Electronic letter

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Cytosine methylation confers instability on the cardiac troponin T gene in hypertrophic cardiomyopathy

EDITOR—Hypertrophic cardiomyopathy (HCM) is an inherited disease (MIM 192600, 115195) of the heart muscle, characterised by unexplained left ventricular hypertrophy. HCM is also one of the major causes of sudden cardiac death,[1] sometimes occurring in young asymptomatic people.[2–4] Although sporadic forms do rarely occur,[5] generally HCM has an autosomal dominant pattern of inheritance caused by mutations of the genes coding for proteins of the cardiac sarcomere. Subjects with HCM caused by mutations in the cardiac troponin T (*cTNT*) gene have been clinically shown to be at increased risk of sudden death,[6] which may occur even in the absence of marked morphological abnormalities.[7] Since incomplete penetrance of the clinical phenotype, measured by ECG and echocardiographic parameters, is one of the hallmarks of "troponin" disease, the identification of *cTNT* mutation in probands would facilitate identification of "at risk" relatives who may not fulfil clinical diagnostic criteria.

In the course of a study undertaken to characterise the *cTNT* mutation profile in HCM patients, we identified a cluster of mutations in exons 8 and 9. Five out of the 11 mutations published to date in this gene have been found in exons 8[8] and 9.[7–10] We report here a novel Arg94Cys de novo mutation in a female patient presenting with HCM bringing the total of *cTNT* mutations to 12.

Four of the mutations found in exon 9, Arg92Trp, Arg92Gln, Arg94Cys, and Ala104Val, involve C→T transitions (or G→A transitions in the opposite strand) within CpG dinucleotides. Approximately 70% of the cytosines within CpG dinucleotides in the mammalian genome contain highly mutable 5-methyl-cytosine (5mC) residues. These residues are not randomly distributed and the majority of the genome is CpG depleted.[11] Although some CpG dinucleotides are found within coding regions, most CpG residues are in CpG islands, at the 5' end of genes, upstream of the transcription start site and usually in the promoter region.[12 13] Cytosines within CpG islands, however, are not normally methylated and it should also be noted that methylated cytosines can occur in other dinucleotides.[14–16]

Both cytosine and 5mC spontaneously deaminate in single and double stranded DNA to form uracil and thymine, respectively.[17] However, unlike G:U mismatches, which are repaired by uracil deglycosylase, G:T mismatches, resulting from deamination of 5mC, involve different, less efficient mechanisms of repair.[18] It is estimated that 30-40% of point mutations which occur within the genome are a result of C→T transitions (or a corresponding G→A transition in the opposite strand) within CpG dinucleotides. Since cytosine methylation could account for the high rate of such transition mutations observed in *cTNT*, we investigated the methylation profile of exons 8 and 9 of this gene with the aim of determining regions of genetic instability.

Patients attending a HCM clinic in a tertiary referral centre (St George's Hospital, London), who fulfilled established clinical, electrocardiographic, and echocardiographic criteria for HCM,[19] were included in this study.

Table 1 PCR primers to amplify exons 8 and 9 following bisulphite conversion of genomic DNA

Primer name	*Primer sequence (5' → 3')*
First round degenerate primers	
8-9Fa	agt ttt tgg gt**t** t**a**g aat ggg g
8-9Fb	agt ttt tgg gt**t** c**a**g aat ggg g
8-9Fc	agt ttt tgg gt**c** t**a**g aat ggg g
8-9Fd	agt ttt tgg gt**c** c**a**g aat ggg g
8-9Ra	gaa tat taa ata aac aaa ct**a** a**a**c acc tac
8-9Rb	gaa tat taa ata aac aaa ct**g** a**a**c acc tac
8-9Rc	gaa tat taa ata aac aaa ct**a** g**a**c acc tac
8-9Rd	gaa tat taa ata aac aaa ct**g** g**a**c acc tac
2nd round inner nested primers	
8-9innerF	gcg gaa ttc ttg atg ttg att att ttt ttt ttt aat agg t
8-9innerR	cgc gcg aat tcc gcg atc cta tct tta aaa aaa ac

The research was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association with informed consent from patients and previous approval from local ethical committees. Relatives who fulfilled recently proposed diagnostic criteria for disease within affected families[19] were also included.

Genomic DNA was extracted from peripheral blood using the Nucleon BACC kit (Amersham Life Sciences). Exons 8 and 9 of *cTNT* were amplified together as a single PCR fragment using primers LAPAN2F (5'cgg ggc agg gct gga aga tt 3') and 9REV (5'atg tta ggt ggg cag act 3'). PCR amplification with *Taq* polymerase (Qiagen) and 1.5 mmol/l $MgCl_2$ was carried out using a step down protocol with an initial denaturation at 95°C for four minutes, two cycles of 95°C for one minute, 63°C for one minute, and 72°C for two minutes, and two cycles of 95°C for one minute, 60°C for one minute, and 72°C for two minutes, followed by 28 cycles of 95°C for one minute, 58°C for one minute, and 72°C for two minutes. A final extension step of 72°C for 10 minutes was carried out at the end of the amplification. PCR products were then purified using the GFX PCR purification kit (Amersham-Pharmacia Biotech) and sequenced using the Thermo Sequenase kit (Amersham Life Sciences) with an IRD800 (MWG-Biotech) labelled LAPAN2F primer on the LICOR 4000L (MWG Biotech) automated DNA sequencer.

DNA fingerprinting for confirmation of paternity in the case of the novel de novo *cTNT* mutation (Arg94Cys) was carried out by Southern analysis using the minisatellite fingerprint probe FP15 (a generous gift from A J Jeffreys). Further confirmations were carried out by microsatellite analysis using markers IVS 17BTA in the *CFTR* gene and DXS1684.

Sodium bisulphite treatment of genomic DNA, for determination of methylated sites, was carried out as previously described by Clark *et al*[20] with some modifications. Briefly, 10 µg of genomic DNA from healthy subjects were denatured with 3 mol/l NaOH, ethanol precipitated, and resuspended in 50 µl of sterile DEPC treated water. Then 208 µl of solution A (6.24 mol/l urea (BDH) and 3 mol/l sodium metabisulphite (Sigma), pH 7.0) and 12 µl of a freshly made solution of 10 mmol/l hydroquinone (Sigma) were added to the DNA in a microcapped tube wrapped in foil. The addition of urea to the sample as previously described[21] ensured complete and reproducible conversion of the DNA. The reaction was allowed to proceed at 55°C for 24 hours. DNA was recovered by absorption to a silica-gel matrix (QIAEXII kit, Qiagen) and eluted with 50 µl of sterile H_2O. NaOH, at a final concentration of 0.5 mol/l, was added to the eluted DNA and incubated for

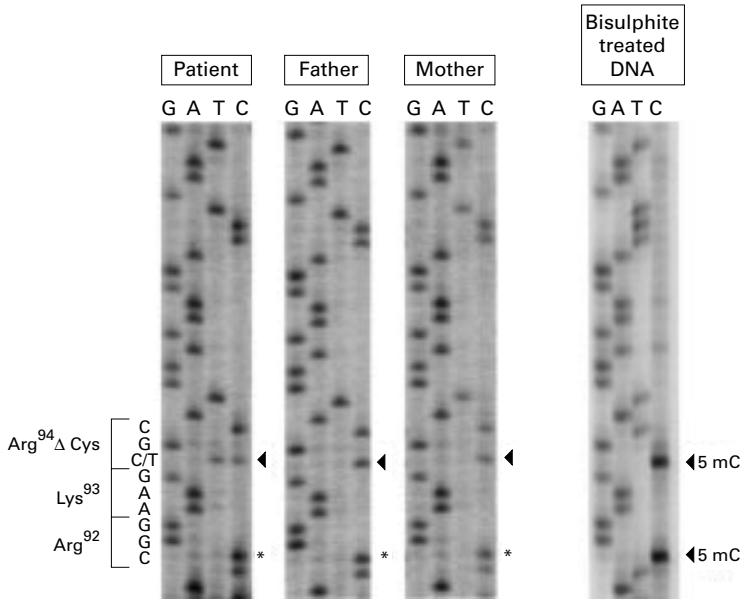

*Figure 1 3.75% acrylamide sequencing gel, showing the Arg94Cys de novo mutation (both parents are negative for the mutation). The cytosine residues at Arg94 and Arg92 (marked with *) are methylated as shown in the sequencing of normal wild type bisulphite treated DNA.*

10 minutes at 55°C. The DNA was neutralised with 50 µl of sodium acetate (pH 5.0), ethanol precipitated, and resuspended in 40 µl of sterile DEPC treated H_2O.

Bisulphite treated DNA was subjected to PCR using degenerate primers (table 1) designed using the Primer Premier 4.0 software (Premier Biosoft, CA) to amplify exons 8 and 9 together. The primers annealed to a region poor in cytosine residues and hence were less affected by the bisulphite conversion step. The degeneracy only affected the 10th and 11th bases from the 3' end, leaving a significant length of nucleotides to confer the 3' end stability needed for the annealing step in PCR reactions. Four sets of reactions, each containing an individual forward primer (either 8-9Fa, 8-9Fb, 8-9Fc, or 8-9Fd) and four of the degenerate reverse primers, were set up. Step down PCR amplification with *Taq* polymerase (Qiagen), 2.5 mmol/l $MgCl_2$ was carried out with initial denaturation at 95°C for four minutes, two cycles of 95°C for one minute, 60°C for one minute, and 72°C for two minutes, two cycles of 95°C for one minute, 58°C for one minute, and 72°C for two minutes, followed by 30 cycles of 95°C for one minute, 56°C for one minute, and 72°C for two minutes. A final extension step of 72°C for 10 minutes was carried out at the end of the amplification.

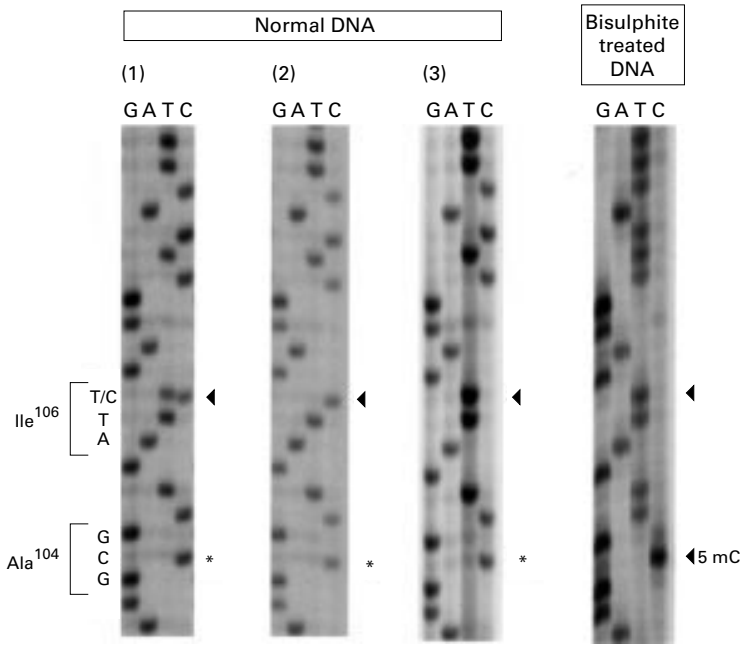

*Figure 2 3.75% acrylamide sequencing gel showing the Ile106 polymorphism (marked with arrowhead) occurring in three genotypically distinct subjects. The cytosine methylation pattern was not determined for codon 106_{Ile} since the test samples for the bisulphite sequencing experiment had the att/att genotype. The cytosine residue at codon 104_{Ala} where a mutation had been described previously (marked with *) is methylated.*

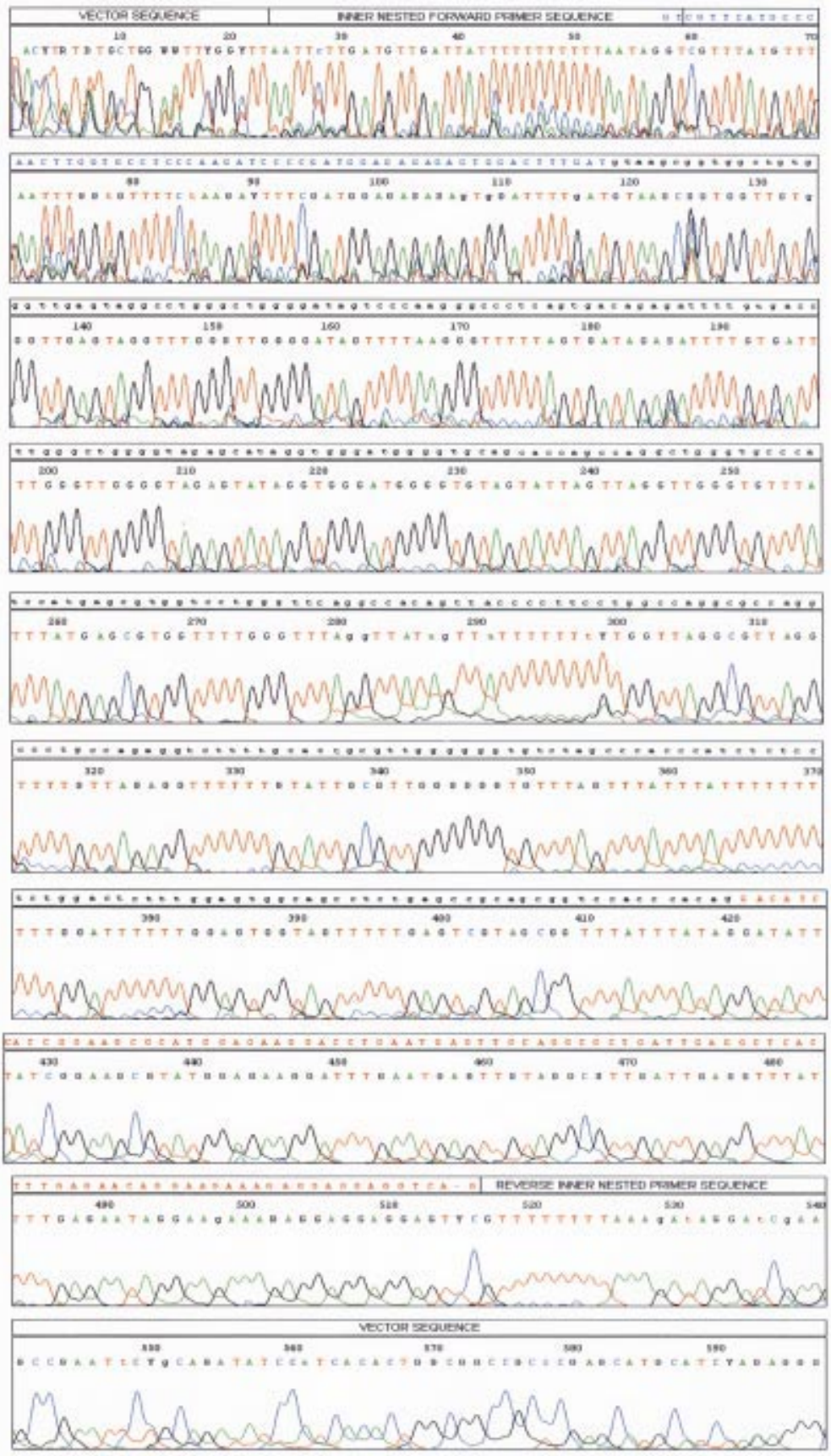

Figure 3 Cytosine methylation profile of exons 8 and 9 and intron 8 of cTNT shown by the bisulphite sequencing reaction which converts all non-methylated cytosines to thymines, hence the remaining cytosine peaks (in blue) are the 5mC residues. The 5' and 3' ends indicate the pCR2.1 vector sequence (Invitrogen) into which the amplified fragment of the bisulphite treated DNA was cloned. The normal DNA coding is boxed above the raw sequence data; exon 8 coding in blue upper case, intron 8 in black lower case, and exon 9 in upper case red letters.

A total of 2 µl of the products from the first round of PCR amplification were used in an inner nested reaction using the primers 8-9 inner F and 8-9 inner R (table 1). These primers were also designed to anneal within regions of the gene poor in cytosine residues. Amplification was carried out using *Taq* polymerase (Qiagen), 1.5 mmol/l $MgCl_2$ with an initial denaturation at 95°C for four minutes, followed by 32 cycles of 95°C for one minute, 56°C for one minute, 72°C for two minutes, and a final extension step of 72°C for 10 minutes. PCR products were analysed by agarose gel electrophoresis to confirm the presence of the amplimers. The inner nested PCR amplimers were purified (GFX PCR purification kit, Amersham Pharmacia Biotech) and eluted in 50 µl of autoclaved H_20.

The nested amplimers were then ligated into the pCR2.1, TA cloning vector (Invitrogen). Competent NM522 *E coli* cells were transformed with 2 µl of the ligation mix and plated on 2X Ty plates containing 50 µg/ml of ampicillin, 40 µg/ml of IPTG, and 50 µg/ml of *Xga*I. After an overnight incubation at 37°C, the plasmids were harvested from the white bacterial colonies and checked for the presence of the correct insert by agarose gel electrophoresis following digestion with *Bst*XI (MBI, Fermentas). Plasmid constructs containing the correct inserts were purified using a plasmid purification kit (Qiagen). Automated sequencing (LICOR 4000L) of the plasmid constructs was carried out using IRD800 labelled (MG Biotech) primers, M13rev: 5'-cag gaa aca gct atg acc-3', and M13for: 5'-tgt aaa acg acg gcc agt-3', and the Thermo Sequenase kit (Amersham Pharmacia Biotech).

A novel de novo Arg94Cys mutation resulting from a single base substitution at a CpG site, C280T (nucleotide numbering according to published cDNA sequences for *cTNT*[22]), was found in exon 9 of the *cTNT* gene of a female patient with 17 mm septal hypertrophy (adjusted normal range on echocardiography 7-11 mm). This patient presented at the age of 15 with recurrent syncope, progressive fatigue, and dyspnoea on exertion. ECG, echocardiography, and cardiac catheterisation confirmed a diagnosis of hypertrophic cardiomyopathy. Her parents had normal ECG and echo and were both negative for the mutation (fig 1). DNA fingerprinting studies confirmed paternity and hence this is a novel sporadic mutation. We did not detect the Arg94Cys mutation in 120 healthy, unrelated subjects from a randomly selected multiethnic population, which is strong evidence that this mutation is not a polymorphism.

Direct sequencing of exons 8 and 9 of the *cTNT* gene from PCR amplimers was carried out on 200 unrelated referral patients with HCM. All HCM probands and samples from healthy donors indicated a polymorphism (genotypes atc/atc, atc/att, or att/att) in exon 9, resulting in a silent mutation (Ile106). This polymorphism (fig 2), involving a C→T transition, occurs within a CpG site with a gene frequency for the atc allele of 0.44.

The complete methylation profile of cytosines between exons 8 and 9 of *cTNT* are presented in fig 3. Bisulphite treatment of lymphocyte genomic DNA from two healthy volunteers yielded consistent and reproducible results when clones generated from each degenerate primer were sequenced. The sodium bisulphite reaction converts non-methylated cytosines to uracil which pair with adenine, and upon subsequent replication produce the observed C→T transition. 5mC, however, are protected and hence remain in the "C" lane of a sequencing gel. Sequencing results showed that all cytosines within CpG dinucleotides of exons 8 and 9, including those in intron 8, were methylated.

Exon 8 contains 15 cytosine residues of which two (corresponding to codons 69_{Ser} and 80_{Pro}) are within CpG

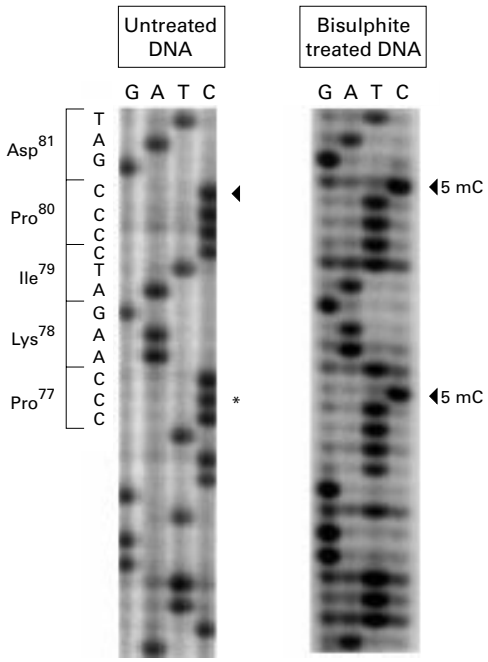

Figure 4 3.75% acrylamide sequencing gel showing that the third cytosine base (marked with arrowhead) in Pro80 is methylated. The middle cytosine in codon 77_{Pro} is a non-CpG methylated cytosine residue.

dinucleotides. Sequencing data as shown in fig 3 (chromatogram base number 60 and 94) proves that both of these cytosines are methylated. In addition, the cytosine residue within codon 77_{Pro} is methylated despite not being adjacent to a guanine residue (fig 4). This was a reproducible finding in all the clones analysed independently.

Of the 23 cytosines in exon 9, five are within CpG dinucleotides. Bisulphite sequencing has shown that the cytosines within codons 92_{Arg}, 94_{Arg}, 104_{Val}, and 120_{Leu} are methylated (fig 3). The methylation status of the cytosine in codon 106_{Ile} was not determined as the site shows the act/att polymorphism and both subjects studied were of the att/att genotype.

A common denominator linking the Arg94Cys mutation (fig 1) described in this paper and the previously published Arg92Trp,[7] Arg92Gln,[8] Ala104Val,[9] and the polymorphism at Ile106 (fig 2) is that they all involve transition mutations within CpG dinucleotides. Spontaneous deamination of 5mC residues leading to C→T transitions might have contributed to the initial founder mutation event. The Arg92Trp,[7] Arg94Cys, and the Ala104Val[9] mutations probably arose from C→T transition at the sense strand, while the Arg92Gln mutation[8] may have resulted from a C→T transition at the methylated CpG dinucleotide in the antisense strand (fig 5A). The cytosine methylation status of these residues confirm their predisposing mutability.

It is thus possible to predict potentially mutable sites within exons 8 and 9 of *cTNT*. Deamination of 5mC (predictive results summarised in fig 5A) resulting in silent mutations in the sense strand occur at positions 80_{pro}, 120_{Leu}, and 127_{Ile}, whereas non-coding strand silent mutations occur in codons 69_{Ser} and 104_{Ala}. If, however, a transition mutation occurs in the antisense 5mC nucleotide (complementary to the guanine of codon 81), a corresponding gat→aat change would occur in the sense strand, which might result in a Asp81Asn mutation in exon 8 of *cTNT* (fig 5A).

Relating to the same strand, a transition mutation in codon 69_{Ser} (tcg→ttg) would give rise to a Ser69Leu muta-

tion (fig 5A). It is interesting to note the amino acid corresponding to the serine in codon 69_{Ser} in humans is actually a leucine molecule in rat and mouse (fig 5B), so there is a possibility that such a substitution, if occurring within the human *cTNT* gene, would be reasonably tolerated and not compromise the function of the molecule in its role within the thin filament. In other species, the residues corresponding to Ser69 in humans have been substituted for a proline (fig 5B).

Deamination of the symmetrical 5mC residue in the antisense strand at codon 94 would give rise to an Arg94His mutation (resulting from the cgc→cac nucleotide change). Codon 120_{Leu} in exon 9 of *cTNT* is methylated (fig 3, base 516 in chromatogram) and a C →T transition in the antisense strand would result in a G→A transition in the sense codon 121, giving rise to a Val121Ile mutation (fig 5A).

Alignment of amino acids encoded by exons 8 and 9 of the human *cTNT* gene with other species shows strong homology (fig 5B). The arginine residues at codon 92_{Arg} and codon 94_{Arg} are highly conserved across species (fig 5B). It is probable that through the course of evolution, the stretch of amino acids shown in fig 5B has developed a crucial role within the contractile apparatus of the cardiac sarcomere, and mutations here could compromise the function of the molecule. Exons 8 and 9 are believed to code for the region in *cTNT* which interacts with α-tropomyosin[6 23–25] and is therefore critical for normal function.

Although *cTNT* methylation patterns in the germ cells have not been determined in our study, it seems likely that

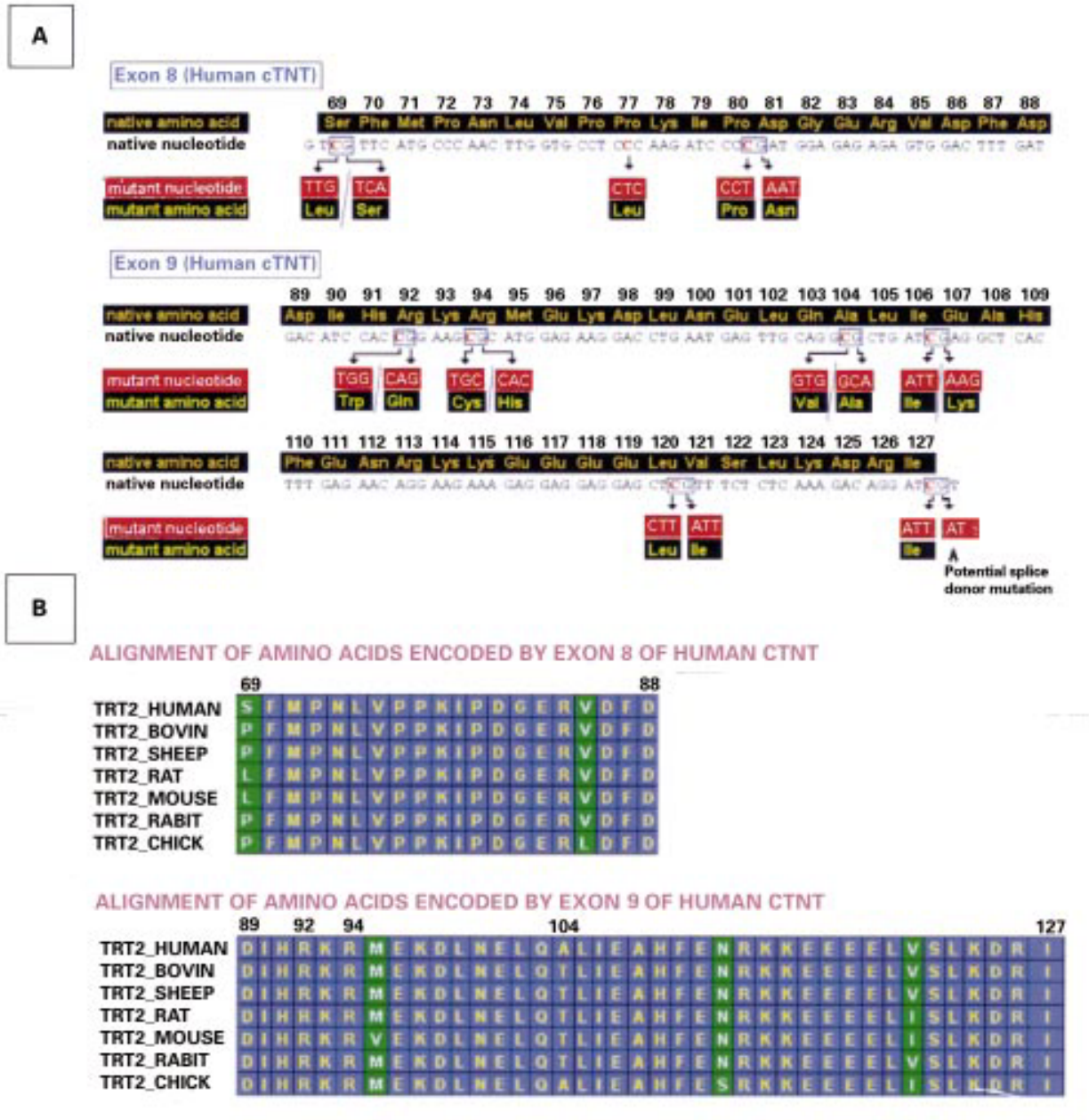

Figure 5 The DNA and amino acid coding of exons 8 and 9 of cTNT. (A) The complete list of possible mutations which can arise from C→T transitions (or a corresponding G→ A substitution in the opposite strand) resulting from deamination of cytosine residues. The CpG dinucleotides are boxed. (B) The amino acid sequence of exons 8 and 9 of human cTNT is conserved across species. Non-homologous amino acids are boxed with residues showing low homology with the human sequence in green. The Arg94 residue (site where the de novo Arg94Cys mutation is described in this paper) shows strong conservation across species.

the lymphocyte methylation profiles described here will be similar in germ cells, particularly since in exons 8 and 9 CpG methylation was found to be 100%. Any male bias in de novo mutation rate probably relates to the markedly high (17×) number of cell cycles in male gametogenesis rather than differential methylation.[26] Factor VII and *FGFR3* genes were both equally highly methylated in human oocytes and spermatocytes, indicating high levels of methylating potential.

We have outlined the regions of genetic instability in this area conferred by potentially mutable 5mC nucleotides and in fact all cytosine residues within CpG dinucleotides found in exons 8, 9, and intron 8 were found to be methylated. In addition, we also noted that the middle cytosine of codon 77_{Pro} was methylated despite not being adjacent to a guanine, indicating that cytosine methylation is not always confined to within CpG. Should a C→T transition occur in this site, it would result in a Pro77Leu (fig 5A). A mutation at this site has not yet been reported.

Although the methylation of cytosine residues in vertebrate DNA seems necessary for normal embryonic development,[12 27] this phenomenon increases the predisposition for mutations to occur within the genome. It has been estimated that a cytosine when methylated at a CpG site increases the possibility for a C→T (or a corresponding G→A) transition by a factor of 12.[28] This supports the observation that approximately one third of all point mutations reported in humans occur at CpG dinucleotides.[29]

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