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ORIGINAL RESEARCH

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Immunoreactivity against fibroblast growth factor 8 in alveolar rhabdomyosarcoma patients and its involvement in tumor aggressiveness

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ABSTRACT

Rhabdomyosarcoma (RMS) is an aggressive pediatric soft tissue sarcoma characterized by a very poor prognosis when relapses occur after front-line therapy. Therefore, a major challenge for patients' management remains the identification of markers associated with refractory and progressive disease. In this context, cancer autoantibodies are natural markers of disease onset and progression, useful to unveil novel therapeutic targets. Herein, we matched autoantibody profiling of alveolar RMS (ARMS) patients with genes under regulatory control of PAX3-FOXO1 transcription factor and revealed fibroblast growth factor 8 (FGF8) as a novel ARMS tumor antigen of diagnostic, prognostic, and therapeutic potential. We demonstrated that high levels of FGF8 autoantibodies distinguished ARMS patients from healthy subjects and represented an independent prognostic factor of better event-free survival. FGF8 was overexpressed in ARMS tumors compared to other types of pediatric soft tissue sarcomas, acting as a positive regulator of cell signaling. Indeed, FGF8 was capable of stimulating ARMS cells migration and expression of pro-angiogenic and metastasis-related factors, throughout MAPK signaling activation. Of note, FGF8 was found to increase in recurrent tumors, independently of PAX3-FOXO1 expression dynamics. Risk of recurrence correlated positively with FGF8 expression levels at diagnosis and reduced FGF8 autoantibodies titer, almost as if to suggest a failure of the immune response to control tumor growth in recurring patients. This study provides evidence about the crucial role of FGF8 in ARMS and the protective function of natural autoantibodies, giving new insights into ARMS biology and laying the foundations for the development of new therapeutic strategies.

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Introduction

Cancer evolves as a result of an accumulation of mutations and chromosomal aberrations. The immune system senses genomic and proteomic changes that tumor cells sustain, recognizing them as exogenous and no longer as self. This triggers an adaptive immune response which includes the production of circulating autoantibodies against the so-called tumor-associated antigens (TAAs). Starting from this assumption, we propose that circulating autoantibodies in blood of cancer patients may provide clues to define clinically relevant targets of tumor growth, development, and progression.

Rhabdomyosarcoma (RMS) is a rare and often lethal disease that mainly affects children and young adults. It is the most common soft tissue sarcoma and a major cause of cancer-related death in childhood. The two main subtypes are alveolar RMS (ARMS, about 20%) and embryonal RMS (ERMS, 60–70%). They occur in children under 10 years of age and in adolescents or young adults, respectively, showing differences in genomics and tumor behavior that account for their diverse clinical course and therapeutic management.[1] In this regard, ARMS are characterized by pathognomonic *PAX3-FOXO1* or *PAX7-FOXO1* gene fusions, a higher propensity for early dissemination and a poor response to therapy, while ERMS exhibit a more pronounced genomic instability, a higher mutational burden, but a significantly better outcome.[2] Multimodal therapy has improved the survival of RMS patients with localized disease at diagnosis (>70%), whereas outcome of recurrent and refractory tumors remains measly (<30%).[3–6]

Herein, we took advantage of our previously adopted immunoproteomic approach to search for new relevant tumor markers and therapeutic targets in very high risk PAX3-FOXO1 fusion-positive (PF+) ARMS patients, identifying FGF8 as the most interesting candidate among all. FGF8, originally described as an androgen-induced growth factor from the conditioned medium of mouse mammary carcinoma cells SC-3,[7] is a secreted glycoprotein belonging to the large family of human fibroblast growth factors (FGFs). The *FGF8* gene undergoes alternative splicing leading to the generation of four isoforms in humans (a, b, e, and f). Among them FGF8b possesses the greatest mitogenic and transforming

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activity.[8–11] It plays an important role during embryogenesis,[12] particularly in the development of the craniofacial, pharyngeal, brain, cardiac, kidney, urogenital organs, and limbs.[13–16] Likewise, it has been shown to be overexpressed in several hormone-related and non-related cancers, including prostate and breast cancer among the formers,[17–19] and colorectal cancer, oral squamous cell carcinoma and hepatocellular carcinoma among the latter.[20–22] Clinically, its association with advanced-stage tumors and its expression in metastasis has been described, as its ability to facilitate tumor progression and spreading throughout the stimulation of cancer cells invasion and angiogenesis, respectively.[23,24] Moreover, sustained activation of downstream receptor substrates and signaling pathways by autocrine FGF8 expression correlates with reduced response to therapy and diminished survival of cancer patients.[25–28] Recently, FGF8 upregulation has been reported in a myoblast murine model of MYCN/PAX3-FOXO1-induced rhabdomyosarcoma, where it has been shown to be necessary and sufficient to rescue oncogenicity and simulate tumor recurrence after PAX3-FOXO1 silencing. Until now, FGF8 expression in pediatric RMS patients has not been investigated and its presence in recurrent and refractory tumors remain to be ascertained. Herein, we demonstrated that FGF8 is a novel ARMS oncoantigen, capable of stimulating a humoral immune response in patients as indicated by the presence of circulating anti-FGF8 autoantibodies in the blood. Autoantibodies found in patients' plasma samples resulted of good prognostic significance in event-free survival. Accordingly, FGF8 was overexpressed in PF+ ARMS primary tumors, further increasing at recurrence, independently of PAX-FOXO1 expression dynamics. *In vitro*, FGF8 stimulated FGFR-related markers of cell signaling, metastatic potential, migration, and invasion, further supporting its involvement in PF+ ARMS aggressive behavior and likelihood of recurrence.

Materials and methods

Patients

Forty-eight blood plasma samples and 50 tumor biopsies of RMS patients were analyzed in this study. Samples were collected between July 2004 and March 2015, at the time of diagnosis, from RMS patients enrolled in the pediatric sarcoma protocols RMS 4.99, EpSSG RMS 2005, and EpSSG MTS-2008. In addition, 12 matched tumor samples were collected at the relapse of the disease. Studies on human specimens were approved by Padua Hospital Ethics Committee and patients were included in this study after obtaining institutional review board approval (No. 191P, 20 June 2000; No. 988P, 31 March 2005). Diagnosis of RMS was reviewed by the Italian Association of Pediatric Hematology and Oncology (AIEOP) reference pathologists and supported by molecular investigation of *MyoD1* and *PAX3/7-FOXO1A* transcripts. Clinical parameters, molecular features, and follow-up data (last update in May 2021) of all the participants are summarized in Table S1. Fifteen pediatric healthy subjects (HS), 12 males and 4 females with a median age of 9,48 ± 3,75 years, were also included in the analysis and used as controls.

Blood samples

Forty-eight blood specimens were collected in a sodium citrate tube at the time of diagnosis, prior to any type of treatment. Plasma samples were obtained by centrifugation of peripheral blood at 820 × g for 10 minutes, followed by a further centrifugation step at 16000 × g for 10 minutes to avoid any type of contamination by blood cells. Plasma was aliquoted and stored at −80°C until use.

Enzyme-linked immunosorbent assays (ELISA)

Secreted FGF8 was quantified in patient's plasma samples and serum-free cell supernatant of RMS cell lines by using the FGF8 ELISA assay kit (Mybiosource), according to the manufacturer's instructions. Plasma samples were diluted 1:25 in PBS 1X, while cells medium were used undiluted. Each assay was performed in triplicate and the mean concentration of FGF8 was calculated for each sample by interpolation from the standard curve of recombinant FGF8 provided by the kit and by multiplying by the dilution factor.

Indirect ELISA assay for anti-FGF8

Plasmatic autoantibodies (Abs) against FGF8 were assessed by a home-made indirect ELISA assay. Human recombinant FGF8 (Gibco) diluted in 50 mM carbonate-bicarbonate buffer (Sigma-Aldrich, St Louis, MO, USA) to a final concentration of 0,5 µg/mL were coated overnight at 4°C on immulon 4HBX microtiter plates with extra-high binding surface (Dynex Tecnologies Inc., Chantilly, VA). Serially diluted purified human IgG (5 ng/mL – 640 ng/mL) (Sigma-Aldrich, St Louis, MO, USA) was used to provide the standard curve. Blocking, washing, probing, development, and quantification procedures were performed as previously described.[29]

Total IgG measurement

To normalize FGF8 autoantibodies levels, total plasma IgG level of each plasma sample was assessed in triplicate by Human IgG ELISA kit (RayBiotech, Inc., Norcross, GA) according to the manufacturer's instructions.

Cell lines

The human RH30, RD, MCF-7, and A549 cell lines were purchased from ATCC (Manassas, VA), while RH4, RH28, RH18, TC32, CHP100, A673, RH1, and TC106 cells were a gift of Dr P.J. Houghton (St Jude Children's Hospital, Memphis, TN). RC2 and CCA cells were a gift from Prof. Pier Luigi Lollini (University of Bologna, IT), while RH36 and SMS-CTR were obtained from Dr. Maria Tsokos (National Cancer Institute, Bethesda, MD). RD-ES and SK-ES -1 were a gift of Prof. Katia Scotlandi (Istituto Ortopedico Rizzoli IRCCS, Bologna, IT). SW982, was kindly provided by Dr. Andrea Ferrari (Istituto Nazionale Tumori IRCSS, Milan, Italy), while the osteosarcoma cell line SAOS-2 was provided by Prof. Luca Scorrano (University of Padova, IT). HT-29 was a gift of Dr. Marco Agostini (University of Padova, IT), SH-

SY5Y and NB1 were provided by Dr. Gian Paolo Tonini (University of Padova, IT), BJ was given by Dr. Michela Pozzobon (University of Padova, IT) and HEK-293 and HEK-293T were obtained from Prof. Dorianna Sandonà (University of Padova, IT). Among non-Hodgkin's lymphoma cell lines Karpas-299, SUP-M2, SU-DHL-1, FE-PD, and MAC2A, were used, while among leukemia cell lines, Jurkat, MO-91, and CEM, were employed. For some cell lines, authentication was confirmed by Short Tandem Repeat (STR) analysis using the Promega PowerPlex Fusion System (Promega, Madison, WI, USA). Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Life Technologies Co., Carlsbad, CA, USA) or RPMI 1640 containing 15% FBS. Both medium were added with glutamine (2mM), penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were maintained at 37C in a humidified atmosphere containing 5% CO_2.

Quantitative RT-PCR

Total RNA was extracted from cell lines and frozen tumor biopsies using TRIzol reagent (Invitrogen). Retrotranscription to cDNA was performed with SuperScript II enzyme (Invitrogen), according to the manufacturer's instructions. As normal controls, mesenchymal stem cells and fetal and adult skeletal muscle samples, were used (Thermo Fisher scientific). Quantitative RT-PCR (qRT-PCR) was performed on Viia7 thermal cycler (Applied Biosystems), using SYBR Green chemistry (Applied Biosystems) and standard protocol of amplification. Gene-specific primer is listed in Table S2. The relative expression of each gene was calculated by the 2^(-ΔΔCt) method, using *GAPDH* as reference gene and the fetal skeletal muscle as external calibrator.

Cell lysis, immunoblotting, and immunoprecipitation

Cells were washed twice in ice-cold PBS 1X and incubated on ice for 30 minutes with a lysis buffer containing Tris-HCl 50 mM at pH 7,5, NaCl 150 mM, EDTA 2 mM, 0,1% SDS, 0,5% sodium deoxycholate, 1% TritonX-100, aprotinin 20 µg/mL, leupeptin 20 µg/mL, and PMSF 1 mM. The lysates were clarified by centrifugation at 14000 rpm for 30 minutes at 4°C, and protein concentrations were determined by the bicinchoninic acid assay (Thermo Fisher scientific) using bovine serum albumin (BSA) as a standard. Cell lysates (60 µg) were diluted with Laemmli loading buffer 5X (2% SDS, 100 mM DTT, 60 mM Tris at pH 6,8, 0,01% blue bromophenol and 10% glycerol), denaturated at 95°C for 5 minutes and fractionated by 10% polyacrylamide gel for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were electrotransferred onto nitrocellulose membranes overnight at 4°C. Membranes were probed with primary antibody properly diluted in a 5% BSA Tris-buffered saline (TBS) 1X solution for 3 hours at room temperature with gentle shaking and then for 1,5 hours with HRP-conjugated secondary antibody (GE Healthcare). Protein bands were visualized by ECL chemiluminescence detection system (Perkin Elmer) and acquired with iBright FL1500 Imaging System (Thermo Fisher scientific). The primary antibodies used were: anti-FGF8 (Peprotech), anti-phospho FRS2 (Cell Signaling), anti-phospho ERK1/2 and total ERK1/2 (Cell Signaling) and anti-γ-Tubulin (Sigma-Aldrich). For immunoprecipitation cells were lysed as above, and 0,2 mg of protein lysates were precipitated overnight at 4°C, with anti-FGFR4 (Cell Signaling) or anti-FGFR2 (Cell Signaling). The immunocomplexes were adsorbed onto 50 µL protein G sepharose beads (GE Healthcare), incubated at 4°C for 120 min and then resuspended in sample buffer before fractionation by SDS gel electrophoresis. Western blotting was performed as above using anti-phospho FGFR (Y653/654) (Cell Signaling) as primary antibody.

Immunohistochemistry

Immunohistochemical staining of tissue biopsies was performed on 5 µm formalin fixed, paraffin-embedded tissue sections using a fully automated system (Bond-maX, Leica, Newcastle Upon Tyne, UK). Sections were de-waxed, rehydrated, and incubated in retrieval buffer solution (Leica, Newcastle Upon Tyne, UK) for antigen recovery. Specimens were then washed with phosphate-buffered saline (pH 7.0) and incubated with the Bond Polymer Refine Detection Kit (Leica, Newcastle Upon Tyne, UK) according to the manufacturer's protocols. Immunostains for FGF8 (Peprotech; dilution 1:50) were performed using an automated immunostainer. Signals were visualized with 3,3′-diaminobenzidine (DAB) and Mayer's hematoxylin (Diapath, Martinengo, Italy) was used as counterstained. Images were acquired using Leica DM4000B microscope with Leica DFC295 camera.

Immunocytochemistry

Cytocentrifuge preparations of RH30 and RD cells were fixed on slides and permeabilized with acetone for 10 minutes and then incubated with anti-FGF8 antibody (Peprotech) diluted in PBS1X for 1 h at room temperature. After washing, slides were probed at room temperature for 40 minutes with HRP-conjugated anti-rabbit secondary antibody (Thermo Fisher scientific) and then with 3,3'-diaminobenzidine (DAB) (Dako) A brief dive in Gill's hematoxylin solution (Sigma-Aldrich) was done for visualization of nuclei in cell preparations. The images were acquired with a Leica DFC420 digital camera, mounted on a Leica DMLB microscope, at 20X magnification. Image analysis was performed with Leica IM1000 software (Leica Microsystem Ltd).

Immunofluorescence

RH30 and RD cells were seeded on 4-well chamber slide (Falcon) and incubated at 37°C for 48 hours. Once reached sub-confluence, cells were fixed with 4% paraformaldehyde for 20 minutes, and permeabilized with 0,2%Triton X-100 in PBS1X for 10 minutes. Slides were then incubated for 10 minutes in 100 mM glycine and for further 10 minutes in 5% BSA in PBS1X. Primary antibody against FGF8 (Proteintech) and in 1%BSA/PBS1X were probed at 37°C for 60 minutes, followed by secondary antibody Alexa Fluor® 546 conjugate (Thermo Fisher scientific) in PBS1X

at 37°C for 60 minutes. Slides were washed and mounted in 1:1 glycerol/PBS1X supplemented with DAPI 1:500 (6,6-diamino-2-phenylindole, dihydrochloride) (Thermo Fisher scientific). The images were acquired with a Leica DFC420 digital camera, mounted on a Leica DM4000B microscope, at 20X magnification. Image analysis was performed with Leica IM1000 software (Leica Microsystem).

Wound healing assay

RH4 and RD cells were seeded on a 12-wells plate at a final density of 50×10^4 cells/well and incubated at 37°C for 24 hours. A single scratch wound was created by dragging a plastic pipette tip across the well surface and then cell culture medium was replaced with or without 100 ng/mL of recombinant human FGF8 protein (Gibco). The images were acquired every 24 hours, up to 48 hours with the Leica DM IRB inverted microscopy (Leica Microsystem Ltd). The width of the scratch was measured using ImageJ software.

Cell viability assay

RMS cell viability was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. Briefly, 50×10^3 cells/mL were seeded in 96-well plates. The cells were grown in the presence or absence of the FGFR inhibitor Infigratinib (BGJ-398) (Selleckchem) at 37°C for up to 72 hours and reduction of the MTT salt (SIGMA Co., USA) was measured every 24 h at 540 nM (Viktor, Perkin Elmer). MTT salt was added for 4 hours. Values represent the mean of triplicate cultures of three independent experiments.

Statistical analysis

ELISA and expression data were analyzed by the Mann–Whitney test or one-way ANOVA test. Data about FGF8 or PAX3/7-FOXO1 expression collected at diagnosis and at the relapse of the disease were compared by Wilcoxon signed-rank test. Spearman rank correlation was used to measure the degree of association between FGF8 autoantibody levels assessed by protein microarray and ELISA assay, and between the expression of FGF8 quantified in tumor biopsies and levels of circulating anti-FGF8 in the blood of the same patients. Survival analysis curves were estimated by the Kaplan–Meier method and overall differences were compared by the log-rank test. The outcome considered was event-free survival (EFS), calculated from the date of diagnosis to the date of the first event (tumor progression or relapse) or the last follow-up, and overall survival (OS), calculated from the date of diagnosis to the date of death for any cause or the last follow-up. Cox uni- and multivariate proportional hazard analysis were carried out to estimate the prognostic impact of FGF8 autoantibodies. All statistical analyses were carried out using GraphPad Prism 8, IBM SPSS Statistics 23 and R statistical version 4.1.0.[30] All *p*-values were two-tailed and considered statistically significant at the alpha level of 0,05.

Results

FGF8 autoantibodies in very high-risk RMS patients and its prognostic role

To identify abnormally regulated proteins in very high-risk RMS patients, we cross-checked tumor antigens, previously identified in metastatic *PAX3-FOXO1*-positive (PF+) ARMS by means of immune response binding profiling (IRBP) analysis,[31] with genes under the regulatory control of PAX3-FOXO1 fusion protein.[32] Among the 55 antigens showing significant difference of immunoreactivity compared to healthy subjects (Figure 1a and Table S3), six were direct targets of PAX3-FOXO1 (Figure 1b). FGF8 was selected for further investigation based on the evidence that it plays important roles during the embryonic development of the muscle system, whereas it is barely detected in adult tissues and frequently upregulated in cancers.[12–22] To validate tumor-derived FGF8 immunoreactivity and rule out inflammation as a general mechanism of autoantibodies production, we used an in-house ELISA assay as the gold standard technique, normalizing anti-FGF8 autoantibodies values to patients' total plasmatic IgGs. We confirmed the presence of FGF8 autoantibodies detected in plasma samples by IRBP analysis (Figure 1c) and substantiated the significant difference between patients and controls (Figure 1d; *healthy subjects*, HS). In contrast, total IgG levels were akin in the two groups (data not shown), supporting an anti-tumor role of FGF8 autoantibodies rather than simply reflecting the presence of an inflammatory response.[33] When compared, the two data set statistically correlated, substantiating these preliminary observations (Figure 1e, *Spearman* $p = 0{,}01$). Next, to analyze the relationship between autoantibodies, clinic-pathological parameters and patients outcome, we measured FGF8 autoantibodies in a larger cohort of PF+ cases (Table S1), for whom clinical information about age, gender, IRS group, fusion status, tumor size, and site at onset were available. No significant association was found (Figure S1), however, when patients were distinguished according to the median abundance of FGF8 autoantibodies, patients with low titers of autoantibodies displayed a significant worse event-free survival than patients with high autoantibody titer (Figure 2a; p=0,02, HR = 2,21, 95% CI = 1,10–4,43), and had a 3-year event-free survival of 13% compared to 45,8% of the latter. In this context, differences in overall survival between the two groups were not significant (Figure 2b; p=0,62, HR = 1,20, 95% CI = 0,56–2,56). Multivariate Cox regression analysis substantiated these findings, as FGF8 autoantibodies at diagnosis were prognostic in event-free survival independently from any other clinical feature assessed (Table 1; $p = 0{,}016$; HR = 0,376; 95% CI = 0,170–0,831). Notably, in patients with high FGF8 IgG level, EFS, and OS were similar, whereas in patients with low FGF8 IgG, OS was better than EFS, due to some patients achieving disease remission despite relapse occurrence. Namely, five patients with localized disease and 1 with metastatic tumor at diagnosis experienced recurrence (2 with metastatic relapse, 3 with regional lymph node relapse and 1 with local relapse) but reached complete

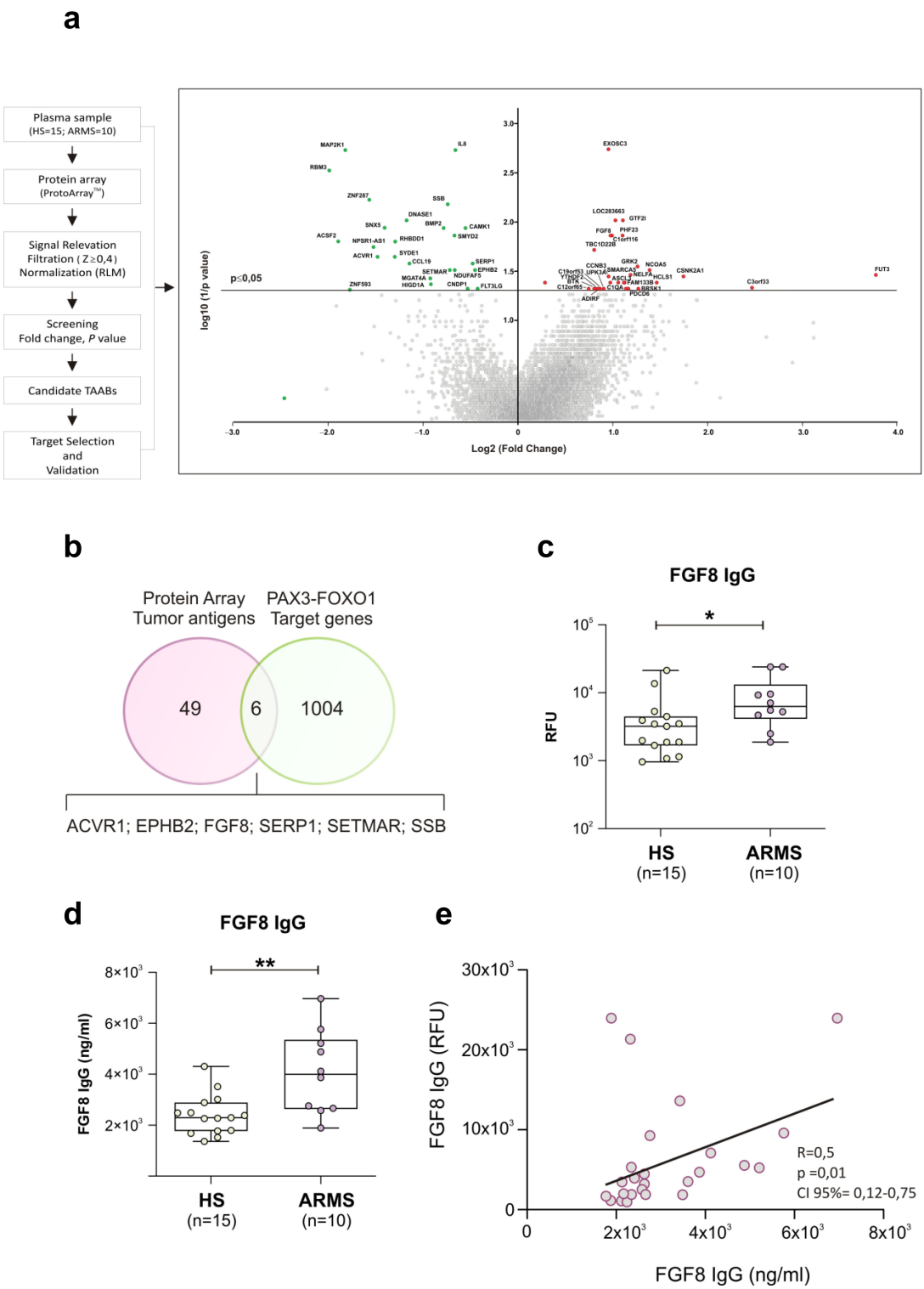

Figure 1. FGF8 autoantibodies detection in very high-risk ARMS patients. (a) *Left*, experimental workflow followed for the identification of autoantibodies in ARMS patients using plasma samples and ProtoArray™ technology.[31] The immune response profile was obtained from 10 metastatic ARMS patients and 15 healthy subjects (HS), probing protein microarray chips with plasma. Reactivity of 9374 spotted antigens was evaluated after signal detection, filtration and normalization using robust linear model (RLM). Antigens median values were calculated for each group, compared and ranked according to significant *p*-value scale. *Right*, volcano plot of protein microarray data showing differentially immunoreactive antigens between patients and healthy subjects, plotted along dimension of fold change (abscissae) and statistical difference (ordinates). Antigens with significant *p*-values (≥0,05) are indicated by colors and names, while antigens with no significant difference in immunoreactivity between patients and controls are plotted uncolored on the bottom of the graph (gray dots). Antigens more reactive in patients or controls are distinguished by red or green dots, respectively. (b) Venn diagram showing the overlap between differential immunoreactive antigens (n = 55) and PAX3-FOXO1 target genes (n = 1010).[32] (c) Box plot of FGF8 signal intensity revealed in patients and controls by protein microarray (*RFU = relative fluorescence unit*) and (d) validation by indirect ELISA assay. (e) Correlation of FGF8 autoantibody signal intensity and FGF8 IgG concentration obtained in the same samples cohort by protein microarrays and ELISA assay, respectively. $p < 0,05$ (*); $p < 0,01$ (**).

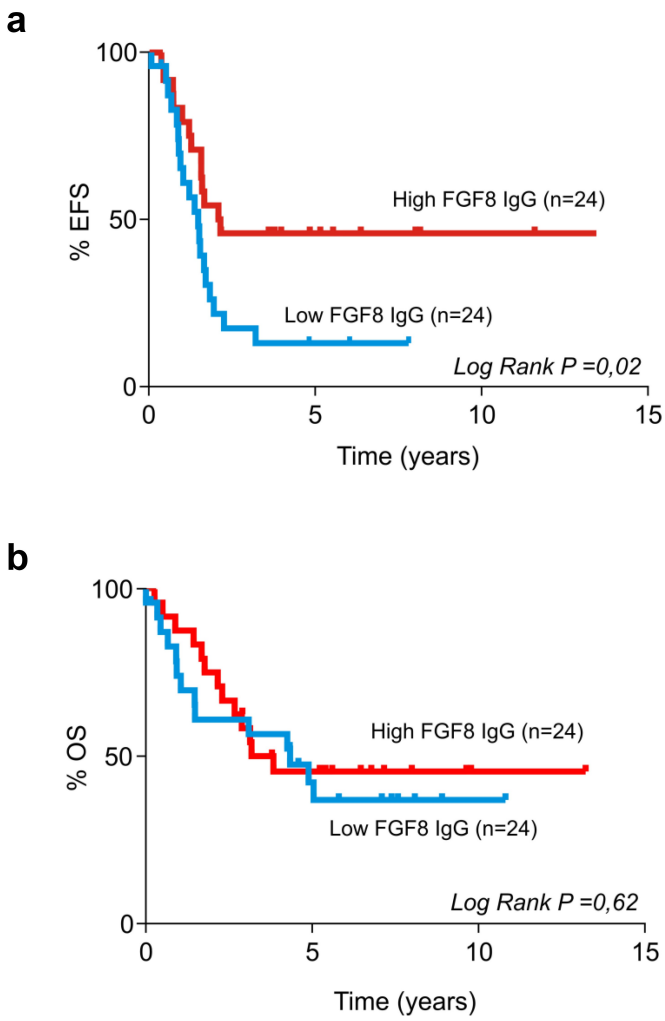

Figure 2. Correlation between humoral immune response against FGF8 and patients' outcome. Kaplan-Meier survival analysis representing (a) event-free survival (EFS) and (b) overall survival (OS) of ARMS patients distinguished according to FGF8 autoantibodies median value.

remission after therapy. All of them benefitted from second-line treatments, including a metastatic patient who developed a second distant metastasis after 6 months from diagnosis. FGF8 autoantibodies, thus, represent surrogate markers of disease progression rather than overall survival.

FGF8 is overexpressed in PF+ RMS cell lines.

To prove that stronger autoantibody reactivity in ARMS patients is associated with higher FGF8 expression in tumors, we first measured FGF8 mRNA levels in normal control cells (mesenchymal stem cells, fetal and adult skeletal muscle cells). As expected, FGF8 was overexpressed in PF+ ARMS cell lines compared to normal muscle and mesenchymal stem cells (*CTR*, $p = 0{,}028$), PF-embryonal RMS (*ERMS*, $p = 0{,}015$), Ewing sarcoma (*EWS*, $p = 0{,}006$), Non-Hodgkin lymphoma (*NHL*, $p = 0{,}015$) and cell lines of various origins, including leukemia, lung, and breast cancer, osteosarcoma, synovial sarcoma, neuroblastoma (*Others*, $p = 0{,}002$) (Figure 3a). This was true for FGF8b/e/f isoforms, whereas FGF8a was uniformly expressed in all cell lines (Figure S2). To examine FGF8 protein expression in RMS cells, both PF+ (RH30, ARMS) and PF– (RD, ERMS) cell lines were used. Corresponding to RNA data, stronger cytoplasmic staining of FGF8 protein was observed in RH30 cells, whereas signal in RD cells was weak and restricted to perinuclear regions (Figure 3(b,c)). Differences were confirmed when both intracellular and secreted FGF8 proteins were assessed, in the lysate and culture media of both cell lines after 48 hours of serum starvation (Figure 3(d,e)). Finally, since FGF8 belongs to a wider family of growth factors with paracrine and autocrine functions, we extended expression analysis to other FGF family members, reporting data for the most relevant of them. With this respect, we found that FGF8-subfamily member FGF17 was also significantly upregulated in PF+ RMS cell lines compared to normal controls (Figure S3, $p = 0{,}028$), though the difference was far less than that of FGF8 (4,6 *vs.* 1671), while not observed between PF+ and PF– RMS cell lines. In contrast, FGF18, the third member of the FGF8 subfamily and a prognostic marker in some of the most aggressive adult epithelial cancers,[34,35] was not expressed in RMS cells at all. Expression of FGF7 in PF+ ARMS cells was also significantly higher compared to PF–ERMS cells ($p = 0{,}015$), as recently reported by others,[36] but similar to that measured in normal cells. FGF1 and FGF2, instead, two important family members with broad mitogenic activities in normal and pathological conditions, were expressed in tumor cell lines as much as in normal cells.

FGF8-induced signaling is active in PF+ RMS cells

FGF8 exerts its effects through the binding to fibroblast growth factor receptors (FGFR1-4) at the plasma membrane, and several lines of evidence suggest that aberrantly expressed FGFRs play an important role in RMS tumorigenesis.[37–40] Therefore, to explore the signaling downstream to FGF8, we first measured FGFRs expression in our panel of PF+ and PF– RMS cell lines. As for FGF8, and consistent with previous findings,[41] significant FGFR2 and FGFR4 protein expression was observed in PF+ ARMS cells compared to PF– ERMS cells (FGFR2, $p = 0{,}016$; FGFR4, $p = 0{,}031$) and the majority of the non-RMS cell lines analyzed (Figure 4a and S4). When assessed under normal growth culture conditions, basal FGFR4 and FGFR2 phosphorylation was also higher in PF+ ARMS cells, indicating a stronger activation of both receptors (Figure 4b). Growth factor-dependent signaling was investigated by adding exogenous FGF8 to culture medium of both PF+ ARMS (RH4) and PF– ERMS (RD) cells. As expected, FGF8 induced a rapid, but transient, activation of fibroblast growth factor receptor substrate 2 (FRS2), the membrane-anchored protein responsible for receptor signal transduction, and a more sustained phosphorylation of ERK1/2 kinase in PF+ RH4 cells (Figure 4c, *left panel and graph*). In RD cells, treatment-dependent phosphorylation of FRS2 was not observed, while phospho-ERK1/2 status changed modestly under the same experimental conditions (Figure 4c, *right panel and graph*). In line with these findings, cell motility, which is a growth factor-induced mechanism that occurs in cancer cells via MAPK activation,[42,43] was stimulated by FGF8 in PF+ RH4 cells only, as observed in wound-healing

Table 1. Uni- and multivariate Cox regression for event-free survival in 41 ARMS patients.

		No. of Patients (n = 41)	Univ. p-value	Multiv. p-value	HR	CI 95%
Gender	Male	15	0,423			
	Female	26				
Age	≥ 10 yrs	23	0,126			
	<10 yrs	18				
Site of tumor onset	Favourable*	4	0,612			
	Unfavourable#	37				
Size of tumor	≤ 5 cm	15	0,302			
	> 5 cm	26				
IRS group†	I–III	20	**0,012**	**0,005**	0,319	0,144–0,705
	IV	21				
FGF8 IgG	≥ 2174,67 ng/ml	20	**0,040**	**0,016**	0,376	0,170–0,831
	< 2174,67 ng/ml	21				
Fusion Status	*PAX3-FOXO1*	35	0,332			
	PAX7-FOXO1	6				

* Favorable site: orbit, urogenital non-bladder/prostate (i.e., paratesticular and vagina/uterus) and head and neck non-parameningeal; # unfavorable site: head and neck parameningeal, urogenital bladder/prostate, extremities, and all "other site" (i.e., thorax, abdominal, retroperitoneal, perianal, pelvis); † IRS, International Rhabdomyosarcoma Study Group, IRS group I defines completely excised tumors, group II grossly resected tumors with microscopic residual disease and/or regional lymph node involvement, group III gross residual disease after incomplete resection of biopsy and group IV metastatic disease; *PAX3*, paired box 3; *PAX7*, paired box 7; *FOXO1*, forkhead box protein 1; Univ., univariate analysis; Multiv., multivariate analysis; HR, hazard ratio; CI, confidence interval.

assay performed 48 hours after growth factor exposure (Figure 4d). Dose-dependent FGF8-induced ERK1/2 phosphorylation was then assessed in the presence or absence of NVP-BGJ398 (Infigratinib), a pan-FGFR inhibitor with no reported off-target effect and cross-reactivity against any other receptor tyrosine kinase. As expected, ERK1/2 phosphorylation was totally abrogated in PF+ RH4 cells by NVP-BGJ398, whereas no changes were observed in PF– RD cells (Figure 4e). Further, when PF+ ARMS (RH30; RH4) and PF– ERMS cells (RD, RH36) were grown in the presence of NVP-BGJ398, drug sensitivity strongly correlated with FGF8 mRNA expression levels, as ARMS cell lines demonstrated an extreme drug sensitivity, while ERMS cells were totally unaffected (figure 4f). Finally, to determine the effect of FGF8-related cell signaling at gene expression level, we measured time-dependent *DUSP6, SPRY4, GDF15*, and *ETV5* transcription upon recombinant FGF8 treatment.[43] As expected, *DUSP6, SPRY4, GDF15*, with the exception of *ETV5*, were all rapidly up-regulated by FGF8 in PF+ RH4 cells, whereas in PF– RD no further transcription was induced (Figure 5(a,b)). Administration of FGF8 increased also plasminogen activator urokinase (PLAU) and matrix metallopeptidase-9 (MMP9) gene expression in RH4 cells, two important genes for invasion, metastasis, and angiogenesis of several cancers (Figure 5c).

FGF8 is expressed at very high levels in PF+ RMS patients

To assess FGF8 expression in RMS patients, 50 tumor biopsies were collected (Table S1) and FGF8 mRNA measured by qRT-PCR. In line with the above-reported findings, FGF8 was overexpressed in RMS tumor tissues (Figure 6a, p=0,0003), particularly in PF+ tumors (Figure 6b, p<0,0001), as substantiated by the paraffin-embedded tissue section staining of PF+ ARMS and PF– ERMS cases (Figure 6c). By querying online accessible genomic data repositories[44,45] FGF8 overexpression in *PAX3/7-FOXO1*-bearing RMS was confirmed when compared to both PF– RMS (Figure S5a, p < 0,0001) and other pediatric soft tissue and bone sarcomas characterized by pathognomonic gene fusions (FigureS5b, p<0,0001). Such a difference was validated in a small cohort set of our cases that included desmoplastic small round cell tumors (DSRCT), osteosarcomas (OS) and Ewing sarcomas (ES) (FigureS5c), providing final evidence that FGF8 is a reliable tumor marker of PF+ ARMS. Beside the association of FGF8 with *PAX3/7-FOXO1* fusion status, we observed that patients older than 10 years and younger than 1 displayed also higher levels of FGF8 compared to children with age between 1 and 10 years (FigureS6). Patients with metastatic disease at diagnosis (IRS group IV), instead, had higher FGF8 levels respect to patients with localized disease (IRS group II, III), but this difference was not statistically significant. Of note, matching FGF8 expression in tumor tissues with the autoantibodies titer revealed a significant inverse correlation between the two variables (Figure 6d, p=0,0002; r = −0,0602; 95% CI = −0,787–0,316). When patients were grouped according to FGF8 IgG and mRNA median values (group I to IV), those with high autoantibody titers and low transcript levels (Figure 6d, group IV, *upper left*) had significantly more chances of not experiencing adverse events, such as recurrence or progressive disease, than any other mRNA/autoantibodies combination (Figure 6d, IV = 42% *v.s.* I–III = 5%; Fisher's exact test p = 0,016).

FGF8 is upregulated in recurrent RMS tumors

FGF8 has been shown to contribute to chemotherapy and radiation therapy resistance, in colon and rectal cancer cells, respectively.[25,27] More recently, in a mouse model of fusion-positive RMS, FGF8 has been found to be maintained at high level in recurrent tumors despite PAX3-FOXO1 deinduction, and used as a key growth- and survival-promoting factor in the absence of the oncogenic fusion protein.[46] However, since a PAX3/7-FOXO1 target therapy is not contemplated among the treatment approaches used to cure RMS patients, to understand whether FGF8 upregulation observed in mice might invoke a more broad phenomena of RMS drug resistance, we measured its expression in matched primary tumors and relapses, alongside with analysis of PAX-FOXO1 expression dynamics. As hypothesized, we found that in recurrent ARMS

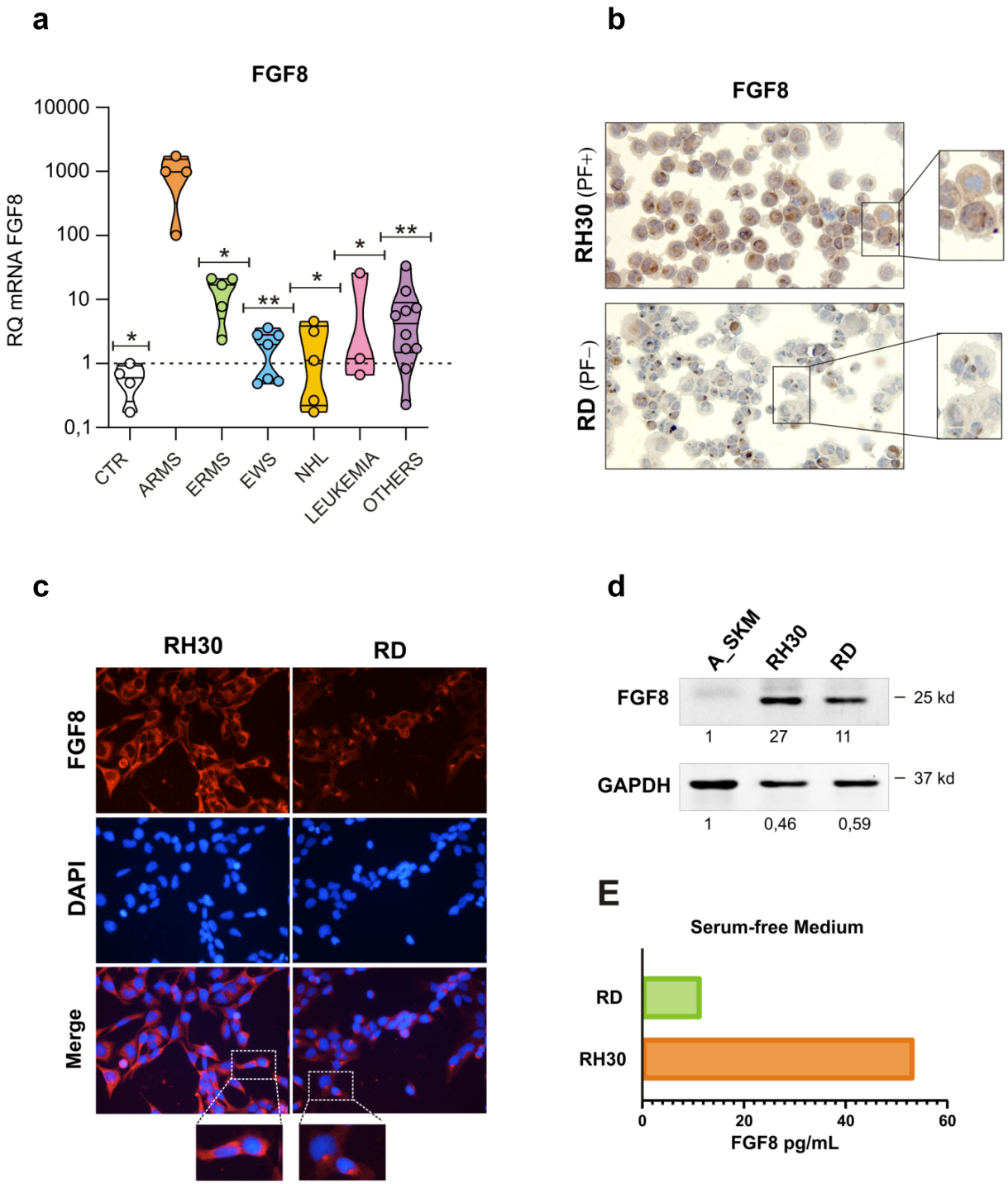

Figure 3. Expression of FGF8 in RMS cell lines. (a) Relative quantification of *FGF8* mRNA by qRT-PCR in normal control cells (CTR, n = 4), alveolar RMS (*ARMS*, n = 5), embryonal RMS (*ERMS*, n = 5), Ewing sarcoma (*EWS*, n = 7), Non-Hodgkin lymphoma cell lines *(NHL*, n = 5), leukemia cell lines (*Leukemia*, n = 3) and cell lines of various origins (*Others*, n = 10). Statistical significance was calculated by Mann-Whitney U test, comparing each group of cell lines with ARMS group. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene was used for normalization, while normal skeletal muscle tissue extracts were used as external calibrator. (b) FGF8 protein expression and localization by immunocytochemistry and (c) immunofluorescence analysis in RH30 (PF+ ARMS) and RD (PF– ERMS) cell lines (magnifications of selected areas are shown apart). (d) Western blotting and (e) direct ELISA assay performed using serum-starved RH30 and RD total lysate and growth medium, respectively, to assess FGF8 protein at intracellular and at secreted level. A_SKM, adult skeletal muscle; $p < 0,05$ (*); $p < 0,01$ (**).

tumors FGF8 expression increased further compared to matched primary tumors (Figure 7a, *left;* $p = 0,048$), in the presence of irrelevant PAX-FOXO1 expression changes (Figure 7a, *right*). Namely, while FGF8 levels at relapse increased in all patients, PAX-FOXO1 mRNA increased in two patients only (ARMS_5 and ARMS_6), decreasing (ARMS_2, ARMS_3, and ARMS_7) or remaining the same (ARMS_1 and ARMS_4) in the others. Likewise, the very low baseline amount of FGF8 in primary fusion-negative ERMS tumors increased at recurrence in two of five patients (Figure 7b; ERMS_1 and ERMS_4),

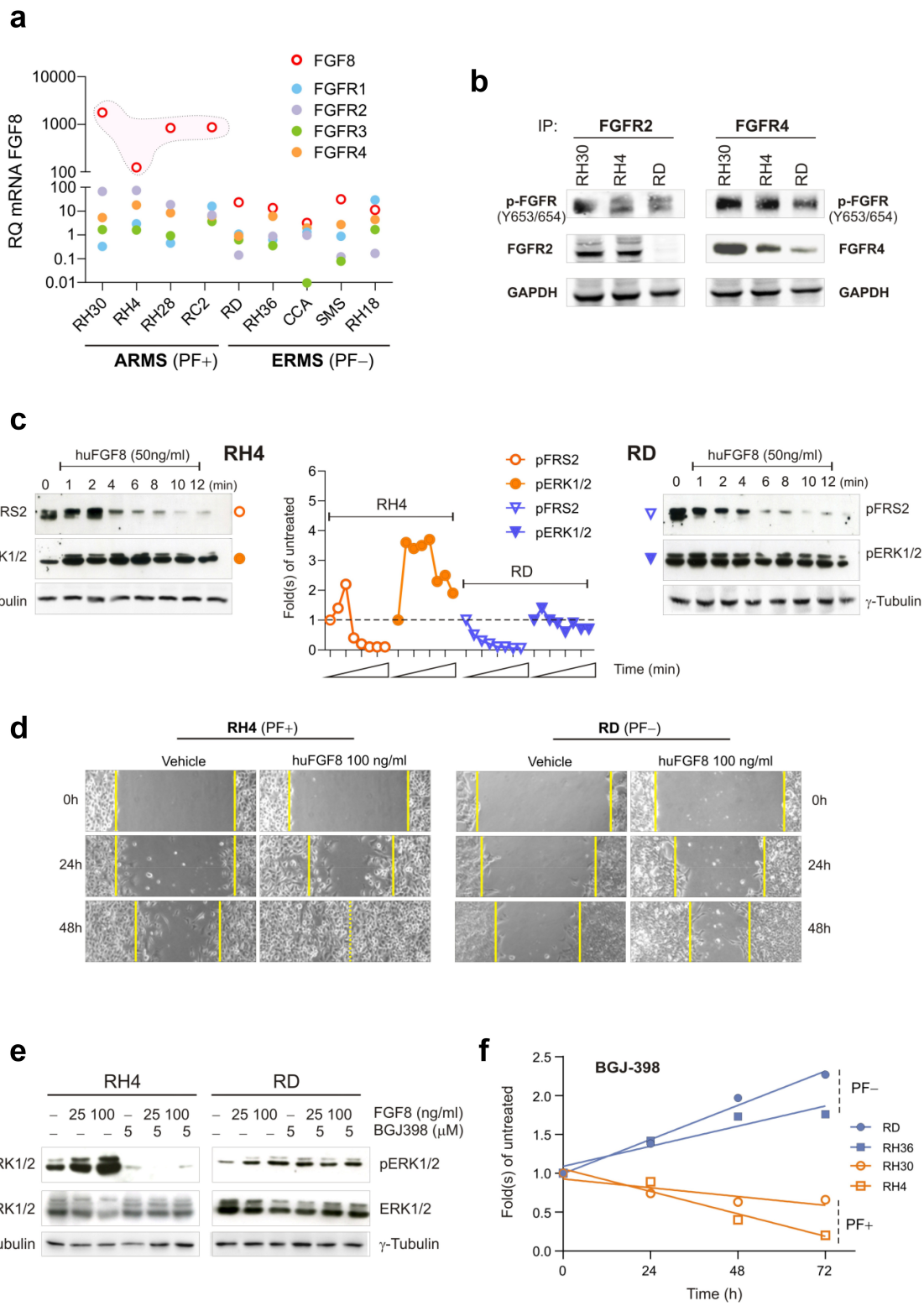

Figure 4. FGF8 signaling in RMS cells. (a) Relative quantification of *FGFR1-4* receptors mRNA in ARMS and ERMS cell lines. *FGF8* mRNA levels are also displayed in graph (red open dots). Glyceraldehyde- 3-phosphate dehydrogenase (*GAPDH*) was used for signal intensity normalization, while skeletal muscle extracts were used as external calibrator. (b) FGFR2 and FGFR4 expression and phosphorylation in PF+ (RH30, RH4) and PF– (RD) cell lines, performed through immunoprecipitation of total FGFR2 and FGFR4 receptor proteins. GAPDH protein was used as gel-loading control. (c) Western blotting showing time-dependent phosphorylation of FRS2 and ERK1/2 proteins induced in RH4 and RD cells upon exposure to 50 ng/mL human recombinant FGF8 protein for the indicated time periods. Between blots a graph displaying phosphorylated FRS2 and ERK1/2 band densities, quantified using ImageJ software, was included. γ-Tubulin was used as gel loading control. (d) RH4 and RD cell lines wound healing assay performed in presence and absence of 100 ng/ml human FGF8. Images were taken up to 48 hours after the treatment. (e) Immunoblot analysis of phosphorylated ERK1/2 kinase in RMS cells exposed to increasing concentration of human FGF8 (25, 100 ng/mL), pretreated or not for 2 hours with 5 μM of NVP-BGJ398. γ-Tubulin was used as gel loading control. (f) MTT assay showing PF+ ARMS (RH30, RH4) and PF– ERMS (RH36, RD) cell viability in the presence of 5 μM of NVP-BGJ398 up to 72 hours.

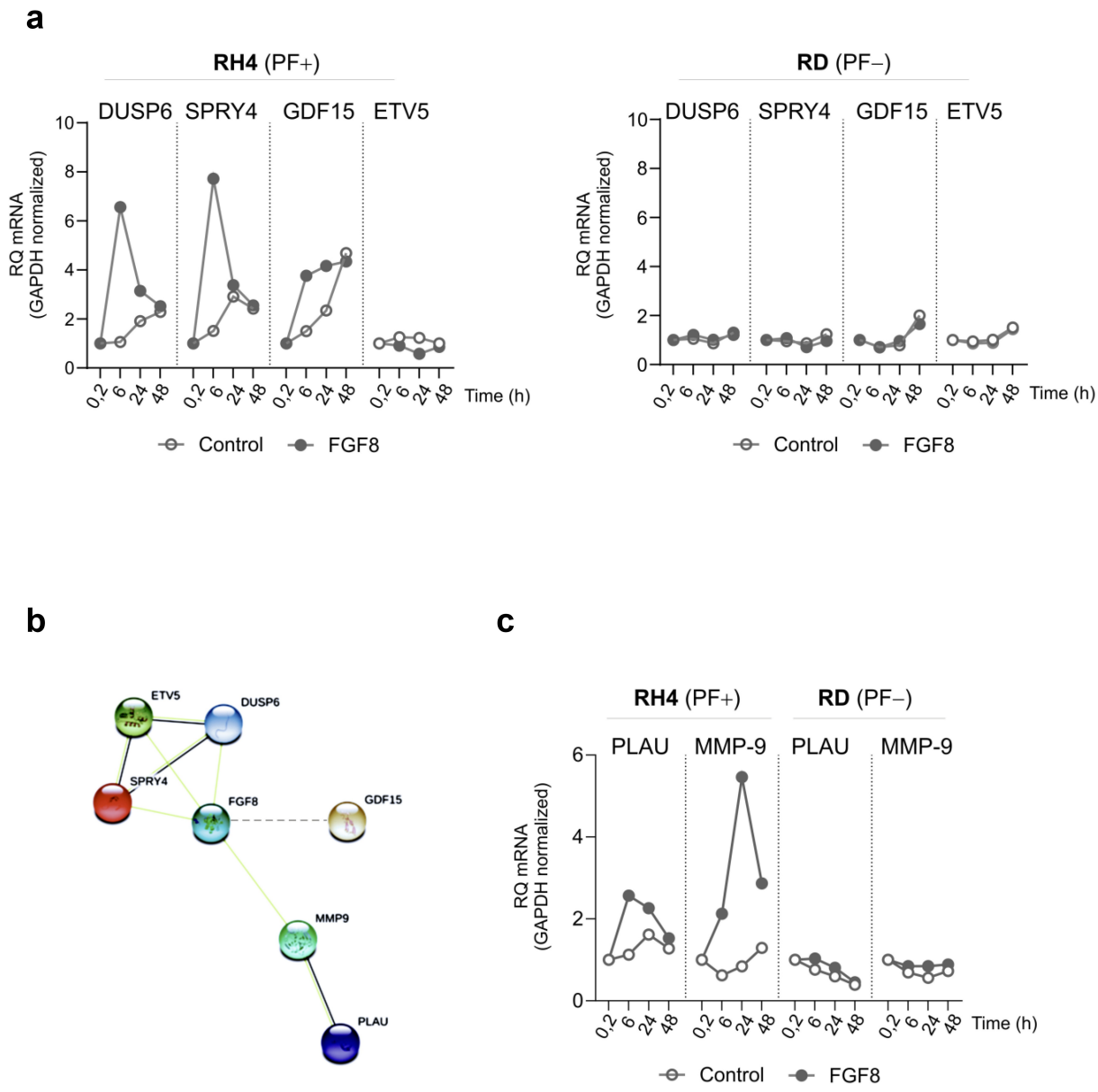

Figure 5. FGF8-induced gene expression in RMS cells. (a) Time-dependent expression of *DUSP6, SPRY4, GDF15* and *ETV5* FGF-target genes, upon treatment of RH4 and RD cells with 100 ng/ml of human recombinant FGF8. Glyceraldehyde- 3-phosphate dehydrogenase (*GAPDH*) housekeeping gene was used for normalization. (b) STRING analysis. (c) Time-dependent expression of *PLAU* and *MMP*-9 genes after treatment of RH4 and RD cell lines with 100 ng/ml of human recombinant FGF8.

though the difference was not statistically significant. Finally, when matching between solid and liquid biopsies was feasible both at diagnosis and at relapse the trend of FGF8 mRNA and anti-FGF8 autoantibodies levels was clearly the opposite. Indeed, while the former increased at relapse the latter decreased (Figure S7), further supporting the inverse correlation of FGF8 mRNA and autoantibodies previously reported (Figure 6d) and the hypothesis that FGF8-expressing RMS would benefit of an impaired humoral response to progress and spread afar.

Discussion

Major challenge in RMS cure is the identification of markers associated with recurrence and metastatic dissemination, since they represent the most critical factors responsible for treatment failure. Nowadays, at least one-third of all RMS patients experience progressive disease or fast recurrence, and 95% of them relapse within the first 3 years from diagnosis.[47,48] Novel markers and targets for personalized treatment are thus needed, likewise strategies capable of using former and latter at once. Immunotherapy fulfills this requirement, being able to exploit the immune system in perceiving the changes that cancer cells sustain and use such changes for killing. One of the events of cancer patients' immune response is the production of circulating autoantibodies: markers of disease onset and tools for therapy when capable of recognizing antigen-bearing cancer cells.[49] Autoantibodies mediate opsonization and complement-mediated lysis of cancer cells, as they can induce cytotoxicity and phagocytosis by natural killer cells and macrophages, respectively.[50] Antibodies targeting tumor-specific GD2, B7-H3, CD99 and EGFR surface antigens have been used in pediatric soft tissue and bone sarcomas, showing efficacy when added to chemotherapy and radiotherapy, or in

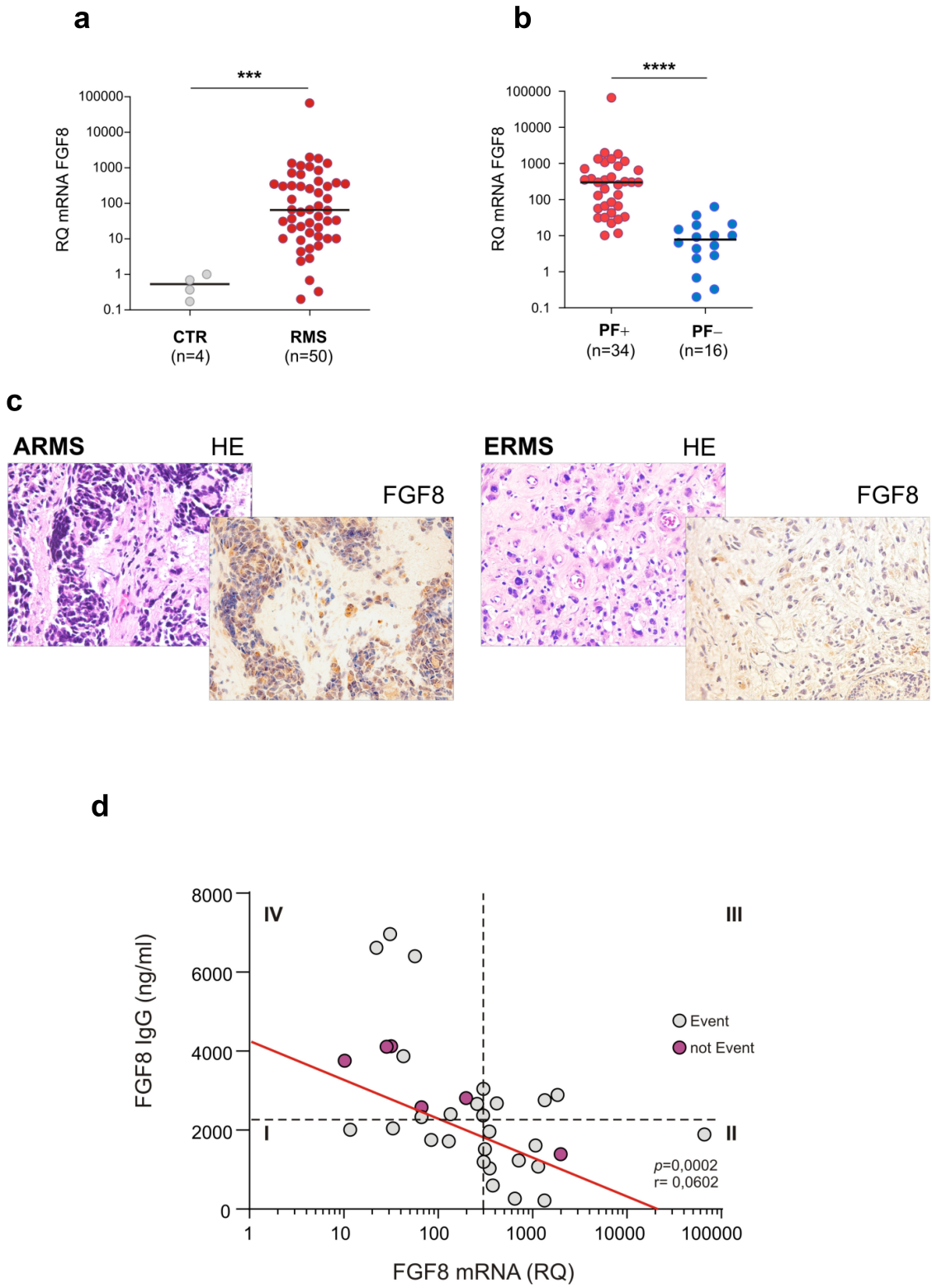

Figure 6. FGF8 expression in RMS tumor biopsies. (a) Relative quantification of *FGF8* mRNA in RMS primary tumors (n = 50) and normal controls (n = 4), and (b) in RMS primary tumors divided according to fusion status. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used for normalization. (c) Hematoxylin/Eosin (HE) and FGF8 staining of representative ARMS and ERMS cases. (d) Scatter plot showing the correlation between *FGF8* mRNA levels and autoantibodies titer in 33 PF+ ARMS primary tumors and plasma samples, respectively. Vertical and horizontal dashed lines represent FGF8 mRNA and autoantibody median values, respectively, used to divide the plot in four regions (I–IV). Dots represent patients, labeled with different colors based on event occurrence (*gray*) or not (*red*) after frontline chemotherapy. $p < 0{,}001$ (***); $p < 0{,}0001$ (****).

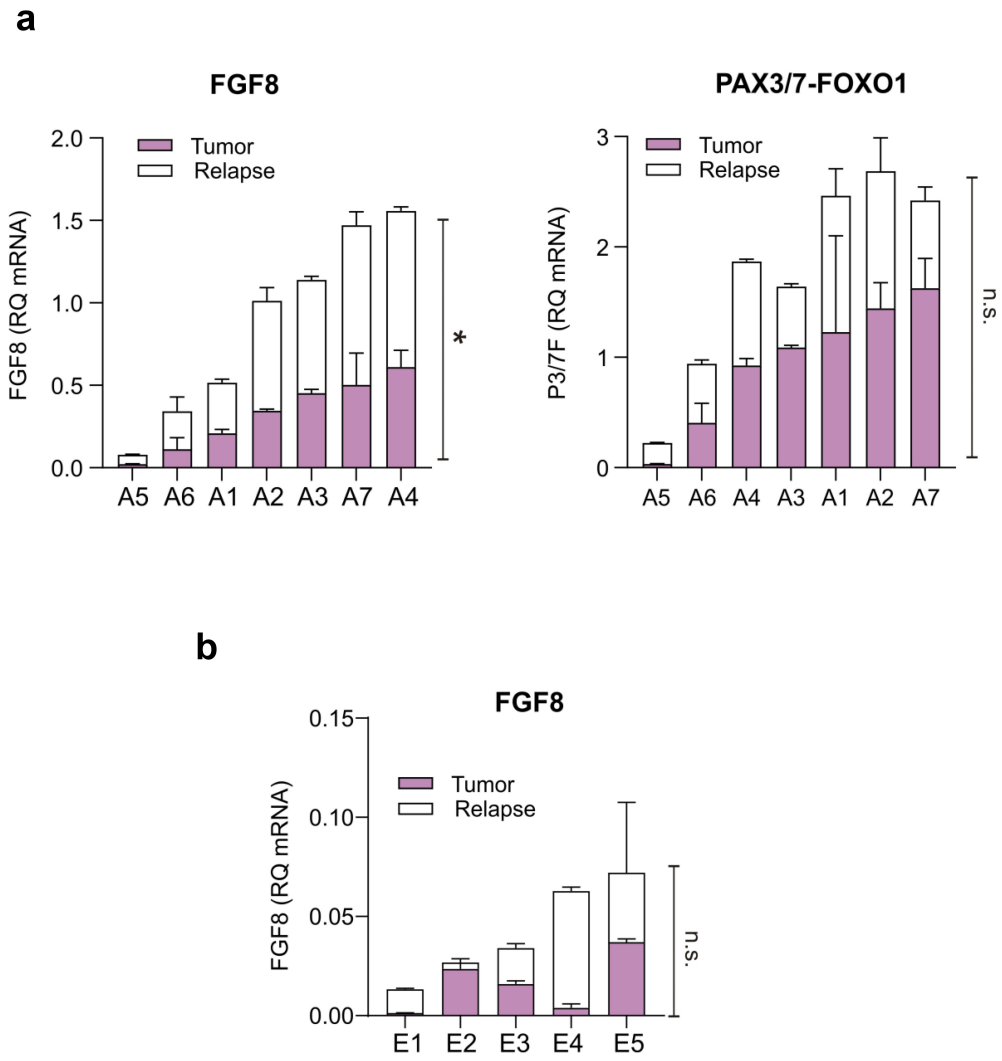

Figure 7. FGF8 expression in RMS recurrent tumors. (a) *FGF8* and *PAX3-FOXO1* or *PAX7-FOXO1*, mRNA at diagnosis and at relapse in 7 cases of ARMS, and (b) 5 ERMS cases. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used for normalization. *n.s.*, not significant; $p < 0,05$ (*).

combination with other immunotherapeutic approaches.[51] Despite these exceptions, tumor antigens for antibody-based therapy in children with RMS remain scarce and methods for their large-scale profiling often rely on indirect gene expression and sequencing data. Progresses have been made using protein microarrays to study and measure host humoral response, as we did to characterize plasma autoantibodies in very high risk ARMS patients. We choose to assess the autoantibody profiling of very high-risk RMS patients (metastatic alveolar RMS, ARMS) because they represent the group of patients with less chances to survive. By employing human proteomic chips for antibody screening and patients plasma profiling we identified 55 autoantibodies capable of clustering sick children from healthy ones.[31] Among them, six were reactive against known PAX3-FOXO1 target genes, including FGF8, a growth factor that has been recently found to support RMS recurrence in mice after PAX3/FOXO1 oncogene deinduction.[46] With the aim of identifying tumor antigens involved in RMS dissemination and recurrence we selected FGF8 for further investigation and validation, since it plays a role in early myogenesis during embryonic development, while it has been shown to be expressed at a low level in normal adult epithelial cells and at an increased level in cancer cells. Transforming capability and pro-angiogenic activity of FGF8 has been widely reported in cancer, mostly in human hormonal tumors of patients at high risk of relapse and poor outcome.[17–22] Herein, we demonstrated that FGF8 is an antigen able to elicit an immune response in ARMS patients, via the production of circulating autoantibodies at levels significantly higher than in healthy children. By matching outcome and FGF8 autoantibody titer, we demonstrated that high levels of autoantibodies significantly correlate with better prognosis in event-free survival, independently of any other known prognostic factor. As for the presence of distal metastasis at diagnosis, the magnitude of autoantibody production was an independent prognostic factor of event occurrence, with low FGF8 autoantibody titers defining patients at higher risk of recurrence, while high levels of FGF8 IgG was associated with better outcome in event-free survival. Consistent with these other findings, a protective role of autoantibodies has been postulated in patients with early-stage cancers who never develop metastasis. In these cases, antigen-specific high-affinity autoantibodies have been found

to correlate with no relapse occurrence rather than with reduction of the primary tumor mass, and considered for therapeutic purposes.[52–54] Herein, titration of FGF8 autoantibodies helped to improve ARMS patients stratification, since the new prognostic groups defined by autoantibody levels included patients with both localized and metastatic disease at diagnosis, hence patients with predicted different outcome according to initial tumor staging, but akin risk of recurrence based on FGF8 autoantibodies level.

Previous studies have shown that an important prerequisite for proposing antibodies as anti-cancer therapy remains the high and specific expression of the corresponding antigen by cancer cells. Therefore, we assessed FGF8 mRNA and protein expression in ARMS cell lines and tumor biopsies, and compared it to that of normal cells and other pediatric bone and soft tissue sarcomas. As expected, FGF8 overexpression was characteristic of PAX3/7-FOXO1-positive ARMS cells, compared to all other cancerous and non-cancerous specimen assessed ($p < 0{,}0001$), including normal muscle and mesenchymal stem cells from which RMS are known to originate. Web-based gene and protein dataset interrogation substantiated these findings, proving that FGF8 expression is limited to normal skeletal muscle tissues, though at too low levels that make it a *bona fide* ARMS tumor antigen. Intriguingly, when we combined FGF8 expression data and patients' autoantibodies level a statistically inverse correlation was observed: children with tumors highly expressing FGF8 were characterized by low levels of autoantibodies and had more chances of experiencing relapse or progressive disease. Our findings suggest a possible immunosuppressive role of FGF8, consistent with that of other FGF family member proteins, receptors, and co-receptors.[55–57]

At protein level previous studies have shown that FGF8 plays a role as potent pro-angiogenic mediator and inducer of metastasis. In breast cancer FGF8 was shown to repress thrombospondin 1 (TSP-1) inhibitor of angiogenesis,[58] whereas in metastatic prostate cancer FGF8 is able to induce MMP9-mediated tumor cells migration and invasion upon FGF inhibitor Sef (*similar expression to FGF*) downregulation.[59] Anomalous FGF8 expression in oral squamous cell carcinoma and hepatoblastoma, instead, promotes metastasis through epithelial-to-mesenchymal transition and modulation of related lineage markers expression.[23,56] Moreover, FGF8 induces the expression of *PLAU* gene in HUVEC cells,[60] that encodes the urokinase-type plasminogen activator (uPA) which plays a major role in tumor progression and metastasis in several cancers, besides in promoting angiogenesis and influencing immune response mechanisms.[61–63] In all these cases, FGF8 acts by linking cell surface receptors and receptor substrates to downstream signal transduction pathways MEK/ERK, PI3K/AKT or STAT/JAK, in a paracrine/autocrine manner that can be prevented by either receptor inhibition, ligand trapping, or neutralizing antibodies treatment.[64–66] Recently, autocrine/paracrine mechanisms have been described for FGF18/FGFR2 and FGF19/FGFR4 axis in gastric cancer and hepatocellular carcinoma, respectively, and clinical trials contemplating the administration of FGFR drug inhibitors have been proposed.[67–69] Direct targeting of FGF8 has also been considered a potential anti-cancer strategy in tumors addicted to its autocrine/paracrine signaling loop, using neutralizing monoclonal antibodies or trap molecules to hamper binding and stimulation of aberrantly expressed receptors and tumor cells growth both *in vitro* and *in vivo*.[70,71]

In RMS the therapeutic potential of FGF receptors has been investigated, while the biological and prognostic role of FGFs remains uncertain. Overexpression of wild-type FGFR4, for example, has been observed in fusion-positive ARMS tumors,[32] while activating mutations have been found more frequently in fusion-negative RMS.[72] Further, in ARMS cells FGFR4 is important for cell survival, while in ERMS for cell proliferation.[73] FGFR2 overexpression has also been reported in fusion-positive alveolar RMS, alongside to its ligand FGF7.[34] A correlation with sensitivity to FGFR inhibitors has been demonstrated, likewise the existence of a FGF7/FGFR2 autocrine loop has been proposed. In our study, we assessed the expression of all four FGFR receptors and several FGF family members, including FGF1, FGF2, FGF7, FGF9, and FGF8-subfamily members FGF17 and FGF18. A significant correlation with RMS fusion status was demonstrated for both FGFR2 and FGFR4 and it was consistent with basal receptor activation.[74] Importantly, FGF8 protein was detected in serum-free conditioned medium of PF+ ARMS cells five times more than in culture medium of serum-starved ERMS cells, suggesting that in fusion-positive ARMS an autocrine/paracrine FGF8/FGFR system is likely. Administration of recombinant FGF8 protein stimulated the FGF/FGFR axis, resulting in a rapid FGFR/FRS2 phosphorylation, a strong MEK/ERK pathway activation and an increased migration capacity of PF+ ARMS cells. Transcriptionally, exposure to recombinant FGF8-induced genes involved into embryonic development and myogenic differentiation (*DUSP6, SPRY4*, and *GDF15*),[44] and more importantly, genes playing a role in tumor invasion and angiogenesis (*PLAU* and *MMP9)*. Consistent with our findings, using a PAX3-FOXO1-induced xenograft model FGF8 was shown to be retained in recurrent ARMS tumors that have lost PAX3-FOXO1 oncogene.[46] Our study added additional evidence to this scenario, as we demonstrated that this occurs in recurrent human ARMS tumors as well, and it was independent of PAX3-FOXO1 expression dynamics. We also reported that FGF8 increased in relapses of ERMS tumors, suggesting that its upregulation may be a general phenomenon of resistance and recurrence that benefit cancer cells independently from their context of origin. Consistent with these results, FGF8 has been shown to elevate in colorectal cancer (CRC) xenografts exposed to chemotherapy, as well as in non-responder rectal cancer patients to neoadjuvant radiochemotherapy.[25,27] In both cases, resistance correlated with increased FGF8 ligand and FGFR3 receptor expression, and concurrent bcl-xl and survivin antiapoptotic proteins transcription.

In conclusion, our study demonstrates for the first time that high-risk PAX3/7-FOXO1 fusion-positive ARMS patients bear circulating autoantibodies capable of improving risk stratification at diagnosis. Namely, we demonstrated that tumorigenic and angiogenic FGF8 growth factor is a novel oncoantigen for PF+ ARMS diagnosis and therapy, early perceived by the host

immune system as the presence of specific anti-FGF8 autoantibodies at diagnosis suggests. By carrying out survival analysis we showed that FGF8 autoantibodies titer can accurately predict recurrence in high-risk ARMS patients, despite clinical diagnosis of localized or metastatic disease. Given the noninvasive and easy detection of circulating FGF8 autoantibodies, this approach may help to consider more accurately patients with apparent akin tumor staging, but potentially diverse outcome, defining patient-specific differences in immune response predictive of recurrence and treatment failure. With respect to this, our study indicates that chance of relapse in RMS patients correlates with increasing FGF8 expression and reduced autoantibodies production. FGF8 expression, in fact, was shown to be higher in relapses of matched primary tumors, providing further insights into the mechanisms that drive ARMS progression and for the identification of candidate patients benefitting of potential anti-FGF8 targeted therapies.

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Author contributions

EP was responsible for conceptualization, designing, and performing experiments, data analysis, interpretation of results and writing original draft. VB and SL contributed to perform experiments. MC was responsible for high-throughput data analysis. LS was responsible for immunohistochemical experiments and related analysis. AZ was responsible of patients' samples collection. GMM and IZ provided patients' clinical data. GB was responsible for funding acquisition and contributed to supervision of the work. PB was responsible for supervision of the work, contributed with EP in conceptualization, designing, and performing experiments, data analysis, interpretation of results, writing original draft, review, and editing. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Data availability statement

Additional data that support the findings of this study are available from the corresponding author, EP, upon reasonable request.

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