MERTK rs4374383 polymorphism affects the severity of fibrosis in non-alcoholic fatty liver disease

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Abstract

BACKGROUND & AIM: Homozygosity for a common non-coding rs4374383 G>A polymorphism in MERTK (myeloid-epithelial-reproductive tyrosine kinase) has been associated with the protection against fibrosis progression in chronic hepatitis C. The main study objective was to assess whether MERTK AA genotype influences liver fibrosis, and secondarily MERTK expression in patients with non-alcoholic fatty liver disease (NAFLD). We also investigated whether MERTK is expressed in human hepatic stellate cells (HSC) and in murine models of fibrogenesis.

METHODS: We considered 533 consecutive patients who underwent liver biopsy for suspected non-alcoholic steatohepatitis (NASH) without severe obesity from two Italian cohorts. As controls, we evaluated 158 patients with normal liver enzymes and without metabolic disturbances. MERTK rs4374383 genotype was assessed by 5'-nuclease assays. MERTK expression was analysed in mouse models of fibrosis, and the effect of the MERTK ligand GAS6 were investigated in human HSC.

RESULTS: Clinically significant fibrosis (stage F2-F4) was observed in 19% of patients with MERTK AA compared to 30% in those with MERTK GG/GA (OR 0.43, CI 0.21-0.88, p=0.02; adjusted for centre, and genetic, clinical-metabolic and histological variables). The protective rs4374383 AA genotype was associated with lower MERTK hepatic expression. MERTK was overexpressed in the liver of NAFLD patients with F2-F4 fibrosis and in in vivo models of fibrogenesis. Furthermore, exposure of cultured human HSC to the MERTK ligand GAS6, increased cell migration and induced procollagen expression. These effects were counteracted by inhibition of MERTK activity, which also resulted in apoptotic death of HSC.

CONCLUSIONS: The rs4374383 AA genotype, associated with lower intrahepatic expression of MERTK, is protective against F2-F4 fibrosis in patients with NAFLD. The mechanism may involve modulation of HSC activation.

Keywords

- NASH;
- MERTK;
- Fibrosis

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a leading cause of chronic liver disease worldwide [1] ; [2], affecting about 20–30% of the general population [3]. The clinical relevance of NAFLD arises from the evidence that NAFLD patients are at risk of progression to cirrhosis and its complications [1] ; [4], presenting also a high rate of cancer and cardiovascular events [5] compared to subjects without fatty liver.

The major risk factors for liver disease severity and its progression are obesity, insulin resistance (IR) and necroinflammation [6]; [7]; [8] ; [9]. However, several pathogenic factors affecting liver damage in NAFLD are emerging, including immune response, apoptosis, and heritability, which play a key role in the susceptibility towards progressive disease [10]. The patatin-like phospholipase-3 (PNPLA3)/adiponutrin rs738409 C>G polymorphism, and the TM6SF2 rs58542926 G>A polymorphism, are the major common genetic risk factors for NAFLD development and progression [11] ; [12], but their determination is not sufficient to accurately stratify the risk of disease progression at an individual level [12] ; [13].

In this complex picture, a recent genome-wide study in patients with chronic hepatitis C, identified several susceptibility loci for severity and progression of liver fibrosis. The strongest was homozygosity for rs4374383 G>A single nucleotide polymorphism, a non-coding variant in the myeloid-epithelial-reproductive tyrosine kinase (MERTK) locus [14]. MERTK is a receptor of the tumour-associated macrophage (TAM) family, with a key role for the initiation of efferocytosis, a process by which dying cells are removed by phagocytes [15] ; [16]. MERTK was overexpressed in activated mouse hepatic stellate cells (HSC) in vitro and in an experimental model of liver fibrosis [17]. Agonists of LXR, a nuclear receptor favouring lipogenesis, increases MERTK expression in monocytes [18], and mice deficient for GAS6, a ligand of MERTK, had an attenuation in hepatic steatosis – via modulating beta-oxidation, inflammation and fibrosis [19]. Therefore MERTK and its variants could act as central players in the control of apoptosis, immune response, HSC activation, and steatosis modulation, i.e. all factors involved in the pathogenesis of NAFLD and of its progression to non-alcoholic steatohepatitis (NASH) and cirrhosis.

The aim of this study was to assess whether the rs4374383 polymorphism influences histological damage (fibrosis as the main objective), and secondarily MERTK expression in patients with NAFLD. We also investigated whether MERTK is co-regulated with fibrogenesis, in murine models of fibrogenesis, and its expression in HSC.

Patients and methods

Patients

We analysed data from 533 Italian prospectively recruited patients, who underwent liver biopsy for suspected NASH without severe obesity, and with blood samples available for genetic analyses. The study cohort included 233 patients from the Gastrointestinal & Liver Unit of the Palermo University Hospital, and 300 patients from the Metabolic Liver Diseases outpatient service, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, in Northern Italy. Other causes of liver disease were ruled out, including alcohol intake (>20 g/day) evaluated by a

questionnaire, viral and autoimmune hepatitis, hereditary hemochromatosis, and alpha1-antitrypsin deficiency. Patients with advanced cirrhosis, hepatocellular carcinoma, severe obesity (body mass index (BMI) ⩾40 kg/m2), and current use of steatosis-inducing drugs were excluded.

The control group included 158 Italian subjects; 120 were consecutive healthy blood donors without obesity, diabetes and chronic diseases, and with normal ALT levels (<32/28 U/L in M/F), where NAFLD was rule out by fatty liver index (n = 120) [20]; 38 cases were obese subjects with normal liver biopsy (steatosis <5% of hepatocytes) at routine liver biopsy performed at the time of bariatric surgery (gastric banding). Mean age was 54.2 years, and 46 (29.1%) were females. Mean total and HDL cholesterol, as well as triglycerides were in the normal range, and mean HOMA value was 2.37. All were negative for viral infection (anti-HCV, anti-HIV, and HBsAg negative, viral infection was excluded by determining viremia in blood donors).

The study was carried out in accordance with the principles of the Helsinki Declaration, and with local and national laws. Approval was obtained from the hospital Internal Review Boards and their Ethics Committees, and written informed consent for the study was obtained from all controls and patients.

Clinical and laboratory assessment

Clinical and anthropometric data were collected at the time of liver biopsy. BMI was calculated on the basis of weight in kilograms and height in meters. The diagnosis of type 2 diabetes was based on the revised criteria of the American Diabetes Association, using a value of fasting blood glucose ⩾126 mg/dl on at least two occasions [21]. In patients with a previous diagnosis of type 2 diabetes, current therapy with insulin or oral hypoglycemic agents was documented.

A 12-h overnight fasting blood sample was drawn at the time of biopsy to determine serum levels of alanine aminotransferase (ALT), total cholesterol, HDL cholesterol, triglycerides, plasma glucose and insulin concentrations. IR was assessed by homeostasis model assessment (HOMA) using the following equation: Insulin resistance (HOMA-IR) = Fasting insulin (μU/ml) × Fasting glucose (mmol/L)/22.5. [22].

Genetic analyses

DNA was purified using the QIAmp blood Mini Kit (Qiagen, Mainz, Germany) and DNA samples were quantified using spectrophotometric determination. Genotyping for PNPLA3 (rs738409 C>G), TM6SF2 (rs58542926 C>T) and MERTK (rs4374383 G>A) variants was carried out using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems, Foster City, CA, USA).

The genotyping call was done with SDS software v.1.3.0 (ABI Prism 7500, Foster City, CA, USA). Genotyping was conducted in a blinded fashion relative to patient characteristics.

Assessment of histology in human NAFLD

Slides were coded and read at each clinical centre by one expert pathologist, who was unaware of the patients' identity and history. A minimum 15 mm length of the biopsy specimen or the presence of at least 10 complete portal tracts was required [23]. Steatosis was assessed as the percentage of

hepatocytes containing fat droplets (minimum 5%). The NAFLD clinical research network Kleiner classification [24] was used to compute steatosis, ballooning and lobular inflammation, and to stage fibrosis from 0 to 4. NASH was considered to be present when steatosis, lobular inflammation and ballooning or fibrosis were present.

In 20 cases randomly selected (10 from each centre), we tested the inter-observer agreement between pathologists for fibrosis, steatosis, lobular inflammation and ballooning by using weighted kappa scores.

Evaluation of liver cells expressing MERTK in human NAFLD by immunohistochemical and immunofluorescence analyses

Immunohistochemistry was performed using a polymer detection method. Briefly, tissue samples were fixed in 10% buffered formalin and paraffin embedded. Four-micrometer thick tissue sections were deparaffinized and rehydrated. The antigen unmasking technique was performed using pH9 Tris/EDTA buffer (Novocastra, UK) in a PT Link Dako at 98 °C for 30 min. Subsequently, the sections were brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidase with 3% H2O2 and Fc blocking by a specific protein block (Novocastra) the samples were incubated 1 h with the primary antibodies rabbit monoclonal [Y323] to MERTK anti-human, Abcam code ab52968 (1:1000 pH9) at room temperature. Staining was revealed by polymer detection kit (Novocastra) and revealed by AEC (3-amino-9-ethylcarbazole, Dako, Denmark), substrate-chromogen. The slides were counterstained with Harris hematoxylin (Novocastra).

Immunofluorescence was performed by using the following primary and secondary antibodies: anti-human CD68 (clone PGM1, diluition 1:100, Dako Cytomation); anti-human MERTK [Y323] 1:1000, Abcam). After Fc blocking, primary antibodies binding was revealed by fluorochrome-conjugated secondary antibodies: Alexa 568-conjugated goat anti-rabbit (Invitrogen Molecular Probes, Carlsbad, CA); Alexa 488-conjugated goat anti-mouse (Invitrogen Molecular Probes, Carlsbad, CA). The slides were counterstained with DAPI Nucleic Acid Stain (Invitrogen, Molecular Probes, Carlsbad, CA). All the sections were analysed under a Leica DM3000 optical microscope (Leica Microsystems, Germany) and microphotographs were collected using a Leica DFC320 digital camera (Leica).

Evaluation of MERTK hepatic mRNA levels in human NAFLD

MERTK hepatic expression (mRNA levels) was evaluated by quantitative real-time PCR in available tissue samples immediately frozen at the time of liver biopsy. Liver biopsies were collected in RNAlater (Ambion, Carlsbad, CA, USA), immediately frozen in liquid nitrogen and stored at −80 °C. When an additional tissue sample was available, this was lysed for protein extraction in RIPA buffer, containing 1 mmol Na-orthovanadate, 200 mmol PMSF and 0.02 mg/ml apoprotinin. RNA was isolated from liver biopsies using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer protocol. RNA quality was evaluated by measuring the 260/280 nm absorbance ratio (>1.8) and by electrophoresis. First-strand complementary DNA (cDNA) was synthesized using SuperScript VILO cDNA synthesis kit (Invitrogen), starting from 0.5 mcg of total RNA. Gene expression was evaluated by quantitative real-time PCR using SYBR Green FAST master mix (Applied Biosystems, Foster City, CA, USA), ran in triplicate on ABI PRISM 7500 fast (Applied Biosystems). Primers sequences used were, for MERTK, forward: 5′-

CCTTCAGCATAACCAGTGTGC and reverse: 5′-TGACAGGTGAGGTTGAAGGC, whereas for beta-actin, forward 5′-GGCATCCTCACCCTGAAGTA, reverse: 5′-GGGGTGTTGAAGGTCTCAAA. Gene expression levels were normalized for beta-actin.

Animal experimental protocol

Two different models of fibrogenesis were employed. Mice were purchased from Charles River Laboratories (Calco, Italy). For carbon tetrachloride (CCl4)-induced fibrosis, male C57BL/6 mice were injected intraperitoneally with CCl4 (0.5 μl/g bw) twice a week for 6 weeks to induce liver fibrosis. Control mice received an equivalent volume of olive oil. Mice were sacrificed 2 days after the final CCl4 injection. Experimental fibrogenic steatohepatitis was induced by administration of a methionine and choline-deficient diet. Male BALB/c mice were fed either a high-fat diet deficient in methionine and choline (MCD diet), or a control diet supplemented with methionine and choline, as previously described [25]. Diets were prepared by Dottori Piccioni Laboratories (Milan, Italy) and stored at 4 °C until used. In both experiments mice, 8 weeks of age at the beginning of this study, weighing between 20 and 25 g were kept under a controlled temperature of 22 ± 2 °C, 50–60% relative humidity and 12 h light/dark cycles. Mice had free access to food and water ad libitum. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23 revised 1985) and experiments were performed after permission of the local IACUC.

Culture of human HSC and measurement of cell migration

HSC were isolated from normal liver tissue unsuitable for transplantation as previously described [26]. Cells were cultured on plastic and used after complete transition to a myofibroblast-like phenotype. Sub-confluent HSCs were serum-starved for 24 h, washed, trypsinized, and re-suspended in serum-free medium at a concentration of 1 × 105 cells/ml. Chemotaxis was measured in modified Boyden chamber equipped with 8 μm pore filters (Millipore Corp, MA, USA) and coated with rat tail collagen (20 μg/ml) (Collaborative Biomedical Products, Bedford, USA), as described in detail elsewhere [26]. At least ten high-power fields per filter were counted in each experiment.

Immunoprecipitation and Western blot analysis

Procedures for preparation of cell lysates, sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis and Western blotting have been described elsewhere [26]. One hundred μg of cell proteins were used for MERTK immunoprecipitation, performed with 15 μl of anti-MERTK (R&D Systems) or the appropriate control IgG followed by addition of protein A-Sepharose, as described by Di Maira et al. [27]. The resulting immunoprecipitates were analysed by Western blotting using anti-MERTK (AbCam). Quantification of the signal was obtained by chemiluminescence detection on an Image Quant Las4000 (GE Healthcare Life Sciences) and subsequent analysis with ImageJ software.

Measurement of cell viability

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT) assay, as previously described [27].

Gene silencing

All siRNAs used were purchased from Dharmacon (Lafayette, USA). Transfection of human HSC was performed using the Amaxa nucleofection technology (Amaxa, Koln, Germany) as previously described, with 100 nM smart Pool siRNA specific for human MERTK (sequence accession no. # NM_006343) or non-targeting siRNA [28].

Statistics

The study had the power to include 12 potential predictors in the multivariate model for F2-F4 fibrosis in a cohort of NAFLD with at least 120 patients with F2-F4 fibrosis, i.e. the expected 25% of the entire cohort. Continuous variables were summarized as mean ± standard deviation, and categorical variables as frequency and percentage. The t test and chi-square test were used when appropriate. Univariate and multiple logistic regression models were used to assess the factors independently associated with significant fibrosis (main objective), and with NASH and severe steatosis (secondary objectives). In the first model, the dependent variable was fibrosis, coded as 0 = no or mild fibrosis (F0-F1) or 1 = clinically significant fibrosis (F2-F4); in the second model, the dependent variable was NASH coded as 0 = absent and 1 = present; in the third model the dependent variable was steatosis coded as 0 = mild-moderate (steatosis grade 1–2), and 1 = severe (steatosis grade 3).
As candidate risk factors, we selected age, gender, BMI, the baseline levels of ALT, triglycerides, total and high-density lipoprotein (HDL) cholesterol, blood glucose, insulin, HOMA score, the presence of diabetes, PNPLA3 rs738409 variant, TM6SF2 rs58542926 C>T, MERTK rs4374383 AA genotype, steatosis, lobular inflammation, ballooning, NASH, fibrosis, and enrolling centre.
In all models, according to literature data, we compared patients homozygous for MERTK AA protective allele to all other variants [14], the TM6SF2 variant was coded in a dominant genetic model because of its relatively low allele frequency [12] ; [13], and an additive model was used for PNPLA3 [11].
To avoid the effect of co-linearity, diabetes, HOMA score, blood glucose and insulin levels, or steatosis, ballooning, lobular inflammation and NASH were not included in the same multivariate model. Regression analyses were performed using SAS [29].

Results

Patients' features

The baseline characteristics of the 233 Sicilian and of the 300 Northern Italian NAFLD patients are shown in Supplementary Table 1. In the group of 20 cases with histological features revised by pathologists from both Northern and Southern Italian centres, the k inter-observer agreements for fibrosis, steatosis grade, lobular inflammation and ballooning were 0.89, 0.76, 0.60 and 0.55, respectively, similar to those reported in the literature [24].

The prevalence of MERTK rs4374383 AA, AG and GG genotypes was 14.6%, 47.8% and 37.6% in the entire cohort. Genetic frequencies did not violate with Hardy–Weinberg equilibrium.
The frequency distribution of the MERTK genotype was similar in patients with NAFLD respect to age and sex-matched individuals with normal liver enzymes and without metabolic abnormalities ($p = 0.90$; Supplementary Fig. 1). As a positive control, the rs738409 G and rs58542926 T alleles, were over-represented in patients vs. controls ($p < 0.0001$).
No associations were found between MERTK genotypes and anthropometric, metabolic and biochemical parameters in the entire cohort (Table 1).

Association of MERTK genotype with histological severity of human NAFLD
Clinically significant fibrosis

Clinically significant fibrosis (stage F2-F4) was associated with older age, higher ALT, type 2 diabetes, NASH diagnosis, PNPLA3 variant and the absence of MERTK AA genotype.
Specifically, significant fibrosis was observed in 19% of patients with MERTK AA compared to 30% of those with MERTK GG/GA ($p = 0.04$). By multiple logistic regression analysis, MERTK AA genotype remained associated with clinically significant fibrosis after correction for genetic and clinical-metabolic variables (OR 0.40, CI 0.20–0.81, $p = 0.01$), and also for the above indicated factors and histological features of NASH (OR 0.43, CI 0.21–0.88, $p = 0.02$; Table 2, upper panel). Notably, when the term of interaction between PNPLA3 and MERTK variants was included in the model it was not significant ($p = 0.18$).

To account for a possible effect of MERTK AA genotype on fibrosis via induction of severe steatosis, MERTK AA genotype remained significantly associated with clinically significant fibrosis when NASH was replaced in by severe steatosis in the model (OR 0.46, CI 0.23–0.92, $p = 0.03$).
When considering severe fibrosis (F3-F4) as histological outcome, similar results were observed: MERTK AA genotype was associated with a lower prevalence of F3-F4 fibrosis after correction for genetic and clinical-metabolic variables (OR 0.37, CI 0.14–0.96, $p = 0.04$) as well as for the above quoted risk factors and NASH (OR 0.38, CI 0.16–0.99, $p = 0.04$).
NASH and severe steatosis
The prevalence of NASH was not affected by MERTK genotype (39.7% in MERTK AA vs. 44.1% in MERTK GG/GA; $p = 0.46$), while NASH was linked to higher ALT, type 2 diabetes, PNPLA3 variant, and enrolling centre. Along this line, by multivariate logistic regression analysis, type 2 diabetes (OR 2.16, 95% C.I. 1.11–4.19 $p = 0.02$) and Southern Italian enrolling centre (OR 31.9, 95% C.I. 19.0–53.6; $p < 0.001$) were independently linked to NASH, but not MERTK AA genotype (OR 0.63, 95% C.I. 0.31–1.30; $p = 0.21$).
Severe steatosis was observed in 8% of patients with MERTK AA compared with 21% with MERTK GG/GA genotype ($p = 0.006$). The other variables associated with severe (grade 3) steatosis were higher BMI, higher ALT levels, both PNPLA3 and TM6SF2 variants, and enrolling centre. By multivariate logistic regression analysis MERTK AA genotype (OR 0.24, 95% C.I. 0.09–

0.63; p = 0.004) remained significantly associated with severe steatosis together with ALT levels (OR 1.00, 95% C.I. 1.00–1.01; p = 0.007), BMI (OR 1.11, 95% C.I. 1.05–1.17; p <0.001), PNPLA3 variant (OR 2.09, 95% C.I. 1.49–2.93; p <0.001), TM6SF2 variant (OR 2.64, 95% C.I. 1.45–4.81; p = 0.002), and Southern Italian enrolling centre (OR 3.03, 95% C.I. 1.79–5.12; p <0.001).

MERTK is expressed in hepatic stellate or monocytoid cells from human NAFLD samples

MERTK was found to be expressed in cells with stellate or monocytoid morphology scattered throughout the hepatic parenchyma and loosely aggregated within inflammatory foci (Fig. 1, upper panels) while being not expressed in hepatocytes. Since the morphology of MERTK-expressing cells was suggestive of stellate cells/macrophages double labelling immunofluorescence analysis was performed for the CD68 macrophage marker and MERTK. Consistent with the morphology observed on immunohistochemically-stained sections, MERTK-expressing cells also expressed CD68 (Fig. 1, lower panels), which confirmed their macrophagic lineage.

MERTK in situ hepatic expression in human NAFLD. MERTK in situ expression was investigated by immunohistochemistry on NAFLD bioptic samples using a specific monoclonal primary antibody. MERTK was found to be expressed in cells with stellate or monocytoid morphology scattered throughout the hepatic parenchyma and loosely aggregated within inflammatory foci (upper panels) while being not expressed in hepatocytes. Since the morphology of MERTK-expressing cells was suggestive of stellate cells/macrophages double labelling immunofluorescence analysis was performed for the CD68 macrophage marker and MERTK. Consistent with the morphology observed on immunohistochemically-stained sections, MERTK-expressing cells also expressed CD68 (lower panels), which confirmed their macrophagic lineage. (This figure appears in colour on the web.)

Figure options

Hepatic expression of MERTK in patients with NAFLD

In 94 morbidly obese consecutive patients from Northern Italy (male gender 30%, mean age 44 ± 11 years, mean BMI 41 ± 8 kg/m2), and at a very low prevalence of F2-F4 fibrosis, 13% [30], the protective rs4374383 AA genotype was associated with lower MERTK expression levels (p = 0.049; Fig. 2A), after correction for age, gender, and lobular inflammation (factors linked with MERTK expression with p <0.1 at univariate analysis together with MERTK genotype). Similarly, in 80 NAFLD patients from the Southern Italian cohort (male gender 30%, mean age 48 ± 11 years, mean BMI 29 ± 4 kg/m2, F2-F4 fibrosis 42.5%) we confirmed lower MERTK expression levels in those carrying the MERTK AA genotype compared with all the others (p = 0.02; Fig. 2B).
To further establish a possible link between MERTK expression and fibrogenesis in NAFLD, hepatic MERTK expression was assessed in a function of fibrosis severity in 27 patients from the FLIP study (male gender 75%, mean age 42 ± 11, mean BMI 27 ± 4), and in the above quoted Southern Italian cohort of 80 patients with NAFLD. Of note, patients with F2-F4 fibrosis had significantly higher MERTK mRNA levels than those with F0-F1 in both FLIP and Southern Italian cohorts (Fig. 2C, D). No association was found between hepatic MERTK expression and other histological features (data not shown).
MERTK expression is upregulated in murine models of fibrosis

We also investigated the expression of MERK in two well-established models of fibrogenesis in the mouse (Fig. 3). After a 6-week administration of CCl4, intrahepatic expression of MERTK was more than 6-fold higher than in control mice. Similarly, 8-week administration of a MCD diet resulted in a 2.5-fold increase in MERTK mRNA levels ($p < 0.05$). This latter model is more representative of fibrogenesis associated with steatohepatitis, and the lower degree of MERTK expression reflects the less abundant fibrosis observed in this model. We also tested hepatic MERTK expression in a murine high-fat diet (HFD) model showing at 16 weeks no different expression of MERTK compared to controls (data not shown). Overall, these data provide additional support to the profibrogenic role of MERTK in models driven by inflammatory and metabolic/inflammatory pathways.

Fibrogenic effects of a MERTK ligand in cultured stellate cells

HSC represent the final effectors and coordinators of the fibrogenic response, and we explored whether MERTK could affect the biology of this cell type. Based on the evidence that MERTK mRNA expression was significantly induced in mouse HSC activated by culture in plastic, and also after induction of chronic liver damage in response to CCl4 administration or bile duct ligation [17], we analysed whether activation of MERTK was expressed in cultured human HSC and if its activation modifies the fibrogenic phenotype of these cells. Immunoprecipitation of HSC lysates with anti-MERTK antibodies and blotting for MERTK revealed a clear band of the expected molecular weight, which was not present when lysates were incubated with non-immune, control antibodies (Fig. 4A). Exposure of human HSC activated by culture on plastic to GAS6, a MERTK ligand, induced a time-dependent increase in the activation of ERK1/2, a member of the mitogen-activated protein kinase family activated by different transmembrane receptors including tyrosine kinases and involved in HSC activation and migration (Fig. 4B). In line with these findings, exposure to GAS6 resulted in an increase in cell migration (Fig. 4C), to an extent similar to that induced by foetal bovine serum, used as a positive control. Collectively, these data indicate that culture-activated HSC express functional MERTK.

We next evaluated the specificity of the effects of GAS6 using UNC569, a specific small molecule inhibitor of MERTK [31]. Induction of cell migration in response to GAS6 was reverted in HSC co-treated with UNC569 (Fig. 5A), providing evidence that the effects of GAS6 are mediated by MERTK. Exposure of HSC to UNC569 also resulted in a significant decrease in cell viability, indicating that MERTK is involved in the maintenance of fibrogenic cell survival (Fig. 5B). To rule out that the effects of UNC569 were due to non-specific inhibition of other molecules, we silenced MERTK using specific siRNAs. Also with this approach, viability of HSC was significantly reduced, essentially replicating the effects of UNC569 (Fig. 5C). To establish whether the reduction of cell viability was due to programmed cell death, HSC were exposed to increasing concentrations of UNC569 (Fig. 5D). Similar to the effects of doxorubicin in HuH7 cells, used as a positive control, UNC569 resulted in PARP cleavage and in the generation of active caspase-3, compatible with induction of HSC apoptosis.

To obtain further evidence for a profibrogenic role of MERTK, GAS6-stimulated cells were assayed for procollagen I gene expression in the presence or absence of UNC569 (Fig. 5E). GAS6 slightly, but significantly increased the expression of procollagen I. These effects were inhibited by co-exposure of HSC to UNC569, indicating that the effects of GAS6 are mediated by MERTK (Fig.

5E). A reduction in procollagen I gene expression was also obtained after MERTK silencing (data not shown). Taken together, these data indicate a profibrogenic role for MERTK activation.

Discussion

The main finding of this study is that the MERTK rs4374383 AA genotype is associated with a lower prevalence of clinically significant fibrosis in patients with NAFLD, and the mechanism may be mediated by decreased expression of MERTK. We also showed that MERTK is expressed in human HSC at the protein level, where mediates profibrogenic actions, and is overexpressed in patients with NASH and severe fibrosis, and in murine models of fibrogenesis.

MERTK is a TAM receptor known to be highly expressed in M2-polarized macrophages, and has a key role in efferocytosis [15] ; [16]. In genome-wide association studies, the rs4374383 gene variant of MERTK has been recently associated with liver fibrosis progression in patients with chronic hepatitis C [14], this association being confirmed in another independent cohort of chronic hepatitis C patients [32]. To the best of our knowledge, this is the first study to demonstrate the potential association between MERTK polymorphisms and the fibrotic phenotype in NAFLD patients, and to show a potential direct profibrogenic action.

The most relevant result from our analysis is the protective effect of the MERTK AA genotype on significant fibrosis. Notably, this association was maintained after correction for well known clinical-metabolic risk factors, and for both PNPLA3 and TM6SF2 gene variants, the strongest genetic determinant of NAFLD [11] ; [12]. Another relevant finding of our study is the association of MERTK AA genotype and lower prevalence of severe steatosis in NAFLD patients. Therefore, we suggest that the AA genotype may represent a moderator of the effect of other risk factors on steatosis severity in NAFLD. Published genome-wide association studies assessing gene variants at risk for NAFLD did not identify MERTK rs4374383 G>A as one at risk. This data are not in contrast with the results of the present study, because we confirmed MERTK gene variant as not associated with NAFLD, while we firstly report in this clinical setting its role as a modulator of liver damage.

Although this study was not designed to fully clarify the pathogenic link between MERTK AA genotype and the severity of liver fibrosis, several hypotheses may be put forward to mechanistically explain this association. In NAFLD patients, where MERTK is mostly expressed in macrophage and HCS, we observed a significantly lower hepatic expression of MERTK in subjects carrying the protective AA genotype, which may therefore have a functional counterpart on the abundance of this receptor. Along this line, we found higher hepatic expression of MERTK in NAFLD patients with significant fibrosis compared to those with none or mild fibrosis (F0-F1). Remarkably, increased expression of MERTK was also present in two independent murine models of fibrogenesis, one of which – the MCD diet – is associated with a histological picture similar to the one of human NASH, while MERTK was not overexpressed in the HFD model (data not shown), where the role of inflammatory cells in liver damage is minimal.

A very intriguing finding is that MERTK may modulate the fibrogenic process via direct actions in HSC. Gene expression profiles of HSC undergoing transactivation showed that MERTK is induced more than 4-fold following activation on plastic, and MERTK hepatic expression is also induced by chronic liver injury in vivo [17]. We demonstrated herein that cultured human HSC express a robust signal for MERTK at the protein level. Moreover, we provide additional, functional data on a possible direct action of this tyrosine kinase, exposing HSC to the MERTK ligand GAS6. GAS6

resulted in activation of the ERK1/2 pathway, coupled with induction of directional migration of HSC. Both these actions are relevant for the fibrogenic process, as indicated by the accumulation of activated HSC in discrete regions of the hepatic acinus during fibrogenesis and by the observation that the activation of ERK1/2 positively modulates fibrosis [33]. Moreover, GAS6 induced a significant increase in the expression of type I procollagen. It should be considered that GAS6 is also a ligand of the tyrosine kinase Axl, the expression of which has been previously reported in HSC and in liver fibrosis [34]. To rule out that the effects of GAS6 were not mediated by MERTK, we tested the effects of the specific inhibitor UNC569 [31]. This compound prevented GAS6-mediated effects on cell migration and procollagen expression, indicating that MERTK contributes to mediate the actions of this soluble mediator on HSC. Moreover, UNC569 resulted in a marked reduction in HSC viability via the induction of apoptosis, indicating that MERTK is implicated in the maintenance of HSC survival. Of note, these effects of the MERTK inhibitor were reproduced by genetic MERTK silencing. Further studies in in vivo models are needed to better define the role of this pathway in the pathogenesis of fibrosis in general and during NASH. When considering the association between MERTK variant and the severity of steatosis, data from the literature suggest that MERTK may modulate the mechanisms regulating lipogenesis. First, agonists of LXR, a nuclear receptor involved in the regulation of lipid metabolism, increase MERTK expression in monocyte [18]; second, in mice fed a choline-deficient ethionine-supplemented diet (CDE) GAS6 deficiency attenuated hepatic steatosis, limiting CDE-induced downregulation of genes involved in β-oxidation [19].

If further confirmed in independent cohorts, the association between the MERTK rs4374383 AA genotype and severity of histological features in NAFLD that may further refine our ability to identify patients needing histological evaluation, or, after prospective studies, those at risk of liver disease progression and mortality. Furthermore, the kinase domain of MERTK may represent a therapeutic target to inhibit disease progression.

Some limitations of this study must be acknowledged, such as its cross-sectional nature, which makes it impossible to dissect the temporal relations between the genetic background and progression of liver disease over time. A further methodological question is to what extent these data may be extrapolated to different populations. Our study included a cohort of Italian patients enrolled at two tertiary care centres, which may be different, in terms of both metabolic features and severity of liver disease, from the majority of prevalent cases of NAFLD in the general population and/or in different geographical areas. Along this line another potential limitation is related to the relative heterogeneity of the study cohorts, relative to the different prevalence of metabolic comorbidities and of severity of liver damage between the two studied cohorts, which on the other hand suggest that our results are applicable to different settings. However, the independent association between MERTK genotype and liver damage obtained by combining two cohorts with different baseline characteristics, and after adjusting also for enrolling centre, further strengthens the robustness of our results. The relatively limited downregulation in liver MERTK mRNA expression as a function of both fibrosis and MERTK genotype, may question the biological significance of this finding. However, data were confirmed in three independent cohorts, and replicated in mouse models. Furthermore, a similarly but not impressive difference in hepatic mRNA levels according to fibrosis severity has already been reported as a key factor in NAFLD pathogenesis – PNPLA3 [11]. Finally, we cannot rule out that the association between MERTK rs4374383 and liver fibrosis is not directly related to this intronic variant, but to other variants in

this or other genes, in linkage disequilibrium with the rs4374383, like the rs6726639, located within the binding site of HNF, recently implicated in gene expression regulation [35].
In conclusion, our results, obtained in a large cohort of patients with histological diagnosis of NAFLD, demonstrate that the protection against significant fibrosis associated with the MERTK AA genotype, make this is an appealing new genetic biomarker to be considered in the natural history, pathophysiological and interventional studies in NAFLD.

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Conflict of interest
The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions
S. Petta, L. Valenti, F. Marra, S. Grimaudo, C. Tripodo, C. Bugianesi E., Cammà, A. Cappon, V. Di Marco, G. Di Maria, P. Dongiovanni, S. Fargion, C. Guarnotta, A. Gulino, E. Mozzi, E. Orlando, M. Maggioni, R.M. Pipitone, R. Rametta, and A. Craxì had full control of the study design, data analysis and interpretation, and preparation of article. All authors were involved in planning the analysis and drafting the article. The final draft article was approved by all the authors.

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Tables

Table 1. Baseline demographic, laboratory, metabolic, and histological features of Italian patients, according to *MERTK* genotype.

	Combined cohort (n = 533)		
	MERTK GG/GA N = 455	*MERTK* AA N = 78	*p* value
Mean age, yr	46.5 ± 11.8	47.3 ± 13.1	0.58
Male gender	61.1	57.7	0.56
Mean BMI, kg/m^2	29.9 ± 5.0	29.5 ± 4.6	0.48
ALT, IU/L	58.2 ± 45.0	64.7 ± 72.3	0.29
Type 2 diabetes	17.1	12.8	0.34
Cholesterol, mg/dl	204.1 ± 43.8	212.4 ± 47.6	0.14
HDL cholesterol, mg/dl	49.1 ± 14.7	51.6 ± 16.5	0.17
Triglycerides, mg/dl	145.6 ± 80.7	142.3 ± 76.8	0.74
Blood glucose, mg/dl	97.6 ± 28.1	99.4 ± 25.5	0.72
Insulin, µU/ml	16.4 ± 10.2	16.6 ± 9.4	0.90
HOMA score	4.15 ± 3.36	4.39 ± 3.39	0.62
PNPLA3 GG	15.0	11.5	0.42
PNPLA3 CC/CG/GG	40.4/44.6/15.0	44.9/43.6/11.5	0.64
TM6SF2 CT/TT	13.8	14.2	0.91
Histology			
Lobular inflammation 2-3	22.2	15.4	0.17
Ballooning 1-2	51.6	52.5	0.52
Steatosis grade 3	20.9	7.7	0.006
NASH	44.2	39.7	0.46
Fibrosis stage 2-4	30.3	19.2	0.04

Table 2. Association of the *MERTK* rs4374383 genotype and liver damage as evaluated by unadjusted and adjusted models in 533 patients with biopsy-proven non-alcoholic fatty liver disease.

Variable	Significant fibrosis		
	Unadjusted model[&]	Adjusted model 2*	Adjusted model 3[§]
	OR (95% CI) *p* value		
Mean age, yr	1.03 (1.02-1.05) <0.001	1.05 (1.02-1.07) <0.001	1.04 (1.02-1.06) <0.001
ALT, IU/L	1.01 (1.00-1.01) <0.001	1.01 (1.00-1.01) 0.001	1.00 (1.00-1.01) 0.003
Type 2 diabetes	3.02 (1.92-4.93) <0.001	2.03 (1.16-3.54) 0.01	1.91 (1.06-3.45) 0.03
NASH	10.7 (6.78-17.0) <0.001	-	7.19 (3.81-13.5) <0.001
MERTK GG/GA *vs.* AA	0.54 (0.30-0.99) 0.04	0.40 (0.20-0.81) 0.01	0.43 (0.21-0.88) 0.02
PNPLA3 CC *vs.* CG *vs.* GG	1.29 (0.99-2.69) 0.05	1.05 (0.77-1.43) 0.75	1.01 (0.73-1.40) 0.90
TM6SF2 CC *vs.* CT/TT	1.23 (0.72-2.08) 0.44	1.26 (0.69-2.32) 0.44	1.28 (0.67-2.43) 0.44
Southern Italian cohort	5.58 (3.69-8.46) <0.001	5.07 (3.19-8.08) <0.001	1.41 (0.75-2.66) 0.27

Figures legend

Fig. 1. MERTK in situ hepatic expression in human NAFLD. MERTK in situ expression was investigated by immunohistochemistry on NAFLD bioptic samples using a specific monoclonal primary antibody. MERTK was found to be expressed in cells with stellate or monocytoid morphology scattered throughout the hepatic parenchyma and loosely aggregated within inflammatory foci (upper panels) while being not expressed in hepatocytes. Since the morphology of MERTK-expressing cells was suggestive of stellate cells/macrophages double labelling immunofluorescence analysis was performed for the CD68 macrophage marker and MERTK. Consistent with the morphology observed on immunohistochemically-stained sections, MERTK-expressing cells also expressed CD68 (lower panels), which confirmed their macrophagic lineage. (This figure appears in colour on the web.)

Fig. 2. Hepatic MERTK mRNA expression. MERTK mRNA hepatic levels according to MERTK rs4374383 genotype in 94 bariatric severe obese patients from Northern Italy (A), and in 80 NAFLD patients from Southern Italian cohort (B); MERTK mRNA hepatic levels according to presence/absence of F0-F2 fibrosis in 27 NAFLD patients from FLIP cohort (C), and in 80 NAFLD patients from Southern Italian cohort (D).

Fig. 3. Mice hepatic MERTK mRNA expression. (A) Male mice were injected intraperitoneally with a dose of CCl4 (0.5 ll/g body weight) or olive oil twice a week for 6 weeks, ⁄p <0.05 vs. oil. (B) Male mice were administered with a control diet (CD) or a diet without methionine and choline (MCD) for 8 weeks, ⁄⁄p <0.05 vs. CD.

Fig. 4. Role of MERTK protein on primary HSCs motility and ERK activation. (A) MERTK expression was detected by immunoprecipitation with anti-MERTK or aspecific antibody, as indicated, from HSC lysate. Precipitated proteins were detected by Western blot with anti-MERTK. (B) Cells were serum-starved for 24 h and then treated with 100 ng/ml of GAS6 at different times as indicated in the figure. Total cell lysates from primary HSCs were analysed by Western blotting

using the indicated antibodies. (C) Migration in the presence or absence of the indicated concentrations of GAS6 for 6 h were measured using modified Boyden chambers. As positive control cells were stimulated with 10% foetal bovine serum for the same time. ⁄p <0.05 vs. control.

Fig. 5. Effects of a MERTK inhibitor or of MERTK knockdown on the biology of HSC. (A) Migration of HSC in response to the indicated concentrations of GAS6 and in the presence (dark blue columns) or absence (light blue columns) of 5 lM UNC569 was measured using Boyden chambers. ⁄p <0.05 vs. unstimulated control; ⁄⁄p <0.05 vs. the same GAS6 concentration without inhibitor, hpf, high power field. (B) Serum-deprived HSC were incubated in the presence of the indicated concentrations of GAS6 for 48 h. Cell viability was measured as described in Materials and methods. ⁄p <0.05 vs. control (no UNC569). (C) HSC were transfected with MERTK-specific siRNAs or with non-targeting (siNT) siRNAs, as described in Materials and methods. ⁄p <0.05 vs. non-targeting siRNAs. (D) Lanes 1–2: Cultured HuH7 were incubated with 10 lg/ml doxorubicin or its vehicle for 48 h. Lanes 3–6: serum-deprived HSC were incubated in the presence of the indicated concentrations of UNC569. Total cell lysates were analysed by Western blotting using the indicated antibodies. (E) Serum-deprived HSC were incubated with the indicated concentrations of GAS6 and in the presence or absence of UNC569 for 48 h. At the end of the experiment gene expression of type I procollagen was measured as indicated in Materials and methods. ⁄p <0.05 vs. unstimulated control; ⁄⁄p <0.05 vs. GAS6 without inhibitor; § p = 0.07 vs. GAS6 without inhibitor.

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