The imprinted region on human chromosome 7q32 extends to the carboxypeptidase A gene cluster: an imprinted candidate for Silver-Russell syndrome

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Imprinted gene[s] on human chromosome 7q32-qter have been postulated to be involved in intrauterine growth restriction associated with Silver-Russell syndrome (SRS) as 7-10% of patients have mUPD(7). Three imprinted genes, MEST, MESTIT1, and COPG2IT1 on chromosome 7q32, are unlikely to cause SRS since epigenetic and sequence mutation analyses have not shown any changes. One hundred kilobases proximal to MEST lies a group of four carboxypeptidase A (CPA) genes. Since most imprinted genes are found in clusters, this study focuses on analysing these CPA genes for imprinting effects based on their proximity to an established imprinted domain. Firstly, a replication timing study across 7q32 showed that an extensive genomic region including the CPA genes, MEST, MESTIT1, and COPG2IT1 replicates asynchronously. Subsequently, SNP analysis by sequencing RT-PCR products of CPA1, CPA2, CPA4, and CPA5 indicated preferential expression of CPA4. Pyrosequencing was used as a quantitative approach, which confirmed predominantly preferential expression of the maternal allele and biallelic expression in brain. CPA5 expression levels were too low to allow reliable evaluation of allelic expression, while CPA1 and CPA2 both showed biallelic expression. CPA4 was the only gene from this family in which an imprinting effect was shown despite the location of this family of genes next to an imprinted cluster. As CPA4 has a potential role in cell proliferation and differentiation, two preferentially expressed copies in mUPD patients with SRS syndrome would result in excess expression and could alter the growth profiles of these subjects and give rise to intrauterine growth restriction.
The transcripts proximal to MEST and TSGA14 belong to the carboxypeptidase A (CPA) cluster of genes. The functions of the protein products of the carboxypeptidase gene family range from the digestion of food to the selective biosynthesis of neuroendocrine peptides. Metallo-CPAs are zinc containing exopeptidases, divided into either the N/E or A/B subfamilies based on the overall domain structure and amino acid sequence similarities. All members of the A/B subfamily contain about a 90 amino acid long N-terminal “pro” region that functions as a chaperone to assist with the folding of the active carboxypeptidase (CP) domain. Four members of the A/B subfamily, CPA1, CPA2, CPA4, and CPA5, were found to be clustered within an interval of 120 kb on human chromosome 7q32. CPA1 and CPA2 (together with CPA1 on chromosome 3q24) are referred to as pancreatic carboxypeptidases. Although CPA1 and CPA2 are expressed most highly in pancreas, expression of the genes in other tissues was also reported. CPA4 was identified as a gene upregulated in NaBu-treated PC-3 cells (an androgen independent prostate cancer cell line), suggesting the possibility that CPA4 protein may have functions in cell proliferation and differentiation. Although the enzymatic properties of CPA4 have not been reported, the deduced amino acid sequence encoded by the CPA4 gene shows a high degree of similarity to other CPA proteins. CPA5 was identified as a new member of the CPA gene cluster on 7q32. The gene has been shown to be strongly expressed in testis germ cells. The active site of CPA5 was predicted to cleave substrates with C-terminal hydrophobic residues, as do CPA1 and 2. The four CPA genes are only 100 kb proximal to the cluster of imprinted genes (MEST, MESTT1, and COPG2IT1) on 7q32.

As part of a systematic screening for additional imprinted genes in the vicinity of the MEST/COPG2 locus, we have examined the imprinting status of the four CPA genes.

MATERIALS AND METHODS
Northern blot analysis
Multiple tissue northern blots containing adult human poly (A)+ RNAs (2 μg/lane) were purchased from Clontech. The cDNA fragments used as probes for CPA4 and CPA5 correspond to nt 1440 to 1693 of GenBank acc NM_016352, and nt 1315 to 1420 of NM_080385, respectively. Probes were labelled with α-32P dCTP using random priming. Hybridisation and washing were performed following the protocols described by Sambrook and Russell.

Analysis of replication timing
Replication timing was analysed in S phase cells labelled by BrdU incorporation in a normal lymphoblastoid cell line, according to the methods described by Hitchins et al. Methods used for probe preparation and FISH were based on those described by Harper et al. and the protocols recommended by Vysis (Vysis, Richmond, UK). Minimums of 200 S phase nuclei were scored for one of three replication patterns: two singlets (single signals representing an unreplicated allele), a singlet plus a doublet (two signals less than two signal widths apart, representing a replicated allele), or two doublets. The number of single/doublet nuclei were represented as a percentage of the total number of nuclei counted. This reflects the percentage asynchrony of a given genomic region.

Fetal tissue and maternal DNA samples for imprinting analysis
Fetal tissues were acquired following termination of pregnancy at Queen Charlotte’s and Chelsea Hospital (QCCH), London. A paired maternal peripheral blood sample was obtained for DNA extraction at the same time. Informed consent was obtained for each sample set. Local ethics approval for obtaining fetal and maternal samples for the study of growth related disorders was granted by the Research Ethics Committee of the Royal Postgraduate Medical School (96/ 4955). Fetal pancreas tissue was obtained from the MRC Tissue Bank at Hammersmith Hospital. All tissues were washed in sterile PBS, snap frozen in liquid nitrogen, and stored at −70°C. Genomic DNA was extracted from the fetal placenta and maternal blood using the standard phenol-chloroform technique.

PCR amplification and sequence analysis
PCR for genotyping and RT-PCR for expression analysis were performed according to standard protocols in 25 μl reactions supplemented with 1.5 mol/l betaine to increase specificity. PCR and RT-PCR products were purified using microCLEAN Supersafe 10 reaction kit ( Quiagen). The purified products were sequenced directly using Big Dye terminator kit and resolved on a 3100 Genetic Analyser (Applied Biosystems).

Imprinting analysis in human fetal tissues
Imprinting analysis was carried out by single nucleotide polymorphism (SNP) analysis that requires heterozygous fetal genotypes to differentiate between expression from each of the parental genomes. A homozygous maternal genotype for the same SNP allows identification of the paternal allele since it is the only allele which could have been contributed by the mother. The other fetal allele is therefore paternal in origin. Analysis of fetal RNA by RT, RF-PCR, and DNA sequencing allows us to determine whether both or just one of these alleles is expressed.

CPA1
A C/T polymorphism in the third coding exon (nt173 of BC005279) was found by EST comparison (www.ncbi.nlm.nih.gov/SNP/snpblastByChr.html). Fetal DNA for which matched pancreatic cDNA was available was screened for informative heterozygotes by sequencing genomic PCR products (table 1) with the forward primer used for amplification. RF-PCR products were amplified for informative sample sets. Allele expression was evaluated by sequencing products with the forward RF-PCR primer.

CPA2
A C/T polymorphism for SNP imprinting analysis was identified in exon 7 (nt631 of U19977) by screening the DNA sequence of exons of a set of normal DNA samples. Fetal DNA for which matched pancreatic cDNA was available was screened for heterozygosity by sequencing genomic PCR products (table 1) with a nested forward primer (gtgtgagggtgaatgtacgta 5′-3′) and reverse PCR primer. RF-PCR products were amplified for informative sample sets. Allele expression was evaluated by sequencing with nested primer caagctacggactttcg (5′-3′).

CPA4
A C/T polymorphism in exon 9 (nt 914 of AF095719) was identified using the Celera database. Fetal DNA samples, for which matched maternal DNA and a range of fetal tissues were available, were screened for heterozygosity by sequencing genomic PCR products amplified from genomic DNA (table 1) using a nested forward primer (cacaacctgtctcgaagttg 5′-3′). RF-PCR products were amplified for informative sample sets in as many tissue types as were available. Allele expression was evaluated by sequencing with nested forward primer gaggtaagaaatggttag (5′-3′) and the reverse, RF-PCR primer.

CPA5
C/T and G/T SNPs were identified in exon 9 (nts1007 and 1014 of AF384667) using the Celera database and used to analyse isoform 1 of CPA5. Fetal DNA, for which matched maternal DNA and a range of fetal tissues were available, was screened for heterozygosity by sequencing genomic PCR
products (table 1) using the nested primer agtgaacttcatcacagccc (5′-3′). RT-PCR products were amplified for informative sample sets in as many tissue types as were available. Allele expression was evaluated by sequencing products with nested forward (agtgaacttcatcacagccc 5′-3′) and reverse (agtgaacttcatcacagccc 5′-3′) primers.
For isoform 3, a SNP was analysed which lies on exon 11 at nt1132 of AF384667 or nt 78 of the reverse complement of AI191876. Fetal DNA, for which matched maternal DNA and a range of fetal tissues were available, was screened for heterozygote informativity by sequencing genomic PCR products (table 1) using nested primer aagatgcttcgattctcgg (5-prime-3)’.

RT-PCR products were amplified for informative sample sets in as many tissues as were available. Allele expression was evaluated by sequencing these products with a nested reverse primer (cggaccatcatggagcacac 5-prime-3)’.

Expression analysis of CPA4 using the pyrosequencing system

Pyrosequencing was used as a quick and accurate method of genotyping fetal and maternal genomic DNA samples and subsequently to quantify allelic expression in RT-PCR products amplified from cDNA generated from fetal tissues of informative subjects. Amplification of fetal and maternal genomic DNA was carried out according to standard PCR protocols using caacccttgctccgaagtgt (5-prime-3)’. RT-PCR products were amplified for informative sample sets in as many tissues as were available. Allele expression was evaluated by sequencing these products with a nested reverse primer (cggaccatcatggagcacac 5-prime-3). The forward pyrosequencing primer aaacatgctgggatagacagtggt (5-prime-3)’ and the reverse sequencing primer caggtcgatgacggagc (5-prime-3)’ for genomic DNA was designed using SNP Primer Design from Pyrosequencing AB version 1.0.1. For RT-PCR 5’ biotinylated primer caacccttgctccgaagtgt (5-prime-3)’ and reverse primer caggtcgatgacggagc (5-prime-3)’ flanking exons 10 and 11 were used with a reverse sequencing primer caggtcgatgacggagc (5-prime-3)’. Pyrosequencing was carried out according to manufacturers standard protocols (PyroSequencing AB).

RESULTS

Localisation, genomic organisation, and expression of CPA genes

The four CPA genes, CPA1, CPA2, CPA4, and CPA5, are clustered in an interval of 120 kb on 7q32 (CP3 is not part of this cluster and has been localised to chromosome 3q24). The distance between the CPA genes and a cluster of imprinted genes (MEST, MESTITI1, and COPG2IT1) on chromosome 7 is approximately 100 kb (fig 1A).

Grail EXP was used to highlight potential CpG islands in the human genomic reference sequence AC024083 (RP11-190G13). No CpG islands were localised; however, two small regions were shown to have a slightly higher CpG dinucleotide content than the surrounding DNA (fig 1B, shown by grey circles). The first was located within intron 10 for CPA2 (102788-103093-PCT GC 54.83) and contained three HpaII (ccgg) and six HinfI (cggc) restriction sites. The second was located 650 bp upstream of the first exon CPA4 (109418-109648-PCT GC 51.47) and contains only two HpaII restriction sites. Both regions were subjected to RepeatMasker analysis, showing that these regions both align to SINE/Alu elements. The sequence from within intron 10 of CPA2 consists of two copies of SINE/Alu elements, whereas the second region represents only a single element. Preliminary methylation sensitive PCR showed that both regions are methylated in mUPD(7) and pUPD(7) lymphoblast DNA, as is expected for SINE/Alu elements.

CPA1 has 10 coding exons and the others have 11 coding exons; exon 3 in CPA1 (234 bp) corresponds to two exons (3 and 4) in the others (fig 1C). The introns 3 of CPA2, CPA4, and CPA5 have progressively larger introns containing more repeat elements than one another. CPA4 has a longer 3’UTR containing a repetitive sequence (Alu) (fig 1C). Interestingly, the 3’UTR of mouse Cpa4 is also longer and contains repetitive elements (data not shown). Otherwise, the four genes have similar exon-intron structures. All intron-exon boundaries of the four genes conform to the splicing donor/acceptor consensus sequences (GT/AG). Intron phases are completely conserved among the four genes for all exon-intron boundaries. The putative amino acid sequences encoded by the four genes have a similar domain structure: a signal peptide at the N-terminus, carboxypeptidase activation peptide (pfam00246), and zinc carboxypeptidase domain (pfam02244), and zinc carboxypeptidase domain (pfam00246) (fig 1C). CPA5 has a longer signal peptide than other members (fig 1C)’.

The similarity of amino acid sequences of two of the four proteins ranges from 51% to 68%.

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Figure 3  Sequence electropherograms showing SNP analysis of CPA1, CPA2, and CPA4. (A) Sequence electropherograms across the polymorphic site (shown by arrows) in exon 7 of CPA2 shown for heterozygous fetal and maternal DNA and corresponding cDNA from various tissues as labelled. Expression of both alleles shows biallelic expression for this in all tissues analysed. (B) Sequence electropherograms across the polymorphic site in exon 3 of CPA1 shown for heterozygous fetal DNA and corresponding cDNA from pancreas tissue showing expression of both parental alleles. (C) Pyrosequencing pyrograms for CPA4 of fetal and maternal DNA and cDNA of various tissues available for F71. Pyrosequencing of genomic DNA show informativity with a heterozygous fetus and homozygous maternal sample. The G allele is therefore maternal in origin. cDNA alleles are expressed as percentages A and C alleles since a reverse primer was used for this section of the analysis. Tolerance that a 30% or less contribution of one allele indicates preferential expression, brain is classified as biallelically expressed while eye, heart, intestine, kidney, and lung are preferentially expressed from the maternal allele for this fetus. PCR primers and conditions are shown in table 1.
and muscle (data not shown). CPA1 and CPA2 expression was evaluated by RT-PCR in fetal tissues. CPA1 was expressed exclusively in pancreatic tissue while CPA2 was ubiquitously expressed.

**Imprinting analysis of CPA genes in human fetal tissue**

Maternal and paternal alleles of imprinted genes replicate at slightly different rates. Generally, genes replicate on the homologous chromosomes in a synchronous manner with 10% of nuclei showing background asynchronous hybridisation patterns. In contrast, imprinted genes showed 25-40% asynchronous replication. This approach can therefore be used to assess imprinting across a large region. In a study by Bonora et al., asynchronous replication was shown for genomic clones containing the genes MEST and COPG2. In our study, replication timing was assessed using 11 PAC and BAC clones across a genomic region of approximately 1.5 Mb extending from NRF1 to beyond FRA7H. A PAC containing the cystic fibrosis transmembrane conductance regulator (CFTR) gene (PAC clone RPCI-1 22F22) was used as the synchronous control where, as expected, only 10% of nuclei showed the background level of asynchronous replication patterns. Each clone analysed representing a region centromeric to and including MEST displayed percentage asynchronies of 25% and above (a minimum of 200 nuclei were counted per probe). The PAC clone RPCI-3 502B1, spanning the fragile site FRA7H, showed 29.7% asynchronous replication. Apart from imprinted regions, fragile sites also replicate asynchronously, but not in a parental origin dependent way as is seen in imprinted regions. Replication timing of the BAC clone B277J23 and PAC clone 477N18 from RPCI-3, which lie between the imprinted asynchronous region and the fragile site, most likely corresponds to the telomeric end of the imprinted domain including MEST.

Allelic expression of individual CPA genes was analysed in fetal tissues by single nucleotide polymorphism (SNP) analysis. Fetal pancreas was the only tissue in which expression of CPA1 could be detected by RT-PCR. Three out of nine fetal DNA samples were heterozygous for the SNP to be analysed. Sequence analysis showed expression of both parental alleles (fig 3B). A C/T polymorphism in exon 7 was used to analyse CPA2 for imprinted expression. Four fetal DNA sample sets which included pancreas were tested and two were found to be heterozygous for the SNP. Both of these showed pancreatic expression of both parental alleles by RT-PCR and DNA sequencing. The same SNP was used to analyse allelic expression in sample sets with a wider range of fetal tissues available. Screening for heterozygotes in these fetal DNA samples showed five informative samples out of 22. Between two and five tissues were available per fetus with a total of seven samples showed two informative sample sets with a wider range of fetal tissues available. Screening for heterozygotes in these fetal DNA samples showed five informative samples out of 22. Between two and five tissues were available per fetus with a total of seven separate tissues (eye, brain, intestine, lung, placenta, tongue, stomach) between them. Analysis by RT-PCR followed by sequencing showed biallelic expression in all tissues (fig 3A).

For CPA4 imprinting was assessed for a SNP in exon 9. Preferential expression of one or other parental allele was seen in 75% of the tissue samples analysed (data not shown). Owing to concerns of the sequencing approach not being sufficiently quantitative to measure preferential expression accurately, this analysis was repeated using pyrosequencing as a means of quantifying the molecular contribution of each parental allele to expression in a particular sample (fig 3C). For the purposes of this study preferential expression is defined as a skewed percentage contribution of one allele of 30% versus 70% of the other. Pyrosequencing information was obtained for nine

imprinting has been shown for insulin-like growth factor II receptor gene (IGF2R) and the serotonin-2A (5-HT2A) receptor gene (HTR2A). Throughout these reported studies of unusual imprinting patterns, only isolated samples have been described where one allele is partially repressed resulting in preferential expression. In this study, however, CPA4 SNP analysis has shown a novel pattern of imprinting where the maternally inherited allele is consistently preferentially expressed. Since CPA4 has been suggested to have a role in controlling cell proliferation and differentiation, it may be that a finely controlled balance of expression is required for normal growth. If CPA4 were to act in limiting growth, then over-expression in a subject with mUPD(7) could have a growth restriction phenotype such as that seen in SRS. This is in agreement with the conflict theory where maternally expressed genes have a growth restricting function and paternally expressed ones act as growth stimulants.

For SNP analysis of CPA5, 48 cycles of RT-PCR was required to detect preferential expression of the two transcripts (isoforms 1 and 3) expressed in fetal tissues. This number of cycles of exponential amplification would theoretically result in $1.4 \times 10^{10}$ amplicons per original copy of template cDNA. According to Sambrook and Russell, 48 PCR cycles can amplify one target molecule to 10 ng of DNA (based on a 200 ng PCR product) when the amplification efficiency is 65-100%. Since in practice more than 10 ng of product would be required, the primer efficiency would have to be somewhat greater than 65%. It is therefore possible that these erratic imprinting results are the result of amplification of a single copy of gene specific cDNA simply because only a single copy was present in the template used for amplification and not as a result of allele specific expression. These results for CPA4 are therefore not likely to be a true reflection of imprinting expression and are simply an artefact of the limitations of the techniques used.

It is clear that the CPA gene family is differentially imprinted and the gene products have different biological functions. It is interesting, despite biallelic expression for CPA1 and CPA2, that CPA4 is maternally preferentially expressed in a broad spectrum of fetal tissues but not the brain. It is interesting to speculate that a double dose of a gene that is maternally expressed and important in growth and proliferation might cause growth restriction and may be in part accountable for the SRS phenotype. Mutation screening of CPA4's 11 exons is being carried out on DNA from a large cohort of SRS patients.

**ACKNOWLEDGEMENTS**

The first two authors contributed equally to this work.

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MEST/PEG1 region at 7q32.


The imprinted region on human chromosome 7q32 extends to the carboxypeptidase A gene cluster: an imprinted candidate for Silver-Russell syndrome

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Corneal dystrophies and degenerations: a molecular genetics approach


The corneal dystrophies represent a large and varied group of inherited conditions, and the underlying molecular basis of many has been elucidated over the past decade or so. This exciting progress has been rapid and now allows a re-evaluation of our clinical and morphological classifications. This monograph has been produced in association with the American Academy of Ophthalmology, and is published by Oxford University Press. The major body of the text comprises six chapters and a total of 123 pages. In addition, there is a short self-study examination directed towards US CME accreditation. As would be expected from such a collaboration, the production qualities are high. Dr Wang, the editor, is a clinical academic in Nashville with major interests in the cornea, external eye disease, and refractive surgery. He lists the book’s educational objectives: to bridge the gap between the new molecular information and the knowledge base for today’s ophthalmologists; to discuss current understanding of the molecular pathogenesis of these conditions; to outline the use of excimer laser for the treatment of corneal diseases; and to review the most recent literature on corneal dystrophies and degenerations. This monograph, and its two first objectives in particular, are therefore timely in their conception.

The editor is co-author of five of the six chapters. The first chapter, written in collaboration with Dr Francis Munier, discusses the inheritance patterns of the corneal dystrophies in particular covering the range of epithelial and stromal dystrophies caused by defects in TGFBI/BIGH3, including a detailed and well-constructed examination of their molecular pathology. The following three chapters describe, respectively, the epithelial, stromal, and endothelial dystrophies. In general the clinical, histopathological, and ultrastructural features of the disorders are clearly described, illustrated, and referenced. Here, the molecular focus is generally on gene identification and, whereas for certain disorders—for example, Meesmann epithelial dystrophy—there is a clear description of the underlying molecular mechanisms, this is disappointingly covered for many conditions. The final two chapters, concerning corneal and conjunctival degenerations and excimer laser therapies for corneal dystrophies, are likely to be of limited interest to the geneticist and carry little molecular information.

The key difficulty, when producing a monograph such as this, is ensuring that what is produced is as recent as possible and is not simply a replication of information that is available in other ophthalmological texts. In the first regard, the book unfortunately appears to have taken a disappointingly long time from completion to publication. However, the degree of illustration—all figures are included on the excellent CD ROM that accompanies the book—when allied to the molecular details ensures that the phenotypic descriptions of the dystrophies are covered in a manner that will be both familiar and useful for ophthalmic clinicians and trainees alike.

Conflicts of interest: none declared

G C M Black

Nucleotide and protein expansions and human disease


Since 1991, when the CAG repeat expansion causing spinobulbar muscular dystrophy and the CCG repeat expansion in fragile X syndrome were discovered, there has been great progress in understanding the biology of triplet repeat instability and the diseases associated with these types of mutation. The number of diseases and classes of mutations has grown such that there are currently nine CAG repeat diseases where the repeats are translated into polyglutamine tracts, a recessive triplet mutation (Friedreich’s ataxia), more than a handful of different diseases caused by expanded polyalanine tracts (for example, oculopharyngeal muscular dystrophy), diseases associated with untranslated triplet repeats (for example, myotonic dystrophy), and diseases caused by expansion of other micro or minisatellites (for example, progressive myoclonus epilepsy).

Soon after the first group of triplet repeat mutations was discovered, the biological mechanism was revealed behind the previously baffling (and controversial) phenomenon of anticipation, where the disease tends to increase in severity or present at an earlier age in successive generations in families. In many cases there has been rapid progress in developing cell and animal models of disease, and in some cases we have a much better understanding of pathogenesis. Yet many mysteries and controversies remain, even for diseases where the mutation was identified a decade or so ago (for example, myotonic dystrophy and Huntington’s disease) and cures and treatments for the human diseases are still elusive.

This book addresses certain aspects of the field with a collection of freestanding articles covering aspects ranging from trinucleotide repeat instability, through epidemiology of spinocerebellar ataxias, to pathogenetic mechanisms. The nature of the book means that there are a number of places that are duplicated between chapters. This is not a problem, as the book is probably not designed to be read from cover to cover, but rather as a reference source. The topical coverage is not complete and gaps include discussions of the pathogenesis of Friedreich’s ataxia and spinocerebellar ataxia type 1 (both where considerable progress has been made). However, in contrast to many other books where there are collections of chapters, for instance focused on neurological diseases, this book deals with some of the most fascinating very well. There are fine chapters on repeat instability (by Lenzmeier and Freundenreich; and by Cleary and Pearson), oculopharyngeal muscular dystrophy (Brais and on transgenic models of myotonic dystrophy (Wansink and Wieringa), spinobulbar muscular dystrophy (Sobue and colleagues), Huntington’s disease (Hickey and Cheesete) and Fragile X syndrome (Bakker and Oostra).

It was good to see a chapter devoted to the interesting and possibly under recognised cerebellar ataxia syndrome associated with FRAXA premutation carriers (Hagerman et al) and the excellent chapter on SCAS by Kobayashi and Kakunuma (a group that have made a number of key contributions to the polyglutamine disease field). In general, the chapters are written by authorities in their fields (including Brice, Ashizawa, Margolis, La Spada, Nelson, Usdin, and Ranum).

In conclusion, this book includes many chapters that add to and complement existing texts dealing with these diseases. The articles are generally of a high standard and are concisely written. This book would be of particular value to human geneticists, genetic counsellors, and researchers working on this class of diseases.

D C Rubinstein

CORRECTIONS

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The acknowledgements for the original article by Bentley et al (J Med Genet 2003;40:249–56) were omitted and should read:

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We apologise for this error.

doi: 10.1136/jmg.2004.016089corr1

Resniovitis T, Griffiths P G, Birch M, et al. Primary open angle glaucoma is strongly associated with a specific p53 gene haplotype (J Med Genet 2004;41:296–8). An error has been detected in the second paragraph of the Key points box. In the penultimate sentence “arginine residues” and “line residue” should have been transposed. The authors apologise for this error.