PIEZO1-R1864H rare variant accounts for a genetic phenotype-modifier role in dehydrated hereditary stomatocytosis

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SUPPLEMENTAL DATA

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Methods

Collection of patients

Twelve subjects from two independent families were enrolled in this study, an English family (family A) and an Italian family (family B). The clinical data of the index cases were obtained by the clinicians responsible for patient care, and with the approval of local university ethical committees. All blood samples were obtained with informed consent. The diagnosis of DHS was based on history, clinical findings, routine laboratory data, peripheral blood smear, and genetic testing. DNA was obtained for genetic analysis from affected and unaffected family members, and from healthy blood donors after signed informed consent, according to the Declaration of Helsinki, and as approved by local university ethical committees.

Direct sequencing of PIEZO1 gene

Genomic DNA preparation and sequencing analysis was performed as previously described. Our primers were designed to avoid amplification of Piezo2 , Piezo1P1 and Piezo1P2 (Ensembl Gene ID ENSG00000154864, ENSG00000233686, ENSG00000237121).[13] Missense substitution mutations in PIEZO1 (Q92508) were evaluated by PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), and the recently reported classifier M-CAP (http://bejerano.stanford.edu/mcap/).

Cloning, site direct mutagenesis and transfection assay

cDNA encoding full-length wild type PIEZO1 was cloned in pLVX-EF1α-IRES-ZsGreen1 vector. The point mutations c.7473_7478dupGGAGCT, p.E2492_L2493dup and c.5591G>A, p.R1864H, were introduced into pLVX-EF1α-IRES-ZsGreen1-PIEZO1 by site-directed mutagenesis. The constructs obtained were transfected into HEK-293 cells for 72h as previously described.[13]

Measurements of ouabain-plus-bumetanide-resistant Rb+ and K+ fluxes in PIEZO1 transfected HEK-293 cells

HEK-293 cells at 72h post-transfection were maintained for 4h in a K+-free medium containing (in mM) 140 NaCl, 5 RbCl, 2 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose, with added 10 μM ouabain and 10 μM bumetanide. At the end of the incubation, cell viability was determined by trypan blue staining and medium was removed for extracellular K determination. Cells were gently washed in buffer containing (in mM) 150 choline chloride, 1 MgCl2, 10 Tris MOPS, then lysed for intracellular Rb measurement. Cell Rb content and medium K content were determined by atomic

absorption spectroscopy (ANALYST 2000, Perkin-Elmer) as previously described[20] and normalized on PIEZO1 protein quantity.

Molecular cloning of PIEZO1

To detect and determine the inheritance pattern of the two mutations in the proband B-II2, the DNA fragment encompassing the two mutations, R1864H and E2492_L2493dup, was PCR-amplified and cloned into PCR Cloning Vector pSC-A-amp/kan (StrataClone PCR Cloning Kit, Agilent).

Osmotic gradient ektacytometry

Deformability of the red blood cells (RBC) of the patients and relative control subjects were evaluated by osmotic gradient ektacytometry using the Laser-assisted Optical Rotational Cell Analyzer (LORCA) as previously described.[3]

RNA isolation, cDNA preparation, and quantitative qRT-PCR

Total RNA was extracted from cell lines and peripheral blood samples from patients and healthy controls using Trizol reagent (Life Technologies). cDNA synthesis from total RNA (2 μg) was performed using Super Script II First Strand kits (Life Technologies). Quantitative RT-PCR (qRT-PCR) was by the SYBR-green method, following standard protocols with an Applied Biosystems ABI PRISM 7900HT Sequence Detection system. Relative gene expression was calculated using the $2^{(-DCt)}$ method, where DCt indicates the differences in the mean Ct between selected genes and the normalization control (b-actin). qRT-PCR primers for each gene were designed using Primer Express software version 2.0 (Life Technologies).

Immunoblotting

Total cell lysates (80 μg protein) electrophoresed on SDS-polyacrylamide gels were electroblotted onto polyvinylidene difluoride membranes (BioRad, Milan, Italy), incubated with the following antibodies: anti-PIEZO1 (1:500; Proteintech), anti-β-actin antibody (1:1000; Sigma, used as loading control), then imaged with HRP-conjugated anti-rabbit Ig (1:5000) (GE Healthcare, UK) and enhanced chemiluminescence substrate (Supersignal West Pico Chemiluminescent Substrate Kit, ThermoScientific, Miami USA). Labeled bands were visualized and densitometric analysis performed with the BioRad Chemidoc using Quantity One software (BioRad).

Immunofluorescence analysis

HEK-293 cells ($2x10^6$) on coverslips were transfected with PIEZO1 cDNAs as previously described.[13] After 72 hrs, cells were incubated with CellMask Deep Red Plasma membrane Stain (C10046, Thermo Scientific), then fixed, and immunostained with anti-PIEZO1 (1:200; AbCam) and secondary antibodies (Alexa Fluor 488 goat anti-rabbit; Life Technologies) were incubated at 1:200 dilution in PBS for 30 min at room temperature. Nuclei were stained with 1 μg/ml DAPI in PBS for 15 min at room temperature. The coverslips were mounted in 50% glycerol (v/v) in PBS and imaged by Zeiss LSM 510 Meta confocal microscope equipped with an oil immersion plan Apochromat 63× objective 1.4 NA, Green channel excitation of Alexa488 by the argon laser 488 nm line was detected with the 505-550 nm emission bandpass filter. Red channel excitation of Alexa546 by the Helium/Neon laser 543 nm line was detected with the 560-700 nm emission bandpass filter (using the Meta monochromator). Blue channel excitation of DAPI by the blue diode laser 405 nm line was detected with the 420-480 nm and emission bandpass filter.

Statistical analysis

Data are presented as mean ± standard error (SE). Statistical significance between two means was calculated by Student's t test. Statistical significance of multiple comparisons was calculated by ANOVA test and Bonferroni post-hoc correction test. P value < 0.05 was considered as statistically significant.

Table 1S. Ektacytometric parameters of the two families here analyzed

	ID	Elmax	Omin	Ohyper
Affected subjects	A-I1	0.58	137.50	435.00
	A-II1	0.58	128.00	437.00
	A-II2	0.58	139.50	439.00
	A-II4	0.58	128.00	424.00
	B-I1	0.61	142.00	410.00
	B-II2	**0.58**	**97.00**	**413.00**
	B-II2	**0.58**	**96.00**	**419.00**
Unaffected subjects	A-I2	0.59	150.50	520.00
	A-II3	0.60	148.00	498.00
	B-I2	0.62	160.50	485.50
Healthy subjects	HC1	0.62	146.00	479.00
	HC2	0.59	163.00	456.00

In bold is highlighted the proband carrying *PIEZO1* modifier variant R1864H.
EI max, Omin and Ohyper values are the average of two ektacytometric analysis.

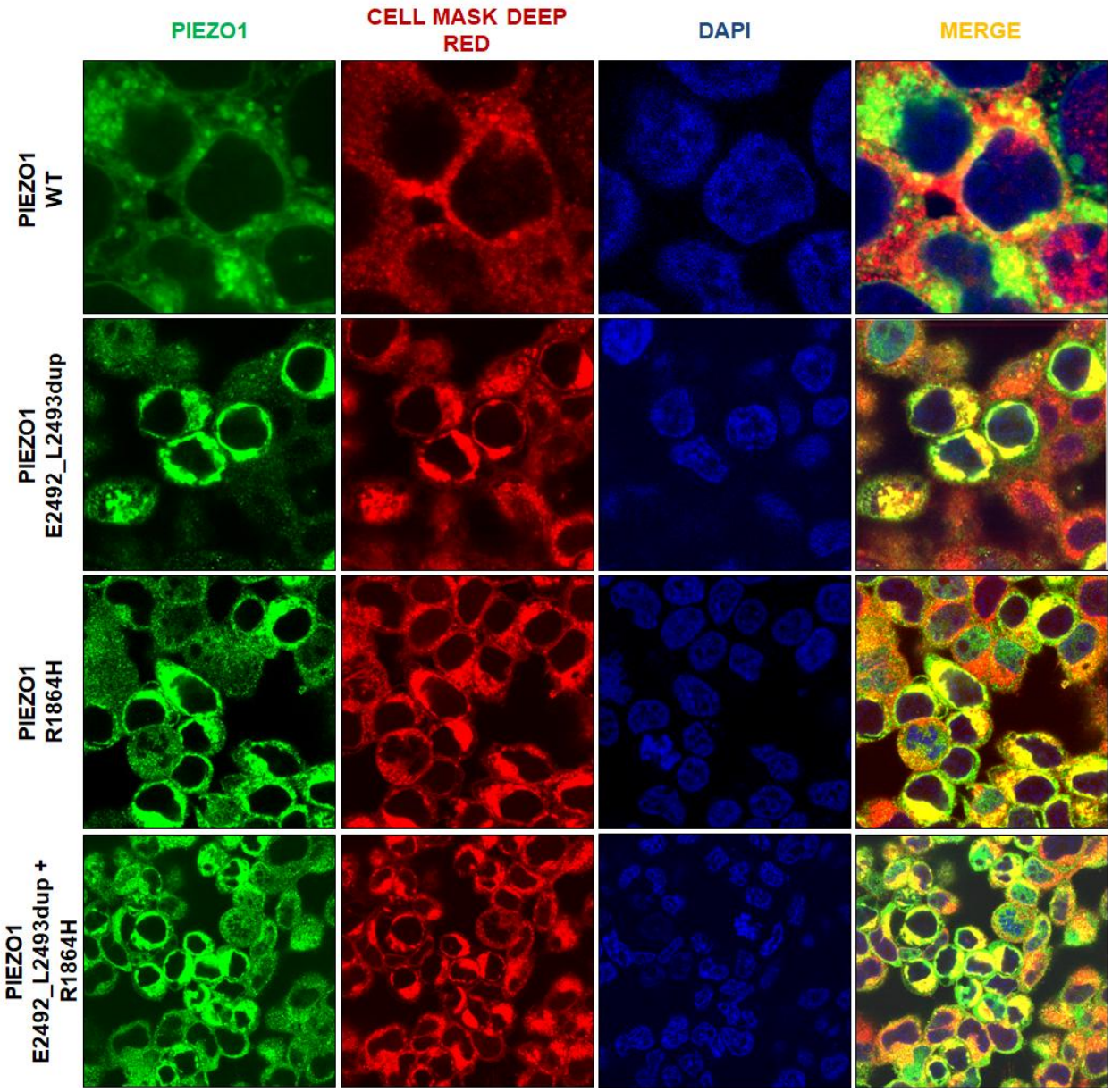

Figure 1S. Subcellular localization of PIEZO1 mutants

Laser-scanning confocal microscopy images of HEK-293 cells transfected with WT or mutant PIEZO1 variants, analyzed by immunofluorescence with rabbit polyclonal anti-PIEZO1 antibody (green) and cell mask (membrane marker, staining the nuclear envelope, the membranes of cytoplasmic compartments and the plasma membrane, red), with merged signal showing regions of colocalization in yellow. Cells were imaged with a Zeiss LSM 510 meta confocal microscope equipped with a 1.4 NA oil immersion plan Apochromat 100× objective. Intensity and contrast were adjusted with Axiovision software. Representative of three independent experiments.