, but this does not explain the clustering around Kuopio. This clustering suggests that some of these patients may have ancestors in common, resulting in an enhanced susceptibility for spontaneous abortion. Such a phenomenon would not be exceptional in Finland due to the population history. A small number of original founders, followed by isolation and rapid expansion in regional subisolates, have resulted in enrichment of some otherwise rare disease alleles and the absence of others. This has increased the local incidence of rare recessive disorders, and regional clustering of cases can still be observed in some Finnish diseases (Norio, 2003a,b). For example, mutations in the FSH receptor (*FSHR*) gene cause infertility due to ovarian failure. FSH-resistant ovaries (FSH-RO) is an autosomal recessive trait which shows geographical enrichment of ancestral birthplaces (Aittomäki *et al*., 1995, 1996).

One limitation of the study may be the number of couples studied. The aetiology of RSA is likely to be very heterogenous, and mutations in *AMN* may cause fetal loss in only a small subpopulation of couples with spontaneous abortions. Mutations in *AMN* seem to be rare, and therefore one may argue that more couples would be needed to determine

the exact role of these genes in RSA. Another limitation is the fact that some sequence variations in the studied samples may have gone undetected. Even though DHPLC has been shown to be a highly sensitive and specific method for mutation detection (sensitivity and specificity of DHPLC exceed 96%), there are also rare instances in which mutations may be missed (Xiao and Oefner, 2001). Mutations located in a high-melt GC-rich pocket in a fragment with otherwise normal nucleotide ratios may in some cases not be detected, and some variations can only be detected at one unique temperature.

In conclusion, the results of this study suggest that homozygous mutations in the *AMN* gene are not a major cause of RSA. However, the phenotypic effects of the potentially deleterious variations in homozygous or compound heterozygous fetuses cannot be determined without further investigations. The genealogic studies of our patients' ancestral birthplaces indicate that there may be a subset of patients with an underlying genetic cause for spontaneous abortions. Therefore, other candidate genes need to be considered. It is, however, particularly complicated to study conditions in which homozygous mutations are expected to be lethal in early pregnancy because of the need to obtain DNA from spontaneously aborted fetuses. Furthermore, there are no clinical features by which subgroups of affected pregnancies could be identified for further studies. While classical linkage studies cannot be performed, further candidate genes identified in animal models should be screened to identify new genetic factors contributing to spontaneous abortions.

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References

Aittomäki K, Lucena JL, Pakarinen P, Sistonen P, Tapanainen J, Gromoll J, Kaskikari R, Sankila EM, Lehväslaiho H, Engel AR *et al*. (1995) Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. Cell 82,959–968.

Aittomäki K, Herva R, Stenman UH, Juntunen K, Ylöstalo P, Hovatta O and de la Chapelle A (1996) Clinical features of primary ovarian failure caused by a point mutation in the follicle-stimulating hormone receptor gene. J Clin Endocrinol Metab 81,3722–3726.

Cartegni L, Wang J, Zhu Z, Zhang MQ and Krainer AR (2003) ESEfinder: a web resource to identify exonic splicing enhancers. Nucleic Acids Res 31,3568–3571.

Clifford K, Rai R, Watson H and Regan L (1994) An informative protocol for the investigation of recurrent miscarriage: preliminary experience of 500 consecutive cases. Hum Reprod 9,1328–1332.

Fyfe JC, Madsen M, Hojrup P, Christensen EI, Tanner SM, de la Chapelle A, He Q and Moestrup SK (2004) The functional cobalamin (vitamin B12)-intrinsic factor receptor is a novel complex of cubilin and amnionless. Blood 103,1573–1579.

Jeffery CJ (2003) Moonlighting proteins: old proteins learning new tricks. Trends Genet 19,415–417.

Jeffery CJ (2004) Molecular mechanisms for multitasking: recent crystal structures of moonlighting proteins. Curr Opin Struct Biol 14,663–668.

Kalantry S, Manning S, Haub O, Tomihara-Newberger C, Lee HG, Fangman J, Disteche CM, Manova K and Lacy E (2001) The amnionless gene, essential for mouse gastrulation, encodes a visceral-endoderm-specific protein with an extracellular cysteine-rich domain. Nat Genet 27,412–416.

Katz VL and Kuller JA (1994) Recurrent miscarriage. Am J Perinatol 11,386–397.

Krawczak M, Reiss J and Cooper DN (1992) The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. Hum Genet 90,41–54.

Nakai K and Sakamoto H (1994) Construction of a novel database containing aberrant splicing mutations of mammalian genes. Gene 141,171–177.

Norio R (2003a) Finnish Disease Heritage I: characteristics, causes, background. Hum Genet 112,441–456.

Norio R (2003b) Finnish Disease Heritage II: population prehistory and genetic roots of Finns. Hum Genet 112,457–469.

Plouffe L, White EW, Tho SP, Sweet CS, Layman LC, Whitman GF and McDonough PG (1992) Etiologic factors of recurrent abortion and subsequent reproductive performance of couples: Have we made any progress in the past 10 years? Am J Obstet Gynecol 167,313–321.

Stephenson MD, Awartani KA and Robinson WP (2002) Cytogenetic analysis of miscarriages from couples with recurrent miscarriage: a case-control study. Hum Reprod 17,446–451.

Strope S, Rivi R, Metzger T, Manova K and Lacy E (2004) Mouse amnionless, which is required for primitive streak assembly, mediates cell-surface localization and endocytic function of cubilin on visceral endoderm and kidney proximal tubules. Development 131,4787–4795.

Tanner SM, Aminoff M, Wright FA, Liyanarachchi S, Kuronen M, Saarinen A, Massika O, Mandel H, Broch H and de la Chapelle A (2003) Amnionless, essential for mouse gastrulation, is mutated in recessive hereditary megaloblastic anemia. Nat Genet 33,426–429.

Tomihara-Newberger C, Haub O, Lee HG, Soares V, Manova K and Lacy E (1998) The amn gene product is required in extraembryonic tissues for the generation of middle primitive streak derivatives. Dev Biol 204,34–54.

Tulppala M, Palosuo T, Ramsay T, Miettinen Salonen R and Ylikorkala O (1993) A prospective study of 63 couples with history of recurrent spontaneous abortion: contributing factors and outcome of subsequent pregnancies. Hum Reprod 8,764–770.

Wang X, Bornslaeger EA, Haub O, Tomihara-Newberger C, Lonberg N, Dinulos MB, Disteche CM, Copeland N, Gilbert DJ, Jenkins NA *et al*. (1996) A candidate gene for the amnionless gastrulation stage mouse mutation encodes a TRAF-related protein. Dev Biol 177,274–290.

Xiao W and Oefner PJ (2001) Denaturing high-performance liquid chromatography: a review. Hum Mutat 17,439–474.

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