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This work is dedicated to the memory and in honor of Renzo Galanello, who had conceived the study and continues to inspire us every day.

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Transferrin Receptor 2 mutations in patients with juvenile hemochromatosis phenotype

To the Editor: Type 3 Hereditary Hemochromatosis (HH) is a rare disorder of iron metabolism caused by mutations in Transferrin Receptor 2 (TFR2), a transmembrane protein able to bind holo-transferrin through its RGD motif [1]. It belongs to an iron-sensor complex together with Transferrin Receptor 1 (TFR1) and HFE and acts as a body iron sensor of diferric transferrin resulting in the upregulation of hepcidin production through a not yet fully understood signaling mechanism [1]. Although HH Type 3 clinical picture was initially described as adult onset HH, mimicking Type 1, other patients with early onset have been reported. In the present paper, we described three women with severe clinical presentation simulating juvenile HH (JH). We identified five mutations (three novel) severely affecting TFR2 function as shown by *in silico* and/or *in vitro* testing.

Patient's iron overload was assessed by transferrin saturation (TS), serum ferritin (SF), and liver iron by MRI [2] or liver biopsy. Genomic DNA was extracted from peripheral blood leukocytes, amplified by PCR using sequence-specific primers (available on request), and directly sequenced. Different available online tools (DNA to protein sequence converter, ClustalW2, Human Splicing Finder, PolyPhen, ExPASy, SIFT, and MUTPRED) were used to analyze mutations at genomic and protein levels. Mutant-TFR2 [p.A621_Q624delAVAQ (ΔAVAQ) and p.Arg678Pro (R678P)] plasmids were generated from pcDNA3.1-TFR2, with the FLAG-tag at the N-terminus (kindly provided by Hiroshi Kawabata); for minigene assay, a fragment of h*TFR2* DNA (exon 12–14) was cloned in pTB-NDE vector (kindly provided by Franco Pagani). HuH7 (human hepatoma) and HeLa (human epithelial) cells were transiently transfected with wild-type or mutant h*TFR2* constructs and then harvested for (a) protein isolation and Western blot analysis and (b) RNA extraction, cDNA synthesis, and minigene assay (details available on request).

We identified three novel (p.Asp680Glufs*111; IVS13 + 1G>A; and p.Arg678Pro) and two already known [3,4] (p.Arg30Profs*31 and p.A621_Q624delAVAQ) *TFR2* mutations associated with a juvenile-like clinical presentation in three young women (clinical, biochemical, and molecular data at diagnosis are summarized in Supporting Information Table S1). None of the probands had other known factors able to aggravate iron accumulation. Similar to women affected by JH [5], they manifested the disease in the third life decade showing very high levels of TS, SF, and liver iron. Patient 1 presented p.Asp680Glufs*111 in exon 17 in compound heterozygosity with p.A621_Q624delAVAQ in exon 16 [4]. She showed high serum iron indices since the age of 12 years and began phlebotomy treatment at 18 years. At 24 years, SF rose to 1500 μg/L (TS = 88%); however, early diagnosis and intensive phlebotomy program prevented further iron loading and

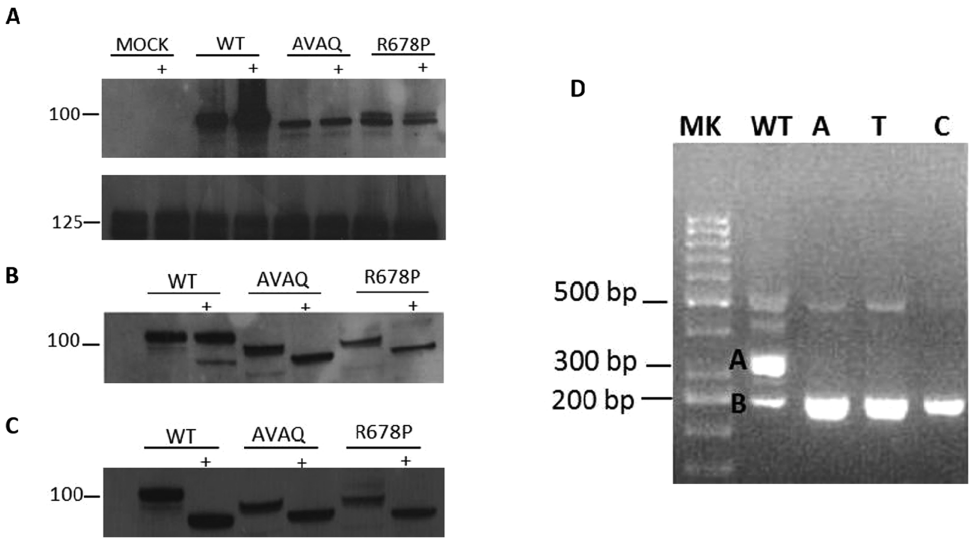

Figure 1. **A**: Western blot analysis of cell surface protein in HuH7 cells. Cells were transiently transfected with empty pcDNA vector (MOCK), wild-type hTFR2-FLAG (WT), and mutant hTFR2-FLAG (ΔAVAQ and p.Arg678Pro) and untreated or treated (+) with human holo-transferrin for 24 h. After cell surface biotinylation and protein extraction, Western blot analysis of human TFR2 was performed. The results were normalized with pan-cadherin expression. **B**: Glycosylation pattern of membrane-bound wild-type and mutant TFR2 (ΔAVAQ and p.Arg678Pro) in HuH7 cells treated (+) or not with Endo Hf. **C**: Glycosylation pattern of membrane-bound wild-type and mutant TFR2 (ΔAVAQ and p.Arg678Pro) in HuH7 cells treated (+) or not with PNGase F. **D**: Minigene assay for *TFR2* IVS13 + 1G>A mutation Agarose gel of PCR on wild-type (WT) or mutant (A, T, C) cDNA was cloned into minigene (pTB-NDE) vector in HuH7. Cells were transiently transfected with pTB-NDE vector with wild type or mutant *TFR2* DNA. After RNA extraction and cDNA retrotranscription, a PCR was performed, using wild type or mutant cDNA as template to analyze splicing. Band A represents the right splicing of exons 12-13-14, and Band B represents the skipping of exon 13. The 500-bp band was a nonspecific genomic DNA band.

organ damage. Patient 2 (IVS13 + 1G>A in exon 13 in compound heterozygosity with p.Arg30Profs*3 in exon 2 [3]) and Patient 3 (p.Arg678Pro in exon 17 at the homozygous state) presented with amenorrhea due to pituitary hypogonadism and liver cirrhosis. In addition, Patient 3 developed severe arthropathy, whereas Patient 2 quickly developed heart failure and diabetes. *In silico* analysis revealed that all *TFR2* mutations we reported are deleterious and lead to truncate or dysfunctional proteins. c.2039_2040insG insertion disrupts the RGD domain at amino acid Asp680 and creates a premature stop codon leading to a 790-amino acid protein. IVS13 + 1G>A affected a donor splicing site, and the substitution created an abnormal splicing site with a probability of 38% (Human Splicing Finder). p.Arg678Pro substitution affects the RGD domain likely limiting the binding of holo-transferrin to TFR2. It was predicted to be a deleterious mutation with PolyPhen score of 1.00, SIFT score of 0.04, and MutPred probability of 0.75.

We performed functional *in vitro* studies to assess ΔAVAQ, p.Arg678Pro, and IVS13 + 1G>A mutations causality. We evaluated membrane expression of wild type and mutants ΔAVAQ- and p.Arg678Pro-TFR2 in both HuH7 (Fig. 1A) and HeLa (Supporting Information Fig. S1A) cells. In the wild-type construct, we found large amount of protein composed by two closely migrating bands that increased after holo-transferrin treatment, indicating protein stabilization at cell membrane, as expected. In ΔAVAQ mutant cells, only the lower form of TFR2 was evident, whereas in p.Arg678Pro mutants, both bands were visible, although in smaller amounts than the wild-type construct. Protein level did not change after holo-transferrin administration in both mutants, suggesting that they do not correctly interact with holo-transferrin. Moreover, in HuH7 (Fig. 1B,C), which are representative of the tissue (hepatocytes) where TFR2 is functionally expressed *in vivo*, we observed a different glycosylation pattern between wild-type and mutant proteins: although the majority of wild-type TFR2 was composed of complex oligosaccharides [6], mutant TFR2s were composed only of hybrid oligosaccharides, suggesting that they were not properly glycosylated. These results suggest that ΔAVAQ and p.Arg678Pro mutations alter the trafficking and function of TFR2. Only small amount of mutant proteins reached the cell membrane; however, they were unable to correctly act as holo-transferrin sensor, likely due to an improper glycosylation. This is also confirmed by their inability to release TFR2 soluble form that originates from a cleavage of the cell surface protein (data not shown) [7]. The functional study performed on IVS13 + 1G>A mutation confirmed that the G>A substitutions abolished the splicing site at position +1 causing the skipping of exon 13 in both HuH7 (Fig. 1D) and HeLa (Supporting Information Fig. S1D) cells, suggesting a nonliver-specific splicing. Surprisingly, the analysis of the wild-type cDNA also revealed a shorter band of lower intensity, corresponding to the skipping of exon 13, in addition to the correct splicing fragment. This raises the possibility that alternative splicing of exon 13 might occur in the normal protein as well.

Overall, our results indicate that the *TFR2* mutations identified affect important functional domains of TFR2 or lead to dysfunctional or truncated protein. Type 3 HH is a fully penetrant disorder; however, our and previous findings suggest that expression may vary from adult- to juvenile-like HH. Interestingly, a recent study described three Chinese subjects with adult-onset HH due to mutations in *HFE2* [8]. Although this could also be ascribed to different ethnicity and genetic background, all these findings suggest that the term juvenile hemochromatosis might indicate a phenotypic rather than a genetic entity (e.g., a form of HH exclusively caused by *HAMP* or *HFE2* mutations) and that *TFR2* mutations should be considered in the genetic workup of individuals presenting early in life with hemochromatosis.

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RNA-seq is a valuable complement of conventional diagnostic tools in newly diagnosed AML patients

To the Editor: Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults. The identification of specific genetic alterations in AML is useful for both prognosis and treatment choice. In recent years, thanks to the development of "*Next-Generation Sequencing*" technologies (NGS), the list of molecular markers able to impact on prognosis and treatment of AML grew very quickly. Moreover, the number of clinically available targeted inhibitors increased at a very fast pace. Despite the availability of these new tools, cytogenetic analysis remains the standard de facto to detect chromosomal abnormalities. However, this technique suffers from several limitations, such as the requirement of an adequate number of mitotic cells, the limited resolution, and the inability to detect cryptic fusions. Similarly, genotyping performed with standard techniques, such as PCR/Sanger sequencing, is time consuming, expensive and allows to detect only a predefined set of known variants. The result is that only a limited fraction of the known oncogenic lesions is routinely tested in clinical laboratories.

In the present study, 20 AML patients at diagnosis were subjected to RNA-Seq to identify gene fusions, *NPM1* insertions, *FLT3* internal tandem duplications, and single nucleotide variants (SNV) and the results were compared with those obtained by the routine clinical approach.

Samples were processed as described in the Supporting Information Methods. The libraries were sequenced on an Illumina HiSeq 2500 or a Genome Analyzer IIx with 76bp paired-end reads. FastQ sequences were aligned to the human genome (GRCh38/hg38) using Star [1] and processed with Samtools [2]. Bam files were analyzed using CEQer2, an evolution and integration of FusionAnalyser [3] and CEQer [4]. Given that RNA-Seq analysis was performed only on leukemic samples, variant analyses were restricted to a predefined list of genes involved in AML pathogenesis (*CEBPA, NPM1, FLT3, RUNX1, KMT2A, WT1, EZH2, NF1, EVI1, KIT, H-RAS, K-RAS, TET2, IDH1, IDH2, DNMT3A, BCOR, BCORL1, NUP98, ASXL1, ABCB5, BAALC, CEP72, DIP2C, ROBO1, KLC1, TP53, IGFBP7, SETBP1, JAK2, NRAS, NOTCH1, CDKN2A, MPL, SF3B1, BRAF, PTPN11, SRSF2, IKZF1, GATA1, MYD88, ATM, CBL, PHF6, BCL2)* to minimize the risk of identifying rare SNPs.

In 20 patients, a total of nine fusions were identified (Table I). Of them, five were previously detected by cytogenetic analyses (one RUNX1-RUNX1T1 [AML-ETO], three PML-RARA, and one CBFB-MYH11). Of the four fusions detected only by RNA-Seq, one was completely new (ETV6-KDM2B) and three were reported in previous works (ETS2-ERG, KMT2A-MLLT10, and ZMYM2-FGFR1). ETV6-KDM2B, resulting from t(12;12)(p13;q24), is a *bona fide* intrachromosomal inversion involving the telomeric regions of the long (*KDM2B*) and short (*ETV6*) arm of chromosome 12 and defining a previously unreported in-frame cryptic translocation (Supporting Information Fig. 1). ETS2-ERG is a cryptic, in-frame intrachromosomal translocation occurring in chromosome 21 [3]. KMT2A-MLLT10, also known as MLL-MLLT10, is an in-frame recurrent translocation resulting from t(10;11)(p12;q23) and characterized by poor prognosis [5]. ZMYM2-FGFR1 is particularly