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Hemochromatosis Due to Mutations in Transferrin Receptor 2

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ABSTRACT: A rare recessive disorder which leads to iron overload and severe clinical complications similar to those reported in HFE-related hemochromatosis has been delineated and sometimes called hemochromatosis type 3. The gene responsible is Transferrin Receptor 2 (TFR2), which maps to chromosome 7q22. The TFR2 gene presents a significative homology to transferrin receptor (TFRC) gene, encodes for a transmembrane protein with a large extracellular domain, is able to bind transferrin, even if with lower affinity than TFRC. The TFR2 function is still unclear. The transcript does not contain IRE elements and is not modified by the cellular iron status. At variance with TFRC, interactions between TFR2 and HFE do not occur, at least in their soluble forms. TFR2 is spliced in two alternative forms, alfa and beta. The alfa form is strongly expressed in the liver. The beta form, codified from a start site in exon 4 of the alpha, has a low and ubiquitous expression. Using anti-TFR2 monoclonal antibodies we have confirmed expression of the protein in the liver but also in duodenal epithelial cells, and studied the protein functional behaviour in cell lines, in response to iron addition, iron deprivation and olo-transferrin exposure. Our results suggest a regulatory role of TFR2 in iron metabolism. Five TFR2 homozygous mutations have been documented in HFE3 patients: a nonsense mutation (Y250X); a C insertion that causes a frameshift and a premature stop codon (E60X); a missense mutation (M172K); a 12 basepair deletion in exon 16, that causes 4 aminoacid loss (AVAQ 594-597del) in the extracellular domain of TFR2; a missense mutation in exon 17 (Q690P). The mutation analysis supports the hypothesis that all are private mutations. The pathogenetic role of TFR2 in hemochromatosis has been recently further demonstrated through the targeted expression of the Y250X human mutation in mice, which develop sings of iron overload identical to the human disease. Although the rarity of TFR2 mutations limits their usefulness in diagnostic/screening programs, their study can contribute to a better understanding of the protein function. © 2002 Elsevier Science (USA)

Hereditary hemochromatosis has demonstrated to be a genetic heterogeneous disorder. Four different loci have been characterized. The first one is localized on chromosome 6p21, where the HFE gene has been identified (1). The HFE gene is involved in the pathogenesis of the most common form of hemochromatosis, which shows a frequency of 2-3/1000 affected subjects in the general population. A juvenile form of hemochromatosis has also been characterized; the responsible gene is still unknown but the locus maps to chromosome 1q21 (2). Furthermore, an atypical form of hemochromatosis has been recently identified. It is an autosomal dominant disease due to mutations in SLC11A3 gene codifying for ferroportin protein (3–6).

The last type of hemochromatosis is represented by a rare disease with clinical features similar to HFE, but with a different genetic background. The cloning of the responsible gene was obtained through an homozygosity mapping approach in a large consanguineous family originated from Southern Italy (7). After the genome scan analysis a consistent homozygosity region was identified in several patients of the family on 7q22, where the Transferrin Receptor 2 gene was previously mapped by radiation hybrids (8). The finding of nonsense mutations in patients con-

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firmed that TFR2 was the gene for this type of hemochromatosis.

TFR2

The TFR-2 gene spans for approximately 21 Kb, is organized in 18 exons and codes for different alternative spliced forms: the α-form, approximately 2.9 kilobase pairs long (AF067864), has been cloned from a cDNA library of TF-1, an erythroid leukemia cell line; the β-form, approximately 2.5 kilobase pairs long, has been cloned from a cDNA library of HL60 (8). A different transcript 2.5 kilobase pairs long (AF053356), has been independently isolated by Glöckner *et al.* (9) during sequencing of a large 7q22 region.

The two cDNAs (AF067864 and AF053356) are very similar, with some differences in the exon-intron boundaries. The α transcript contains additional 81 nucleotides in exon 8 and lacks 18 nucleotides in exon 18. Also, the α form contains additional 298 nucleotides in the 3′-untranslated region (nucleotides 2580-2877). The β-form lacks exons 1, 2 and 3 and its first exon (exon 4 of the α-form) has additional 142 nucleotides at the 5′-end.

The TFR2 gene belongs, together with TFRC (Transferrin Receptor) and PSMA (Prostate Specific Membrane Antigens), to the TfR protein family. Based on the predicted amino acid sequence, TFR-2 α-form is a type II trans-membrane protein with high similarity with TFRC and with PSMA, 66% and 60% respectively, in the extracellular domain. As TFRC and PSMA, the transmembrane domain (hydrophobic stretch from 81 to 104 residues) is located close to the amino terminus. The TFR-2 β-form lacks the amino-terminal portion, including the putative trans-membrane domain and part of extracellular domain, and could be an intracellular protein. Translation probably starts at nucleotide 542, in frame with the α-transcript open reading frame. The TFR2 murine orthologue (AF222895) cDNA is 50% homologous to the murine TFRC, but, as human TFR2, does not contain iron-responsive elements in 3′ or 5′ untranslated regions (10). As human TFR2 also the murine tfr2 shows alternative transcript forms visible in Northern blot (8). The murine tfr2 and human TFR2 amino acid sequences are highly conserved, sharing 85% identical and 92% similar amino acids. The promoter regions of human TFR2 and murine (AF207742) are considerably conserved. Both human and murine promoters contain 2 typical GATA-1 (an erythroid-specific transcription factor) consensus sequences, putative C/EBP binding sites, a doublet of CAAT at around –240, and a CCAAT sequence in the reverse direction at around –190. In addition, several CACCC sequences are present, which can be EKLF consensus sequences. (11).

Analysis by Northern Blot revealed expression of the TFR2 α-transcript predominantly in liver and, to a lesser degree, in the stomach among human tissues, and in K562 (erythroleukemia) and HepG2 (hepatoblastoma) among a panel of different cell lines. TFR2-α mRNA is detectable at high levels in erythroid precursor cells and is down regulated during erythropoietin-induced differentiation *in vitro.* Its decrease is inversely related to the increase of TFRC, that indicates different functions of the two proteins (12). The β form seems to be expressed at low level in all tissues and its expression is detectable only by RT-PCR (8).

Mechanisms involved in the regulation of TFR2 expression at transcriptional and translational levels are only partially known. No iron-responsive elements are found in 5′ or 3′ untranslated regions of TFR2 mRNA, suggesting that iron does not control TfR2 expression by the classical pathway involving iron-regulatory proteins. This hypothesis has been confirmed by *in vitro* and *in vivo* experiments: expression of TFR2 mRNA was not altered by adding DFO and Ferric Nitrate to K562 cell line medium, while TFRC mRNA clearly increase and decrease respectively in the two conditions (13). In mouse $hfe^{-/-}$, the murine model of Hemochromatosis, the TFRC expression is undetectable because of the iron loading, by contrast TFR2 mRNA is abundant both in control and in $hfe^{-/-}$ mouse liver (10). Conversely, cell cycle status regulates TFR2 mRNA synthesis, with maximal levels reached at late G1 phase (13).

The physiological functions of TFR2 remain

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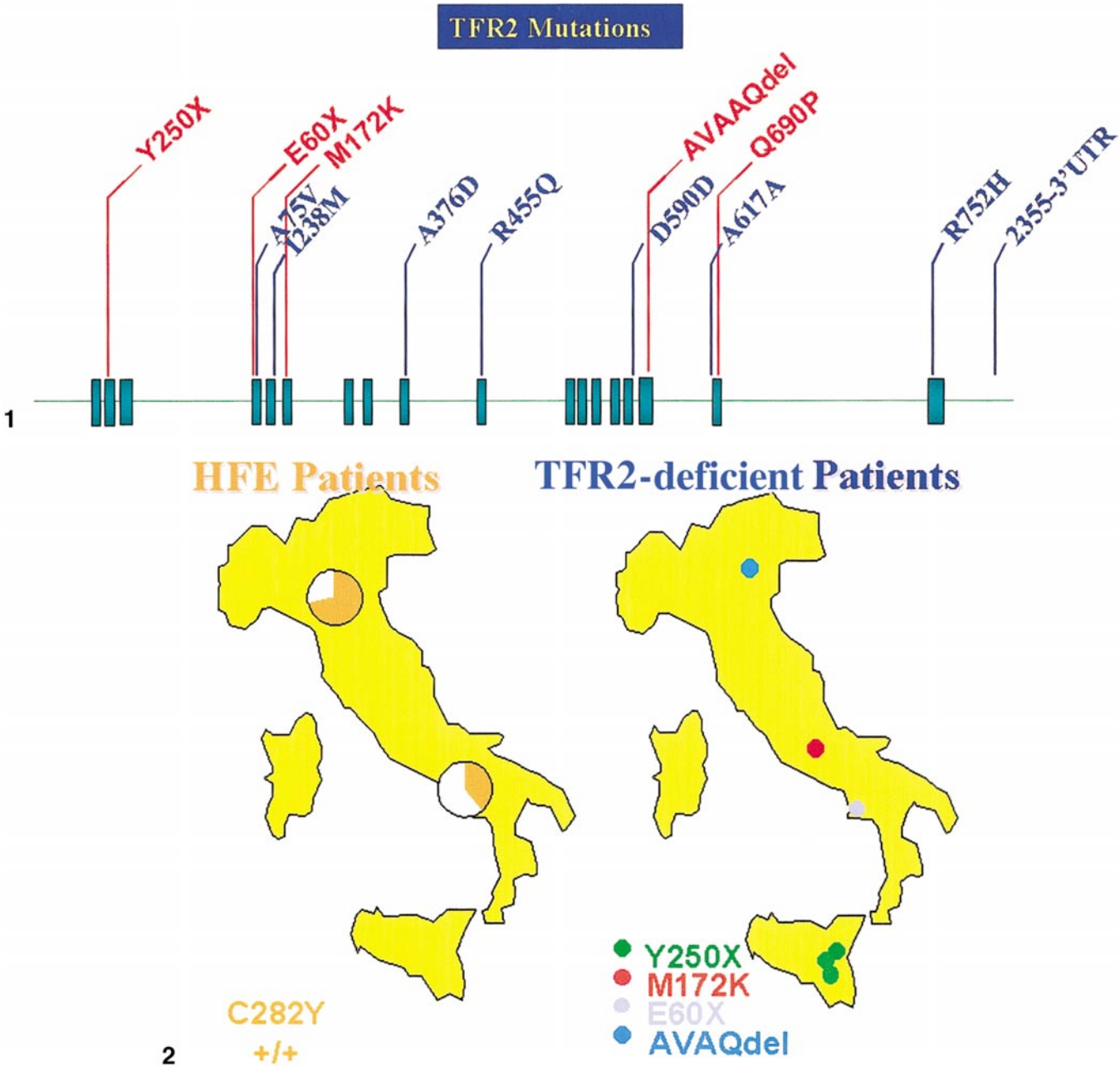


FIG. 1. TFR2 mutations characterized until now and their localization in the gene sequence. The causal mutations are showed in red, the polymorphic changes in blue.

FIG. 2. Geographical origin of Italian TFR2 deficient patients compared to C282Y HFE patients.

to be elucidated. TFR2 is able to bind transferrin and to mediate its uptake in transfected cells, but with lower affinity compared to TFRC (9). The high level of TFR2-α mRNA expression in a tissue with high iron requirement such as erythroid precursor cells is an indirect evidence of a possible physiological role of TFR2 in iron uptake by cells which need it for metabolic purposes. Furthermore, the phenotype of TFRC knock-out mouse, that dies *in utero* with defective erytropoiesis and neurological abnormalities (14), indicates that TFR2 does not compensate for the loss of the functions of TFRC.

In contrast to TFRC, TFR2 does not interact with HFE, at least *in vitro.* Binding experiments fails to detect any interaction between the ectodo-

mains of HFE and TFR2. Moreover, about half of the TFRC residues, that form contacts with HFE, are replaced by different amino acids in TFR2 (15). It remains to be determined whether interaction between TFR2 and HFE can be identified *in vivo.*

Hemochromatosis Due to TFR2 Deficiency

Hereditary Hemochromatosis due to TFR2 deficiency is a form of iron overload distinct from the most common hereditary hemochromatosis form (HFE) and from Juvenile Hemochromatosis (JH). The clinical symptoms are very similar to those of HFE, with increased serum iron parameters due to increased iron absorption at the duodenal level that leads to parenchymal iron overload. TFR2 gene mutation analysis in 2 unrelated Italian families, revealed the presence of an homozygous nonsense mutation Y250X in the affected subjects (7). Since that, four additional mutations affecting TFR2 coding sequence have been identified in different patients: E60X, M172K (16), AVAQ 594-597del (17), Q690P (18). Furthermore, the Y250X mutation has been found in another family originating from the same very small geographical area of the previously two characterized families (unpublished data).

Several groups in the world screened the TFR2 gene for mutation in iron loaded patients without finding any causal mutations, but only polymorphic changes (19–22). The TFR2 hemochromatosis mutations found until now have been illustrated in Fig. 1. These mutational data, together with the results of a screening study on the frequency of Y250X mutation in Italy (23), support the hypothesis that the TFR2 mutations are rare and most of them are private of the family in which they have been found.

Italian TFR2 hemochromatosis families are usually from Central Southern part of the country, a trend common to JH and opposite to the HFE C282Y mutation prevalence (Fig. 2). Very recently the mouse model of TFR2 hemochromatosis has been developed by Fleming *et al.* (24). The $tfr2^{-/-}$ mouse has been obtained through the insertion of the murine homologue of the Y250X human mutation ($tfr2^{Y245X}$ mouse). The animals present a phenotype fully overlapping to Tfr deficiency clinical symptoms, with increased iron loading in the liver and low iron concentration in the spleen. In order to address the biological function monoclonal and polyclonal antibodies have been recently developed (25). Immunohistochemical analysis with monoclonal antibody showed that TFR2 protein is highly expressed in hepatocytes and in the duodenal cells, where it is distributed along the entire villus.

When the TFR2 expression in K562 cell line is analyzed in cytofluorography in comparison to TFRC, the two proteins present an opposite pattern of expression when the cells are exposed to iron loaded transferrin: while there is a decreasing expression of TFRC, TFR2 protein expression significatively increases and clusters in selective cell membrane areas (25). Also, it has been evidenced that TFR2 expression does not change in condition of iron overload/deprivation. This results support the hypothesis that the two proteins have a different role in iron metabolism.

Linking all the experimental data available at the moment: the TFR2 high tissue specific expression, the ligand binding properties of TFR2, which shows a lower affinity for transferrin compared to that of TFRC, and the fact that mutations inactivating the gene cause an iron overload disease, we argue that these features are more consistent with a regulatory function than with a simple iron carrier or receptor function. Furthermore, the similar pattern of expression suggests that TFR2 protein could be involved in the same functional pattern of Hepcidin, a novel protein involved in iron metabolism (26–29).

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