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A recurrent intragenic genomic duplication, other novel mutations in *NLRP7* and imprinting defects in recurrent biparental hydatidiform moles

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A complete hydatidiform mole (CHM) is an abnormal pregnancy with hyperproliferative vesicular trophoblast and no fetal development. Most CHM are sporadic and androgenetic, but recurrent HM have biparental inheritance (BiHM) with disrupted DNA methylation at differentially methylated regions (DMRs) of imprinted loci. Some women with recurrent BiHM have mutations in the *NLRP7* gene on chromosome 19q13.42. Using bisulfite genomic sequencing at eight imprinted DMRs on DNA from two BiHMs, we found a pattern of failure to acquire or maintain DNA methylation at DMRs (*PEG3*, *SNRPN*, *KCNQ1OT1*, *GNAS exon 1A*) that normally acquire CpG methylation during oogenesis, but not at *H19*, which acquires CpG methylation during spermatogenesis. Secondary imprints at the *GNAS* locus showed variable abnormal patterns with both gain and loss of CpG methylation. We found novel missense and splice-site mutations in *NLRP7* in women with non-familial recurrent BiHM. We identified and characterized a homozygous intragenic tandem duplication including exons 2 through 5 of *NLRP7* that results in a predicted truncated protein in affected women of three unrelated Egyptian kindreds, suggesting a founder effect. Our findings firmly establish that *NLRP7* mutations are a major cause of BiHM and confirm presence of a complex pattern of imprinting abnormalities in BiHM tissues.

Keywords: biparental; hydatidiform mole; imprinting; mutation; *NLRP7*

Introduction

Hydatidiform moles are abnormally developing pregnancies with hyperproliferative vesicular trophoblast and defective fetal development. Sporadic HM are classified into complete hydatidiform mole (CHM) or partial hydatidiform mole (PHM). Sporadic CHM have a 46,XX or 46,XY karyotype but are androgenetic concepti in which all the genetic material is paternally derived (AnCHM), whereas PHM have a diandric triploid genome, with three copies of each chromosome, two of which are paternally inherited (Van den Veyver and Al-Hussaini, 2006). A rare class of highly recurrent hydatidiform moles is pathologically indistinguishable from AnCHM or PHM (Moglabey *et al*., 1999; Al-Hussaini *et al*., 2003; Panichkul *et al*., 2005). They are however not androgenetic or diandric but have normal diploid biparental inheritance (BiHM) (Helwani *et al*., 1999; Fisher *et al*., 2002; Judson *et al*., 2002; Hodges *et al*., 2003). DNA methylation analysis of differentially methylated regions (DMRs) at imprinted loci (Judson *et al*., 2002; El-Maarri *et al*., 2003) and gene expression studies (Fisher *et al*., 2002) showed generalized defective reprogramming of imprinting in BiHM tissues. Absent methylation of imprinted DMRs that normally acquire DNA methylation on the maternally inherited allele in BiHM tissues suggests a defect in establishment or maintenance, rather than in erasure of maternal imprints (Fisher *et al*., 2002; Judson *et al*., 2002; El-Maarri *et al*., 2003). One study concluded that the primary defect lies in failure to acquire or maintain DNA methylation marks in the maternal germline during oogenesis because DMRs that normally acquire a primary DNA methylation imprint on the paternally inherited allele show normal patterns (Judson *et al*., 2002). A second study also found gain of paternal-specific DNA methylation on the maternally inherited allele of loci, such as the *H19* DMR, that normally remain unmethylated on this allele (El-Maarri *et al*., 2003). Hence, the nature of the imprinting defect in BiHM tissues appears complex and is still incompletely understood.

Linkage to chromosome 19q13.42 (Moglabey *et al*., 1999; Sensi *et al*., 2000; Hodges *et al*., 2003; Panichkul *et al*., 2005) and autosomal recessive mutations in the NALP-like receptor (*NLR*) family, pyrin domain containing 7 genes (*NLRP7*, formerly called *NALP7*) on chromosome 19q13.42 have been found in a small number (Murdoch *et al*., 2006; Qian *et al*., 2007), but not all (Judson *et al*., 2002; Slim *et al*., 2005; Zhao *et al*., 2006) affected women, supporting genetic heterogeneity. *NLRP7* encodes a member of the *NLRP*-family of CATERPILLER proteins that has not been extensively studied yet,

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but similar to other *NLRP*s, may play a role in innate immunity (Kinoshita *et al*., 2005; Drenth and van der Meer, 2006; Ting *et al*., 2006). Whether it has a direct role in the establishment of imprinting marks during oogenesis or in their maintenance in the early embryo is currently unknown and it has been proposed that the imprinting abnormalities seen in BiHM are secondary to a more generalized immune-driven disruption in oogenesis or early embryo implantation (El-Maarri and Slim, 2006; Murdoch *et al*., 2006). Nevertheless, the methylation defects are very specific for imprinted loci, and DNA methylation at non-imprinted genes, as well as allelic methylation at genes subject to X-inactivation, is intact (Djuric *et al*., 2006). Affected women themselves also have normal DNA methylation patterns at imprinted loci (El-Maarri *et al*., 2005).

We report novel missense mutations, splice-site mutations and a genomic duplication in *NLRP7* that results in a truncated protein in women with recurrent BiHM. We also find, in two BiHM tissues, deficient CpG methylation at imprinted DMRs that normally acquire a primary CpG methylation imprinting mark on the maternal allele during oogenesis, but normal haplomethylated patterns at the *H19* DMR, which acquires its DNA-methylation imprinting mark on the paternally inherited allele during spermatogenesis. Our data at secondary DNA-methylation imprints at the complex imprinted *GNAS* locus show differences with two previous studies (Judson *et al*., 2002; El-Maarri *et al*., 2003). This supports that autosomal recessive *NLRP7* mutations in women lead to complicated patterns of abnormal imprinting in trophoblast tissues of their BiHM pregnancies that likely originate both in oogenesis and post-zygotically.

Materials and methods

Subjects and samples

Subjects were from two previously described Egyptian families (RHM 1 and 2) with recurrent BiHM (Al-Hussaini *et al*., 2003; Panichkul *et al*., 2005), but additional affected and unaffected individuals have been included since our prior reports (Al-Hussaini *et al*., 2003; Panichkul *et al*., 2005). HM42 and HM43 are sisters from a new Caucasian family RHM3. Updated pedigrees are in the online Supplementary Fig. S1. Six other single affected women, HM19 (Pakistani), HM33 (Egyptian), HM24, HM70 and HM73 (all Mexican Latino) were also included. Some of these have been previously reported (Panichkul *et al*., 2005). Written informed consent was obtained from all subjects with BiHM, participating family members and control subjects (with normal term pregnancies or sporadic AnCHM) prior to collection of clinical information, venous blood samples, molar pregnancy tissue and control term placenta (TP) samples according to a protocol approved by the Baylor College of Medicine Institutional Review Board for Human Subject Research. Epstein Barr-virus transformed lymphoblastoid cell lines (LCL) were established from a portion of the venous blood samples. DNA was extracted from peripheral blood leukocytes (PBL) and LCL using the PureGene purification kit (Gentra Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

Bisulfite treatment and DNA methylation analysis

Aliquots of 1 μg of genomic DNA from trophoblast tissue samples BiHM70 and BiHM73 (from subjects HM70 and HM73, respectively), AnCHM and TP (TP1, TP2 and TP3) were sheared by repeated up-and-down pipetting, denatured in 3 M NaOH for 10 min at 37°C and subsequently incubated with 30 μl 10 mM hydroquinone and 520 μl 3 M sodium bisulfite at 50°C for 16 h (Frommer *et al*., 1992). After purification using a Qiaquick DNA purification kit (Qiagen Inc., Valencia, CA, USA), another denaturization step using 5 μl of fresh 3 M NaOH for 5 min and repurification, the eluted samples were PCR amplified using primers specific for the DMRs of the studied imprinted genes, as previously described (Judson *et al*., 2002), but with minor modifications. Primer sequences are in the online Supplementary Table S1A). PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA), transformed into competent cells and between 8–20 individual clones from at least two independent reactions were picked for sequencing with M13 primers.

Mutation analysis by direct sequencing

Primers were designed for amplification of all coding exons and flanking intronic sequences of *NLRP7* using the genomic sequence from GeneID 199713 (NT_011109). PCR was performed for 35 cycles on 50 ng of genomic DNA using standard conditions. Primer sequences and amplification conditions are in the online Supplementary Table S1B. Purified PCR products were directly sequenced in forward or reverse orientation, and all identified mutations were verified by sequencing in both orientations of a separate PCR product. Sequences were compared with the reference genomic sequences (NT_011109 region: 60126690-60150679) for mutation detection. Identified sequence variants and mutations were named according to the Human Genome Variation Society recommendations (den Dunnen and Antonarakis, 2000). To describe mutations, we chose reference transcript variant 2 of *NLRP7* (NM_206828) to be consistent with prior reports (Murdoch *et al*., 2006; Qian *et al*., 2007), but we note that the alternate reference transcript variant 1 (NM_139176) is longer by 87 nucleotides, contains the alternatively spliced exon 10 (which is not present in NM_206828), but lacks the first 84 nucleotides of exon 5 because of alternative splicing. For comparison, we provide the online Supplementary Table S2 which lists the identified mutations according to the two alternate reference transcripts.

To evaluate identified mutations in controls, restriction enzyme digestion of PCR products was performed with BccI or MnlI and fragments were separated by gel electrophoresis.

RNA isolation, reverse transcription and RT–PCR

Total RNA was isolated using RNABee (IsoTex Diagnostics, Inc., TX, USA) from LCL cultures. First-strand cDNAs were synthesized from 2.5 μg of total RNA using oligo-dT primers and Powerscript II (Invitrogen, Carlsbad, CA, USA) reverse transcriptase. PCR amplification of reverse-transcribed cDNA was performed with *Taq* polymerase in the presence of 1.5 mM $MgCl_2$ and Q solution (Qiagen Inc.) (primer sequences are in the online Supplementary Table S1C). Selected RT–PCR products were subsequently cloned into pCR2.1-TOPO for sequencing.

Southern analysis

Aliquots of 5 μg of genomic DNA samples from affected women, heterozygous controls and affected controls were digested with each of the restriction enzymes EcoRI, HincII, HindIII, XhoI and XcmI, electrophoresed through a 0.8% (w/v) agarose gel and blotted onto a Magnacharge nylon membrane Hybond-*N*+ (Amersham Biotech Inc., UK), which was hybridized with α^{32}P random-labeled *NLRP*7 cDNA probe (GenBank ID: BC109125) and visualized by autoradiography after stringent washing.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) for duplication detection within the *NLRP7* locus was performed using SYBR-green chemistry on 20 ng of genomic DNA as previously described (del Gaudio *et al*., 2006; Wang *et al*., 2007). Primer sequences were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Each reaction was performed in triplicate using the ABI7450 real-time PCR system, absolute gene copy number was determined by the standard curve method (Applied Biosystems user bulletin #2), data were normalized against an endogenous reference gene (*GAPDH*), and a melting curve analysis was performed to verify PCR product specificity. Primer sequences are provided in online Supplementary Table S1D.

Results

Loss of DNA methylation at maternally methylated DMRs in BiHM

We examined the DNA methylation at DMRs of imprinted loci on DNA extracted from freshly evacuated tissues from the fourth and fifth recurrent molar pregnancy, respectively, of HM70 and of HM73, two unrelated Latino women from Mexico with recurrent

BiHM, who carry mutations in *NLRP7* (see below). The chorionic villi were carefully cleaned from contaminating decidual tissue and maternal blood under a dissecting stereoscope prior to DNA extraction. Biparental inheritance of the tissues was then confirmed by genotyping with ten genome-wide polymorphic short tandem repeat (STR) markers as described (data not shown) (Panichkul *et al*., 2005).

Aliquots of DNA from these tissues, a sporadic confirmed androgenetic CHM (AnCHM), two different TPs and one PBL sample were used for bisulfite genomic sequencing of eight imprinted DMRs. The DMRs of paternally expressed genes *PEG3*, *SNRPN* and *KCNQ1OT1*, which normally acquire CpG methylation on the maternally inherited silenced allele, were mostly unmethylated in the two BiHM samples (Fig. 1A). The *SNRPN* DMR showed a few methylated clones in AnCHM, which was unexpected but also present in a previous study (Judson *et al*., 2002). The DMR 5′ to *H19* which acquires DNA methylation on the paternally inherited alleles during spermatogenesis showed a normal haplomethylated DNA methylation pattern in BiHM, whereas it was completely methylated in AnCHM tissues. This is similar to reported findings in BiHM tissue from a patient with recurrent biparental HM not linked to 19q13.42 (Judson *et al*., 2002), but is different from the increased DNA methylation on the maternal allele seen in another study on BiHM tissues from women with mutations in *NLRP7* (El-Maarri *et al*., 2003).

We also analyzed four DMRs at the complex *GNAS* imprinted locus (Fig. 1B). The maternal methylation at the *GNAS exon 1A* (*1A*) DMR is thought to be a primary imprint established in oogenesis (Judson *et al*., 2002). The pattern observed at the *1A* DMR was exactly as expected with absence of methylation in both BiHMs and in the AnCHM, but with haplomethylation in TP and PBL. Methylation on the paternal allele of the *NESP55* DMR is a secondary imprint, i.e. dependent on the correct establishment of the primary maternal methylation imprint at *1A* (Judson *et al*., 2002). As expected, the *NESP55* DMR was completely methylated in both BiHM tissues and in the AnCHM, likely secondary to the absence of methylation at *1A*. Interestingly, all 19 sequenced clones of one TP sample were methylated and all 9 clones of the PBL sample were unmethylated, suggesting that even in normal tissues, methylation patterns at this locus are not always as predicted. At the *GNAS* antisense (*AS*) DMR, the results were also complex. Bisulfite sequencing of both TPs showed only a few unmethylated clones. In contrast to a theoretically predicted loss of methylation in AnCHM and BiHM, our data on AnCHM and BiHM70 were more consistent with haplomethylation, although all 14 sequenced BiHM73 clones were completely methylated. Furthermore, CpG methylation at the DMR in the *XLαS* first exon (*XLαS*), which normally shows methylation on the maternal allele, i.e. established later in post-zygotic development, also showed a mixed pattern. Whereas the pattern was consistent with predicted haplomethylation in PBL and TP, and absent methylation in AnCHM, haplomethylation was found in both BiHM tissues. The complexity of the bisulfite sequencing data may have resulted in part from the presence of chimerism for both parental alleles in

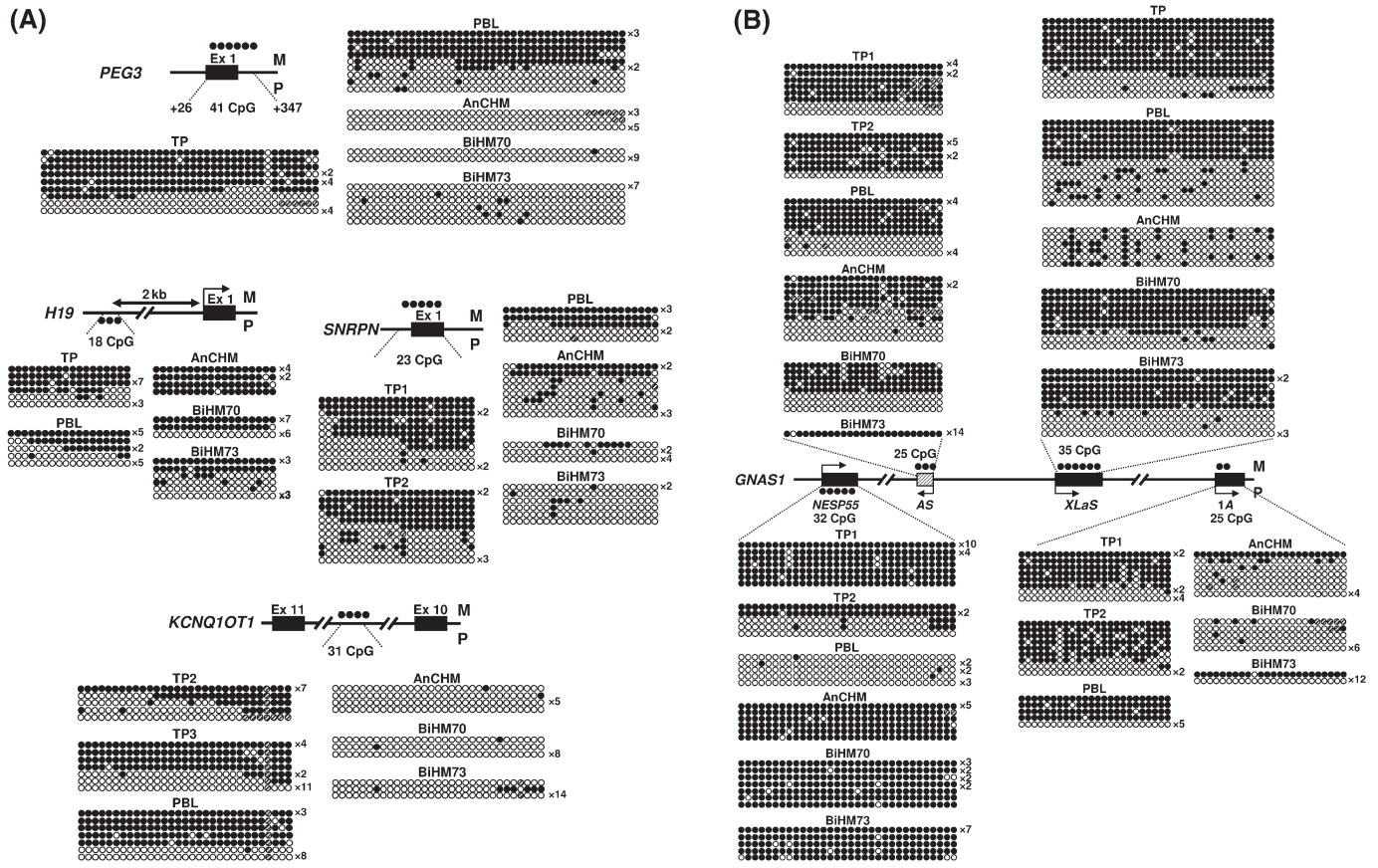

Figure 1: Bisulfite genomic sequencing at imprinted DMRs
(**A**) DMRs at the *PEG3*, *H19*, *SNRPN*, *KCNQ1OT1*. (**B**) Four DMRs at the complex *GNAS* imprinted locus. Schematic maps for each gene show the number of analyzed CpG sites and their position relative to the respective imprinted gene. Black filled circles above the map indicate that a specific DMR is normally methylated on the maternally inherited allele (M) and black filled circles below the map indicate that the DMR is normally methylated on the paternally inherited allele (P). Each row represents methylated, unmethylated and unscorable CpG sites represented as black, white or hatched circles, respectively. Between 8 and 20 clones were sequenced for each tissue and DMR region

some of the cloned bisulfite-PCR products. This may explain the unexpected results in the control PBL and TP samples for some of the analyzed loci at the analyzed *GNAS* DMRs. We were not able to analyze multiple single nucleotide polymorphisms (SNPs) to determine the presence of chimeric clones.

Mutation analysis of NLRP7 in women with BiHM

Homozygosity mapping with STR markers in 19q13.42 on additional subjects from the BiHM pedigrees, as well as detailed analysis with 6 SNPs at the revised centromeric boundary of the candidate region confirmed that *NLRP7*, which during the course of our studies was reported to be mutated in other women with BiHM (Murdoch *et al.*, 2006), was included in the region of homozygosity in affected patients in this study (see online Supplementary Figs S2 and S3).

We then PCR-amplified and sequenced all coding exons and flanking introns of *NLRP7* on LCL-derived genomic DNA of 11 affected individuals with recurrent BiHM and their unaffected family members from a total of eight families of different ethnic backgrounds.

We identified a homozygous missense mutation c.2248C>G (p.L750V) in subject HM73 (Fig. 2A) that changes a conserved leucine to valine in the second leucine-rich repeat (LRR) of *NLRP7*. Individual HM70 is a compound heterozygote for the c.2248C>G (p.L750V) mutation and for a c.2810+2T>G mutation, which changes the invariant T of the GT splice donor sequence of exon 9. We further found a homozygous splice donor mutation c.2471+1G>A in subject HM19, who is

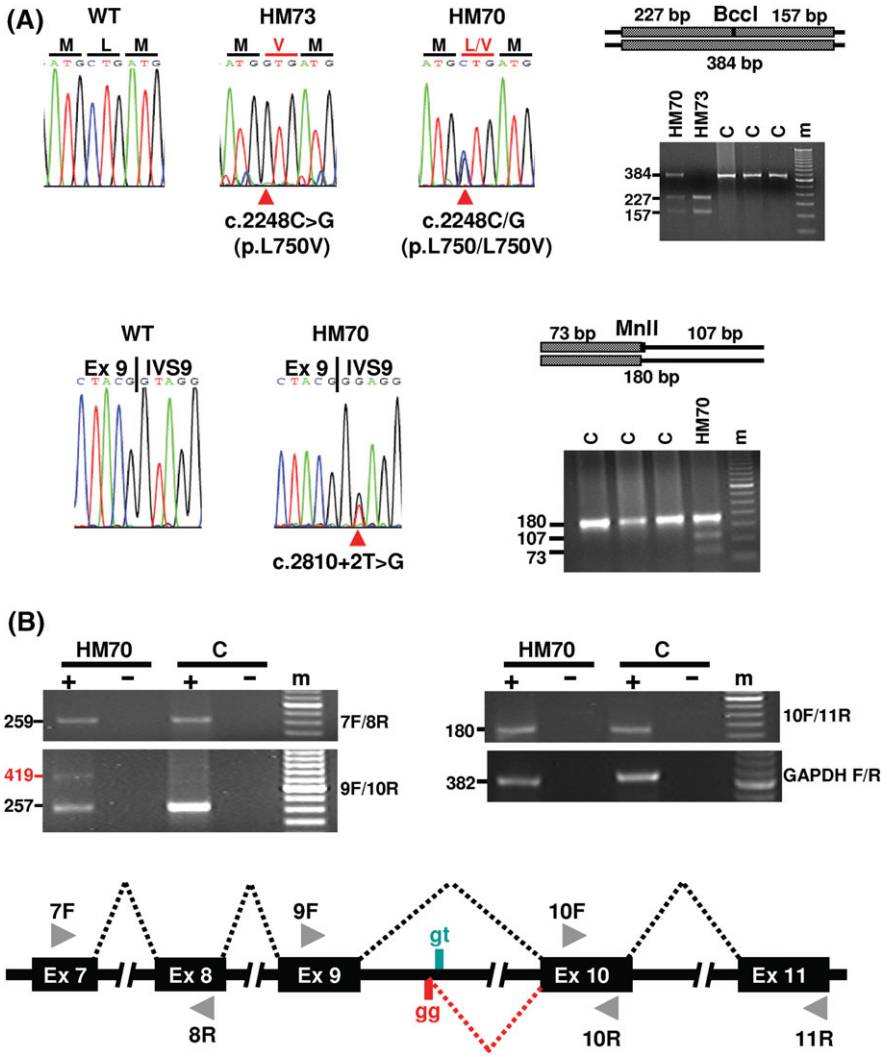

Figure 2: Novel autosomal recessive *NLRP7* point mutations in women with BiHM
(**A**) Top left: partial sequencing profiles of exon 6 of *NLRP7* showing a homozygous c.2248C>G (p.L750V) mutation in HM73 and a heterozygous c.2248C>G in HM70, compared with the normal sequence (WT). Top right: analysis of the c.2248C>G mutation in HM70 (heterozygous), HM73 (homozygous) and normal controls (C) by restriction enzyme digestion: the mutant 'G' allele creates a BccI restriction site, resulting in a 227 and 157 bp band. The undigested 384 bp band represents the wild-type 'C' allele. Bottom left: partial sequencing profiles of exon 9 and intron 9 showing the heterozygous c.2810+2T>G mutation in HM70. Bottom right: the c.2810+2T>G mutation introduces a MnlI site, digesting the original 180 bp PCR product into 107 and 73 bp fragments. Control lanes (C) only contain the undigested 180 bp fragment whereas the HM70 lane contains all three bands, indicating heterozygosity for this mutation. (**B**) Exon 9 splicing in control (C) and HM70 analyzed by RT–PCR on LCL-derived RNA. Primer combinations 7F/8R and 10F/11R amplify the expected 259 and 180 bp PCR product, respectively, in both HM70 and C. Primers 9F and 10R amplify an expected 257 bp product from control (C), but two products from HM70 RNA: a weak 257 bp and a novel 419 bp fragment. The diagram shows exons 7 through 11 of *NLRP7* with their normal splice patterns shown by grey dashed lines. The blue 'gt' shows the predicted mutant splicing, based on splice site prediction analysis; the red 'gg' and red dashed line show the observed mutant splice pattern in HM70, based on sequencing of the 419 bp PCR product (*GAPDH F/R*: RT–PCR control: '+' reaction with RT enzyme; '−' reaction without RT enzyme; m = 100 bp ladder)

of Pakistani origin, i.e. identical to one identified in an unrelated Pakistani patient (Murdoch *et al*., 2006).

The p.L750V missense mutation in exon 6 introduces a BccI restriction site and the c.2810+2T>G mutation introduces a MnlI site (Fig. 2A). PCR amplification followed by restriction enzyme digestion of control DNA samples failed to demonstrate these mutations in 260 control chromosomes from women of various ethnic groups, including 120 chromosomes from Latino individuals. We established that patient HM70 is a true compound heterozygote for the c.2248C>G and the c.2810+2T>G mutation by analyzing the DNA of the BiHM70 trophoblast tissue, which only contained the c.2810+2T>G mutation, confirming that these mutations were not on the same allele (data not shown).

We then performed RT–PCR on RNA extracted from LCL cultures of subject HM70 to determine whether the c.2810+2T>G mutation affects splicing of exon 9 to exon 10 (Fig. 2B). Products of the predicted size were generated with a forward primer in exon 7 (7F) and reverse primer in exon 8 (8R), and with forward primer 10F and reverse primers 11R from RNA of HM70 and unaffected controls. However, amplification with primers 9F and 10R resulted in two products when amplified from the reverse-transcribed cDNA of HM70: the expected 257 bp fragment and a fainter larger fragment of 419 bp. Direct sequencing of these fragments after gel-purification revealed the expected normally spliced exon 9 and exon 10 sequences for the 257 bp product, whereas the larger product included an additional 162 bp from intron 9. This abnormally spliced mRNA was not present in controls and is predicted to result in an in-frame insertion of 54 amino acids in the translated mutant protein. Interestingly, splice-site prediction software (NetGene2 at http://www.cbs.dtu.dk/services/NetGene2/) predicted that the next best 'GT' donor splice site was at position +183 in intron 9, whereas the sequence following nucleotide 162 in intron 9 is 'GG', which is not a canonical splice-donor site. We cannot rule out that splicing after nucleotide 182 in intron 9 occurs, but that the resulting mutant mRNA may be undetectable because it is predicted to contain a frameshift and premature stop codon upstream of the 3′-most exon of *NLRP7* that may trigger nonsense-mediated mRNA decay.

Intragenic tandem duplication containing exons 2 through 5 in Egyptian women with recurrent BiHM

We were not able to find mutations in coding exons of *NLRP7* in any of the affected women with BiHM from Egypt. To our surprise, we detected apparent heterozygous changes in exon 4 in DNA from these women which was inconsistent with our mapping data that indicated homozygosity by descent for the genomic region containing *NLRP7*. Suspecting a possible ancestral duplication of exon 4 with accumulated sequence alterations in the duplicated exon, we performed Southern analysis of genomic DNA from Egyptian women with BiHM and from selected family members, as well as unrelated BiHM patients and unaffected controls (Fig. 3A). Hybridization with a *NLRP7* cDNA probe revealed novel fragments in genomic DNA of all Egyptian affected women and carriers, as well as missing fragments in DNA of all Egyptian affected women with all restriction enzymes tested. These changes tracked with the disease haplotypes and were not detected in DNA of non-Egyptian subjects with BiHM, unaffected non-carrier Egyptian family members or unrelated controls. The combined Southern data suggested the presence of an intragenic duplication that included at least exon 4 and possibly flanking exons 2, 3 and 5. Real-time qPCR on LCL-derived genomic DNA of subjects from Egyptian families and controls using primers that amplify exons 1 through 6 and selected introns of *NLRP7* (Fig. 3B) showed the presence of two extra copies of exons 2 through 5 of *NLRP7* in affected subjects HM34, 35, 28, 46 and 33 (subject/control ratio of ∼2.00), whereas there was one extra copy of these exons in all carrier subjects of the RHM 1 and RHM2 kindreds (subject/control ratio of ∼1.5) (Fig. 3B). Exons 1 and 6 were not duplicated and PCR amplification of fragments in introns 1 and 5 established that the duplication involved a region of ∼4 kb from intron 1 to intron 5.

PCR amplification with a forward primer in intron 4 and a reserve primer in intron 2 resulted in amplification of a 951 bp PCR product from genomic DNA of all homozygous affected women and heterozygous carriers, but not from unaffected family members (Fig. 3C) or 150 control DNA samples (300 chromosomes) of individuals from various ethnic groups, confirming that the duplication was intragenic and in tandem. Sequencing of this PCR product revealed that the recombination between intron 5 and intron 1 occurred in a region of identical sequence of 32 nucleotides (Fig. 3C).

RT–PCR amplification with a forward primer in exon 5 (5F) and reverse primer in exon 2 (2F) (Fig. 4A) yielded a 267 bp fragment from RNA of patient HM35, but not from control RNA (Fig. 4B). The sequence and size of the 267 bp RT–PCR product confirmed that exon 5 splices to exon 2, using the canonical splice donor and acceptor sites of both exons. Amplification with primers combinations 4F/5R, 5F/6R and 5F/7R followed by direct sequencing of PCR-products indicated that the mutant mRNA was detectable at low levels. Translation of the mutant mRNA is predicted to result in a truncated protein containing a frameshift after Val709 of six amino acids followed by a stop codon. This deletes the entire LRR region of *NLRP7*, indicating that this mutation results in loss-of-function.

Discussion

Most of the mutations in *NLRP7* that we identified in women with recurrent BiHM disrupt the LRR, an important functional domain of *NLRP7* that may play a role in protein–protein interactions or pattern recognition. The p.L750V changes a leucine to valine in the second LRR. Alignment of human *NLRP7* with other proteins of the *NLRP* family and with *NLRP7* sequences of the chimpanzee and cow genome indicates that this leucine is evolutionary conserved. Although leucine-to-valine substitutions are relatively conservative changes, they can be pathogenic (Ishiko *et al*., 2001). Furthermore, leucine residues are essential components of LRR motifs. The c.2810+2T>G splice-site mutation is also predicted to affect the LRR, whereas the intragenic duplication is predicted to result in a frameshift and truncated protein that, if translated, lacks the entire LRR domain. To the best of our assessment, women with this and other mutations we identified had only molar pregnancies and no other reproductive outcome (Al-Hussaini *et al*., 2003; Panichkul *et al*., 2005). This contrasts with other reports of spontaneous abortions, and even normal pregnancies interspersed with the recurrent BiHMs (Qian *et al*., 2007; Slim and Mehio, 2007). Whether the mutations we identified cause a more severe loss of function of *NLRP7* than those previously reported, or whether these differences in reproductive outcome are due to genetic modifier alleles in the population is currently unknown.

NLRP7 belongs to the NLRP-family of CATERPILLER proteins and contains a NACHT associated domain (NAD), a NACHT domain and a LRR region with 9 LRRs. CATERPILLER proteins, especially NLRPs, are intracellular sensors of microbial and viral pathogens with some functional similarity to membrane-bound Toll-like receptors (*TLR*s) which recognize extracellular pathogens (Ting *et al*., 2006). Other members of the *NLRP* family of proteins are major components of the inflammasome, a recently discovered

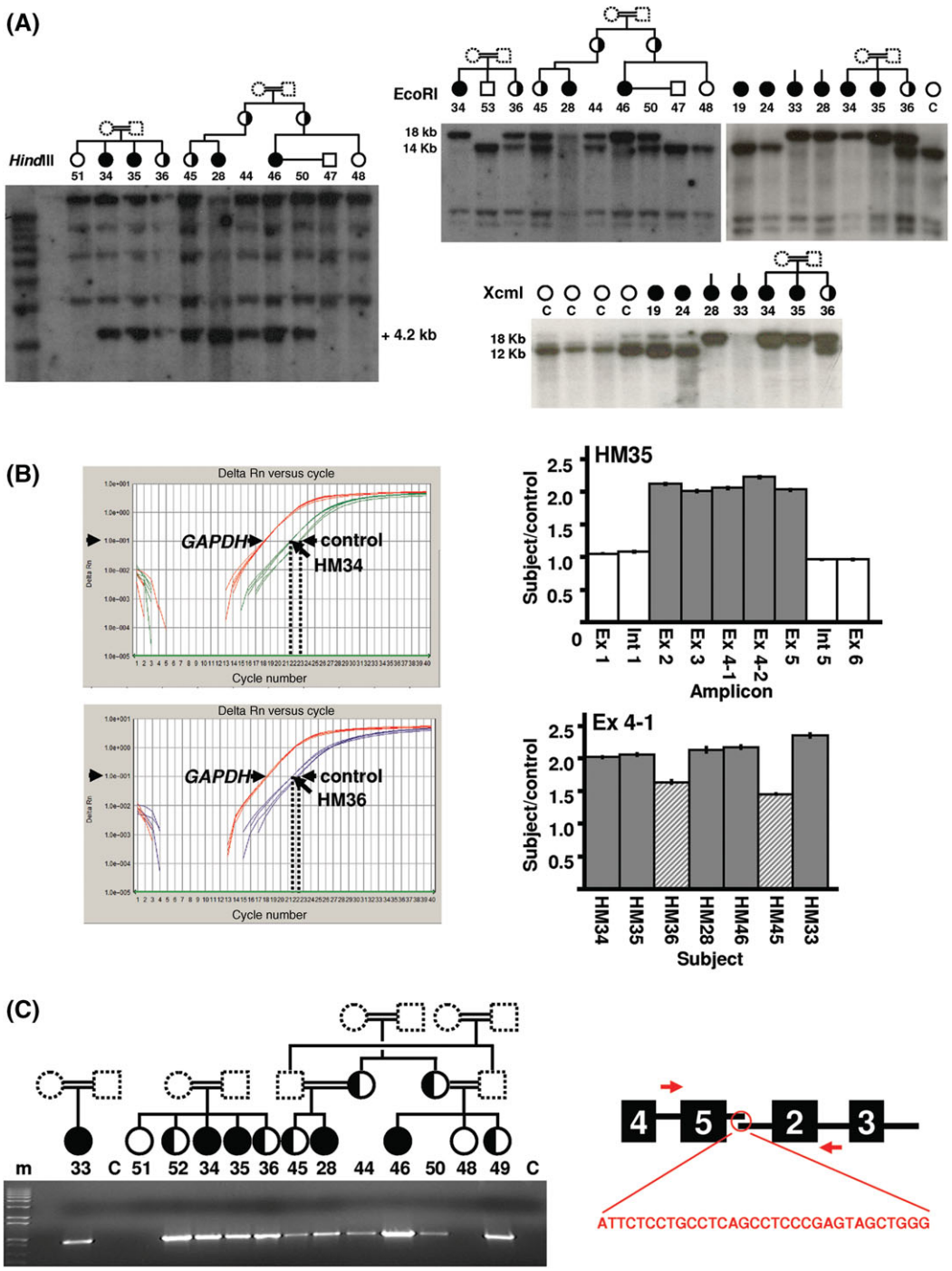

Figure 3: Intragenic 4 kb duplication in *NLRP7* in Egyptian women with BiHM

(**A**) Southern analysis of leukocyte- and lymphoblast-derived total genomic DNA of affected women with BiHM (28, 34, 35, 46), unaffected carriers (36, 44, 50) and non-carriers (47, 48, 51, 53) from the Egyptian kindreds and of healthy controls (C) and non-Egyptian women with BiHM (19, 24). DNA was digested with different restriction enzymes and hybridized with a *NLRP7* cDNA probe. The HindIII digest yielded an extra 4.2 kb band in all affected women and carriers, the XcmI digest resulted in an extra 18 kb band in affected women and carriers and loss of a 12 kb band in affected women, the EcoRI digest resulted in an extra 18 kb band in affected women and carriers and loss of a 14 kb band in affected women of Egyptian kindreds. None of these aberrant restriction patterns were seen in non-carriers, controls or women with BiHM from other ethnic backgrounds. Pedigree symbols drawn in hatched lines were not analyzed. (**B**) Real-time qPCR analysis of *NLRP7* exons 1 through 6, intron 1 and 5 and *GAPDH* control in a duplex assay. Actual qPCR results are shown on the left for a control and a homozygous patient, HM34 ($\Delta\Delta$Ct$\sim$1) and a heterozygous patient, HM36 ($\Delta\Delta$Ct$\sim$0.5) (*y*-axis is Delta Rn; *x*-axis is cycle number). The top graph on the right shows the subject to control ratio of all amplicons in one homozygous individual HM35, indicating the duplicated region contains exons 2 through 5 of *NLRP7*. The bottom graph shows the results on Ex 4-1 for homozygous affected (HM28, 33, 34, 35) and heterozygous carrier (HM36, 45) women. (**C**) Amplification of a junction fragment indicates the presence of a tandem intragenic duplication in Egyptian families. Left panel shows the results of PCR-amplification with a forward primer in intron 4, near exon 5 and reverse primer in intron 2, near exon 2 yielded a 915 bp junction fragment in all affected Egyptian women and their heterozygous carrier sisters or mothers. The junction fragment could not be amplified from non-Egyptian BiHM patient HM24 (not shown), non-carrier family members (51, 48) and unaffected control DNA (C). Direct sequencing of the junction fragment showed that recombination occurred between intron 5 and intron 1 in a region of identical sequence, represented in the diagram on the right

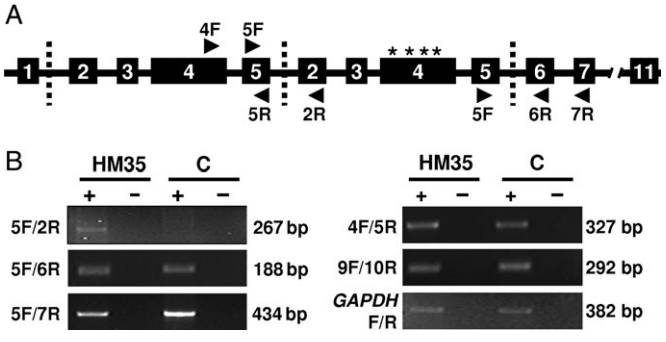

Figure 4: RT–PCR analysis of the tandem intragenic duplication (**A**) Schematic diagram of the mutant *NLRP7* genomic region showing the location of the RT–PCR primers used. (**B**) RT–PCR with indicated forward and reverse primers on LCL-derived reverse-transcribed RNA from HM35 and unaffected control. A novel 267 bp product was amplified from reverse-transcribed RNA of HM35 with primers 5F and 2R, but not from the control. This indicates the presence of a tandem intragenic duplication. Primer pairs 5F/6R, 5F/7R, 4F/5R and 9F/10R yielded the expected 188, 434, 327 and 292 bp products from HM35 and from control, indicating that the *NLRP7* mutant mRNA was present in the homozygous patient HM35. '+' lanes and '−' lanes represent reactions with and without reverse transcriptase, respectively. *GAPDH* F/R is the RT–PCR control amplification; hatched lines on diagram shows approximate position of the boundaries of the tandem duplication; stars indicate the position in the duplicated exon 4 of the 'apparent heterozygous' nucleotides

intracellular protein complex of macrophages and neutrophils that senses extracellular pathogens and intracellular noxious compounds and generates a caspase-1 dependent activation of the pro-inflammatory cytokine interleukin-1β (IL1β) (Drenth and van der Meer, 2006; Ogura *et al*., 2006). Studies in cultured cells suggest that NLRP7 may have an anti-inflammatory role as a feedback regulator of lipopolysaccharide-stimulated caspase-1-dependent IL1β secretion in lymphoid cells (Kinoshita *et al*., 2005), but its role *in vivo* has not been characterized.

The expression of *NLRP7* is however not restricted to the immune system. The mRNA is detected in a variety of tissues (Kinoshita *et al*., 2005), including oocytes, early preimplantation embryo, endometrium and uterus (Murdoch *et al*., 2006), and it was found to be up-regulated in germ cell tumors (Okada *et al*., 2004), suggesting that it may have a specific role in these reproductive organs. There is no rodent homologue for *NLRP7* and the gene is found in only a few genomes (human, primate and cow), supporting that it may have a specialized function in those mammals, perhaps related to imprinting. Parent-of-origin specific reprogramming of imprinting is a complex process initiated during gametogenesis. In female mice, parental DNA methylation marks are first completely erased in primordial germ cells. This is followed in the growing oocyte by remethylation of maternally methylated DMRs. Post-zygotically, these imprinted DNA methylation marks are largely mitotically maintained and protected from the global DNA demethylation and remethylation of the rest of the genome. DNA methyltransferases, other chromatin-associated factors, microRNAs as well as motifs in the DNA sequence near imprinting control regions (ICRs) have all been implicated in the establishment and maintenance of imprinting marks, but it is incompletely understood which factors direct the recognition of the parental identity of imprinted alleles. The DNA methylation patterns observed in this study indicate that BiHM are characterized by failure to acquire primary DNA methylation imprints in the maternal germline, or maintain these acquired maternal DNA methylation patterns post-zygotically. For the *H19* DMR which normally acquires primary paternal DNA methylation imprints, we found no evidence for increased methylation on maternally inherited alleles, although we could not establish the parental origin of the methylated clones. The results at DMRs with secondary imprints are more complex. While at the *XLαS* DMR, our findings in BiHM and AnCHM are similar to those predicted and previously reported (Judson *et al*., 2002), they are nearly the opposite to what is predicted at the *AS* DMR. Combined with data from previously published reports (Judson *et al*., 2002; El-Maarri *et al*., 2003), this indicates that in addition to a failure to acquire or maintain primary maternal DNA methylation imprints set during oogenesis, there is a failure to correctly establish and/or maintain secondary post-zygotic imprinting marks in an unpredictable pattern in BiHM tissues.

Considering the role of NLRP7 in inflammation, it has been proposed that the defects in DNA methylation observed in BiHM trophoblasts are a secondary phenomenon associated with a more generalized immune-response-driven dysfunction of the oocyte, early embryo or intrauterine milieu. This could potentially explain other adverse reproductive outcomes seen in some women with recurrent BiHM (Slim and Mehio, 2007; Qian *et al*., 2007). It is however striking that the DNA methylation defects in BiHM tissues are highly specific for imprinted genes (Djuric *et al*., 2006) and do not affect other methylated loci, whereas women with BiHM have normal DNA methylation patterns themselves (El-Maarri *et al*., 2005). It is possible that *NLRP7*, in addition to its role in the regulation of the cellular immune response, may have been recruited to carry out a specialized different function in oogenesis and early pregnancy that involves correct establishment or maintenance of parent-of-origin-specific DNA methylation on imprinted genes. Several indirect observations support the possibility of a direct interaction of NLRP7 with DNA. First, although NLRP7 does not have a clear nuclear localization signal, the reprogramming of imprinting marks during meiosis and their propagation to daughter cells during mitosis occurs at stages in the cell cycle when the nuclear envelope is not present. Second, *NLRP3* (*CIAS1*) was reported to bind nucleic acid (RNA), likely via its LRR (Kanneganti *et al*., 2006). Recent analysis of *Cias1*−/− mice has shown that it is activated in response to the synthetic compounds R837 and R848 (which resemble nucleic acids), to viral and bacterial ssRNA, to dsRNA (but not mammalian RNA) and to purines (Kanneganti *et al*., 2006; Mariathasan *et al*., 2006; Martinon *et al*., 2006). Third, the LRR domains of *TLR7* and *TLR8* bind RNA (Koski *et al*., 2004; Kariko *et al*., 2005), whereas the LRR domain of *TLR9* recognizes unmethylated bacterial CpG motifs (Hemmi *et al*., 2000; Bauer and Wagner, 2002).

Further experiments will be needed to study the normal function of *NLRP7* and to address whether it is primarily the establishment or maintenance of parent-of-origin-specific DNA methylation at DMRs at imprinted genes that is disrupted in BiHMs of women with *NLRP7* mutations, or whether there is a more general inflammatory disturbance that affects oogenesis or embryogenesis, and secondarily imprinting marks.

Supplementary Data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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