Report of four new patients with protein truncating mutations in *C6orf221*/*KHDC3L* and co-localization with NLRP7

Running title: *KHDC3L* mutation and subcellular localization

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Abstract

To date, two maternal-effect genes have been shown to play causative roles in recurrent hydatidiform moles (RHMs); *NLRP7* that is mutated in 48-60% of patients with RHMs and *C6orf221* (HUGO approved nomenclature is now *KHDC3L*), a recently identified gene, that is mutated in 14% of patients with RHMs who are mostly negative for *NLRP7* mutations. We sequenced *KHDC3L* in 97 patients with RHMs and reproductive loss who are mostly negative for *NLRP7* mutations. We identified three unrelated patients, each homozygous for one of the two protein truncating mutations, a novel 4-bp deletion resulting in a frameshift, c.299_302delTCAA, p.Ile100Argfs*2, and a previously described 4-bp deletion, c.322_325delGACT, p.Asp108Ilefs*30, transmitted on a shared haplotype to three patients from different populations. We show that five HM tissues from one of these patients are diploid and biparental similar to HMs from patients with two defective *NLRP7* mutations. Using immunofluorescence, we show that KHDC3L protein displays a juxta perinuclear signal and co-localizes with NLRP7 in lymphoblastoid cell lines from normal subjects. Using cell lines from patients, we demonstrate that the *KHDC3L* mutations do not change the subcellular localization of the protein in hematopoietic cells. Our data highlight the similarities between the two causative genes for RHMs, *KHDC3L* and *NLRP7,* in their subcellular localization, the parental contribution to the HM tissues caused by them, and the presence of several founder mutations and variants in both of them indicating positive selection and adaptation.

Keywords: *KHDC3L*, 4-bp deletion, diploid biparental hydatidiform mole, *NLRP7*, co-localization.

Introduction

Hydatidiform mole (HM) is an aberrant human pregnancy with absence of, or abnormal embryonic development, hydropic degeneration of chorionic villi, and excessive proliferation of the trophoblast[1]. Sporadic HMs are common, not recurrent, and affect 1 in 600 pregnancies (all forms combined) in western countries [2] but have higher frequencies in developing countries. [3,4] Recurrent HMs (RHMs) affect 1-6% of women with sporadic HMs[5-8] and occur either in patients with no family history of HMs (singleton cases) or occasionally in sisters or related females from the same family (familial cases). Histologically, HMs can be categorized into two types, complete hydatidiform mole (CHM) and partial hydatidiform mole (PHM). CHMs are characterized by excessive trophoblastic proliferation and absence of fetal tissues beside the chorionic villi. PHMs are characterized by less pronounced trophoblastic proliferation and may contain embryonic tissues[1]

Two maternal-effect genes responsible for RHMs are known, *NLRP7* and *KHDC3L*. *NLRP7* is mutated in 88% and 60% of analyzed familial and singleton cases of RHMs, respectively.[9-14] Recently, we demonstrated that *ex-vivo* lipopolysaccharides (LPS) stimulated peripheral blood mononuclear cells (PBMCs) from patients with mutations and rare variants in *NLRP7* have defective Interleukin 1 beta (IL-1β) and Tumor necrosis factor (TNF) secretion but normal to higher intracellular levels of pro- and mature- IL-1β.[13,15] The requirement of NLRP7 for normal IL-1β secretion by

macrophages was also confirmed in *in-vitro* studies after *NLRP7* silencing using small interfering RNA (siRNA).[16] Within hematopoietic cells, we showed that *NLRP7* co-localizes with the Golgi apparatus and the microtubule organizing centre (MTOC) and associates with microtubules suggesting that mutations in this gene may decrease cytokine secretion by affecting microtubule structure and consequently intracellular cytokine trafficking.[15]

Recently, mutations in a second maternal-effect gene, *KHDC3L*, were shown to be responsible for RHMs. [17] Mutations in this gene were found in one familial case and in two out of 14 analyzed singleton cases of RHMs (14.2%) that are negative for *NLRP7* mutations by conventional Sanger sequencing[17]. Among the identified *KHDC3L* mutations, a 4-bp deletion in exon 2, c.322_325delGACT, p.Asp108Ilefs*30, that was found in a homozygous and heterozygous state in one Tunisian and one Iranian patient, respectively[17].

Here, we report two *KHDC3L* mutations, including one novel 4-bp deletion, in two new singleton cases and one familial case of RHMs. We confirm the causality of *KHDC3L* mutations in 10% of our patients with RHMs who are negative for *NLRP7* mutations and exclude the causative role of *KHDC3L* in our cohorts of other forms of reproductive losses. We show that five HM tissues from one patient with two defective alleles in *KHDC3L* are diploid and have biparental contribution to their genomes similar to HMs from patients with two defective alleles in *NLRP7*. We demonstrate that KHDC3L protein co-localizes with NLRP7 in lymphoblastoid cell lines and that this localization is not affected by the two protein truncating mutations. Our data shed

new lights on our understanding of HMs and indicate positive selective pressures underlying their increased incidences from North to South and from West to East.

Materials and Methods

Patients

The study was approved by the McGill Institutional Review Board. Patients were ascertained through various collaborative hospitals and written consents were obtained. DNA was isolated from whole blood cells using Flexigene DNA Kit (Qiagen). Mutation analysis was performed by conventional Sanger sequencing on DNAs from 97 patients with no mutations in *NLRP7* with the exception of 8 patients who have one *NLRP7* defective allele each. Patients were divided into three categories according to their reproductive histories, (i) 29 patients with at least 2 HMs (≥ 2 HMs), (Supp. Table S1) (ii) 27 patients with at least two reproductive losses including 1 HM (≥ 2 RL including 1 HM) (Supp. Table S2), and (iii) 41 patients with at least 3 spontaneous abortions and no HMs (≥3 SAs) (Supp. Table S3). DNA from a total of 142 subjects of African origin were used as controls, fifty of African-American origin obtained from the Corriell Institute from the Human Variation Panel (HD50AA) and 92 of from the general Senegalese population. Both cohorts of controls included males and females and no data about their reproductive outcomes were available.

Mutation and Haplotype Analyses

The three exons of *KHDC3L* were PCR amplified on genomic DNA as previously described[17]. Sequence analysis was performed using DNASTAR and the sequences

were compared with reference sequence NM_001017361. DNA mutation numbering is based on cDNA sequence with a 'c.' symbol before the number and uses the A of the ATG translation initiation start site as nucleotide +1. Protein numbering starts from the initiation codon (codon 1).We refer to non-synonymous variants for DNA changes leading to amino acids changes that are present in the general population. Two additional primers, Ex2del_forward, 5'-CGTGAATCGATTGGACCCTA-3' and Ex2del_reverse, 5'-GCTCCAGGTAGCCCTATTCC-3' that amplify a 156-bp PCR fragment spanning the 4-bp deletion in exon 2 were used. A total of 24 single nucleotide polymorphisms (SNP) spanning a 22-kb interval surrounding *KHDC3L* were selected from the NCBI Database of Single Nucleotide Polymorphisms (dbSNP) and used to determine the haplotypes carrying mutation c.322_325delGACT.

Flow cytometry and Fluorescent microsatellite genotyping of HM **tissues**

Flow cytometry was performed on one paraffin-embedded tissue containing substantial amount of chorionic villi from each available product of conception (POC) according to standard methods. [18] Five serial 8 □m sections were performed on one paraffin block from each POC and stained with hematoxylin and eosin. Pinpoint solution (Zymo Research, Orange CA) was applied to the areas containing only chorionic villi defined by stereomicroscopy. The tissues were then removed and used to extract DNA that was used in multiplex PCR to amplify sixteen highly polymorphic microsatellite loci in a single PCR reaction using PowerPlex® 16 HS System (Promega Corporation). Amplified PCR products were resolved by capillary electrophoresis using an ABI 3130

Genetic Analyser. The genotype of the HM tissue was compared with that of the patient to determine the parental contribution to the HMs.

Constructs, cell culture, and transfection

GFP (PS100010, Origene), GFP wild-type wt-*KHDC3L* (RG215631, Origene), and FLAG-tagged wild type wt-*NLRP7* constructs [15] were used for transfections. One day prior to transfection HEK293T cells were seeded at a density of 1×10^5 cells per well using four-well chamber slides (Ultident) in 500µl of Dulbeco's modified Eagle's medium (DMEM) (Invitrogen, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, CA). The cells were transfected as previously described.[15]

Immunofluorescence

Immunofluorescence was performed as previously described[15]. The following primary antibodies were used: Rabbit anti-KHDC3L (1:100) [HPA043699, Sigma prestige raised against the last 70 amino acids at the C-terminus of the protein (C-term)], Rabbit anti-KHDC3L from Abgent (AP11238a) raised against amino acids 27 to 56 at the N-terminal (N-term) part of the protein, Goat anti-NLRP7 (1:100) (sc-50642 from Santa Cruz) and Rabbit anti-NLRP7 (1:100) (IMG-6357A from Imgenex). The secondary antibodies were Alexa Fluor® 568 conjugated Donkey anti-Rabbit (1:500) (A10042, Invitrogen) and Alexa Fluor® 488 Donkey anti-Goat (1:500) (A11055, Invitrogen). Finally, the slides were mounted using Vectashield hard-set mounting medium with 4–

6 diamidino-2-phenylindol-2-HCl (DAPI) (Vector Laboratories). Fluorescence images were captured on an Axioskop 2 plus microscope.

Results

KHDC3L mutations in 3 unrelated patients and a novel protein truncating mutation

KHDC3L mutation analysis in a cohort of 29 unrelated patients, 26 singleton and 3 familial cases, each with at least two hydatidiform moles revealed two mutations in three patients (Table 1). The first mutation was a novel 4-bp deletion, c.299_302delTCAA, p.Ile100Argfs*2, in a homozygous state in patient 481 of African-American origin, who had 7 HMs with three partners (Fig 1A). This 4-bp deletion was not found in 142 control subjects, 50 of African-American origin who were screened by PCR amplification and electrophoresis on Nusieve:agarose (3:1) gel and 92 of Senegalese origin who were screened by DNA sequencing. The second mutation was another 4-bp deletion, c.322_325delGACT, p.Asp108Ilefs*30, previously reported to be causative of RHMs[17] and was seen in a homozygous state in two unrelated patients, 654 and 1096 (Fig 2). Patient 654 is of Turkish origin and had 5 HMs from natural conceptions and two failed cycles of *in-vitro* fertilization followed by pre-implantation genetic screening for aneuploidies. In the two cycles, a total of 11 embryos were analyzed. One embryo showed a monosomy for chromosome 16, another displayed monosomies for chromosomes 1 and 16, and all the other embryos had complex aneuploidies except for one that was transferred to the patient but did not result in a positive pregnancy test. Screening of this mutation in available family

members of patient 654 using Nusieve:agarose gel electrophoresis and DNA sequencing showed that both her parents and a sister with 2 normal pregnancies were heterozygous for the mutation while her brother has two normal copies of the *KHDC3L* gene (Fig 2). Patient 1096 is of Indian origin and had 10 RHMs and is from a familial case of RHMs. Screening the other family members revealed the same mutation, c.322_325delGACT, in a homozygous state in her sister with 3 RHMs and in a heterozygous state in her two parents, but not in her brother who does not carry any copy of the mutation (Fig 2). The two mutations were not reported in the 1000 Genomes database (www.1000genomes.org), in which DNA variants found in 1488 individuals from five different populations, including 245 from sub-Saharan Africa, are listed.

Prior to the identification of the *KHDC3L*, a SNP microarray, Illumina 610 Quad, had been performed on DNAs from patient 654, her two parents, and unaffected sisters. This analysis revealed a large homozygous region encompassing *KHDC3L* and spanning a total of 44-Mb, from rs9267522 to rs3777505 in patient 654. Altogether, the SNP microarray data combined with the homozygous state of the mutation are in favor of the causality of *KHDC3L* mutation in the recurrent HMs in this patient.

A shared small haplotype carrying c.322_325delGACT in *KHDC3L* in patients of different countries of origin

The presence of the same mutation, c.322_325delGACT, in two patients of Tunisian and Iranian origins[17] and in two of our patients from different countries, Turkish and Indian, prompted us to look for shared alleles at markers around the common mutation

in our patients and in the previously reported Tunisian patient[17] whose DNA was available to us. The analysis of a total of 24 SNPs spanning 21-kb around *KHDC3L* revealed shared alleles at 5 to 11 adjacent SNPs around the mutation (Table 2). These data indicate the presence of an old founder effect for the c.322_325delGACT mutation and its inheritance from a common ancestor to the Tunisian, Turkish, and Indian patients.

Absence of *KHDC3L* mutations or associated variants in patients with sporadic HMs and recurrent spontaneous abortions

To investigate the potential involvement of *KHDC3L* in sporadic HMs and recurrent spontaneous abortions, we screened this gene in 27 unrelated patients with at least two reproductive losses including one HM (≥ 2 RL including 1 HM) (Supp. Table S2), and 41 unrelated patients with at least three spontaneous abortions (≥ 3 SAs) (Supp. Table S3). However, we did not find any mutation in these 68 patients.

In the three analyzed categories of patients, only two non-synonymous variants (NSVs) in *KHDC3L* that are reported in public databases were observed, c.289G>C leading to p.E97Q and c.602C>G leading to p.A201G, and were found in the same frequencies in patients and controls indicating that these two NSVs do not predispose our patients for reproductive loss (Table 3).

Among the 97 patients included in this study, eight had one defective allele in *NLRP7* and these patients did not have *KHDC3L* mutations. We note that the four patients with *KHDC3L* mutations do not have any of the rare *NLRP7* NSVs that confer susceptibility for reproductive loss.[13]

HM tissues from one patient with two defective alleles in *KHDC3L* are diploid biparental

Flow cytometry analysis on five different POCs from patient 481 (Fig 1B) showed the presence of a single diploid peak demonstrating that the five POCs are diploid (Fig 1B). Fluorescent microsatellite genotyping on the five POCs demonstrated that the analyzed tissues are biparental based on one to seven informative markers, in each POC, where a second allele of non-maternal origin was identified (Fig 1C). These data demonstrate that the five HM tissues from patient 481 with two *KHDC3L* defective alleles are diploid biparental similar to HMs from patients with two defective *NLRP7* alleles.

KHDC3L expression and co-localization with NLRP7 in hematopoietic cells

KHDC3L is reported to be transcribed in a wide range of human tissues and cell lines including hematopoietic cells (http://biogps.org/). PCR after reverse transcription on RNA extracted from EBV-transformed cells from the 2 patients, 481 and 654, and 2 controls was performed using exonic primers located in exons 2 and 3. This analysis revealed the amplification of a 284-bp cDNA fragment of the expected size (Supp Fig. S1) whose identity was also confirmed by DNA sequencing. This demonstrates the transcription of *KHDC3L* in EBV-transformed cells and the presence of *KHDC3L* transcripts in cells from the two patients with homozygous protein truncating mutations. Also, these data indicated that EBV-transformed cells can be used to perform subcellular localization of KHDC3L protein.

Before investigating the subcellular localization of KHDC3L, we first validated a commercially available antibody (Sigma, prestige HPA043699) that detects the C-terminal part of the protein (C-term) using transfected HEK293T cells with a GFP-tagged wild-type *KHDC3L* cDNA (GFP-wt*KHDC3L*) construct and simultaneous revelation of the transfected protein with GFP immunofluorescence and the KHDC3L C-term antibody (Fig. 3 A to D). Using the GFP-wt*KHDC3L*, the C-term antibody signal overlapped exactly with that of GFP and both revealed a peri-nuclear staining that was not obtained with the transfected empty vector carrying only GFP (Fig 3E and F). This analysis demonstrated that C-term antibody recognizes the exogenous transfected wild-type *KHDC3L* protein. We next used the C-term antibody to detect endogenous KHDC3L. This antibody revealed a juxta peri-nuclear signal that co-localized with NLRP7 protein in EBV-transformed cells from controls (Fig. 3G-I). The validation of one NLRP7 antibody (Santa-Cruz, sc-50642) was previously reported [15] and additional validations of this antibody (Santa Cruz, sc-50642) and of another one (Imgenex, IMG-6357A) were performed during this study (Supp. Fig S2). The juxta and peri-nuclear signal obtained with the C-term KHDC3L antibody on EBV-transformed cells from controls was also confirmed on the same cells with another antibody that detects the N-terminal (N-term) part of KHDC3L (Abgent, AP11238a) (Fig. 3J-L). Analysis of EBV-transformed cells from the two patients, 481 and 654, revealed their truncated proteins with the KHDC3L N-term antibody but not with the C-term antibody. With the N-term antibody, the frequency of the signal in patients' cells was lower than in control cells and many cells displayed diffuse signal. In both patients, when present the signal with the N-term antibody overlapped with that of

NLRP7 (Fig 3M-X). Altogether, our immunofluorescence data demonstrate first that KHDC3L co-localizes with NLRP7 in EBV-transformed cells and second that the two *KHDC3L* 4-bp deletions identified in our patients do not change the subcellular localization of the protein in hematopoietic cells.

Discussion

Here we report a novel 4-bp deletion resulting in a frameshift in exon 2 of *KHDC3L*, c.299_302delTCAA, p.Ile100Argfs*2, in a patient with 7 HMs. We also report the identification of a previously reported 4-bp deletion in exon 2, c.322_325delGACT, p.Asp108Ilefs*30, in two sisters and in another unrelated singleton patient with RHMs.

To date and including this study, six unrelated patients with mutations in *KHDC3L* have been described[17]. Of these patients, two have two different DNA substitutions leading to a shift in the initiation codon and four have two different 4-bp deletions leading to frameshift and premature termination of the protein. In the reported patients, the presence of small deletions (20-bp or less) in *KHDC3L* in four out of six patients is comparatively higher than the average observed in human diseases (15.6%) (http://www.hgmd.org/). In addition, the fact that both deletions occurred in exon 2 and one of them was found in four unrelated patients from different populations, Iranian, Tunisian, Turkish and Indian, prompted us to search for direct repeat DNA, palindromes, and mirror repeat sequences that could lead to hotspots of deletions and promote such mutations using publicly available softwares[19]. However, we did not find any such elements. On the contrary, haplotype analysis revealed that our Turkish and Indian patients and the previously reported Tunisian patient[17] share the

same haplotype around the mutation indicating its inheritance from a common ancestor. Although this finding was surprising, it is not inconceivable because the Ottomans conquered Tunisia in 1574 and assimilated it to their Empire for more than 300 years until the establishment of the French colonization in 1881. Also, geneflow and haplotype sharing through maternal and paternal lineages have been reported between the Iranian, Turkish and Indian populations[20,21].

In our study, we found *KHDC3L* mutations in about 10% of analyzed patients with at least 2 HMs. Our finding is in agreement with those reported by Parry et al. 2011[17] describing *KHDC3L* mutations in 14% of analyzed patients and also confirms that *KHDC3L* is a minor gene responsible for RHMs. In addition, our data demonstrate that *KHDC3L*, at least in our patients, does not play a causative role in other forms of reproductive losses, ≥ 2 RL including 1 HM and ≥ 3 SAs. Analysis of additional cohorts of patients with these conditions will allow validating our observations in other populations.

KHDC3L is the second identified maternal-effect gene in humans after *NLRP7*.[17] Maternal-effect genes are a subset of genes whose products, mRNA and proteins, are coded by the maternal genome and are stored in the oocyte to sustain normal postzygotic development until the activation of the fetal genome. [22] The maternal-effect of *KHDC3L* is supported by the facts that one of our patients, 481, had HMs with three different partners, and a second one, patient 654, had a high rate of postzygotic aneuploidies after two cycles of IVF performed at the age of 32. These data are in agreement with our previous observations in some patients with *NLRP7* mutations [11,23] and suggest that the two genes might have similar or overlapping

functions in early development. This suggestion is corroborated by the sub-cellular localization of endogenous KHDC3L and its co-localization with NLRP7 whose strongest signal localizes to the Golgi apparatus and the MTOC. Others and we demonstrated the requirement of wild type NLRP7 for normal IL-1β secretion by *ex-vivo* LPS-stimulated PBMCs from patients with *NLRP7* mutations and by macrophages in which *NLRP7* was knocked-down using small interfering RNA studies.[15,16] At the present time, we do not know exactly how defects in *NLRP7* cause all the features of HM pregnancies, but we believe that at least two independent events that occur at different times during embryonic development are required for HM manifestation. The first event is an oocyte defect that is leading to an early arrest of the pregnancy before the establishment of a functional fetal vascular system.[24] The second event is the retention of the arrested pregnancy and its delayed rejection by the mother until the end of the first trimester. At this point in time, we do not know what causes the early pregnancy arrest, but we believe that the impaired cytokine secretion by patients' cells prevents them from mounting appropriate inflammatory reaction to timely reject these early arrested pregnancies. The delayed rejection of these conceptions, in which fetal vessels do not form inside the chorionic villi, leads to their hydropic degeneration and HM manifestation. Our proposal is in agreement with a recent case where a patient with two *NLRP7* defective alleles conceived a healthy baby from a donated oocyte.[25] The co-localization of KHDC3L with NLRP7 suggests that KHDC3L may also have a role in the intracellular trafficking and secretion of cytokines that will be interesting to assess in PBMCs from the patients which unfortunately could not be done in this study because of the lack of accessibility to fresh blood samples from our two patients.

To date, the parental contribution to four HM tissues from patients with two defective alleles in *KHDC3L*, three from patient L1[14,26] and one from a second patient[27], were reported to be biparental. In this analysis, we demonstrated that five HMs from one patient are diploid by flow cytometry and have biparental contribution to their genomes. In line with these findings the fact that all characterized HMs to date from patients with two defective *NLRP7* alleles, approximately a total of 45 HMs from 30 patients, have been found diploid biparental and indicate common mechanisms leading to HMs caused by mutations in *KHDC3L* and *NLRP7*.

Hydatidiform moles is a condition known to display wide geographic variations in its frequencies with up to 10-fold increase in the rates of HMs from West to East and a 2-fold increase from North to South (Fig. 4A).[3,4] One of the factors that could explain these gradients is the increased frequencies of some mutations and NSVs predisposing to HMs in Eastern and Southern populations. In line with these data is the presence of the same small haplotype carrying the same mutation in *KHDC3L* in patients of Middle Eastern and Asian origins. Similarly, we previously reported the presence of several founder mutations in *NLRP7* in the Indian, Chinese, and Pakistani populations based on haplotype analyses.[10] Also, we and others previously reported the presence of the same rearrangement and missense in unrelated patients from Egypt and Mexico, respectively.[23,28] Re-reviewing SNPs in the sequenced *NLRP7* amplicons in our patients and in those found in patients with identical mutations, reported by our collaborators, revealed more shared haplotypes, not only in patients from regions with high rate of consanguinity, but also in patients from different populations. For instance, mutation p.Arg693Trp in *NLRP7* was found in seven patients from six different

populations, five of which share the same haplotype 5' of the mutation, and two others share another haplotype (Supp. Table S4). In addition, we previously showed that four *NLRP7* NSVs, associated with recurrent reproductive loss in Europeans, display gradients of increasing frequencies from Europe to sub-Saharan Africa (Fig 4B-E) with the Tunisian population (Mediterranean) having intermediate frequencies.[29] Consistent with these data is the global distribution of the countries of origin of patients with mutations in *NLRP7* and in *KHDC3L* with most of them originating from southern, eastern and Asian countries (Fig 4F). This is further corroborated by the presence of several founder mutations in both *NLRP7* and *KHDC3L*, which is surprising for a condition that prevents the reproduction of women with two defective alleles and indicates some selective advantages for males and/or heterozygous females.

We previously showed that LPS-stimulated PBMCs from patients with *NLRP7* mutations and rare NSVs have low cytokine secretion. We suggested that this will lower the inflammatory response of the patients and make them more tolerant to the growth of aberrant HM conceptions. By the same token, the low inflammatory response of patients with *NLRP7* mutations and NSVs may increase their tolerance for various pathogenic microorganisms and could confer selective advantages for heterozygous carriers that may explain the persistence of old mutations and NSVs across several populations. Our data are in agreement with previous reports showing that mutations and susceptibility alleles for autoimmune diseases such as celiac disease and type 1 diabetes have been maintained in human populations due to past positive selection and adaptation.[30-32] Immune response and reproduction are believed to be the main selective pressures that have shaped human evolution.[30,33] Exploring further the

geographic distribution of variants in these two genes will provide a unique model to dissect evolutionary constraints on genes with roles in both immune responses and reproduction.

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Conflict of interest.

The authors declare no conflict of interest.

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Legends to figures

Figure 1. Pedigree structure of patient 481, reproductive outcomes, mutation analysis, histopathology, and parental contribution to five HMs. (A) Mutation analysis showing a novel 4-bp deletion in a homozygous state in patient 481. (B) The upper row shows the ploidy analysis of five products of conception (POC) from patient 481 showing a single diploid peak in each. Microphotographs showing the histopathology of five HMs and the diagnosis based on independent evaluations performed by two pathologists. From each POC, 4 to 9 different slides were available. HM, indicates hydatidiform mole and is used when the two pathologists did not reach a conclusion as to whether the HM is partial or complete (CHM). Arrows, indicate abnormal circumferential trophoblastic proliferation around chorionic villi (CV). We note that, in general, trophoblastic proliferation in the five HMs was milder than that seen in common sporadic HMs. (C). Genotypes of the five POCs using the PowerPlex® 16 HS System (Promega Corporation) demonstrating the biparental contribution to the five analyzed HMs. Informative alleles showing biparental contribution are shown in bold.

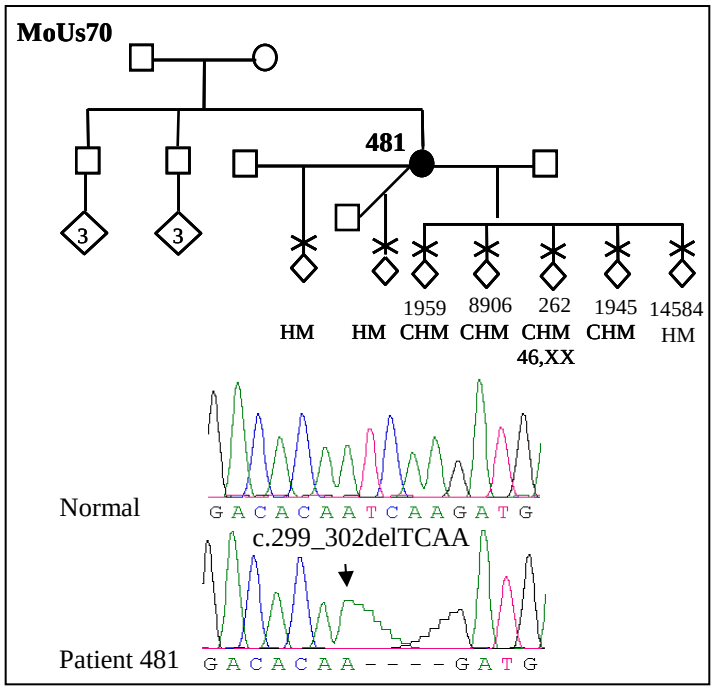
Figure 2. Pedigree structure, reproductive outcomes, and *KHDC3L* mutation analysis of one singleton (case MoTu98, patient 654) and one familial case of RHMs (MoIn355, patients 1094 and 1096). In both cases, a previously described mutation was identified. IVF, stands for *in-vitro* fertilization; ET, embryo transfer; PGS, pre-implantation genetic screen**.**

Figure 3. Sub-cellular localization of KHDC3L and co-localization with NLRP7. (A-D) Simultaneous revelation of GFP-tagged wild-type *KHDC3L* (GFP-wt*KHDC3L*) construct with GFP signal (green) and KHDC3L C-terminal (C-term) antibody (Sigma, prestige) (red) in transfected HEK293T cells showing that the antibody recognizes the exogeneous protein found close to the nucleus. DNA was counterstained with DAPI (blue). (E-F) Absence of peri-nuclear staining in HEK293T cells transfected with the empty vector carrying GFP. (G-I) KHDC3L C-term antibody detected a juxta and peri-nuclear signal that co-localizes with NLRP7 (green) in EBV-transformed cells from a control subject. (J-L) A similar signal that co-localizes with NLRP7 was also detected by another N-terminal (N-term) KHDC3L antibody (Abgent). (M-X) Presence of KHDC3L signals with the N-term antibody and absence of signals with the KHDC3L C-term antibody in EBV-transformed cells from patients 481 and 654 with protein truncation at positions 100 and 108 amino acids, respectively. The truncated proteins in both patients co-localize with NLRP7 (M-O and S-U).

Figure 4. Global distribution of frequencies of hydatidiform moles, four *NLRP7* non-synonymous variants, and countries of origin of unrelated patients with mutations in *KHDC3L* and *NLRP7* genes. (A) Epidemiology of HMs. Countries with low incidences are in white, countries with middle incidences (2-fold increase) are in yellow, and those with high incidences (up to 10-fold) are in red. (B-E) Distribution of the minor allele frequencies of four non-synonymous variants in *NLRP7* according to data from the 1000 Genomes database. Ancestral alleles are indicated by their colors. (F)

609 Distribution of the countries of origin of patients with *NLRP7* (black dots) and
610 *KHDC3L* (red dots) mutations.

A

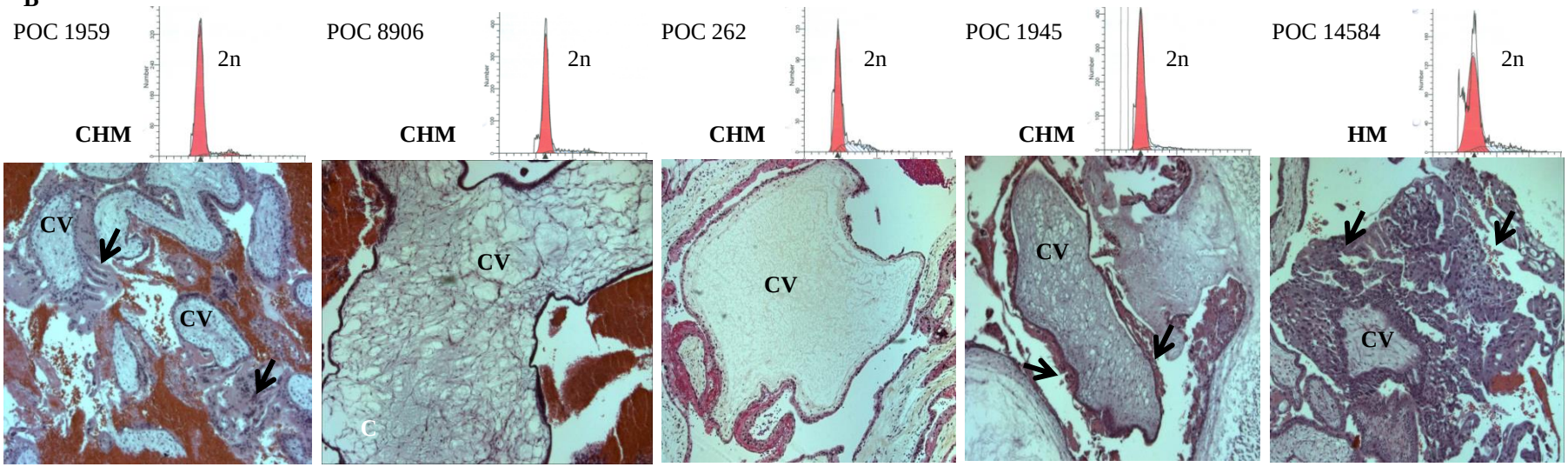


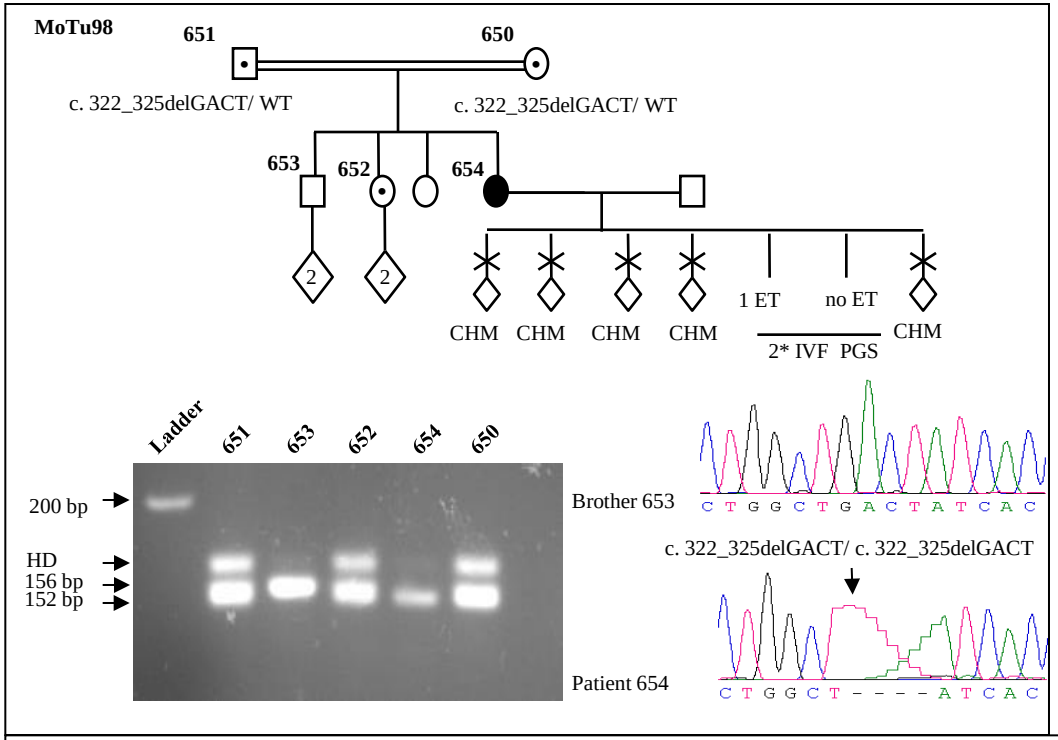
C

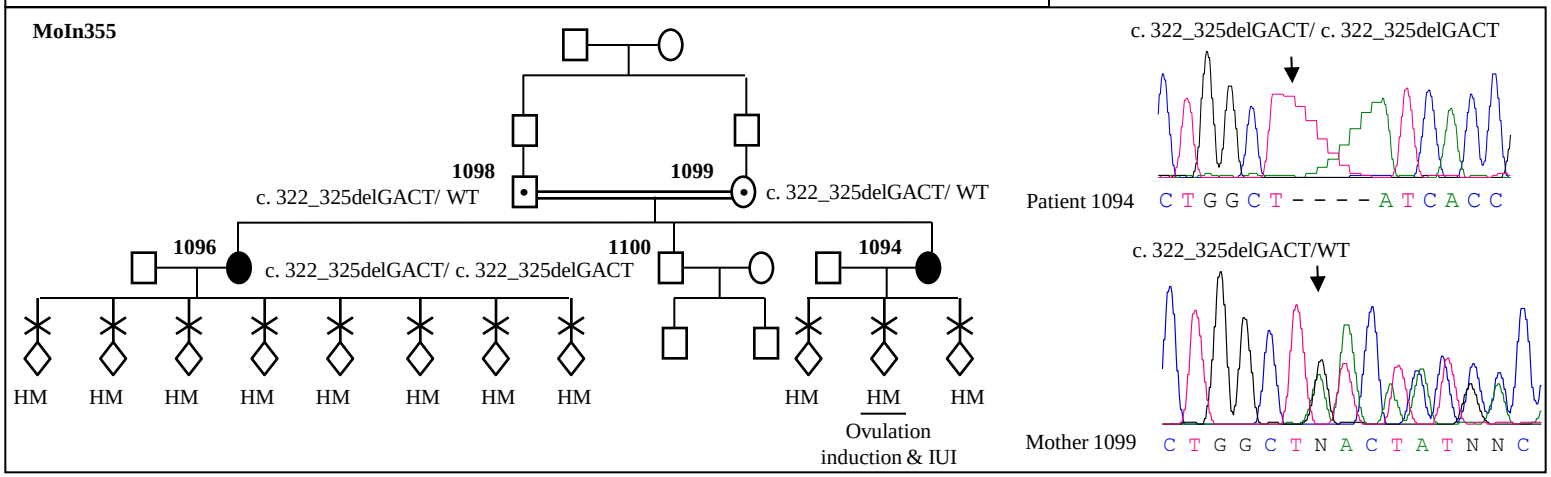
Locus	Chr.	Patient 481	POC 1959	POC 8906	POC 262	POC 1945	POC 14584
D18S51	18	310/314	n.a	**310/318**	n.a.	n.a	**314/318**
D21S11	21	218/230	218	**218/214**	**218/214**	230/218	230/218
TH01	11	165/169	165	**165/177**	**165/177**	169/165	**169/177**
D3S1358	3	126/138	**126/122**	126/138	126/138	138	126/138
FGA	4	350/353	n.a.	350/353	350/353	n.a	350/353
TPOX	2	272/276	n.a.	272	272	n.a	272
D8S1179	8	234/238	234/238	**238/230**	**234/230**	**238/230**	**234/230**
AMEL	X/Y	107	**107/113**	**107/113**	107	107	**107/113**
Penta E	21	414/419	n.a.	419/419	n.a.	n.a	n.a.
CSF1PO	5	338	**338/342**	338	n.a.	n.a	n.a.
D16S539	16	282/290	282/290	**290/298**	282/290	n.a	**290/298**
D7S820	7	223	**223/231**	**223/231**	223	223	**223/231**
D13S317	13	195	**195/191**	195	195	195	195
D5S818	5	137/141	137/141	137	137/141	137	141

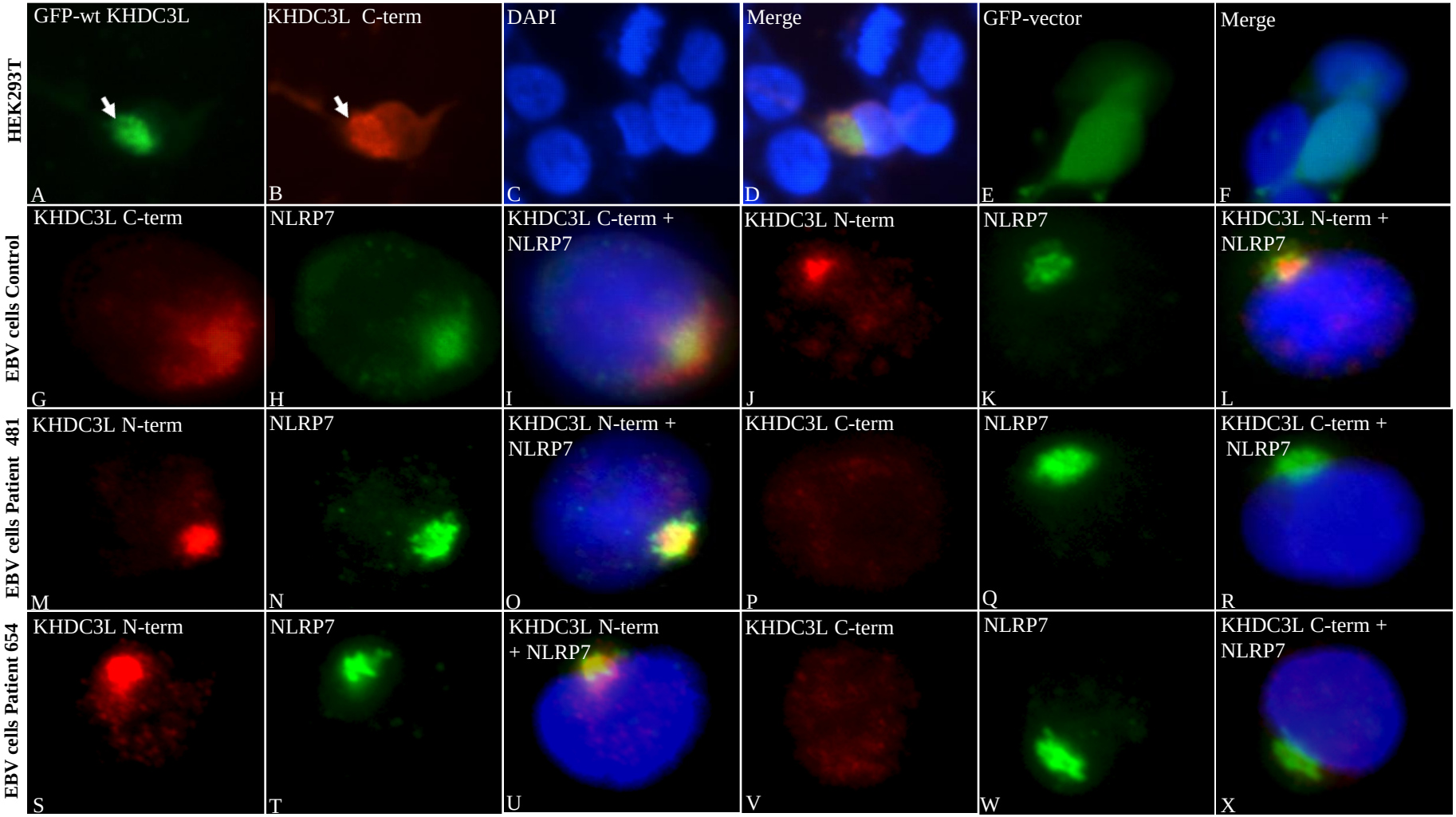
Chr. stands for chromosome; n.a. stands for not available.

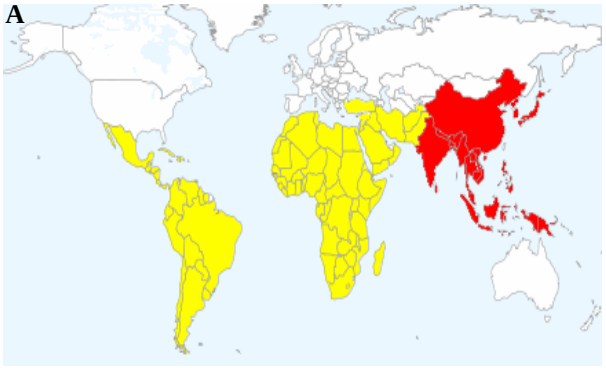
B



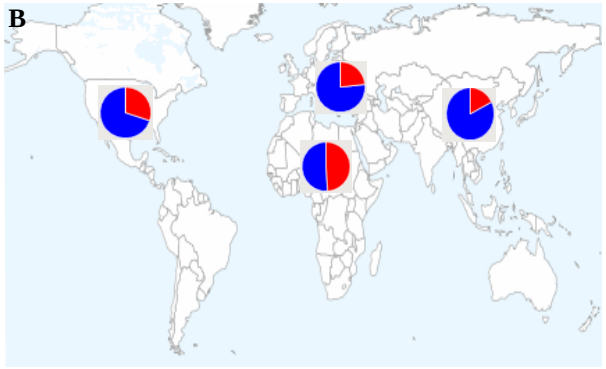

MoTu98
651
650
c. 322_325delGACT/ WT
c. 322_325delGACT/ WT
653
652
654
2
2
CHM
CHM
CHM
CHM
1 ET
no ET
CHM
2* IVF PGS
Ladder
651
653
652
654
650
200 bp
HD
156 bp
152 bp
Brother 653
C T G G C T G A C T A T C A C
c. 322_325delGACT/ c. 322_325delGACT
C T G G C T - - - - A T C A C
Patient 654


MoIn355
1098
1099
c. 322_325delGACT/ WT
c. 322_325delGACT/ WT
1096
c. 322_325delGACT/ c. 322_325delGACT
1100
1094
HM
HM
HM
HM
HM
HM
HM
HM
HM
HM
HM
Ovulation
induction & IUI
c. 322_325delGACT/ c. 322_325delGACT
Patient 1094
C T G G C T - - - - A T C A C C
c. 322_325delGACT/WT
Mother 1099
C T G G C T N A C T A T N N C

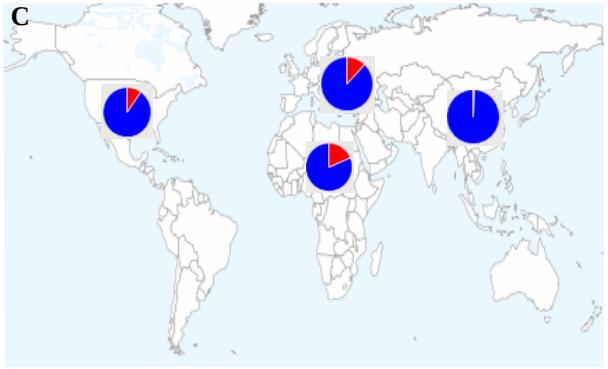

HEK293T
EBV cells Control
EBV cells Patient 481
EBV cells Patient 654
GFP-wt KHDC3L
KHDC3L C-term
DAPI
Merge
GFP-vector
Merge
A
B
C
D
E
F
KHDC3L C-term
NLRP7
KHDC3L C-term + NLRP7
KHDC3L N-term
NLRP7
KHDC3L N-term + NLRP7
G
H
I
J
K
L
KHDC3L N-term
NLRP7
KHDC3L N-term + NLRP7
KHDC3L C-term
NLRP7
KHDC3L C-term + NLRP7
M
N
O
P
Q
R
KHDC3L N-term
NLRP7
KHDC3L N-term + NLRP7
KHDC3L C-term
NLRP7
KHDC3L C-term + NLRP7
S
T
U
V
W
X



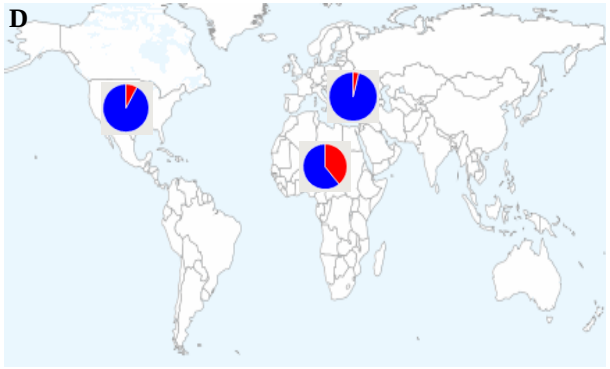
Epidemiology of HMs



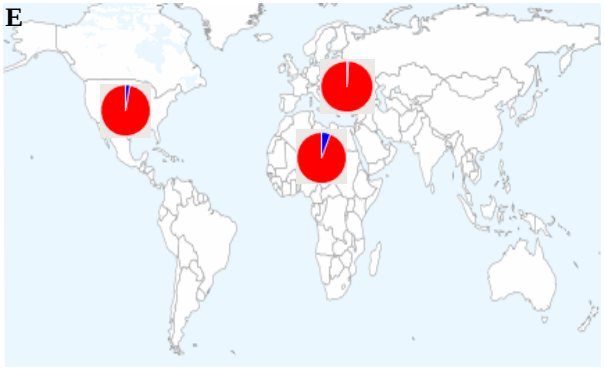
V319I, SNP rs775882, ancestral allele C



A481T, SNP rs61747414, ancestral allele C



G487E, SNP rs775881, ancestral allele T



K511R, SNP rs61743949, ancestral allele T

Distribution of *NLRP7*and *KHDC3L* mutations