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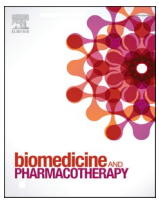

FGF/FGFR inhibitors downmodulates c-Myc oncoprotein and hampers the growth of adrenocortical carcinoma

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ABSTRACT

Adrenocortical carcinoma (ACC) is a rare endocrine neoplastic disease that originates from the cortical cortex of adrenal gland. Unfortunately, after complete resection of the tumor, ACC relapses either locally or with distant metastasis in about 74 % of patients and for these patients the therapeutic options are still severely limited. In this study we demonstrate that the system composed by the fibroblast growth factors (FGFs) and their receptors (FGFRs) might represent a promising therapeutic target for ACC. Indeed, human ACC specimens and cell lines express FGF ligands and FGF receptors and show FGFR activation, suggesting the presence of an autocrine FGF/FGFR loop of stimulation able to sustain ACC growth. Accordingly, inhibition of FGFR activation by TK inhibitors (erdafitinib and infigratinib) or FGF trapping (by NSC12) significantly hampered ACC growth and survival in vitro. Importantly, oral administration of erdafitinib strongly affected tumor growth in vivo by reducing tumor cell proliferation/survival and tumor angiogenesis. Mechanistically, FGF/FGFR inhibition in ACC cells strongly decreased the levels of the oncoprotein c-Myc and induced oxidative stress and DNA damage, leading to reduced tumor cell proliferation and increased tumor cell apoptosis. Altogether these results demonstrate for the first time the impact of FGF/FGFR blockade on ACC cell growth and survival both in vitro and in vivo. This study may set the rationale to start clinical trials investigating the therapeutic potential of FDA approved FGFR-TK inhibitors for the treatment of aggressive ACC.

1. Introduction

Adrenocortical carcinoma (ACC) is a rare endocrine neoplastic disease with an incidence in adults ranging from 0.7 to 2 million cases per year [1,2]. The prognosis for ACC patients attests around a median overall survival of 3–4 years, heterogeneously distributed according to stage based on ENS@T classification and other prognostic factors [1,2]. ACC originates from the cortical cortex of adrenal gland, physiologically deputed to the production of a variety of hormones [3]. Indeed, in addition to non-specific symptoms related to the presence of an abdominal mass, ACC also manifests symptoms due to hormonal excess in 50–60 % of patients. Hypercortisolism is the most frequent condition, followed by pure hyperandrogenism and, rarely, oestrogen or mineralocorticoid excess [4–6]. The metastasis process of ACC involves the peritoneum, liver, and lung more frequently [1,2,7]. The disease can also affect the bones and, occasionally, the brain [8]. Surgery performed in highly specialized center is the gold standard treatment for localized resectable disease, possibly followed by adjuvant treatment with the

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adrenolytic drug mitotane (M) in case of high risk of recurrence [9]. In case of non-resectable/metastatic disease, the first-line systemic therapy is based on mitotane (M), either administered alone or in combination with the chemotherapy agents etoposide (E), doxorubicin (D), and cisplatin (P) (EDP-M scheme) [10,11]. Even though EDP-M can achieve complete pathological remissions in almost 7 % of patients, it is common for those receiving this regimen to experience disease relapse and resistance [12]. New therapeutic targets and strategies are therefore urgently needed [13], even though the scarcity of preclinical models so far available to study ACC make the development of new therapies extremely difficult [14].

The fibroblast growth factor (FGF) family includes 18 secreted members and 4 intracellular FGF homologous factors. The secreted members are grouped into six subfamilies based on phylogenetic analysis and sequence homology and include canonical (FGF1/2/5, FGF3/4/6, FGF7/10/22, FGF8/17/18, FGF9/16/20) and endocrine (FGF19/21/23) FGFs. Secreted FGFs interact with tyrosine kinase (TK) FGFRs (FGFR1–4) that trigger intracellular signaling cascades [15]. An aberrant activation of the FGF/FGFR system has been found in different types of cancer where it plays a crucial role by affecting tumor cell proliferation and survival, angiogenesis, and drug resistance [15,16]. Indeed, several FGFR TK inhibitors as well as anti-FGFR/FGF monoclonal antibodies and FGF ligand traps have been developed and tested in clinical trials involving mostly patients with solid tumors [17,18]. At present, four drugs belonging to the FGFR TK inhibitor (FGFR TKi) class (i.e., erdafitinib, pemigatinib, futibatinib and infigratinib) have received FDA approval for the treatment of urothelial cancer and cholangiocarcinoma [19–21].

In the context of adrenal gland, FGF/FGFR axis acts both in physiological conditions (in the development and maintenance of tissue homeostasis) and in the pathogenesis of adrenocortical carcinoma [22]. Indeed, FGFR1 appears to be one of the most differentially expressed genes in ACC. Also, FGFR4 overexpression is observed in 88 % and 47 % of respectively pediatric and adult adrenocortical tumors, related in some cases to gene amplification [22]. Moreover, FGFR4 is higher expressed in late stages of ACC compared to lower grade tumors and associates with worst prognosis [22,23].

On these bases, this work is aimed at investigating the therapeutic potential of FGF/FGFR blockade by using two inhibitory approaches, one targeting FGF receptors with the FDA approved TK inhibitors erdafitinib and infigratinib, and the other one based on the trapping of FGF ligands at the extracellular level with the small molecule NSC12 previously identified in our laboratory [24,25].

2. Materials and methods

2.1. Cell cultures and reagents

H295R (CVCL_0458) cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). H295R cells, derived from a primitive ACC [26], were cultured in DMEM/F-12, HEPES media (Gibco, Life Technologies, Carlsband, CA, USA), supplemented with 1 % ITS + Premix (Corning, NY, USA), 2.5 % Nu-Serum (Corning, NY, USA), and 1 % penicillin–streptomycin (Lonza Group AG, Basel, CH). MUC-1 cell line was kindly provided by Dr. Hantel. MUC-1 cells, established from an ACC neck metastasis of an EDP-M treated male patient [14,27], were cultured with Advanced DMEM/F12 media (Gibco, Life Technologies, Carlsband, CA, USA), supplemented with 10 % FBS and 1 % penicillin–streptomycin. Both human ACC cell lines were tested regularly for *Mycoplasma* negativity and authenticated using STR profiling.

Erdafitinib (JNJ-42756493) and infigratinib (BGJ-398) were obtained from MedChemExpress (Monmouth Junction, NJ, USA) and NSC12 was synthetized as previously reported [25].

2.2. RT-PCR analysis

Total RNA was extracted using TRIzol Reagent (Invitrogen) according to manufacturer's instructions. Two μg of total RNA were retrotranscribed with MMLV reverse transcriptase (Invitrogen) using random hexaprimers. Then, cDNA was analyzed by semiquantitative PCR using the following primers:

FGFR1	For: GGGCTGGAATACTGCTACAA
	Rev: GCCAAAGTCTGCTATCTTCATC
FGFR2	For: GGATAACAACACGCCTCTCTT
	Rev: GCCCAAAGCAACCTTCTC
FGFR3	For: TGGTGTCCTGTGCCTACC
	Rev: CCGTTGGTCGTCTTCTTGT
FGFR4	For: AACCGCATTGGAGGCATT
	Rev: TCTACCAGGCAGGTGTATGT
FGF1	For: TGAATGGGAACTCCCTTCC
	Rev: GCTTTCAAGACTCTTTGCCT
FGF2	For: TGTGTCTATCAAAGGAGTGTG
	Rev: CCGTAACACATTTAGAAGCCA
FGF3	For: ACTCTATGCTTCGGAGCAC
	Rev: GAGGCATACGTATTATAGCCCA
FGF4	For: CCAACAACTACAACGCCTACGA
	Rev: CCCTTCTTGGTCTTCCCATTCT
FGF5	For: ATGCAAGTGCCAAGTTCAC
	Rev: TGTATTGCTGAGGCATAGGT
FGF6	For: GAAAGTGGCTATTTGGTGGG
	Rev: ATTTCCAGCAGGCTGTAGG
FGF7	For: TGACTTTGCCTCGTTTATCA
	Rev: TGGCTACAAATGTGAACTGT
FGF8	For: TCATCCGGACCTACCAACTC
	Rev: AATCTCCGTGAAGACGCAGT
FGF9	For: TGGATTTCACTTAGAAATCTTCCC
	Rev: ATTCCAGAATGCCAAATCGG
FGF10	For: GGAGAAAGCTATTCTCTTTCACC
	Rev: ATCTCCAGGATGCTGTACG
FGF16	For: ACTCTATGGGTCGAAGAAACTC
	Rev: GAGGCATAGGTGTTGTACCA
FGF17	For: CAACAAGTTTGCCAAGCTC
	Rev: TACTTCTCACTCTCAGCCC
FGF18	For: GGACATGTGCAGGCTGGGCTA
	Rev: GTAGAATTCCGTCTCCTTGCCCTT
FGF19	For: TTTGCTGGAGATCAAGGCA
	Rev: CCTCCGAGTACTGAAGCAG
FGF20	For: CATTTCCTGTTGCCTCCTG
	Rev: CCAAGATACCGAAGAGGCT
FGF21	For: ACCTGGAGATCAGGGAGGAT
	Rev: GCACAGGAACCTGGATGTCT
FGF22	For: ATCAGACCATCTACAGTGCC
	Rev: ATCTTCTGCTCATCACACCT
FGF23	For: ATCAGACCATCTACAGTGCC
	Rev: ATCTTCTGCTCATCACACCT

2.3. Western blot analysis

Cells were washed in cold PBS and homogenized in NP-40 lysis buffer (1 % NP-40, 20 mM Tris–HCl pH 8, 137 mM NaCl, 10 % glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin). Protein concentration in the supernatants was determined using the Bradford protein assay (Bio-Rad Laboratories). Expression of specific proteins were detected using specific antibodies indicated in the table below. β-actin or GAPDH were used as loading controls. Chemiluminescent signal was acquired by ChemiDoc™ Imaging System (Bio-Rad) and analyzed using the ImageJ software (http://rsb.info.nih.gov/ij/).

ANTIBODY	SOURCE
Phospho-FGFR (Tyr 653/654)	Cell Signaling Technology
c-Myc	Cell Signaling Technology
Cleaved Caspase 3	Cell Signaling Technology
Phospho-Histone H3 (Ser 10)	Merck Millipore
Nitrotyrosine	Merck Millipore
Phospho-H2AX (Ser 139)	Cell Signaling Technology

(*continued on next page*)

(*continued*)

ANTIBODY	SOURCE
CD31	Dianova
β-Actin	Sigma-Aldrich
GAPDH	Santa Cruz Biotechnology

2.4. Viable cell counting

Cells were cultured under appropriate conditions for 96 or 120 h. Propidium iodide staining (Immunostep, Salamanca, SP, EU) was used to detect PI negative viable cells by flow cytometry. Absolute cell counts were obtained by the counting function of the MACSQuant® Analyzer (Miltenyi Biotec).

2.5. Subcutaneous human xenografts

Experiments were performed according to the Italian laws (D.L. 116/92 and following additions) that enforce the EU 86/109 Directive and were approved by the local animal ethics committee (OPBA, Organismo Preposto al Benessere degli Animali, Università degli Studi di Brescia, Italy). Six- to eight-week-old female NOD/SCID mice were injected subcutaneously (s.c.) with H295R cells (5×10^6 cells/implant) in 200 μl of PBS. When tumors were palpable, mice were randomly assigned to receive everyday treatment with erdafitinib (30 mg/kg) or control/vehicle DMSO in drinking water. Tumor volumes were measured with caliper and calculated according to the formula $V = (D \times d^2)/2$, where D and d are the major and minor perpendicular tumor diameters, respectively. At the end of the experimental procedure, tumor nodules were excised, weighed and processed for histological analysis.

2.6. Histological analyses

Tumor samples were fixed in formalin and embedded in paraffin. Formalin-fixed, paraffin-embedded samples were sectioned at a thickness of 3 μm, dewaxed, hydrated, and processed for immunohistochemistry with rabbit anti-human/mouse pFGFR (Cell Signaling Technology), rabbit anti-human/mouse c-Myc (Cell Signaling Technology), rabbit anti-human/mouse nitrotyrosine (Millipore), rabbit anti-human/mouse pH2AX (Cell Signaling Technology), rabbit anti-human phospho-Histone H3 (Chemicon), rabbit anti-human/mouse cleaved Caspase3 (Cell Signaling Technology), or rat anti-mouse CD31 (Dianova) antibodies.

Sections were finally counterstained with Carazzi's hematoxylin before analysis by light microscopy. Images were acquired with the automatic high-resolution scanner Aperio System (Leica Biosystems, Wetzlar, Germany, EU) and image analysis was carried out using the open-source ImageJ software.

2.7. Statistical analyses

Statistical analyses were performed using Prism 9 (GraphPad Software). Student's *t* test for unpaired data (2-tailed) was used to test the probability of significant differences between two groups of samples. For more than two groups of samples, data were analyzed with a 1-way analysis of variance and corrected by the Bonferroni multiple comparison test. Tumor volume data were analyzed with a 2-way analysis of variance and corrected by the Bonferroni test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Adrenocortical carcinoma cells express FGFs and FGFRs

To assess the expression of secreted FGF ligands (FGFs) and their receptors (FGFRs) in ACC, we first analyzed datasets from The Cancer Genome Atlas (TCGA) by using the cBioPortal software platform. These datasets included genomic and proteomic data from 92 ACC patients (Table S1). Notably, about 66 % of ACC cases analyzed showed alterations of the FGF/FGFR system with about 15 % of patients presenting alterations of both FGFs and FGFRs (Fig. 1A–B, Table S2). Particularly, about 25 % of ACC cases showed high expression of at least one FGFR, FGFR1 and FGFR4 being the most upregulated (10 % and 13 % of all cases, respectively) (Fig. 1C and Fig. S1). Also, 58 % of ACC cases showed high expression of at least one secreted FGF (Fig. 1D), being FGF18 the most upregulated (21 % of all cases) (Table S2 and Fig. S2). High levels of FGFR and FGF expression were due mainly to mRNA upregulation and in some cases to gene amplification, whereas very rare structural genetic mutations were detected (Fig. S1 and S2). In keeping with these findings, H295R and MUC-1 ACC cell lines express all the four FGFRs and several secreted members of the FGF family (Fig. 1E). Also, both cell lines show the constitutive activation of FGFR signaling as demonstrated by the expression of phosphorylated (Tyr653/654) FGFR (pFGFR) in the absence of exogenous stimuli (i.e, without addition of exogenous FGFs) in the cell medium (Fig. 1F). These data strongly indicate the presence of an autocrine FGF/FGFR loop of stimulation in ACC cells (Fig. 1G).

3.2. FGF/FGFR inhibition affects ACC cell growth and survival in vitro

In order to assess the role of the FGF/FGFR system in ACC cell growth, we utilized two inhibitory approaches, one targeting the TK activity of FGFRs and the other one trapping the FGF ligands at the extracellular level. To this aim, H295R and MUC-1 cells were treated with increasing doses of the FGFR selective TK inhibitors erdafitinib and infigratinib (BGJ398) or with increasing doses of the pan FGF trap small molecule NSC12 (Fig. 2). Both FGFR-TK blockade (by erdafitinib or BGJ398) and FGF trapping (by NSC12) significantly reduced the growth and survival of H295R and MUC-1 cells with IC_{50} ranging from 2 to 7 μM (Fig. 2). Of note, FGF/FGFR blockade similarly affected ACC cells sensitive (H295R) and resistant (MUC-1) to the first-line therapy EDP-M [14]. These data indicate that autocrine FGF/FGFR stimulation may play a pivotal role in ACC biology.

3.3. The FGFR selective TK inhibitor erdafitinib hampers ACC growth in vivo

We next assess the therapeutic potential of the FDA approved TK inhibitor erdafitinib in vivo by using an ACC xenograft model. To this aim, H295R cells were subcutaneously implanted into immunodeficient mice. When tumors were palpable (28 days after tumor implantation), mice received everyday treatment with erdafitinib (30 mg/kg) or vehicle in drinking water (Fig. 3). In keeping with in vitro data, in vivo FGFR-TK blockade by erdafitinib significantly reduced the rate of growth of H295R xenografts compared to vehicle-treated animals with a significant decrease of tumor weight at the end of the experiment (day 49 after tumor implantation) (Fig. 3A).

Accordingly, immunohistochemical analysis of H295R tumor xenografts showed high levels of FGFR phosphorylation that were drastically reduced in erdafitinib-treated animals compared to vehicle-treated mice (Fig. 3B). This was accompanied by a significant reduction of tumor cell proliferation as revealed by the staining for the proliferation marker phospho-Histone H3 (pHH3) (Fig. 3B). Various members of the FGF family expressed by ACC cells (e.g. FGF1, FGF2, FGF4, FGF5, and FGF8) have been shown to be endowed with a potent pro-angiogenic activity [28], thus suggesting that FGFs secreted by ACC cells in vivo may also exert a paracrine pro-angiogenic function in ACC. This hypothesis was supported by the presence of elevated levels of FGFR phosphorylation in endothelial cells of H295R xenografts (Fig. 3C). On this basis, we investigated the effect of FGFR-TK blockade on ACC neovascularization in vivo. As shown in Fig. 3C, treatment with erdafitinib strongly reduced

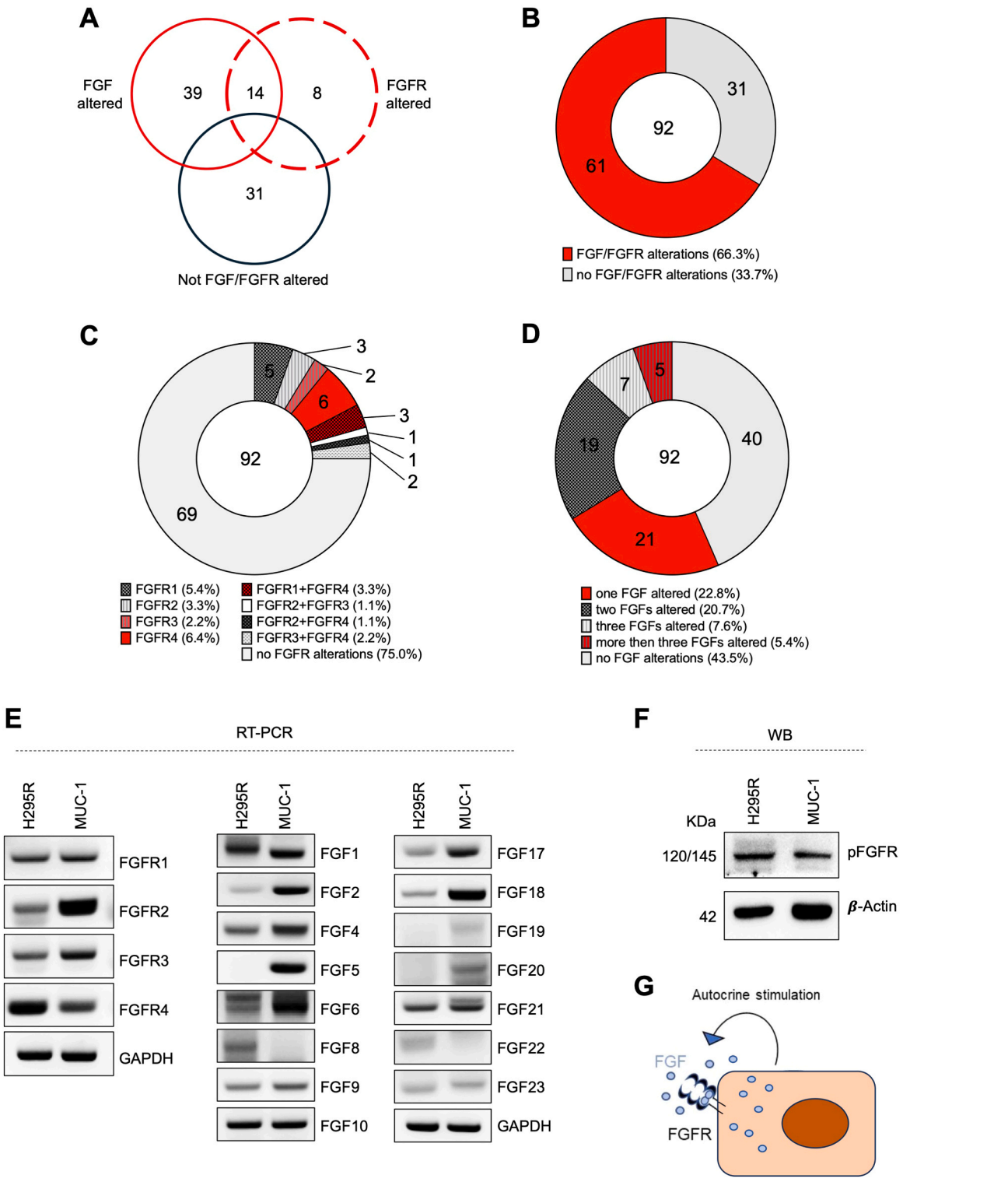

Fig. 1. FGFs and FGFRs are expressed in ACC cells. Venn diagram **(A)** and donut charts **(B-D)** representing the number of ACC patients with or without FGF/FGFR alterations. Data are from 92 ACC patient-derived samples reported in datasets from TCGA and cBioPortal platforms. **E)** RT-PCR analysis for the expression of FGFR1–4 and secreted FGFs in ACC H295R and MUC-1 cell lines (FGF3, FGF7 and FGF16 expression was undetectable). **F)** Western blot analysis to assess FGFR activation in unstimulated H295R and MUC-1 cells by using an antibody that recognizes the phosphorylated form (Tyr653/654) of all the four FGFRs. **G)** Cartoon representing the autocrine FGF/FGFR loop of stimulation in ACC cells.

vascular FGFR phosphorylation in H295R xenografts. This was paralleled by a significant reduction of tumor vascularization, as revealed by immunohistochemical analysis of $CD31^{+}$ vessels that appear also less elongated and anastomized in tumors treated with erdafitinib when compared to controls (Fig. 3D).

3.4. Blockade of FGFR activation strongly reduces c-Myc protein levels in ACC cells

The activation of FGFR signaling is involved in c-Myc stabilization in different tumor types, FGF trapping or FGFR-TK inhibition leading to the

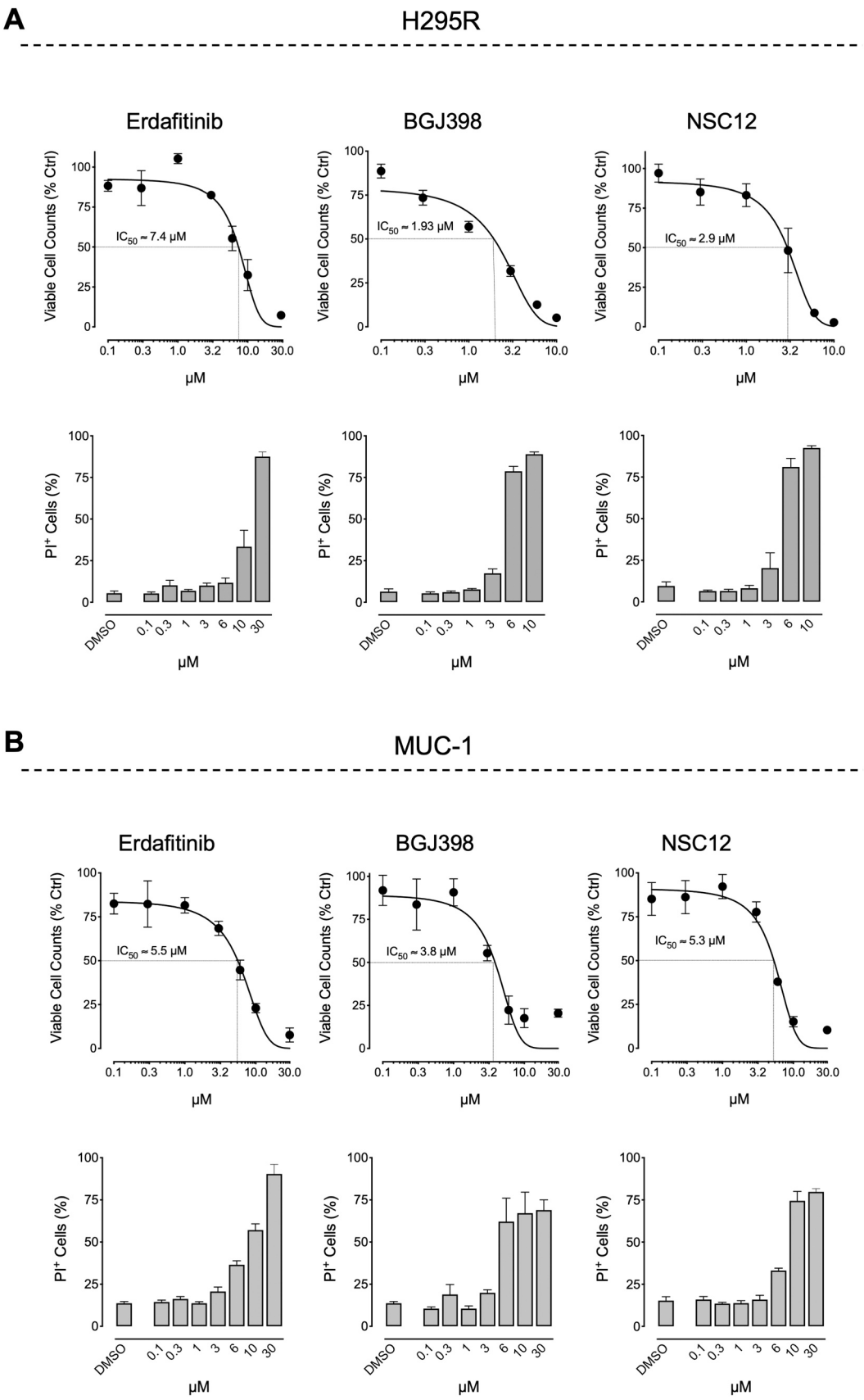

Fig. 2. Selective FGFR-TK inhibitors and FGF trapping hamper ACC cell growth and survival. Viable cell counting (upper panels) and percentage of dead PI positive cells (lower panels) of H295R **(A)** and MUC-1 **(B)** cells treated with increasing doses of erdafitinib, BGJ398, or NSC12 for 96 h (H295R cells) or 120 h (MUC-1 cells). Data are mean ± SEM of three independent experiments.

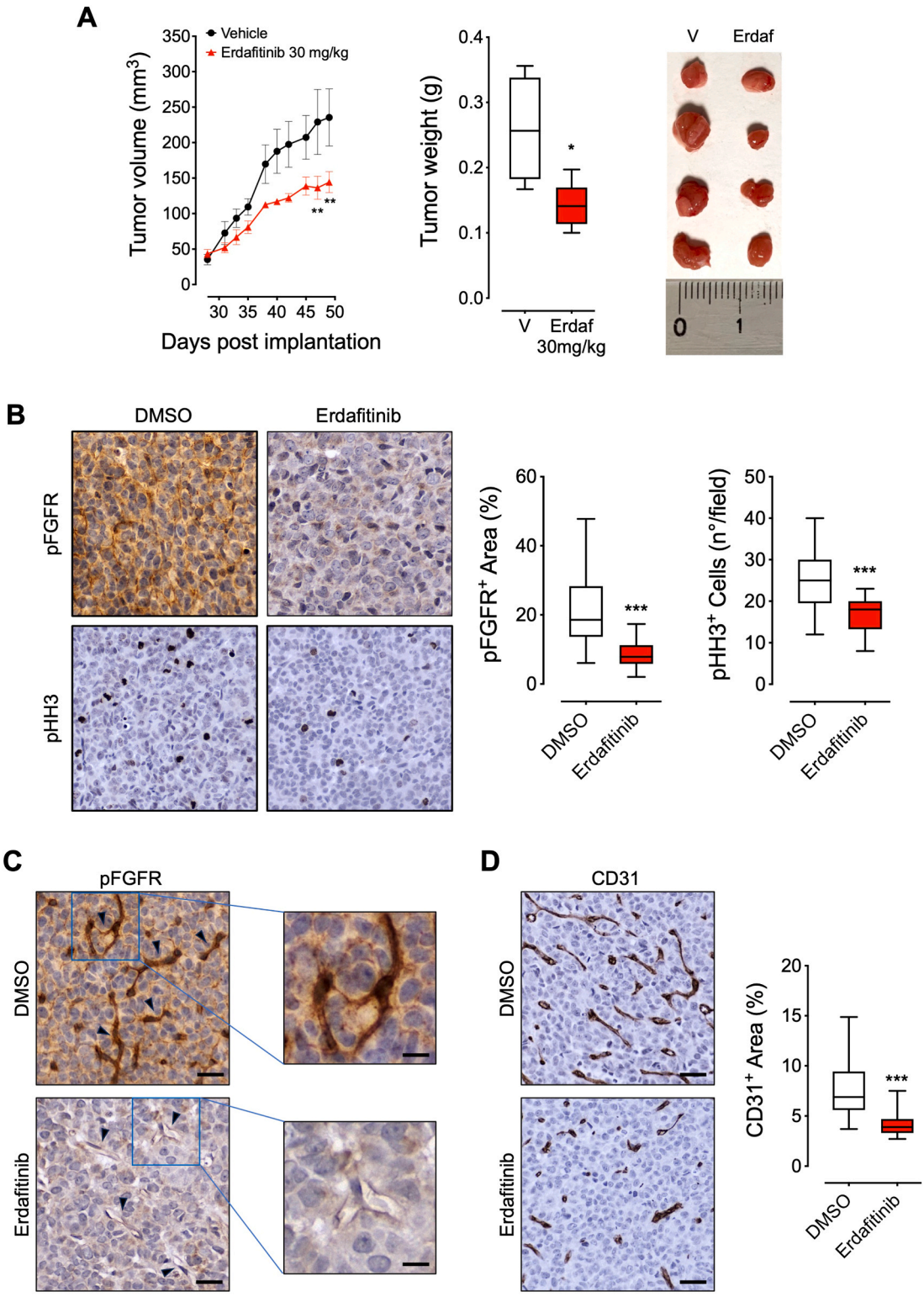

Fig. 3. Erdafitinib inhibits ACC growth in vivo. H295R cells were subcutaneously implanted in immunodeficient mice and, when tumors were palpable (28 days after tumor implantation), mice received vehicle or erdafitinib 30 mg/kg in drinking water (N = 5 mice/group). **A)** *Left panel*: Tumor growth over time. Data are mean ± SEM. *Right panel*: Tumor weight and representative tumor pictures at the end of the experiment (day 49 after tumor implantation). V = Vehicle, Erdaf = erdafitinib. **B)** Immunohistochemical analysis of H295R tumor xenografts reported in Fig. 3A. The levels of phosphorylated (Tyr653/654) FGFR (pFGFR) and pHH3 were quantified as brown positive area by using ImageJ software. **C**) Staining for phosphorylated FGFR. Black arrowheads indicate tumor vessels. Scale bars: 20 μm and 10 μm in magnified images. **D**) Tumor vascularization assessed by CD31 immunostaining and quantified as brown positive area using ImageJ software. Scale bars: 50 μm. In box and whiskers graphs, boxes extend from the 25th to the 75th percentiles, lines indicate the median values, and whiskers indicate the range of values. * $p < 0.05$, *** $p < 0.001$.

proteasomal degradation of the c-Myc protein [29–33]. So far, little is known about the role of c-Myc in ACC growth and progression. Analysis of the same datasets from The Cancer Genome Atlas (TCGA) used for FGF and FGFR expression revealed that about 9 % of ACC patients show altered c-Myc expression due to gene amplification (1.1 %), high mRNA levels (5.4 %), and high protein levels (2.2 %) (Fig. 4A and S3). Interestingly, 100 % of patients with c-Myc alterations also showed altered expression of FGF and/or FGFR (Table S2). Also, a recent work by Pennannen et al. demonstrated that c-Myc protein expression in ACC samples is associated to malignancy and shorter survival [34]. Accordingly, TCGA data analysis revealed that higher c-Myc mRNA expression was observed in recurred/progressed (R/P) patients compared to disease free (DF) patients (Fig. 4B) and correlated with worse overall survival (Fig. 4C).

In keeping with these findings, inhibition of the FGF/FGFR axis by erdafitinib, BGJ398, or NSC12 significantly reduced c-Myc protein levels in ACC cells (Fig. 4D). Accordingly, immunohistochemical analysis of H295R tumor xenografts showed high levels of c-Myc protein that

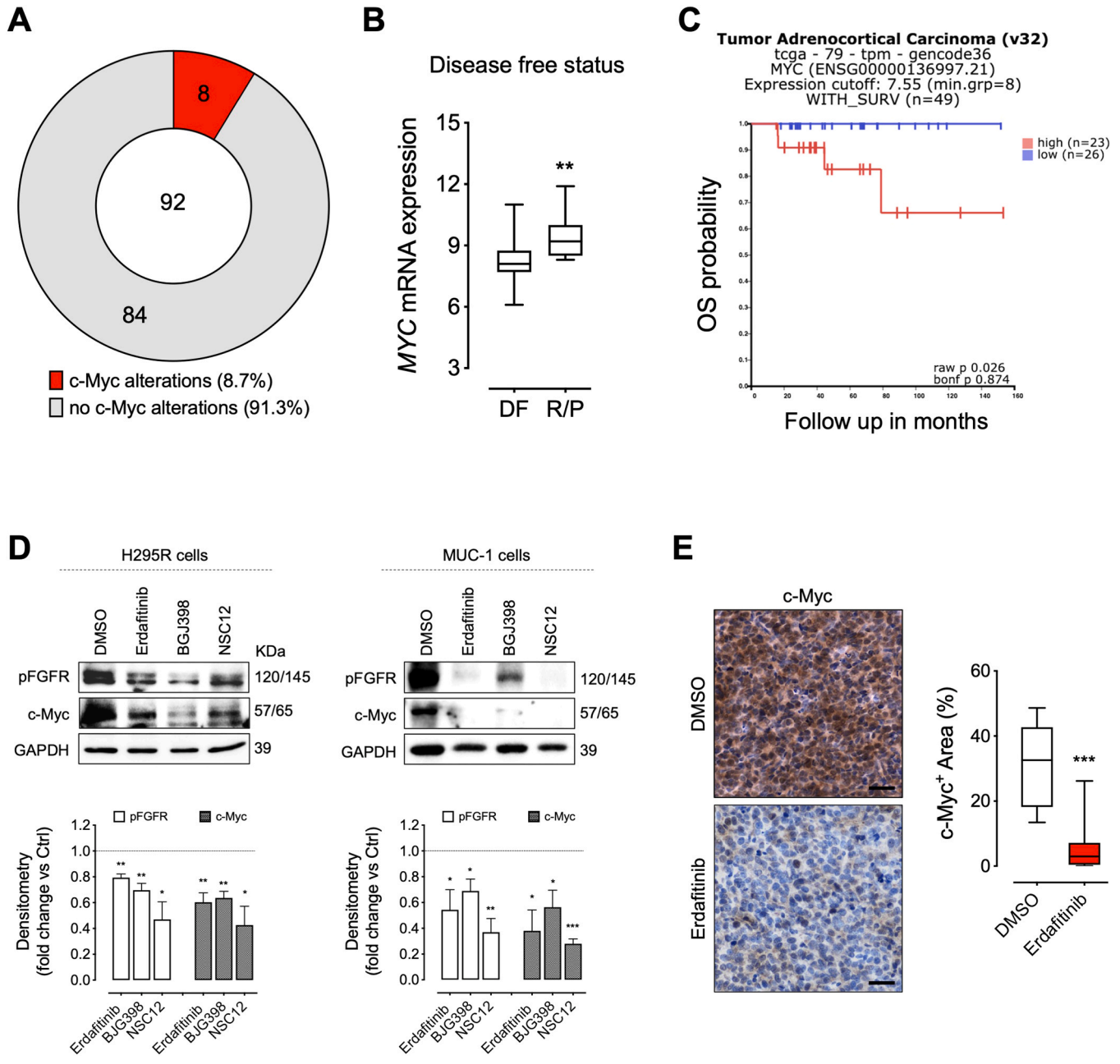

Fig. 4. c-Myc expression is deregulated in ACC and reduced by FGFR inhibition. A) Donut charts representing the number of ACC patients with or without c-Myc alterations. Data are from 92 ACC patient-derived samples reported in datasets from TCGA and cBioPortal platforms. **B)** Correlation between *MYC* mRNA expression and disease-free status in ACC patients. DF, Disease Free (N = 33); R/P, Recurred/Progressed (N = 11); L, living (N = 50); D, Deceased (N = 26). **C)** Kaplan-Meier curve of ACC patients' overall survival correlated with c-Myc expression. **D)** *Upper panel:* Western blot analysis of H295R and MUC-1 cells treated with 10 μM erdafitinib, BGJ398, or NSC12 for 24 (H295R cells) or 48 (MUC-1 cells) hours. Representative images are shown. *Lower panel:* densitometry quantification of western blot bands with ImageJ software. All samples were normalized to GAPDH. Data are expressed as fold changes vs Ctrl (dotted line) and are mean ± SEM of three independent experiments. **E)** Immunohistochemical analysis of H295R tumor xenografts reported in Fig. 3B. The levels of c-Myc protein were quantified as brown positive area by using ImageJ software. Scale bars: 50 μm. In box and whiskers graphs, boxes extend from the 25th to the 75th percentiles, lines indicate the median values, and whiskers indicate the range of values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

were drastically reduced in erdafitinib-treated animals compared to vehicle-treated mice (Fig. 4E).

3.5. FGFR inhibition triggers oxidative stress, DNA damage, and ACC cell death

In previous works we have demonstrated that the decrease of c-Myc protein levels induced by FGF/FGFR blockade triggers oxidative stress and DNA damage, leading to tumor cell death [29,31,32]. On this basis, we investigated whether the same molecular mechanism is recapitulated also in ACC cells. To this aim, H295R cells were treated for 24 h with 10 μM erdafitinib and assessed for oxidative stress and DNA damage. As shown in Fig. 5A, the decrease of FGFR phosphorylation and c-Myc protein levels was paralleled by a significant increase of the oxidative stress and DNA damage markers nitrotyrosine and γH2AX, respectively. These effects eventually led to impaired H295R cell proliferation and induction of cell death after 96 h of treatment with 10 μM erdafitinib (see Fig. 2A).

Similar results were observed in vivo (Fig. 5B). Indeed, immunohistochemical analysis of H295R tumors from the experiment shown in Fig. 3A revealed a strong increase of nitrotyrosine and γH2AX levels in erdafitinib-treated mice when compared to controls. This was accompanied by a significant increased tumor cell death, as assessed by the analysis of the apoptotic marker cleaved caspase 3 (Fig. 5B).

4. Discussion

Adrenocortical carcinoma (ACC) is a rare endocrine neoplastic disease with few therapeutic options. Meagre experimental evidence reported in the literature suggests that the FGF/FGFR system may play a crucial role in the growth of ACC. Indeed, FGFR1 and FGFR4 and their ligands FGF8, FGF9, FGF19 and FGF21 have been found significantly upregulated in patient-derived ACC samples [22,23]. Importantly, a negative correlation between FGFR1 and FGFR4 expression and patient survival endpoints has also been shown [23].

Data reported herein strongly strengthen the studies so far present in the literature supporting a role for the FGF/FGFR system in the biology of ACC and demonstrate the therapeutic potential of targeting FGFRs or FGF ligands in this type of aggressive solid cancer. Indeed, our findings demonstrate that ACC cells express both FGF ligands and receptors and basally show FGFR activation, suggesting the presence of an autocrine FGF/FGFR loop of stimulation able to sustain the growth and survival of ACC cells. Accordingly, inhibition of FGFR activation by the selective FGFR-TK inhibitors erdafitinib and BGJ398 or trapping extracellular FGFs by the FGF trap NSC12 significantly hampered in vitro the growth and survival of ACC cells both sensitive (H295R) and resistant (MUC-1) to the first-line therapy EDP-M [14]. Importantly, oral treatments with erdafitinib strongly affected tumor growth in vivo by reducing tumor cell proliferation/survival and tumor angiogenesis.

Mechanistically, FGF/FGFR inhibition strongly reduced the protein levels of the oncoprotein c-Myc in ACC cells causing oxidative stress, DNA damage, reduced tumor cell proliferation, and eventually leading

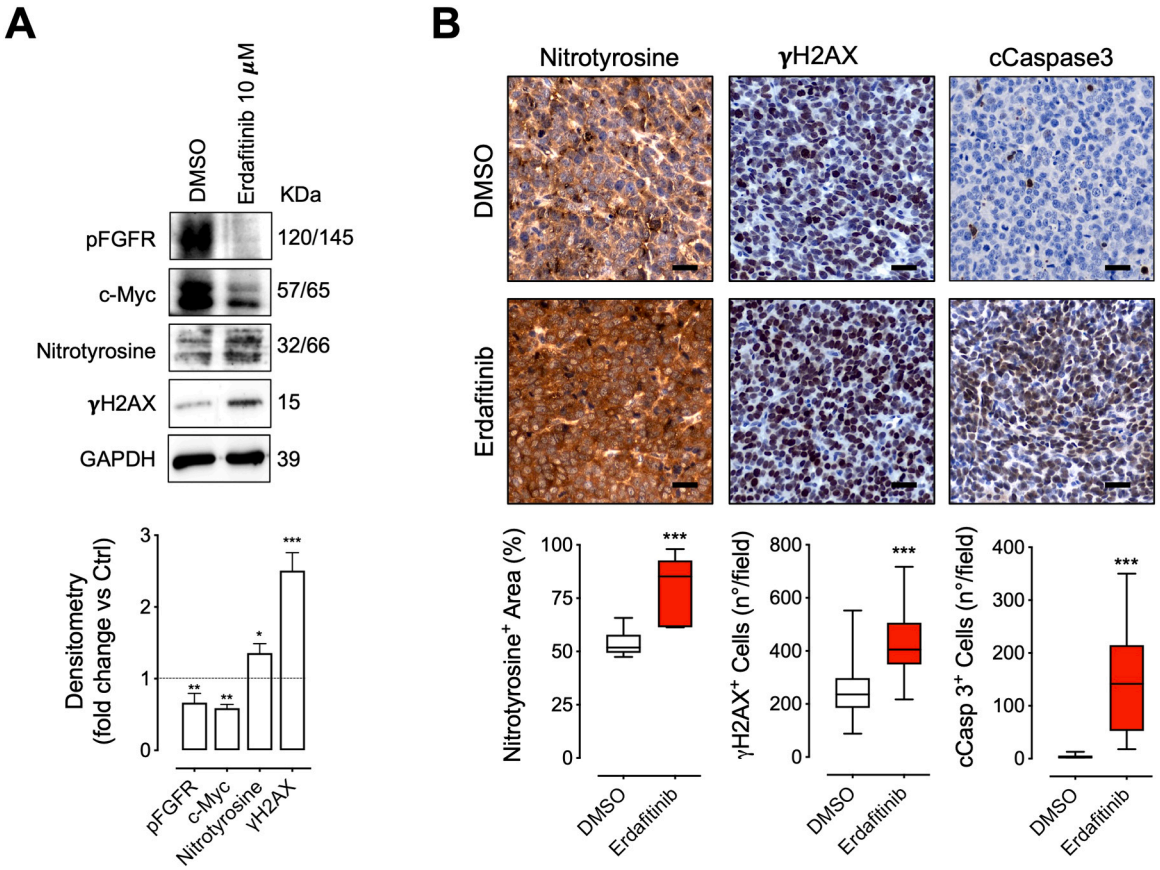

Fig. 5. FGFR inhibition triggers oxidative stress, DNA damage, and cell death in ACC. A) *Upper panel:* Western blot analysis of H295R cells treated for 24 h with 10 μM erdafitinib. Representative images are shown. *Lower panel:* densitometry quantification of western blot bands with ImageJ software. All samples were normalized to GAPDH. Data are expressed as fold changes vs Ctrl (dotted line) and are mean ± SEM of three independent experiments. **B)** Immunohistochemical analysis of H295R tumor xenografts reported in Fig. 3A. All markers analyzed were quantified as brown positive area by using ImageJ software. Scale bars: 50 μm. In box and whiskers graphs, boxes extend from the 25th to the 75th percentiles, lines indicate the median values, and whiskers indicate the range of values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

to tumor cell apoptosis. These findings are in keeping with our previous work demonstrating the pivotal role of FGFR activation in stabilizing the intracellular levels of the oncoprotein c-Myc and the role of c-Myc in preventing mitochondrial oxidative stress [29]. So far little is known about the expression of the oncoprotein c-Myc in ACC cells and its role in ACC growth and progression. However, the Wnt/β-catenin signaling, which is known to sustain the expression of *MYC* gene [35,36], is often found deregulated in ACC cells [37]. Indeed, H295R cells used in this work harbor an activating mutation in the gene codifying for β-catenin (*CTNNB1*) thus causing the constitutive activation of the transcription activity of β-catenin [14]. Accordingly, we observed high expression of c-Myc protein in H295R cells both in vitro and in vivo. Also, our data demonstrate that about 9 % of ACC patients show altered c-Myc expression, due to gene amplification, high mRNA levels, and/or high protein levels. Importantly, higher c-Myc mRNA expression is observed in recurred/progressed patients compared to disease free patients and correlates with worse overall survival. This is in keeping with a recent work by Pennannen et al. demonstrating that c-Myc protein expression in ACC samples associates with malignancy and shorter survival [34].

Altogether these results demonstrate for the first time that FGF/FGFR blockade can represent a promising novel therapeutic strategy for ACC. Although we are aware of the limitation of this study due to the scarcity of preclinical models so far available to study ACC, data herein reported shed a light in the potential efficacy of FGF/FGFR inhibitors for the treatment of ACC and may provide important information that could be rapidly translated into the clinics. Indeed, this study may set the rationale to start clinical trials investigating the therapeutic potential of FDA approved selective FGFR-TK inhibitors (i.e pemigatinib, futibatinib, erdafitinib) for the treatment of aggressive ACC with poor prognosis. Also, our findings highlight the oncoprotein c-Myc as a new target in ACC that can be indirectly inhibited by FGF/FGFR blockade. On these bases and in a translational perspective, it would be worth to assess the levels of FGFR activation and c-Myc expression in patient-derived samples as new predictive biomarkers for the use of FGF/FGFR inhibitors in ACC.

CRediT authorship contribution statement

Luca Mignani: Methodology, Investigation. **Andrea Abate:** Methodology, Investigation. **Constanze Hantel:** Writing – review & editing, Investigation. **Jessica Faletti:** Writing – review & editing, Methodology, Investigation, Data curation. **Marta Laganà:** Methodology, Investigation. **Sara Taranto:** Writing – review & editing, Methodology, Investigation, Data curation. **Marta Turati:** Methodology, Investigation. **Riccardo Castelli:** Methodology, Investigation. **Edoardo Rocca:** Methodology, Investigation. **Marco Presta:** Writing – review & editing, Supervision. **Roberto Ronca:** Writing – review & editing, Methodology, Investigation. **Sandra Sigala:** Writing – review & editing, Supervision. **Mariangela Tamburello:** Methodology, Investigation. **Giorgia Gazzaroli:** Methodology, Investigation. **Arianna Giacomini:** Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Giulia Garattini:** Methodology, Investigation.

Ethics approval and consent to participate

Animal Experiments were performed according to the Italian laws (D.L. 116/92 and following additions) that enforce the EU 86/109 Directive and were approved by the local animal ethics committee (OPBA, Organismo Preposto al Benessere degli Animali, Università degli Studi di Brescia, Italy).

Consent for publication

Not applicable.

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Competing interests

The authors declare no competing interests.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118677.

Data availability

Data will be made available on request.

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