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The role of Matriptase-2

during the early postnatal development in humans

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The hepatic hormone hepcidin is a key regulator of systemic iron homeostasis. It limits both iron absorption from the intestine and iron release from macrophage stores by binding to ferroportin and triggering its internalization and degradation. Hepcidin expression is modulated in response to several physiologic and pathologic stimuli, which include systemic iron loading, erythropoietic activity and inflammation.[1] A type II transmembrane serine protease matriptase-2 (MT-2, encoded by TMPRSS6) was identified as a repressor of hepcidin expression acting through interruption of BMP6/HJV/SMAD signaling.[2-4] Thus, by downregulating hepcidin gene expression, MT2 controls iron availability to avoid systemic iron deficiency.[5,6]

Mutations in TMPRSS6 result in clinical phenotype of iron deficiency iron refractory anemia (IRIDA)[7] characterized by hypochromic microcytic anemia, low transferrin saturation and inappropriate normal/high levels of hepcidin. Up to now 69 different mutations in the *TMPRSS6* gene have been reported in 65 IRIDA families with patients of different ethnic origin. [8,9]

Even considering all cases published so far, experience with the natural history of IRIDA patients is limited, and data are not reported for the neonatal period. Thus, from the clinical histories, it remains unknown whether IRIDA patients are already iron-deficient at birth.

The aim of this study is to clarify whether matriptase-2 has a role in human fetuses/neonates for a better understanding of iron homeostasis during early development.

Four families (with seven probands) were collected whose pedigrees are shown in Figure 1. Results on ethnic origin, clinical, genetic and laboratory tests are shown in *Online Supplementary Table S1*. These studies were approved by the institutional review board of Federico II University Medical School in Naples and conducted in accordance with the Declaration of Helsinki.

The conditions of DNA extraction, polymerase chain reaction, and sequence analysis used were standard. All exons, exon–intron boundaries and a varying amount of the 5' and 3' flanking sequence of the *TMPRSS6* gene were examined using fluorescent chain-terminator cycle sequencing. Detailed protocols and primer used for sequencing sequences are available in *Online Supplementary Method*s.

Serum hepcidin was measured by SELDI-TOF-MS (*see Online Supplementary Methods*).

We identified seven patients from four unrelated families homozygous or compound heterozygotes for mutations in TMPRSS6 gene. Mutations were either missense or frameshift and two were novel (*Online Supplementary Table S1*). Details about the mutations described are in *Online Supplementary Methods*. Three families were of Turkish origin, one was Kurdish, consanguinity was reported in three. All patients displayed the characteristic phenotype of IRIDA, with hypochromic, microcytic anemia, low transferrin saturation and normal/high serum hepcidin values. Anemia in all probands was first diagnosed in infancy. During follow up most of them required iron

treatment, were unresponsive to oral iron and showed only a partial response to parenteral iron administration (*Online Supplementary Table S1*). Thus, the diagnosis of IRIDA occurred during early childhood, confirming that the condition is not recognized until a routine laboratory screening, because of the normal growth and development of the affected individuals.[9] Moreover we diagnosed two adult IRIDA patients (CII2, CII3 in Figure 1). They had indeed a history of refractory anemia in spite of oral iron therapy. CII2 had a history of oral iron supplementation only during pregnancy and her molecular diagnosis of IRIDA was made only during the investigation of her son (CIII1).

For four probands (AII3, BII3, CIII1 and DII1) we succeed in collecting the complete blood count (CBC) performed in the first days of life (Table 1). Furthermore none of the probands had infections during the time in which CBC samples were taken. As reported in Table 1 patients showed normal-borderline hemoglobin (Hb), normal mean corpuscular volume (MCV) and normal mean corpuscular hemoglobin (MCH) indicating that the phenotype of IRIDA was not present at birth. Unfortunately iron parameters (serum iron, transferrin saturation and serum ferritin as well as hepcidin levels) were not performed, since not required for healthy neonates. Normal erythrocyte morphology was documented also by examination of the peripheral blood smear in patient AII2 at 2 days of life (Figure 2B). These findings, along with reports of normal birth weights for all these patients, suggest that in utero iron transfer was normal, with the depletion of iron stores occurring only after birth. In Figure 2A we showed a time course of hematological findings (Hb, MCV, MCH and MCHC) of proband AII2 in whom an IRIDA phenotype appeared at 2 months of life.

Suspicion of IRIDA usually occurs during a routine pediatric evaluation. However, in some patients, the condition is recognized only in adulthood, either because anemia is mild or because it has been misclassified.[9] Remarkably, despite congenital and severe iron deficiency, long-term follow-up of the affected subjects has shown normal growth and intellectual development with no evidence of the cognitive concerns on which iron deficiency screening in infancy have been founded. In healthy fetuses and neonates in mice, because of the rapid growth and expansion of the red cell compartment, hepcidin gene expression is drastically repressed.[10] Very recently, Willemetz *et al* demonstrated that in *Tmprss6*-/- fetuses, liver *Hamp1* mRNA expression was up to 60 times higher compared with control mice, in which hepcidin expression was only barely detectable.[11] Noteworthy, *Tmprss6*-/- fetuses and new-borns had a lower iron content, mean corpuscular erythrocyte volume (MCV) and hemoglobin (Hb), indicating microcytic anemia secondary to iron deficiency in mutant mice. These observations suggest that, at variance with humans, in mice Mt2 is required for hepcidin repression during fetal and postnatal development, and its deficiency leads to

a microcytic anemia in utero and at birth, with persistence into the adulthood. Moreover female Tmprss6 homozygote knockout mice are infertile, reflecting yet another difference in the phenotypes of humans versus mice with Tmprss6 deficiency.[5] So despite animal models provide a useful genetic model for the analysis of molecular mechanisms that underlie human hematologic disorders, the different IRIDA phenotypes at neonatal period, as well as the rescue of anemia and alopecia in *Tmprss6* mutant mice by iron administration, confirm that the iron regulation and the pathophysiology of the disorder in humans are more complex.

Our findings are further supported by our recent publication on a Turkish female infant who had a molecular diagnosis of IRIDA identified through a family screening at the age of 3 months before she developed an overt IRIDA phenotype.[12] At birth her weight was appropriate for the gestational age. The follow-up data of the same infant were later reported, showing that a typical IRIDA phenotype became evident at 4-months of age.[13]

In this paper we describe for the first time the hematological parameters in the neonatal period of four IRIDA patients (Table 1). Data indicate that anemia is not present in utero and at birth and develops during the first months of life. In full-term infants the iron stores, released during the hemolysis of senescent RBCs, support the iron needs of the expanding erythropoiesis and growth until 4-6 months of age[14] when the clinical IRIDA phenotype usually became evident. Follow up of the patient AII3, from the birth until 18 months confirms this observation (Figure 2A). Indeed clinical phenotype in the proband manifests after two months of life and this probably depends on different genotype or other environmental factors. Maternal iron status accounts for only 6% of the variation in infant iron stores at birth, and the remaining causes of the highly variable size of the birth endowment are not known, but likely include low birth weight, intrauterine growth retardation, prematurity, time of cord clamping, maternal smoking, and diabetes in pregnancy.[15] A proof of concept is that maternal-fetal iron delivery in IRIDA probably is not inadequate, as demonstrated by the normal findings in CIII1 who had an affected mother. Moreover this patient (CII2) was the first reported case of a pregnancy of an affected IRIDA woman. Of note because iron homeostasis develops during the period of 6-9 months, MT2 is not essential in hepcidin regulation.[15]

In conclusion, our study indicates that in humans MT2 is dispensable during fetal life tempting to speculate that another hepcidin suppressor is produced during fetal development or hepcidin is overexpressed, but because humans are typically born with substantial iron stores, the overexertion of hepcidin is insufficient to actually cause iron deficient erythropoiesis at the time of birth. Due to the nature of the disease the reported numbers are very low, but our results could help the better understand the role of TMPRSS6 and definitely highlights the differences between men and mice.

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Authorship Contributions

LDF and AI designed and conducted the study, and prepared the manuscript; MBr performed sequencing analysis and contributed to critical review of the manuscript; EY-K, ES, MB, ZK carried out patient ascertainment and recruitment; DG performed hepcidin dosage**.**

Conflict-of-Interest Disclosure

The authors declare no competing financial interests.

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Table 1. Hematological parameters of IRIDA patients in the neonatal period.

					Reference values					
					1-3 days		1 week		2 weeks	
	A II3	B II3	C III1	D II1	mean	±SD	mean	±SD	mean	±SD
Hb, g/dL	13.8	12.5	17.3	13.8	17.3	1.9	16.7	2.2	15.9	1.9
MCV, fL	102	106.8	111.3	101.8	109.1	4.8	107.7	6.2	106.4	3.9
MCH, pg	34.8	33.3	36.1	34.3	34.1	1.3	33.8	1.9	33.7	1.5
MCHC, g/dL	33.8	31.2	32.4	33.7	31.3	0.9	31.6	1.3	31.7	0.9
RDW, %	14.9	15.6	20.4	17.4	16.2	1.1	15.6	1.1	15.5	0.9
RBC, $*10^6$/µL	3.9	3.3	4.8	4.02	5.1	0.6	4.9	0.7	4.7	0.6
WBC, $*10^3$/µL	15.7	14.5	16	6.7	11.8	3.2	10.8	2.5	11	2.4
PLT, $*10^3$/µ/L	452	420	236	301	287	88	306	101	420	122

Data were collected from records for A II3 at 2 days, B II3: 2 weeks, C III1: 1 day, D II1: 1 week; reference values of 204 healthy, term neonates were collected by Dr Yilmaz-Keskin at Samsun Education and Research Hospital in Samsun, Turkey. Details in Supplementary Table 3. CBC testing has been performed by the device Mindray BC-6800 for CBC testing. Birth weight was within normal range for all probands except for C III1 who was prematurely born.

Figure Legends

Figure 1. Family pedigree of the affected subjects. *TMPRSS6* mutations identified by automated sequencing are displayed under the pedigree: open symbols, not affected; closed symbols, affected; the half-filled black symbols denote unaffected carriers. Mutations are indicated for each family, mutations in grey have been previously reported. BI1 was not available for genetic studies. The probands are indicated with an arrow.

Figure 2. Hematological parameters of patients AII3 during postnatal development. Time course of hematological findings of proband AII3 in the perinatal period (A). On x-axis are reported hematological data hallmarked of IRIDA phenotype: hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), on y-axis is reported patient age. Horizontal grey bars indicate normal values; Peripheral blood smears of proband AII3 at 2 days (upper) and 18 months (below) of life (B). At 2 days there are no signs of hypochromic microcytic anemia, at 18 months peripheral smear show hypochromic cells.

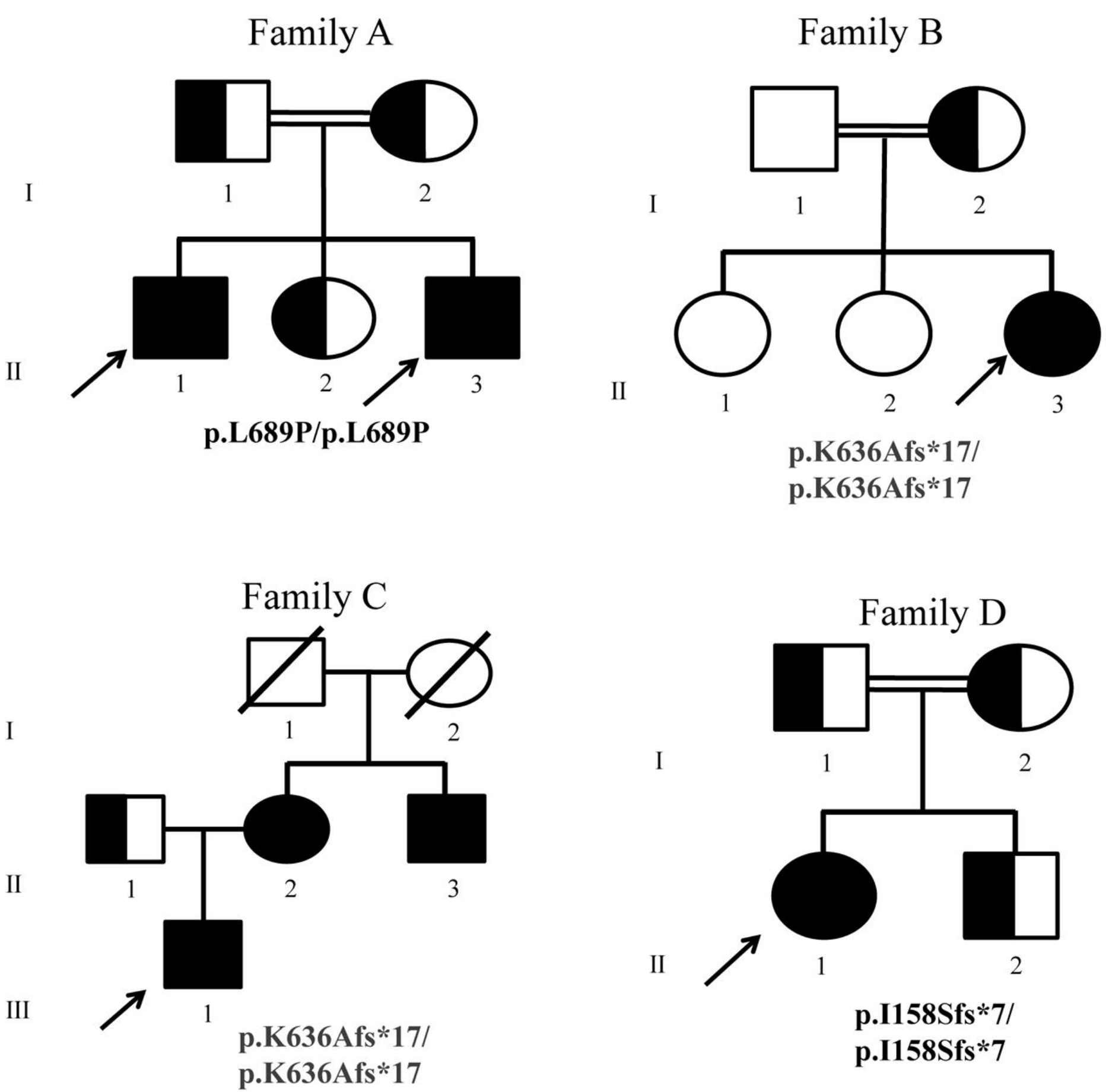
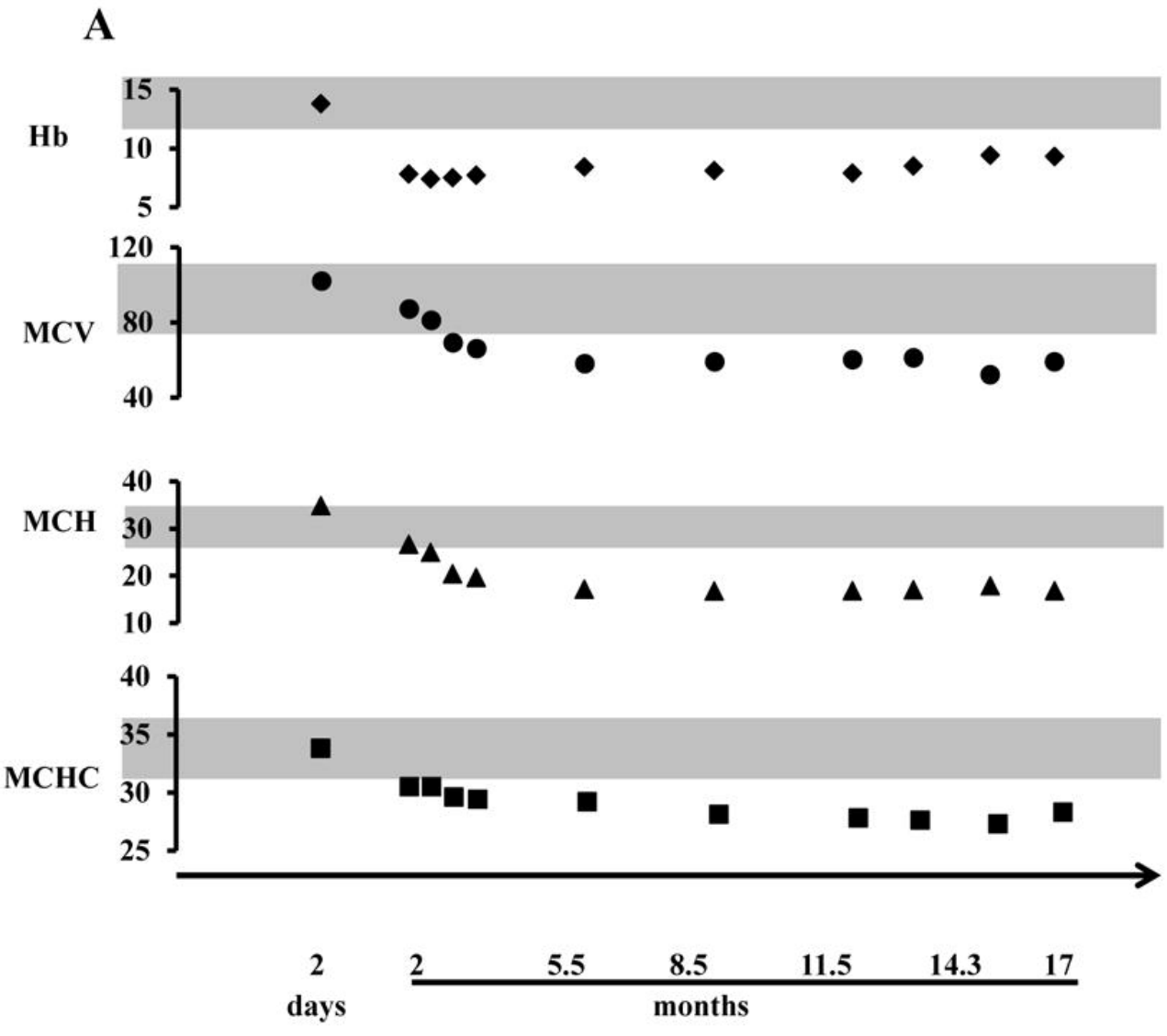
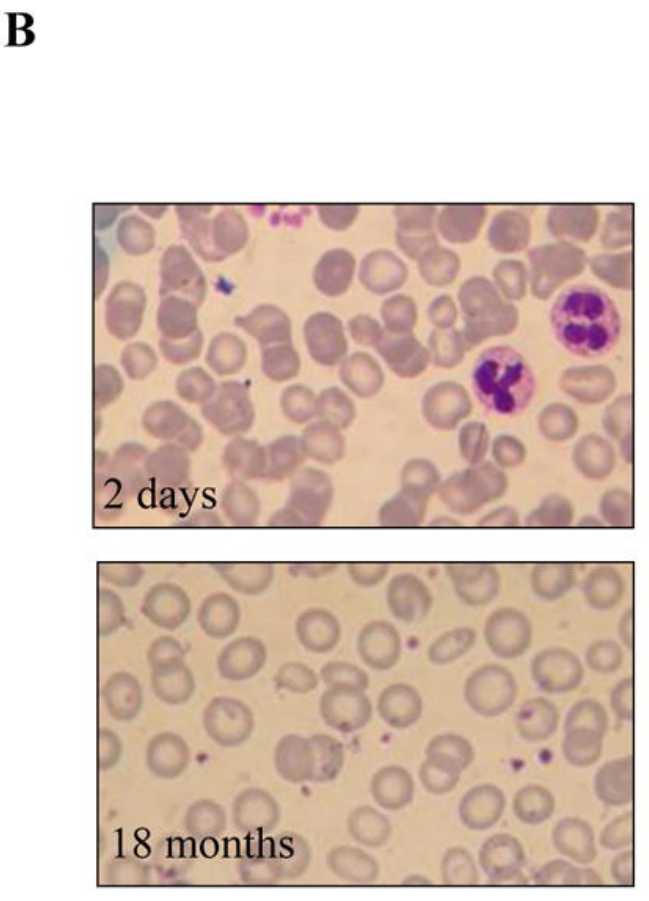

Family A
I
1
2
II
1
2
3
p.L689P/p.L689P
Family B
I
1
2
II
1
2
3
p.K636Afs*17/
p.K636Afs*17
Family C
I
1
2
II
1
2
3
III
1
p.K636Afs*17/
p.K636Afs*17
Family D
I
1
2
II
1
2
p.I158Sfs*7/
p.I158Sfs*7

Figure 2


A
Hb
15
10
5
MCV
120
80
40
MCH
40
30
20
10
MCHC
40
35
30
25
2
days
2
5.5
8.5
11.5
14.3
17
months


B
2 days
18 months

Supplementary Methods

DNA Sequence Analysis

Anticoagulated (EDTA-treated) blood samples were obtained and stored at -20°C. Genomic DNA was isolated by the QIAmp DNA Blood Mini Kit (Promega Corporation, Madison, WI), according to the manufacturer's instructions.

To analyze *TMPRSS*6 gene all coding exons and splice junctions were amplified by PCR and amplified fragments were directly sequenced. The *TMPRSS6* genomic sequence from GenBank accession numbers NC_000022.9 was used as reference sequence. Detailed protocols and primer sequences are available on request.

The amplified products were isolated by electrophoresis on 1% agarose gel and purified using the QIAamp purification kit (Qiagen, Valencia, CA). Direct sequencing was performed using a fluorescence-tagged dideoxy chain terminator method in an ABI 3100 automated sequencer (Applied Biosystem, Foster City, CA), according to the manufacturer's instructions.

Hepcidin assay

Serum hepcidin was measured by means of a recently validated mass spectrometry-based approach, i.e. SELDI-TOF-MS using a PBSCIIc mass spectrometer, copper loaded immobilized metal-affinity capture ProteinChip arrays (IMAC30-Cu2+), and a synthetic hepcidin analogue (hepcidin-24, Peptides International, Louisville, KY) as an internal standard, as described in detail elsewhere.[1,2]

Bioinformatic Prediction Methods

Prediction of possible impact of amino acid substitution on TMPRSS6 protein was done using the commonly used and previously published software SIFT version 4.0.3 (http://sift.jcvi. org)[3] and PolyPhen-2 version 2.2.2 (http:// genetics.bwh.harvard.edu/pph2/)[4] using default parameters. Multiple sequence alignment of TMPRSS6 protein (MT-2) in several species was done using ClustalOmega software using default parameters (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Results

Sequencing analysis of TMPRSS6 gene in 7 IRIDA patients from 4 unrelated families revealed 4 mutations, two were novel: one missense (p.L689P); one frameshift (p.I158Sfs*7) (Table 1S and Figure 1). None of the novel variants has been previously reported in the examined databases (ENSEMBL: http://www.ensembl.org/, NCBI dbSNP: http://www.ncbi.nlm.nih.gov/SNP/, 1000Genomes: http://browser.1000genomes.org/). In two Turkish families with four patients (Table

1S) we identified the same homozygous duplication leading to a frameshift and a pre-mature stop codon (c.1904_1905dupGC, p.K636AfsX17). This mutation has been previously reported in other four unrelated families of Turkish origin at homozygous state yet.[5,6] Hb levels in patients carrying the missense variant L689P are higher compared to patients with frameshift mutations in the TMPRSS6 gene, probably confirming a more clinical severe phenotype for the patients with two frameshift mutations compared to patients with two missense mutations.[7]

Bioinformatic Prediction

The novel missense substitution is bioinformatically predicted to be damaging or deleterious according to two commonly used programs (SIFT and PolyPhen-2). In addition, a multiple sequence alignment of MT-2 proteins among 24 species shows that this mutation is highly conserved through evolution (identical amino acid in 100% of the sequences; Supplementary Figure S1).

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Supplementary Table 1: Clinical, genetics and laboratory data and response to iron treatment of IRIDA patients.

	A II1	A II3	B II3	C II2	C II3	C III1	D II1				
Age at diagnosis, years/sex	6/M	1.5/M	0.75/F	26/F	17/M	3.25/M	1/F				
Genotype	p.L689P/ p.L689P	p.L689P/ p.L689P	p.K636Afs*17/ p.K636Afs*17	p.K636Afs*17/ p.K636Afs*17	p.K636Afs*17/ p.K636Afs*17	p.K636Afs*17/ p.K636Afs*17	p.I158Sfs*7/ p.I158Sfs*7	**Normal values adults (range)**		**Normal values children[b]**	
Consanguinity, Y(es)/N(o)	Y	Y	Y	N	N	N	Y	**M**	**F**	**Mean**	**- 2SD**
Hb, g/dL	9	9.4	6.2	11.5	9.18	7.9	7.2	12.0-17.5	12.0-16.0	12.5	11.5
MCV, fL	52	52	54.9	80.9	57.8	52	53	80-97		81	75
MCH, pg	16.6	17.8	15.4	27.5	17.1	15	15	25-34		33	31
MCHC, g/dL	32	34.3	28.1	33.9	29.6	29.5	28	32-37		34	31
Plt, *10^3/µl	69.1	43.9	42	26.4	46.6	53.9	32.9	130-400		150	303
WBC, *10^3/µl	13.6	10	6.8	5.5	5.4	7.4	6	4.8-10.8		11.4-9.1[c]	5.5-17.5[c]
Reticulocytes count, *10^3/µl	43.2	36.4	37	n.a.	53.7	70	46.4	20- 120		n.a.	n.a.
RBC, *10^6/µl	5.4	5.2	4.07	4.17	5.37	5	5.8	4.2- 5.6	4.0-5.4	4.6	3.9
Ferritin, µg/L	44.2	59.8		135	9.44	11.7	76	18-370	9-120	6	24
Serum Fe, µg/dL	11	13	17	14	9	10	12	16-124		22	136
Transferrin saturation, %	3.9	5.1	6.1	5	2	2	4	15- 35		7	44
Serum Hepcidin[a], nM	7.03	14.53	8.02	4.5	7.3	11	9.1	3-7		2 ± 2.6	
Iron Treatment/ Responce	Oral (No)	Oral (No)	Oral (No) and parenteral (No)	Oral iron during pregnancy	Oral (No)	Oral (No)	Oral (No)/parenteral (Partial)				

[a] Reference range- adults: n=57 normal individuals (median 4.7)
Reference range- children: mean +/- SD (range) = 2 ± 2.6 (0.55-11.3) nM
* Values in iron deficiency anemia are 0,04-0,12 nM.

[b] Reference values reported from Nathan and Oski's, Hematology of infancy and childhood, Nathan DG, Orkin SH, Ginsburg D, Look AT, VI edition.

[c] WBC ranges for children aged 1-5 years

Supplementary Table 2: Time course of iron indices for A III1 proband.

Age (months)	Serum iron (µg/dL)	Transferrin saturation (%)	Serum ferritin (ng/mL)
2.5	**27**	**8**	**238**
3	**16**	**4**	**140**
8.5	**10**	**3**	**154**
12.5	**16**	**6**	**97**
14.3	**13**	**5**	**59**

Reference values from Nathan and Oski's, Hematology of infancy and childhood, Nathan DG, Orkin SH, Ginsburg D, Look AT, VI edition: Serum Ferritin (ng/mL): 1-6 months: male: 6-410; female: 6-340, 7-12 months: male: 6-80; female: 6-45, 1-6 years, male/female: 6-24; Serum Iron (µg/dL) :1-6 years, male/female: 22-136; Transferrin saturation (%), male/female: 7-44.

Supplementary Table 3: Reference values of healthy, term neonates.

Healthy Controls	WBC,* 10^3/µl	Hb, g/dl	RBC, *10^6/µl	MCV, fl	MCHC, g/dl	MCH, pg	RDW, %	PLT, 10^3/µl	AGE (days)	Sex
1	16230	16.9	5.2	104.8	31	32.4	16.9	450000	2	M
2	10960	18.3	5.51	108	30.7	33.2	18	302000	2	M
3	10960	18.2	5.76	104.1	30.3	31.6	16.7	110000	2	M
4	11400	18.9	5.65	106.9	31.2	33.4	17.2	286000	3	M
5	10250	19.4	5.43	116.8	30.6	35.8	16.2	292000	3	M
6	10060	16.7	4.57	116	31.5	36.6	15.9	277000	3	M
7	16950	13.9	4.08	104.9	32.4	34	14.3	327000	3	M
8	10220	18.5	5.32	107.8	32.3	34.8	15.8	329000	3	M
9	10640	14.9	4.43	104.5	32.1	33.5	15.2	317000	3	M
10	10380	20	6.16	108.1	30	32.5	17.8	246000	3	M
11	8690	17.2	4.78	112.9	31.8	35.9	15	232000	3	M
12	10270	13	4.03	99.1	32.5	32.2	16.4	295000	3	M
13	7970	16.5	4.74	114.9	30.3	34.9	16.8	67000	3	M
14	8030	14.3	3.68	124.4	31.3	30.3	12.8	289000	4	M
15	10410	19.5	5.86	111.5	29.8	33.2	16.3	202000	4	M
16	9290	17.1	5	108.5	31.5	34.1	16	209000	4	M
17	15160	17.3	5.07	109	31.3	34.2	15.6	309000	4	M
18	11380	17.7	5.18	111	30.8	34.2	15.2	129000	4	M
19	9060	18.7	5.48	113.9	30.1	34.2	16.4	255000	4	M
20	8290	16.2	4.92	110.4	29.8	32.9	15.6	365000	4	M
21	10400	18.7	5.76	101.9	31.9	32.5	14.4	239000	4	M
22	9740	17.3	4.88	109.6	32.4	35.5	16	256000	4	M
23	8520	8.7	2.95	88	33.4	29.4	14.4	318000	4	M
24	9560	18.7	5.02	114.7	32.5	37.3	15.6	224000	4	M
25	9540	18.8	5.73	109.3	30	32.8	16.7	86000	5	M
26	11870	18.3	5.47	104.9	31.8	33.4	15.3	305000	5	M
27	8060	18.4	6.34	98.2	29.5	28.9	20	275000	5	M
28	10690	14.2	4.12	107	32.1	34.3	14.2	276000	5	M
29	12160	17.2	5.16	107.4	31	33.3	15.4	372000	5	M
30	8920	18.6	4.86	110.7	34.6	38.3	15.5	272000	5	M
31	11270	18.2	4.9	110.8	33.4	37.1	15.6	418000	5	M
32	9540	19.7	5.69	110.2	31.5	34.7	16.7	313000	6	M
33	8560	14.5	4.33	105.8	31.7	33.5	16.7	92000	6	M
34	10800	11	3.04	110.9	32.5	36.1	15.9	477000	6	M
35	14250	13.7	4.06	107.4	31.5	33.8	14.5	319000	6	M
36	11850	20.9	6.37	110.7	29.6	32.8	16.4	170000	6	M
37	12700	13.5	4.11	102	32.2	32.9	14.9	543000	6	M
38	8110	16.6	4.81	105.6	32.6	34.4	15.1	305000	6	M

39	10920	17.7	5.03	112.2	31.4	35.3	16.7	240000	7	M
40	11450	17.7	5.66	95.4	32.8	31.2	14.2	428000	7	M
41	17480	15.7	4.57	106.9	32.2	34.4	14.8	473000	7	M
42	13470	19.1	5.42	110.5	31.9	35.3	16.7	189000	7	M
43	10450	14.7	4.47	101.6	32.4	32.9	15.2	385000	7	M
44	10840	16	4.94	104.1	31.2	32.4	17.2	264000	7	M
45	11770	12.8	4.13	101.3	30.5	31	16.5	572000	7	M
46	9910	19.5	5.52	113.5	31.1	35.3	16	196000	8	M
47	12910	16.6	4.9	106.9	31.7	33.9	16	352000	8	M
48	12860	16.7	5.07	105.8	31.2	33	16.7	252000	8	M
49	9390	15.8	4.38	112.1	32.3	36.2	15.2	466000	9	M
50	11180	14.7	4.18	112.5	31.3	35.3	14.5	197000	9	M
51	12090	14.9	4.26	106.7	32.8	35	15.4	412000	9	M
52	18910	17.5	5.09	110.8	31	34.4	15.8	447000	9	M
53	10390	14.5	4.24	106.2	32.1	34.1	14.5	488000	9	M
54	9590	18.5	5.26	108.8	32.3	35.2	16.3	352000	9	M
55	10640	16.2	5.08	103.5	30.8	31.8	15.4	348000	10	M
56	10600	14.5	4.24	108.2	31.6	34.2	13.9	277000	10	M
57	12210	17.1	4.99	110.7	30.9	34.2	14.6	700000	10	M
58	9110	16.2	5.04	102	31.6	32.2	15.3	454000	10	M
59	8640	15.2	4.53	104.9	32	33.6	14.2	251000	11	M
60	9560	15.9	4.54	107.4	32.5	35	15.5	353000	11	M
61	17080	15	4.56	103.6	31.7	32.9	16.1	331000	11	M
62	9510	19.3	5.67	108	31.5	34	16	387000	11	M
63	10750	16.8	5.02	105.1	32	33.6	16.4	226000	11	M
64	9690	14.7	4.48	107	30.7	32.9	15.5	357000	12	M
65	14160	16.9	5.18	103.3	31.5	32.5	16.5	294000	12	M
66	9740	17	5.21	106.7	30.6	32.7	16.9	296000	12	M
67	14880	16.4	4.69	110	31.7	34.9	15.7	369000	13	M
68	3750	17.2	4.92	109.2	32	35	15.4	360000	13	M
69	8510	14.9	4.58	101.8	31.9	32.4	16	481000	14	M
70	12600	15.5	4.68	105.5	31.4	33.1	15.5	332000	15	M
71	9900	14.1	4.13	104.2	32.8	34.2	14.9	541000	15	M
72	10760	12.3	3.57	101.2	33.9	34.3	14.9	496000	15	M
73	10710	19.6	5.97	106.3	30.8	32.8	15	419000	15	M
109	11770	16.9	5.02	109.6	30.6	33.6	15.9	310000	2	F
110	19830	20	6.1	108.5	30.2	32.7	16.7	260000	2	F
111	10280	18.5	5.59	110.8	29.9	33.1	16.7	228000	2	F
112	15250	15.1	4.56	104.1	31.8	33.1	15.9	297000	2	F
113	11090	16.8	4.81	111	31.5	34.9	15.8	368000	2	F
114	9890	17.3	4.94	113.2	30.9	35	15.7	514000	3	F
115	15760	17.9	5.38	108.3	30.7	33.2	15.7	285000	3	F
116	15860	15.1	4.15	120.7	30	36.2	15.9	337000	3	F

117	13040	18.1	5.23	110.8	31.2	34.6	19.4	298000	3	F
118	17510	16.9	5.04	109.3	30.6	33.5	15.3	416000	3	F
119	7750	14.1	4.1	108.7	31.7	34.4	14.8	352000	3	F
120	11750	18.4	5.23	110.8	31.7	35.1	15.2	256000	3	F
121	6600	16.4	4.77	110.8	31	34.4	15.2	198000	3	F
122	14200	18.1	5.6	99	32.7	32.4	16.8	244000	3	F
123	9230	17.3	4.86	113	31.4	35.5	15.9	303000	3	F
124	10000	19.9	5.67	108.4	32.4	35.1	17.2	234000	3	F
125	8850	20.2	5.7	107.8	32.8	35.4	15.6	195000	3	F
126	12410	16.4	4.72	110.2	31.5	34.7	15.3	314000	4	F
127	9400	17.6	5.07	107.8	32.1	34.6	15.6	131000	4	F
128	10340	18.4	5.72	105.6	30.4	32.1	17.1	328000	4	F
129	9900	19	5.53	110	31.3	34.4	16.2	406000	4	F
130	13480	15.7	4.28	113.9	32.1	36.6	16	292000	4	F
131	7630	13.6	3.94	106.4	32.4	34.5	14.6	314000	4	F
132	10910	16.3	4.46	107.8	33.8	36.5	14.9	384000	4	F
133	7300	16.7	4.84	107.9	34.9	34.5	15.8	316000	5	F
134	13890	18	5.15	110.1	31.8	35	16.4	372000	5	F
135	11030	16.3	5	115.7	28.1	32.5	15.5	199000	5	F
136	8280	16.4	4.77	110.9	30.9	34.3	15.7	338000	5	F
137	11760	15.9	4.78	107.2	31.1	33.4	16.2	348000	5	F
138	15190	12.1	4.37	80.7	34.3	27.7	12.1	328000	5	F
139	6330	17.4	5.64	101.6	30.4	30.9	16.4	200000	5	F
140	8930	16.3	5.35	101.9	29.9	30.5	14.9	300000	5	F
141	7510	16.8	4.63	118.7	30.6	36.3	15.1	323000	5	F
142	9140	17	5.15	105.9	31.2	33	15.8	290000	5	F
143	16570	16.4	4.83	107.9	31.4	33.9	15.2	335000	5	F
144	12020	19.2	5.8	111.9	29.6	33.1	16.4	311000	5	F
145	8650	17	5.02	110.5	30.7	33.9	15.8	260000	5	F
146	12250	17.8	5.1	111.3	31.4	35	16.3	335000	5	F
147	14930	16.7	5.06	103.9	31.7	32.9	15.5	344000	6	F
148	9320	16.6	4.83	107.9	31.9	34.5	16.1	203000	6	F
149	9160	15.9	4.41	114.2	31.6	36.1	16.4	407000	6	F
150	9550	18.1	5.38	110.3	30.5	33.6	14.5	281000	6	F
151	12150	17.8	5.48	105	30.9	32.5	15.9	270000	6	F
152	10260	14	4.32	102.4	31.8	32.6	14.2	373000	6	F
153	12260	15.1	4.43	108.3	31.4	34	16.5	260000	6	F
154	11930	18	5.14	109.2	32	35	15.5	212000	6	F
155	9380	18.4	5.77	109.3	29.1	31.8	17.1	327000	7	F
156	11730	19.7	5.63	102.6	35	34.1	16.2	489000	7	F
157	8470	16	4.6	111.4	31.3	34.8	15.4	261000	7	F
158	17220	20.3	6.19	102.8	31.9	32.7	15.3	148000	7	F
159	11950	15.5	4.32	111.6	32.2	35.9	14.1	450000	7	F

160	10450	16.3	4.65	110.2	31.8	35.1	15.6	273000	7	F
161	11970	16.4	4.53	119	30.5	36.2	15.1	197000	7	F
162	5730	18.4	5.3	103.6	33.5	34.7	15.4	272000	7	F
163	15750	17.2	5.18	103.7	32	33.2	14.3	370000	7	F
164	9270	18.2	5.17	109.4	32.3	35.3	16.5	317000	7	F
165	7670	11.8	3.33	108.7	32.7	35.5	14.6	586000	7	F
166	8980	15.9	4.59	109.7	31.6	34.7	15.2	450000	8	F
167	8950	16.3	4.98	105.1	31.2	32.8	15.7	459000	8	F
168	12900	16.5	4.57	111.3	32.4	36	16.8	443000	8	F
169	15600	19.5	5.7	106.7	32.1	34.3	15.5	425000	8	F
170	10990	14.1	4.22	110.3	30.2	33.3	16.3	480000	9	F
171	14530	16.3	4.76	109	31.5	34.3	14.6	471000	9	F
172	10720	20	6.09	105.2	31.2	32.8	14.8	439000	10	F
173	10510	17.1	5.21	105.9	31	32.9	16.1	444000	10	F
174	10160	12.8	3.87	105.2	31.5	33.2	14.5	459000	10	F
175	8940	17.9	5.23	107.9	31.8	34.3	15.9	482000	10	F
176	10130	15.8	4.79	99.4	33.2	33	16.2	427000	10	F
177	9890	11.7	3.47	103.6	32.6	33.8	14.5	553000	11	F
179	16910	14.3	4.51	100.3	31.6	31.7	14.9	701000	12	F
180	13250	13.7	3.67	112.3	33.2	37.3	16.4	350000	12	F
181	10400	12.4	3.54	109.8	31.9	35	13.8	575000	12	F
182	12150	17.1	5.23	102.8	31.7	32.6	14.4	378000	12	F
183	10080	15.6	4.43	109.8	32.2	35.3	14.5	318000	12	F
184	8470	18.8	5.42	109.6	31.6	34.6	16.6	325000	12	F
185	9300	15.7	4.27	114.7	32	36.8	19.3	549000	13	F
186	12880	15	4.49	106.2	31.5	33.5	15	266000	13	F
187	11560	16.3	5.04	104.9	30.7	32.3	16	440000	13	F
188	10650	15	4.54	103.5	32	33.1	15.5	771000	14	F
189	13880	15.1	4.73	100.9	31.7	32	16.9	594000	14	F
190	8380	15.9	4.76	107.5	31.1	33.4	15.3	226000	14	F
191	13340	15.8	4.33	113.7	32.1	36.5	15.4	480000	14	F
192	11350	16.9	5.11	103.3	32	33.1	14.8	580000	14	F
193	9920	17.8	5.36	103.1	32.3	33.2	16.2	308000	14	F
194	8970	15	4.55	102.7	32.1	32.9	15.9	504000	14	F
195	9840	15.2	4.57	109.6	30.4	33.3	15.7	373000	15	F
196	8210	15.7	5.06	101	30.8	31.1	15.6	278000	15	F
197	9290	9.9	3.38	104.6	28	29.3	15.1	379000	15	F
198	13540	18.2	5.35	106.8	31.9	34	15.3	404000	15	F
199	13170	19.2	5.81	108.7	30.4	33	15.3	424000	15	F
200	9750	16.1	4.82	107.8	31	33.4	15.1	344000	15	F
201	9570	15.6	4.47	106.8	32.7	31.5	15.2	463000	15	F
202	9910	14.2	4.07	102.9	34	34.9	14	633000	15	F
203	11380	14.5	4.39	104.7	31.5	33	14.5	608000	15	F

204	9080	14.3	4.68	93.2	32.8	30.6	13.7	514000	15	F

Supplementary Figures Legend

Figure S1: Multiple amino acid sequence alignment of MT-2 protein in 24 species. New missense mutation reported in this work is marked with a vertical arrow. Uniprot accession number and entry name are reported for each sequence. Below the alignment, a star indicates that the amino acid at this position is identical for all the species, semicolons and dots indicate amino acids with similar but not identical properties. Species correspond as following (common name is reported): TMPS6_HUMAN= Human, G3SKP5_GORGO= Lowland gorilla, F7HLZ8_MACMU= Rhesus macaque, H2P4A0_PONAB= Pongopygmaeusabelii, F7IFY5_CALJA= White-tufted-ear marmoset, G1RXE8_NOMLE= Northern white-cheeked gibbon, H0WT78_OTOGA= Small-eared galago, F6ZMU8_HORSE= Horse, F1PGA1_CANFA= Dog, TMPS6_MOUSE=Mouse, D3ZF49_RAT = Rat, I3NFA4_SPETR= Thirteen-lined ground squirrel, M3W9I6_FELCA=Cat, M3YMK0_MUSPF= European domestic ferret, F6WLV0_MONDO=Gray short-tailed opossum,G3WMX0_SARHA=Tasmanian devil, F7FHE7_ORNAN= Duckbill platypus, F1NDU7_CHICK=Chicken, G1NJ34_MELGA=Common turkey, H0ZK80_TAEGU=Zebra finch, K7FBU9_PELSI=Chinese softshell turtle, F6X0X4_XENTR=Western clawed frog, H3DNB6_TETNG=Spotted green pufferfish, M4A0S3_XIPMA=Southern platyfish.

Figure S1