Mutations in *TMPRSS6* Cause Iron-Refractory, Iron Deficiency Anemia (IRIDA)

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SUPPLEMENTARY NOTE

Recruitment of IRIDA Kindreds. All kindreds were recruited via ascertainment of affected index cases. Kindreds B, C, D, and E have been previously reported[1-5]. We obtained informed consent from affected individuals or their parents, as appropriate. The Children's Hospital Boston Committee on Clinical Investigation approved this study.

Clinical Characterization of IRIDA Kindreds. In all cases, anemia was present on the earliest complete blood count performed, which was typically before the age of two years. Platelets were typically elevated. Peripheral blood smears demonstrated hypochromic, microcytic anemia with anisocytosis and poikylocytosis; red cell distribution widths (RDWs) were concomitantly increased. Hemoglobin electrophoreses excluded a hemoglobinopathy. Bone marrow biopsies/aspirates, when performed, excluded a sideroblastic anemia. Serum ferritin levels were low or low-normal, even when assessed following initial empiric oral iron therapy. When measured, whole blood lead levels and plasma C-reactive protein (CRP) were normal and free erythrocyte (zinc) protoporphyrin levels were elevated. No patient had clinical evidence of a chronic inflammatory disorder. Despite their anemia, affected subjects showed normal growth and development and lacked obvious dysmorphology, except for one index case, in whom anemia was diagnosed incidentally during an evaluation for craniosynostosis.

Urinary hepcidin levels were determined by ELISA at Intrinsic LifeSciences (La Jolla, CA).

SUPPLEMENTARY METHODS

DNA Extraction and Amplification. We isolated genomic DNA from whole blood of study subjects using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN), and quantified genomic DNA using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). We performed whole genome amplification (WGA) of purified genomic DNA using the REPLI-g Mini Kit (Qiagen, Valencia, CA).

Haplotype Analysis. Using WGA DNA from disease family members as template, we amplified polymorphic microsatellite loci from chromosome 22q by PCR using custom, sequence-specific primers with a fluorescent-dye end-label incorporation strategy[6]. We fractionated pooled fluorescent products on an ABI Prism 3130xl genetic analyzer (Applied Biosystems, Foster City, CA) in the Diagnostic Molecular Pathology Laboratory of Massachusetts General Hospital. We determined product sizes using GeneMapper 4.0 software (Applied Biosystems, Foster City, CA).

DNA Sequencing. Using WGA DNA of affected family members as a template, we individually amplified all exons of *TMPRSS6* by PCR using primers designed within intronic sequences located at least 30 base pairs from each intron-exon boundary. We designed primer sequences (**Supplementary Table 1**) using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers were synthesized by

Integrated DNA Technologies (Coralville, IA). We purified reaction products by either treating the reaction mixture with Exonuclease I (New England Biolabs, Ipswich, MA) and shrimp alkaline phosphatase (Roche Diagnostics, Indianapolis, IN) or by using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). We determined the sequence of both DNA strands using fluorescent dye-terminator chemistry at the Children's Hospital Boston Molecular Genetics Core Facility. We analyzed sequence results using Sequencher 4.8 DNA Sequence Assembly Software (Gene Codes, Ann Arbor, MI). We confirmed all sequence variants by independent amplification using non-amplified genomic DNA as a template. To determine if *TMPRSS6* sequence variants identified in affected individuals were polymorphisms, we sequenced the corresponding exons in genomic DNA from 50 Caucasian or 50 African American controls (Coriell Institute for Medical Research, Camden, NJ), depending upon the ethnic origin of the family harboring the putative mutation. We generated sequence alignments of TMPRSS6 homologues by the ClustalW method using MegAlign software (DNASTAR, Madison, WI). We performed protein domain analysis using the Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de).

Northern Blotting. We used PCR to generate an [α-P^{32}]dCTP-labeled, 966 bp probe corresponding to the 5' untranslated region through exon 8 of the *TMPRSS6* cDNA (nucleotides 60–1025 of Ensembl transcript ENST00000346753), employing I.M.A.G.E. clone 5164881 as a template (ATTC, Manassas, VA). We hybridized this probe to a human Multiple-Choice Northern Blot (Origene, Rockville, MD) using MiracleHyb hybridization solution (Stratagene, La Jolla, CA).

SUPPLEMENTARY REFERENCES

1. Andrews, N.C. *Yale J. Biol. Med.* **70**, 219-226 (1997).
2. Brown, A.C. et al. *Am J Hematol* **27**, 1-6 (1988).
3. Hartman, K.R. & Barker, J.A. *Am J Hematol* **51**, 269-75 (1996).
4. Mayo, M.M. & Samuel, S.M. Iron deficiency anemia due to a defect in iron metabolism: a case report. *Clin Lab Sci* **14**, 135-8 (2001).
5. Pearson, H.A. & Lukens, J.N. *J. Pediat. Hematol. Onc.* **21**, 412-417 (1999).
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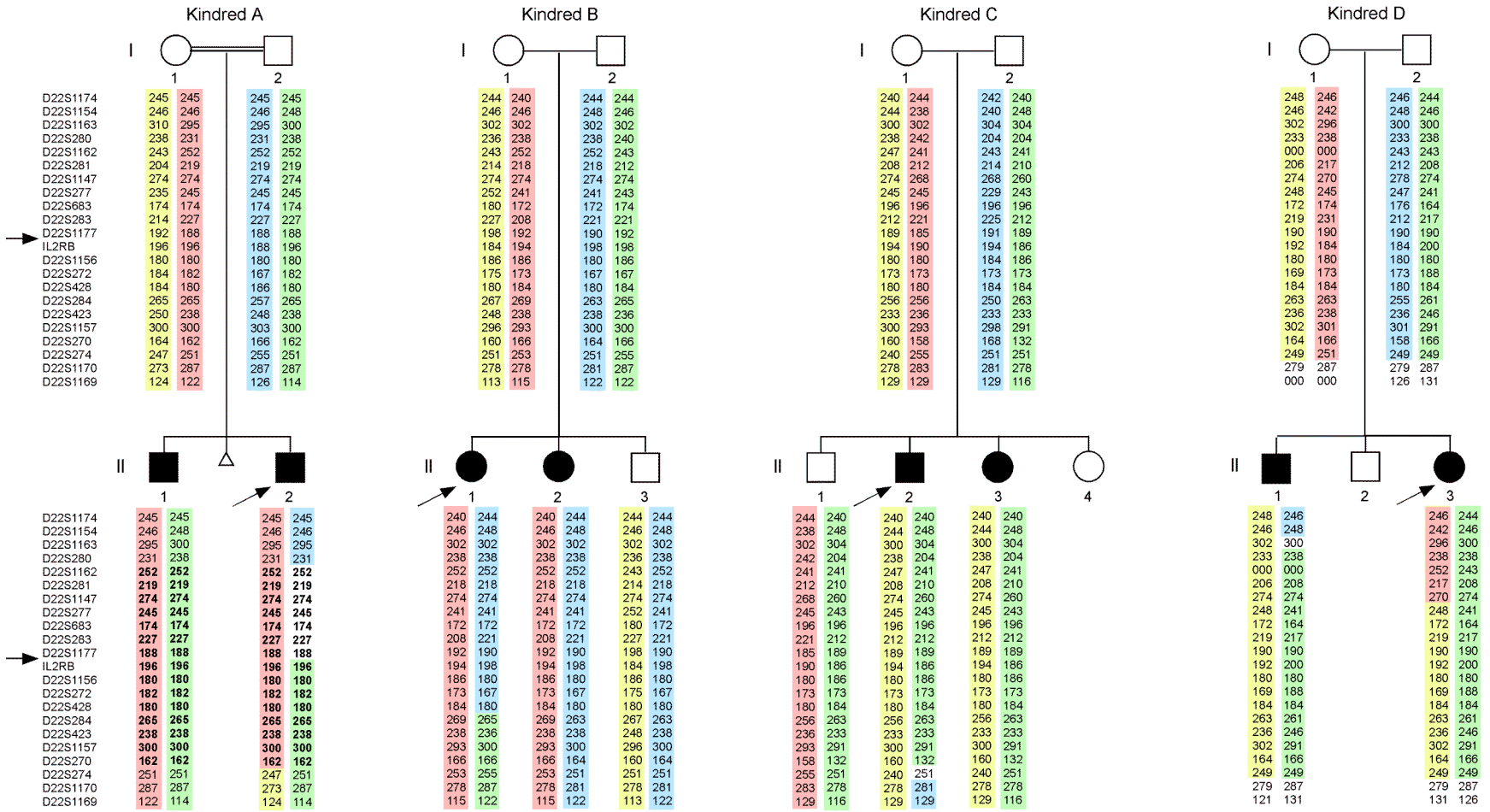
Exon	Forward Primer Sequence (5'→3')	Reverse Primer Sequence (5'→3')	Amplicon Size (bp)
1	CTGAGACCTCCGTCTGTCCTC	TGGAAACAGCCTCGCATTTG	271
2	TGCCGCCTGATGTTGTTACTC	GCCTGCTACAGTCACCCCAAG	395
3	GCAGGAGAAGGCATGGAAGAG	TCCCTGTGAATGCTCCAGATG	323
4	AGTAGGAGCAAAGGGCACCTC	GACATGCAGGAAGCCAAGTTC	301
5	CTTCTGCGTGAAGACGGACAG	GGCCACACCACAGCTTGTTTC	378
6	AGACAAGGCTGGCTCCAAGG	CCCTGCACACACAACAGAAGC	255
7	AGGCGTGAAGCTCAGTGTGTG	CTAGCCGTCCTGTCTCCCAGA	584
8	GATGTCCAGACTCCCGTCCAC	GAATCTTCCCTCTCCCCATCC	364
9	ATTTGCTGGCAGAGGTGGTAG	GGAAACACAGAATCCCAGGTG	458
10	TGTTGTTAGGGAGGTGGGTTCAC	GAGATTGGGGACTTGGGCTTC	287
11	AGGGAGAAATCAGGGCAGAGG	CCTTGGTGGTTCCAGGGATG	356
12	GCCACAAGGGTTTGCAGGAAT	GAGGCTGCATTGCTGGTCTGT	523
13	GTGATTGGTAACGTGCAATACAGC	TGAAGCATGTAGCAGGCCTAGA	285
14	CTCTTCTGGCTCCATCGTTCC	TGAGATTTCCCTCCAGCTTCC	295
15	TCTCCCCCTCCATCATTCTCC	CCACCACCCTTCCCTCTATCTG	399
16	ACCACCAGCTAGGCGACCTTC	GCCCAATTTGAATCCCAGCAC	571
17	GTGGGCAGAGCAGGAGAGAAG	GATGTGAGCAAAGGGCCAGAC	337
18	CCCAGTCAATTCCCAACAGTC	GAATACTTGTCCCCCTGCTTG	344

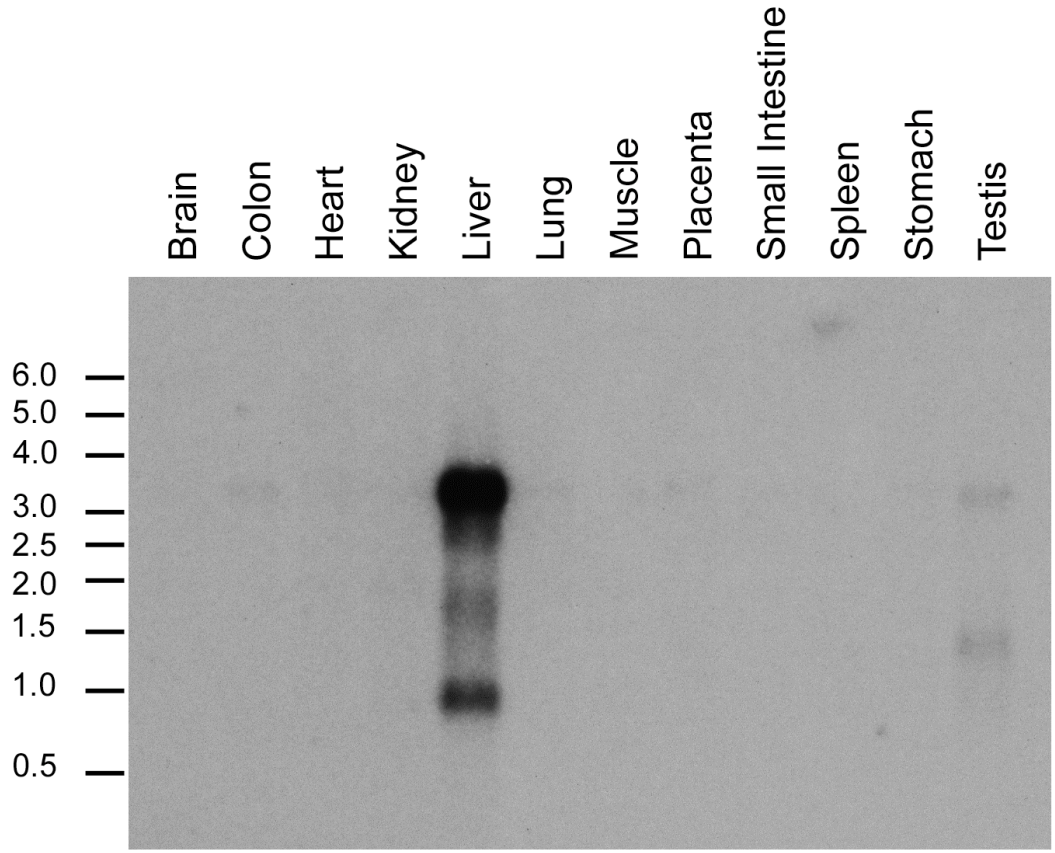
Supplementary Table 1. Primer sequences used for sequence analysis of *TMPRSS6*. Exon number corresponds to Ensembl transcript ENST00000346753. PCR conditions are available upon request.

Kindred	Subject	Sex	Age at Evaluation (y)	Blood				Plasma		Urine	*TMPRSS6* Mutations
				RBC ($x10^{12}$/L)	Hb (g/dL)	MCV (fL)	Retics (%)	Transferrin Saturation (%)	Ferritin (ng/ml)	Hepcidin/ Creatinine (ng/mg)	
A	II-1	M	16	5.1	8.3	63	2.2	4	3	461	p.K636fs/ p.K636fs
	II-2	M	10	5.0	7.7	58	1.2	5	38	1828	p.K636fs/ p.K636fs
B[a]	II-1	F	25	N/A	N/A	N/A	N/A	N/A	N/A	4055	p.605fs/ IVS13+1G>A
	II-2	F	23	N/A	N/A	N/A	N/A	N/A	N/A	3329	p.605fs/ IVS13+1G>A
D	II-3	F	20	4.4	9.3	72	2.1	3	23	113	p.G442R p.D521N

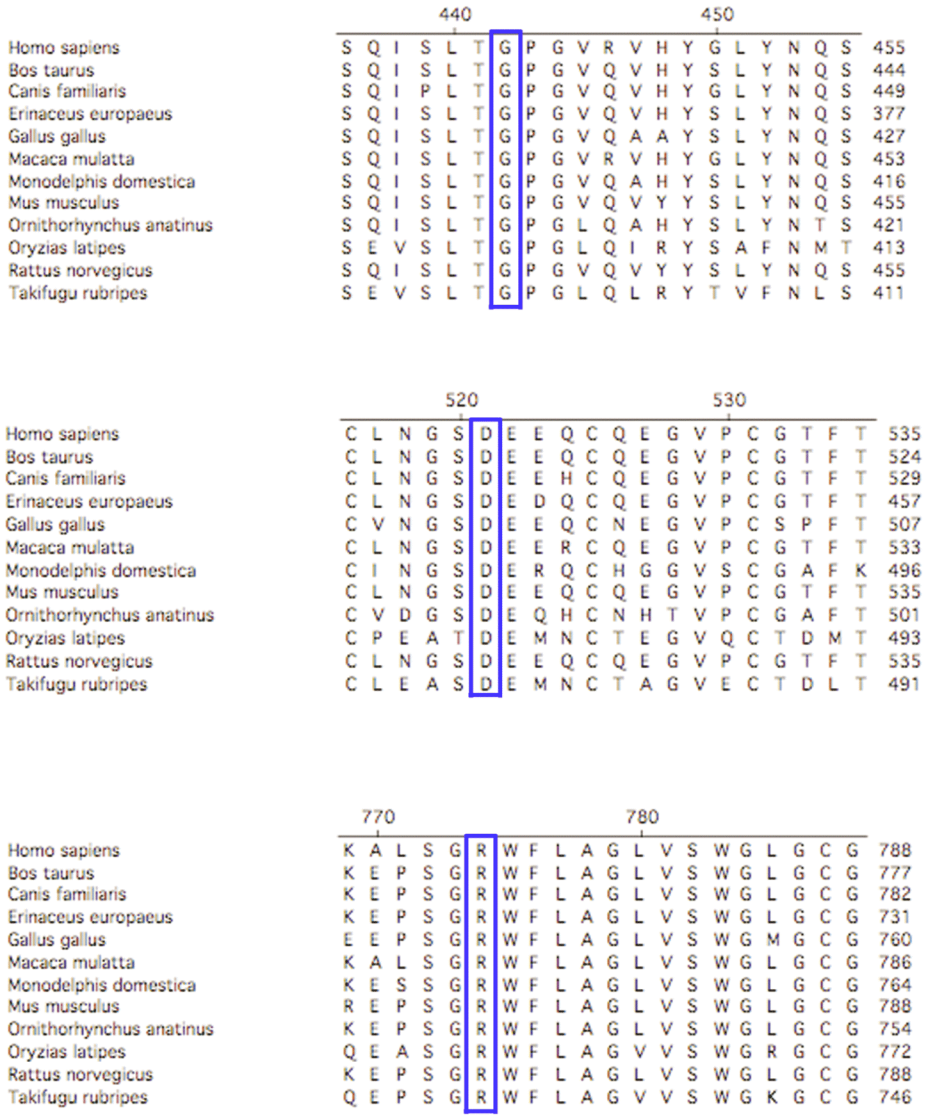
Supplementary Table 2. Clinical and biochemical parameters in affected individuals from IRIDA kindreds in whom urinary hepcidin was measured. Hematological parameters at the time of urine hepcidin determination, as well as the corresponding *TMPRSS6* mutations, are shown. In all five affected individuals, urinary hepcidin/creatinine ratios were either within or above the reference range reported for healthy adult controls (71-1567 ng/mg, M. Westerman, Intrinsic LifeSciences, La Jolla, CA, personal communication). Transferrin saturation (%) was calculated by dividing the serum iron level by the total iron binding capacity and multiplying by 100. RBC, red blood cell count; Hb, hemoglobin; MCV, mean corpuscular volume; Retics, reticulocytes. N/A, not available. [a]In Kindred B, urinary hepcidin/creatinine was also measured in the unaffected sibling and was found to be 400 ng/mg.



Supplementary Figure 1. Haplotype analysis in multiplex IRIDA kindreds. Chromosome 22 microsatellite markers are shown in the following order: cen-D22S1174 to D22S1169 tel. The location of *TMPRSS6* is indicated by the horizontal arrow. Colored boxes indicate inferred haplotypes. Regions where the phase of chromosomal segregation could not be determined are not colored. In kindred A, where I-1 and I-2 are first cousins, genotypes in bold indicate regions of presumed identity by descent among affected individuals. In combination with other mapping data (Maria Antonietta Melis, Milena Cau, Rita Congiu, Gabriella Sole, Antonio Cao, Renzo Galanello, unpublished data), meiotic recombinations in kindreds B and D refine the critical region to a 5.3 Mb region of 22q12-13 limited centromerically by D22S1147 and telomerically by D22S284. In kindred A, subject I-2 is heterozygous for single nucleotide polymorphisms within the *TMPRSS6* gene (data not shown), demonstrating that, although he is homozygous for alleles at several microsatellite loci immediately centromeric to *TMPRSS6*, he carries two distinct haplotypes at the *TMPRSS6* locus. In one additional kindred, DNA was available only from the affected individuals, who were siblings; in this kindred, the affected siblings share identical alleles at all chromosome 22 markers examined telomeric to D22S281 (data not shown). “000” indicates a genotype that was not determined.



Supplementary Figure 2. Northern blot analysis of human *TMPRSS6* expression. Northern blotting of messenger RNA isolated from a panel of human tissues demonstrates a predominant 3.3 kb transcript in the liver that is expressed at much lower levels in other tissues.



Supplementary Figure 3. Amino acid conservation in *TMPRSS6* vertebrate homologues. Residues homologous to human TMPRSS6 amino acid positions 442, 521, and 774, the sites of missense mutations identified in IRIDA patients, are boxed in blue.