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Gefitinib and afatinib show potential efficacy for Fanconi anemia-related head and neck cancer

(**Running title:** Gefitinib and afatinib for HNSCC in Fanconi anemia)

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CONFLICTS OF INTEREST

JM and JS participate in the recently approved EMA orphan drug designations for gefitinib (EMA/OD/090/18; EU/3/18/2075) and afatinib (EMA/OD/141/18; EU/3/18/2110) for the treatment of HNSCC in FA. HM, JM and JS filed a patent for afatinib as a new indication therapy for HNSCC in FA patients (EP18382527.2). JS team has a research agreement with Rocket Pharmaceuticals.

TRANSLATIONAL RELEVANCE

Our work reports for the first time the repositioning of gefitinib and afatinib, two anticancer EMA/FDA approved drugs, to treat head and neck squamous cell carcinoma (HNSCC) in Fanconi anemia, a rare disease whose patients currently have surgical resection as their only therapeutic option. We screened existing drugs for antitumor activity and identified both candidates using a combination of cell-based and *in vivo* mouse models. Our team recently obtained orphan drug designation (ODD) by EMA for gefitinib (EU/3/18/2075) and afatinib (EU/3/18/2110) (FDA ODD pending), with the midterm goal to organize a multicenter, international clinical trial to prove that gefitinib/afatinib improve the follow up of these patients when diagnosed with HNSCC.

ABSTRACT

Purpose: Fanconi anemia (FA) rare disease is characterized by bone marrow failure and a high predisposition to solid tumors, especially head and neck squamous cell carcinoma (HNSCC). FA patients with HNSCC are not eligible for conventional therapies due to high toxicity in healthy cells, predominantly hematotoxicity, and the only treatment currently available is surgical resection. In this work we searched and validated two already approved drugs as new potential therapies for HNSCC in FA patients.

Experimental design: We conducted a high-content screening of 3,802 drugs in a FANCA-deficient tumor cell line to identify non-genotoxic drugs with cytotoxic/cytostatic activity. The best candidates were further studied *in vitro* and *in vivo* for efficacy and safety.

Results: Several FDA/EMA-approved anticancer drugs showed cancer-specific lethality or cell growth inhibition in FA HNSCC cell lines. The two best candidates gefitinib and afatinib, EGFR inhibitors approved for non-small-cell lung cancer (NSCLC), displayed non-tumor/tumor IC50 ratios of ~400 and ~100 times, respectively. Neither gefitinib nor afatinib activated the FA signaling pathway or induced chromosomal fragility in FA cell lines. Importantly, both drugs inhibited tumor growth in xenograft experiments in immunodeficient mice using two FA patient-derived HNSCCs. Finally, *in vivo* toxicity studies in *Fanca*-deficient mice showed that administration of gefitinib or afatinib was well-tolerated, displayed manageable side-effects, no toxicity to bone marrow progenitors and did not alter any hematological parameters.

Conclusions: Our data present a complete preclinical analysis and promising therapeutic line of the first FDA/EMA approved anticancer drugs exerting cancer specific toxicity for HNSCC in FA patients.

INTRODUCTION

Fanconi anemia is a rare genetic disease, caused by mutations in at least 22 genes, which encode for proteins involved in interstrand-crosslink DNA repair. FA patients suffer from bone marrow failure, congenital abnormalities and a high incidence of malignancies, such as solid tumors and leukemias (1,2). The management of the hematological phenotype has been remarkably improved over the last 20 years, thanks to optimized hematological stem cell transplantation protocols, leading to an important increase in FA patient survival, from less than 20 years of age in the 1990s to more than 30 years observed today (3,4). The prevention and treatment of solid malignancies are expected to further impact the survival and quality of life of these patients (5). While there are some studies on chemoprevention, with chronic treatment proposals such as quercetin or metformin (6,7), few therapeutic options are available beyond surgical resection once solid malignancies appear (8,9). The most frequent solid tumors, accounting for up to 50 %, are HNSCC, with an incidence 700-fold higher than in the general population. Patients can tolerate complex surgeries for oral tumor removal, but usually receive mild chemotherapy, radiotherapy, or a combination, that yields moderate to high toxicities, with low survival rates of around 30 months (4,8,9).

In this study, we searched for anticancer drugs approved by the American Food and Drug Administration (FDA) and/or European Medicines Agency (EMA) that could be repositioned to treat HNSCC in FA patients thanks to the induction of cancer specific lethality and identified several approved drugs (10,11). The best drugs from this screening were thoroughly studied *in vitro* and *in vivo*, obtaining complete preclinical data and a solid basis to present the first, non-toxic and potentially therapeutic option for FA patients with HNSCC.

MATERIALS AND METHODS

Cell lines and Reagents

Wild type (PN) and FANCA-deficient (FA551) primary fibroblasts, WT (VU040-T), FA-derived 1131 (VU1131-T2.8, *FANCC*$^{-/-}$), 1604 (VU1604-T, *FANCL*$^{-/-}$) and 1365 (VU1365-T, *FANCA*$^{-/-}$) and SCC25 and Detroit 562 HNSCC cell lines, were grown in DMEM (Biowest) supplemented with 10 % heat inactivated FBS and plasmocin (ant-mpt, Invivogen). WT and FANCA-deficient lymphoblastoid cell lines were grown in DMEM supplemented with 20 % heat inactivated FBS, sodium pyruvate (Gibco), non-essential amino acids (Gibco), beta-mercaptoethanol (Gibco) and plasmocin. HNSCCs were kindly provided by Dr Josephine Dorsman, from VU University Medical Center, in Amsterdam (Netherlands). Non-FA HNSCC cell lines were from ATCC. Diepoxibutane (DEB, 202533), hydroxyurea (HU, H8627) and Mitomycin C (MMC, M0503) were purchased from Sigma. Drugs for *in vitro* studies, gefitinib (HY-508945), AEE788 (14816), afatinib (11492), AZD9291 (16237), ceritinib (19374), CO-1686 (16244) and vandetanib (14706) were from Cayman Chemical and Cetuximab/Erbitux® was from Merck. For *in vivo* studies, drugs gefitinib/Iressa® (AstraZeneca) and afatinib/Giotrif® (Boehringer Ingelheim) were used, and vehicles Tween-80 (P4780), methylcellulose 4,000cP (M0512) and alpha-lactose (L3625) were from Sigma.

Screening validation

A total of 3,800 drugs high-content screening was previously described (Montanuy *et al*, submitted). For non-genotoxic candidate validation, FA primary fibroblasts and FA HNSCC cell lines were seeded in 384 well plates, treated with candidate drugs at 1 micromolar concentration per duplicate and cultured for 7 days. Cells were then fixed, Hoechst stained and nuclei images taken with ImageXpress confocal microscope

(Molecular Devices, representative images in Supplementary Figure 1A). Nuclei in each well were counted with CellProfiler software.

Survival assays

Seeded cells in 96 well plates were exposed to 9 different concentrations of MMC or antitumor drugs and cultured for 3 or 7 days. Cell growth and survival was measured with sulforhodamine B (SRB) staining assay (12). IC50 was determined by calculating logarithmic normalized trend lines with GraphPad. To identify best antitumor candidates, we calculated a ratio from IC50 of non-tumor cell lines (primary fibroblasts) *vs* averaged IC50 of the averaged three FA HNSCC cell lines.

Western blot

Western blot was performed as described earlier (13). FANCD2 (Ab2187), total ERK1 (Ab32537), phosphorylated ERK1/2 (pT202/pY204 for ERK1, pT185/pY187 for ERK2; Ab50011), total AKT (Ab32505) and Vinculin (Ab18058) antibodies were from Abcam. Ser473 phosphorylated AKT (9271T), total EGFR (4267T) and Tyr1068 phosphorylated EGFR (3777T) antibodies were from Cell Signaling.

Chromosome fragility and cell cycle analysis

Chromosome fragility in cell lines was measured for 48 hours with flow cytometric micronucleus (FCM) assay, as described earlier (14–16). Micronuclei (MN) frequency was expressed as the number of MN per thousand nuclei. Percentage of cells arrested in G2/M phase of the cell cycle was obtained from nuclei plots. For *in vivo* chromosome fragility in mice, genotoxicity was measured in erythrocytes and reticulocytes from peripheral blood of wild type and *Fanca*-deficient mice as previously described (17). Briefly, peripheral blood was drawn from mice tail (~100 µL), collected into EDTA containing tubes, fixed in methanol and stored at -80 ºC. Samples were then incubated with anti-CD71-FITC antibody to select reticulocytes from erythrocytes, and stained

with propidium iodide to detect micronuclei. FACS analysis was performed in a FACSCanto cytometer (Becton Dickinson).

Gene sequencing of HNSCC cell lines

To analyze mutations in cancer related genes (including *EGFR*) in HNSCC cell lines, we used TruSight Tumor 15 (Illumina), a next-generation sequencing panel designed to identify sequencing variants in 15 genes commonly mutated in solid tumors and associated with marketed therapeutics (*AKT1, BRAF, EGFR, ERBB2, FOXL2, GNA11, GNAQ, KIT, KRAS, MET, NRAS, PDGFRA, PIK3CA, RET* and *TP53*).

***In vivo* xenograft experiments**

NOD-SCID mice (both sexes, age 6-9 week old, Charles River) were injected subcutaneously in the right flank with a mixture 1:1 of $1x10^6$ FA-HNSCC cells-matrigel (Corning). Animals were monitored twice a week (body weight and tumor volume) until tumors were ≈150 mm^3. Animals were then randomized into 4 experimental groups (n=8 animals/group): 1) Vehicle (0.5 % Tween-80); 2) Gefitinib; 3) Vehicle (0.5 % methylcellulose); 4) Afatinib. Treatments were administered 5 days a week orally (gavage): gefitinib/Iressa® 150 mg/kg and afatinib/Giotrif® 20 mg/kg (18–21). Vehicles were further supplemented with lactose at 98 mg/kg and 117 mg/kg respectively, to pair excipients in the medicinal products. Animals were monitored three times a week (body weight and tumor volume) until tumors were ≈1,000 mm^3. Tumor volume was determined by using the formula: (length × $width^2$) × (pi/6). At end-point animals were euthanized, and tumors were surgically removed. Tumor specimens were formalin-fixed and paraffin-embedded for routine histological analysis. Animal experiments were performed under protocols approved by the Vall d'Hebron Ethical Committee for Animal Experimentation and the appropriate governmental agency and carried out in accordance with the approved guidelines.

Immunohistochemistry

Tumor samples excised from mouse xenograft experiments were fixed in 4 % formalin. For IHC, NovoLink polymer detection system (Novocastra Laboratories) was used. Anti-phopho-ERK1 (pT202/pY204)/ phospho-ERK2 (pT185/pY187) immunostaining (1:200 dilution) was carried out after heat-induced antigen retrieval (4 min, pressure cooker) with 10 mM citrate buffer pH 6.0, and then counterstained with hematoxylin and mounted.

***In vivo* toxicity experiments in *Fanca*-deficient mice**

Fanca-deficient mice were previously described (22). Wild type and *Fanca*-deficient mice (female, age ranging from 8 to 20 weeks) were weight randomized into 4 experimental groups and started to receive treatment (n=6 animals/group): 1) Vehicle (Tween-80); 2) Gefitinib; 3) Vehicle (Methylcellulose); 4) Afatinib. Treatments were administered 5 days a week orally (gavage): gefitinib 150 mg/kg and afatinib 20 mg/kg, for two weeks. Animals were monitored three times a week (body weight), and tail bled at 0 (pretreatment) and 14 days (end-point) of treatment. At end-point, animals were euthanized and bone marrow from femurs extracted for further analysis.

FACS analysis of hematopoietic cell populations

For counting LSK^+ cells from bone marrow, we selected Lin^- (all FITC-labeled: TER-119, from eBiosciences; B220, RA3-6B2 from Biolegend; CD3, 145-2C11 from BD Biosciences; CD11b/Mac1, M1/70 from Biolegend; GR1, RB6-8C5 from Biolegend), C-Kit^+ (C-Kit PE/Cy7, 2B8 from Biolegend) and Sca-1^+ (Sca-1 PE, E13-161-7 from BD Biosciences) cells. For peripheral blood cells, the following antibodies were used: B220-FITC (RA3-6B2), GR1-PE (RB6-8C5), CD4-BV711 (RM4-5) and CD11b/MAC1-AF647 (M1/70) were from Biolegend; CD3-PEvio770 (145-2C11) was from Milteny; CD8-PECy5 (53-6-7) was from BD Biosciences. T lymphocyte ($CD3^+$), B lymphocytes ($B220^+$) and myeloid cells (non-T, non-B cells) were gated in the region of live

leucocytes from FSC-A, SSC-A and DAPI parameters. $CD4^+$ and $CD8^+$ cells were quantified from $CD3^+$ cells. Myeloid cell subpopulations $GR1^+MAC1^+$ (mainly neutrophils and other granulocytes) and $GR1^-MAC1^+$ (mainly monocytes, macrophages and dendritic cells)(23) were quantified from $CD3^-B220^-$ cells.

Blood hematology and bone marrow colony formation unit assays

Peripheral blood was drawn from mice tail (~100 µL), collected into EDTA containing tubes (Sarstedt) and counts were determined using an Abacus Junior Vet hematology analyzer (Diatron). Number of colony forming unit-granulocyte/macrophage (CFU-GM) progenitors present in total bone marrow was performed as described earlier (22).

Statistics

All experiments were performed using triplicate repeats unless otherwise stated, and data present means ± SEM. Statistical significance was tested using Student's T test, and P values were reported as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

RESULTS

EGFR inhibitors selectively inhibit the growth of FA HNSCC cell lines

From a previous screening in *FANCA*-deficient tumor cells (Montanuy *et al.*, submitted) we sought to find non-genotoxic drugs that induce cancer-specific cytotoxicity. We

used primary fibroblasts from FA donors as non-tumor cells and three different FA patient-derived HNSCC cell lines: 1131 (*FANCC* deficient), 1604 (*FANCL* deficient), and 1365 (*FANCA* deficient) (24). From 150 selected candidates, validation analysis at a concentration of 1 micromolar identified seven anticancer drugs: ceritinib, an anaplastic lymphoma kinase (ALK) inhibitor, used to treat NSCLC (25); CO1686 (rociletinib), a second-generation EGFR inhibitor; AZD9291 (osimertinib), a third-generation EGFR inhibitor approved for patients with EGFR T790M mutation-positive metastatic NSCLC (26); vandetanib, a multikinase inhibitor including EGFR, vascular endothelial growth factor 2 (VEGFR2) and RET, approved for thyroid cancer (27); AEE788, also a dual inhibitor of EGFR/ERBB2 and VEGFR2; gefitinib, a first-generation inhibitor of EGFR, also approved to treat NSCLC (28); and afatinib, a second-generation EGFR inhibitor, also used to treat NSCLC (Figure 1A, 1B, and S1A-F) (29). Interestingly, other EGFR and VEGFR inhibitors, such as erlotinib and vatalanib, did not have or had a low non-tumor/tumor ratio in the cell lines tested, probably due to different cell line sensitivities that these drugs may exert (data not shown). In this sense, cetuximab treatment, a highly specific EGFR-targeting antibody used to treat HNSCC in the general population, among other malignancies (30) inhibited growth in all FA HNSCC cell lines, while having no effect in primary fibroblasts, showing specific dependency of EGFR pathway for FA HNSCC growth (Figure S1G). Subsequent cytotoxicity assays with doses ranging from low nanomolar to micromolar concentrations showed, as expected, that the DNA crosslink-inducer mytomycin C (MMC) was highly toxic both in FA HNSCC cell lines as well as primary cells, at less than 1 nM (Figure 1C). In sharp contrast, gefitinib and afatinib were the drugs that best inhibited growth in all three HNSCC cell lines derived from FA patients, while having a much lower effect in primary FA fibroblasts (Figure 1D-E). Gefitinib produced a sensitivity ratio of non-tumor *versus* tumor cell lines of 386 times, and afatinib 112 times, exerting its antitumor effect at a low nanomolar concentration (the IC50 for HNSCCs averaged 25.3 nM for gefitinib and 10.8 nM for afatinib; see Figure

1F). Other drugs with good antitumor profile were AEE788 (with an average IC50 of 28.4 nM), AZD9291 (IC50 64.2 nM) and vandetanib (IC50 of 108.4 nM). However, when compared with primary fibroblasts, only AEE788 showed results similar to afatinib (non-tumor *versus* tumor ratio of 81 times). CO1686 (IC50 of 629.3 nM) and ceritinib (IC50 of 1,246 nM) showed modest differences between malignant and healthy cells (ratios of 2.4 and 1.3 times, respectively; see Figure S1B to S1F). We performed the survival assays at 7 days to better show longterm non-toxicity in primary fibroblasts; 3 day treatments of gefitinib and afatinib also gave similar results (data not shown). We also confirmed gefitinib and afatinib inhibited non-FA HNSCCs in a similar trend (Figure S1H and data not shown). Thus, gefitinib and afatinib were the best anticancer drugs that specifically inhibited the growth of FA HNSCC cell lines at low-nanomolar concentrations.

Gefitinib and afatinib are non-genotoxic in FANCA-deficient cells

EGFR (ERBB-1) is a member of the ERBB family of tyrosine kinase receptors that has a central role in the tumorigenesis of many types of solid tumors, including HNSCC (31). Multiple drugs targeting these receptors have been approved for the treatment of several cancers, such as gefitinib and afatinib, as well as vandetanib and AZD9291 (26–29). These drugs bind to the tyrosine kinase domain and impair kinase activity and downstream signaling pathways, such as PI3K/AKT and the RAS/MAPK axis. Moreover, no genotoxic toxicity is reported from these drugs. To discard any direct or indirect effect on DNA that could be easily repaired by normal cells but compromise FA cell viability, we treated U2OS cells with gefitinib or afatinib to analyze FANCD2 monoubiquitination, a central step in the FA/BRCA pathway, induced by several types of DNA damage (2). As seen in Figure 2A and 2B, neither gefitinib nor afatinib up to 10 μM were able to activate the FA/BRCA pathway as measured by FANCD2 mono-ubiquitination by Western blot, indicating that these drugs do not induce interstrand-crosslinks (ICLs), stalled replication forks or double strand breaks on DNA that would

require processing by the FA pathway. We further analyzed their genotoxic capacity in FA cells, which are highly sensitive to ICLs such as diepoxybutane (32). Again, as seen in Figure 2C to 2G, high concentrations of gefitinib or afatinib were unable to induce chromosome fragility (micronuclei, MN) or G2/M cell cycle arrest (a specific hallmark of FA cells treated with ICL-inducing agents) in WT or FA lymphoblastoid cell lines, which express EGFR (Figure 2D) and are derived from T-cells reported to have a functional EGFR pathway (33,34).. In summary, our *in vitro* results showed that non-tumor FA cells could be safely treated with gefitinib and afatinib at therapeutic concentrations, as they did not activate the FA/BRCA pathway, nor induce chromosome fragility or cell cycle arrest in the absence of the FA pathway.

EGFR pathway in FA HNSCCs

Previous reports indicate that the EGFR pathway is functional in sporadic HNSCCs, and targeting this pathway inhibits tumor growth (35). Thus, we sought to further explore the EGFR pathway inhibition achieved by gefitinib and afatinib in FA HNSCCs. As shown in Figure 3A, 24 hours treatment with gefitinib or afatinib inhibited downstream signaling mediators of the EGFR pathway in all three FA HNSCC cell lines tested, such as phosphorylated AKT or ERK1/2. As previously reported in sporadic HNSCCs (36), we also observed that the EGFR pathway was overactivated in FA HNSCCs in comparison with primary fibroblasts, as detected by total and phosphorylated EGFR expression (Figure 3B). In the general population the majority of HNSCCs have mutations in *TP53* (72 %) or *PIK3CA* (18 %) genes, but few in *EGFR* (4 %) (35,37,38). Interestingly, van Zeeburg *et al* showed a similar TP53 mutation trend in FA HNSCCs (8 out of 13 FA HNSCCs tested, 62%, carried TP53 mutations) (39). Mutation analysis of key tumor-promoting genes showed that all three FA HNSCCs presented mutations in *TP53*, with a variant frequency of almost 100 % in DNA from the 1131 and 1604 cell lines, and 34 % from the 1365 cell line (Figure 3C and (24)). No other genes, such as *EGFR*, *PIK3CA*, *AKT1*, *NRAS*, or *KRAS* were found mutated in

these cell lines. Interestingly, EGFR MLPA assay showed a gain in EGFR copy number for 1131 and 1604 but not for 1365 cell lines (data not shown). These results highlight that FA HNSCC cell lines have a functional EGFR pathway similar to sporadic HNSCCs, with no mutations in key genes, increased EGFR activity and expression, in 2 out of 3 cell lines with EGFR gene copy number gain, and functional AKT and ERK1/2 activities that could be inhibited by gefitinib and afatinib.

Gefitinib and afatinib inhibit growth of FA HNSCCs in mouse xenografts

To further investigate the therapeutic potential of gefitinib and afatinib for FA HNSCC, we used a preclinical mouse subcutaneous xenograft model. The FA HNSCC cell lines 1604 and 1131 were subcutaneously implanted in NOD-SCID immunodeficient mice. Tumor growth was monitored over time, and when the tumors reached approximately 150 mm^3, animals were randomized into vehicle control groups or gefitinib (Figure 4) and afatinib (Figure 5) treatment groups. Importantly, treatment with these two FDA/EMA-approved EGFR inhibitors led to a significant reduction of the growth of the tumors compared to control animals at the end of the experiment (Figure 4A-C, E and Figure 5A-C, E), or a significant shrinkage of the size of the tumors compared to the size at the beginning of the treatment (Figure 4D, G, H and Figure 5D, G, H). Treatment did not have a major impact on mouse weight (Figure S2A-D). The efficacy of the treatment was further confirmed measuring the weight and the average volume change of the tumors at the end of the experiment (Figure S2E-L). Finally, tumors from vehicle-treated mice showed strong phospho-ERK immunostaining (Figure 4I-J, Figure 5I-J and Figure S3), while tumors from gefitinib or afatinib-treated mice had almost no phospho-ERK signal, confirming a high efficiency of either drug in inhibiting the EGFR pathway in both HNSCC *in vivo*.

Gefitinib and afatinib treatment did not produce hematotoxicity in *Fanca*-deficient mice

Our *in vitro* results show gefitinib and afatinib are innocuous in FA fibroblast cells at therapeutic concentrations (Figure 2). The most frequently reported adverse effects (AEs) for these drugs in humans are skin rashes, diarrhea, and nausea and vomiting, among others (40–42). Thus, hematologic toxicity was not expected, but given the extreme fragility of FA patients, we sought to discard toxicity of these EGFR inhibitors in animal models of the disease. After two weeks of chronic administration of gefitinib or afatinib in wild type (WT) and *Fanca*-deficient mice, we monitored weight and general health status three times a week, hematological parameters before and at the end of the experiment, and bone marrow status when mice were sacrificed. As seen in Figure 6A, gefitinib treatment had no effects on body weight either in the WT or in *Fanca*-deficient mice. General health status showed no evident toxicity, specially skin rash or diarrhea, typical adverse effects reported for gefitinib and afatinib. We did not observe any differences in white or red blood cells, platelets, hemoglobin, hematocrit, or leukocyte populations from peripheral blood (CD4 and CD8 T-cells B-cells and myeloid cells), LSK^+ cells or colony forming units (CFUs) from bone marrow (Figure 6, S4-S7). Following afatinib treatment, some *Fanca*-deficient mice showed weight loss during the first week of the treatment (Figure 7A). Clinical trials in HNSCC and NSCLC show that afatinib efficacy is higher than the standard of care but produces more toxicity and AEs than gefitinib. In these cases, a dose adjustment is often chosen with good results (43,44). For this reason, from day 7 we reduced afatinib dosages while maintaining its therapeutic effect (from 20 mg/kg/day to 15 mg/kg/day). *Fanca*-deficient mice progressed favorably after dose reduction and indeed recovered weight at the end of the experiment, also seen in wild type mice (Figure 7A and S4D). Afatinib administration also mildly reduced some hematological parameters, but in both WT and *Fanca*-deficient mice, and blood counts were always within the physiological range (Figure 7, S4, S6, and S7) (45). Notably, we did see an increase in blood myeloid cells in *Fanca*-deficient mice, which could suggest an increase in infection susceptibility, as previously reported for this drug (Figure S7B) (46,47). Finally, to exclude any *in vivo*

genotoxic effects on chromosomal stability, we analyzed MN presence in blood reticulocytes, which reflects acute chromosome fragility, and in erythrocytes, which represents chronic chromosomal instability in bone marrow erythroid precursors *in vivo* (17). *Fanca*-deficient mice spontaneously showed a reduction in reticulocyte counts (Figure S8A), while MN from erythrocytes or reticulocytes increased by more than two-fold respect WT mice (Figure 6F and S8B). Interestingly, neither gefitinib nor afatinib treatment affected these chromosome fragility biomarkers in wild type or *Fanca*-deficient mice, indicating that these EGFR inhibitors do not exert any clastogenic effect in the development of blood cells irrespective of the FA pathway. In summary, as seen in wild type and *Fanca*-deficient mice, gefitinib or afatinib administration is safe *in vivo* as a chronic treatment, with afatinib showing some toxicity that could be balanced by dose adjustment.

DISCUSSION

Twenty years ago, FA was mainly a pediatric disease, as most patients died in the first two decades due to bone marrow failure or leukemias (5). With improved transplantation protocols, FA patients now reach their fourth decade of life. Thus, HNSCC and other solid tumors are arising as the main challenge for their long-term survival, and last efforts in recent decades to treat patients with current therapies have resulted in poor survival rates. Due to its rare condition, few case reports have been published. Beginning in the 2000s, they highlighted the frequent clinical complications and severe toxicities of conventional chemotherapy and radiotherapy in these patients (8,9,48–50). On average, the median age reported at diagnosis has been 31-33 years, with a median follow-up of around 30-35 months, with very low tolerance to radiotherapy and chemotherapy. These case reports and small cohort studies highlight a painful reality and an unmet medical need that FA patients suffer nowadays: beyond tumor resection, there is no safe or effective treatment for FA patients with solid tumors in general, but especially HNSCCs.

Our work describes for the first time comprehensive preclinical data regarding gefitinib and afatinib, two previously approved anticancer drugs, with a strong potential for treating HNSCCs in FA. Drug validation in FA tumor and non-tumor cells identified several approved antitumor drugs inducing FA cancer-specific lethality, with gefitinib and afatinib having the best IC50 non-tumor/tumor ratio (Figure 1 and S1). Antibody-based EGFR inhibitor cetuximab remains the only FDA-approved targeted therapy available for sporadic HNSCC, but it works in combination with radiotherapy or standard chemotherapy, which are not well-tolerated by FA patients (31). Indeed, Wong *et al.* and Kutler *et al.* have reported FA patients who received post-surgery cetuximab and radiotherapy. Two of them displayed lower toxicities and the other two had manageable toxicities, but all died of recurrent or metastatic disease (8,51). Unfortunately, without preclinical evidence of efficacy and safety and controlled studies such as with clinical trials, clinicians may find unsuitable to choose cetuximab as a single therapeutic option for FA patients.

Our work shows that gefitinib and afatinib are effective *in vitro* in three different FA HNSCC cell lines (Figure 1) and more importantly *in vivo*, in xenograft experiments with immunodeficient mice with two different FA-patient derived HNSCC tumors (Figures 4, 5, S2 and S3). In addition, our results also highlight that gefitinib and afatinib are safe in non-tumor FA cells, as they did not activate the FA/BRCA pathway nor induce chromosome instability (Figure 2), and more remarkably in *Fanca*-deficient mice; these drugs did not generate treatment-related hematotoxicity nor bone marrow failure (Figures 6, 7 and S4 to S8).

Jung *et al.* published in 2005 a case report of a FA patient with a large squamous cell carcinoma on the tongue, which was 90% positive on EGFR according to immunohistochemical staining. The patient was then administered gefitinib as a palliative treatment, and after two months the tumor size was reduced by 80%, with no gefitinib-associated AEs such as skin rash or diarrhea (52). As shown here, our data

demonstrate both gefitinib and afatinib have cancer-specific lethality in FA HNSCC, with no toxicity targeting DNA, nor hematotoxicity in mouse models. We did observe some toxicity in afatinib-treated *Fanca*-deficient mice, which was reverted by dose adjustment, maintaining the therapeutic effect (Figure 7A and S4D). We did also observe an increase of myeloid cell populations (Figure S7), which suggests FA patients may need more thorough follow up with afatinib compared to gefitinib.

Given that FA is a rare disease, the repositioning of approved medicines to achieve patient treatment is a viable approach regarding time and the cost/effectiveness ratio to market authorization (53,54). With this in mind, we recently received the orphan drug designation (ODD) status for gefitinib and afatinib by EMA to treat HNSCCs in FA patients (FDA orphan application submitted). ODD gives the sponsors regulatory benefits and facilities regarding reduced fees, scientific advice, protocol assistance, and market exclusivity after authorization, with the purpose to promote clinical trials that demonstrate safety and efficacy of new or repositioned drugs to treat rare diseases. This support from the European and American drug regulatory institutions may help to push current preclinical research to organize, coordinate, and initiate a multicenter, international clinical trial with gefitinib and/or afatinib to treat HNSCCs in FA with the aim to provide the patients a new anticancer therapeutic option and improve their clinical outcomes and quality of life.

AUTHOR CONTRIBUTIONS

JM designed, performed and reviewed the experiments and wrote the manuscript. JS design and reviewed the experiments and wrote the manuscript. AMB and JAC designed and performed the experiments and reviewed the manuscript. HM, MJR, LR, RN, CC, AG, EL, JC and AL performed the experiments and reviewed the manuscript. PR, TH, JB and DA discussed the experiments and reviewed the manuscript. reviewed the manuscript.

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FIGURE LEGENDS

Figure 1. **Drug screening identified gefitinib and afatinib with antitumor activity in Fanconi anemia-derived HNSCCs, non-toxic for FA cells**. **A)** FANCA-deficient U2OS cell line was used to screen for drugs with acute cytotoxicity. Non-genotoxic drugs with potential activity were selected and validated in FA HNSCCs and primary cells. **B)** Validation screening identified 7 potential drugs with high growth inhibition in three different FA HNSCCs while maintaining good viability in FA primary fibroblasts (at 1 μM). Bars show mean of samples performed at least in duplicates. **C-E)** Extended cytotoxicity analysis with gefitinib (D) and afatinib (E) in primary fibroblasts (from wild type, and FANCA-deficient patient) and three different FA HNSCC cell lines. Mitomycin C (C) was used as a control. The mean +/- SEM of at least three independent experiments is shown, with normalized curves in lines. **F)** IC50 (nM) of the candidate drugs used, in FA fibroblasts (green) and FA HNSCC cell lines (averaged, red). Ratio of non-tumor *vs* tumor IC50 (below) is shown to highlight best candidates (e.g. gefitinib, afatinib and AEE788).

Figure 2. Best candidates gefitinib and afatinib are non-genotoxic. A-B) U2OS cells were stimulated for 24 hours with different concentrations of gefitinib (A), afatinib (B) or 2 mM HU (as a positive control). Cells were lysed and FANCD2 ubiquitination

analyzed by Western blot (upper panels) with vinculin used as a loading control. Averaged graphs of two independent experiments are shown in lower panels. **C)** Chromosomal fragility analysis by flow cytometry micronucleus assay (see materials and methods) using a FA lymphoblastoid cell line. Graphs show representative plots of MN (upper) and G2/M cell cycle (bottom). **D-G)** Graphs from experiments performed as in C, with WT (green bars) or FA-derived (red bars) lymphoblastoid cell lines. MN induction (D, F) and G2/M cell cycle arrest (E, G) of cells with different concentrations of gefitinib (D, E), and afatinib (F, G). Diepoxybutane (DEB) was used as a positive control. Bars show mean +/- SEM of three independent experiments with similar results. EGFR expression in lymphoblastoid cell lines is shown in a Western blot inside graph from figure 2D and is representative of two independent experiments.

Figure 3. EGFR pathway in FA HNSCC cell lines. A) 1365 (left), 1131 (middle) and 1604 (right) FA HNSCC cells were stimulated 24 hours with the indicated doses of gefitinib and afatinib, and Western blots for expression and phosphorylation status of key kinases of the EGFR pathway were performed. Vinculin was used as a loading control (p-Vinculin refers to membranes blotted with phospho-antibodies). Images are representative of at least three independent experiments with similar results. **B)** Total EGFR and phospho-EGFR basal expression in FA HNSCC in comparison with WT and FA primary fibroblasts (left panel). Relative expression normalized to WT primary fibroblasts is shown. Middle and right graph show mean +/- SEM of phospho-EGFR and total EGFR, respectively, of three independent experiments. **C)** Gene variants identified and their frequency in FA HNSCCs using TruSight Tumor 15 kit (see materials and methods).

Figure 4. Gefitinib inhibits FA HNSCCs growth *in vivo* in mouse xenograft experiments. FA-derived HNSCC 1604 (A, C, D, G, I) and 1131 (B, E, F, H, J) xenografts are shown. **A-B)** Excised tumors at end-point. **C, E)** Tumor growth by vehicle (black lines) or gefitinib (blue lines) treatment groups. The arrow indicates the

start of the treatment. Graphs show mean +/- SEM. **D, F)** Response Evaluation Criteria In Solid Tumors (RECIST) classification from the percentage of tumor volume change. CR, complete response; PR, partial response; SD, stable disease; PD, progression disease **G-H)** Percentage of tumor volume change at baseline (start of treatment) for individual tumors (black bars, vehicle; blue bars, gefitinib)**.** The percentage of tumor volume change of treated (T) *vs* vehicle (V) is shown. Dashed lines represent 20 % volume above and - 30 % below X axis. **I-J)** Immunohistochemistry of phospho-ERK activation in representative formalin fixed, paraffin-embedded tumors from xenografts treated with vehicle (upper) or gefitinib (lower). Student's T-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 5. Afatinib inhibits FA HNSCCs growth *in vivo* in mouse xenograft experiments. FA-derived HNSCC 1604 (A, C, D, G, I) and 1131 (B, E, F, H, J) xenografts are shown. **A-B)** Excised tumors at end-point. **C, E)** Tumor growth by vehicle (black lines) or afatinib (green lines) treatment. The arrow indicates the start of the treatment. Graphs show mean +/- SEM. **D, F)** RECIST classification from the percentage of tumor volume change, as shown in Figure 4D and 4F **G-H)** Percentage of tumor volume change at baseline (start of treatment) for individual tumors (black bars, vehicle; green bars, afatinib)**.** The percentage of tumor volume change of treated (T) *vs* vehicle (V) is shown. Dashed lines represent 20 % volume above and - 30 % below the X axis. **I-J)** Immunohistochemistry of phospho-ERK activation in representative formalin-fixed paraffin-embedded tumors from xenografts treated with vehicle (upper) or afatinib (lower). Student's T-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 6. Gefitinib is non-toxic in *Fanca*-deficient mice. A) Percentage body weight of wild type and *Fanca*-deficient mice, treated with vehicle or gefitinib (see materials and methods). **B-C)** Red blood cells (B) and platelets (C) at 0 and 14 days of vehicle or gefitinib treatment. Dashed blue lines in B show physiologic range of red blood cells. **D-**

E) LSK^+ cell percentage (D) and colony forming units (CFUs) capacity from bone marrow cells (E) at end-point (14 days). **F-G)** *In vivo* genotoxic analysis in murine blood cells. F) Percentage of erythrocytes with MN in wild type *vs Fanca*-deficient mice. G) Percentage of MN-erythrocytes in mice treated with vehicle or gefitinib. B to G graphs show data for individual mouse (green dots, wild type, red dots, *Fanca*-deficient) and mean +/- SEM. Student's T-test: ns, not significant, * $p<0.05$, *** $p<0.001$.

Figure 7. Afatinib is non-toxic in *Fanca*-deficient mice. A) Percentage body weight of wild type and *Fanca*-deficient mice, treated with vehicle or afatinib. From day 7, afatinib dose was reduced from 20 mg/kg/day to 15 mg/kg/day. **B-C)** Red blood cells (B) and platelets (C) at 0 and 14 days of vehicle or afatinib treatment. Dashed blue lines in B show physiologic range of red blood cells. **D-E)** LSK^+ cell percentage (D) and colony forming units (CFUs) capacity from bone marrow cells (E) at end-point (14 days). CFU graph shows afatinib data in blue dots superimposed to gefitinib data from Fig 6E. **F)** Percentage of MN-erythrocytes in mice treated with vehicle or afatinib. B to F graphs show data for individual mouse (green dots, wild type, red dots, *Fanca*-deficient) and mean +/- SEM. T-test: ns, not significant, * $p<0.05$, ** $p<0.01$.

Figure 1

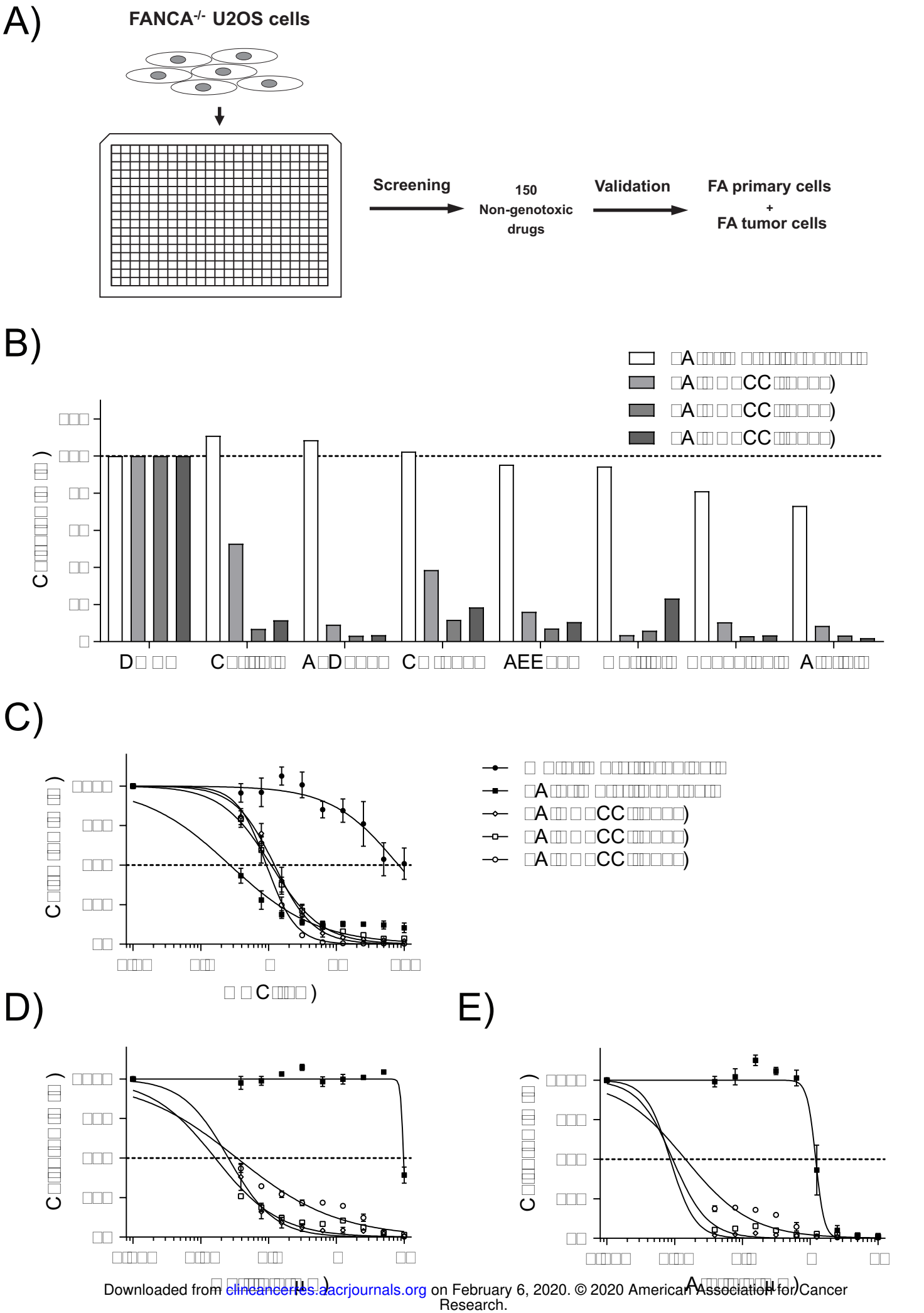


Figure 2

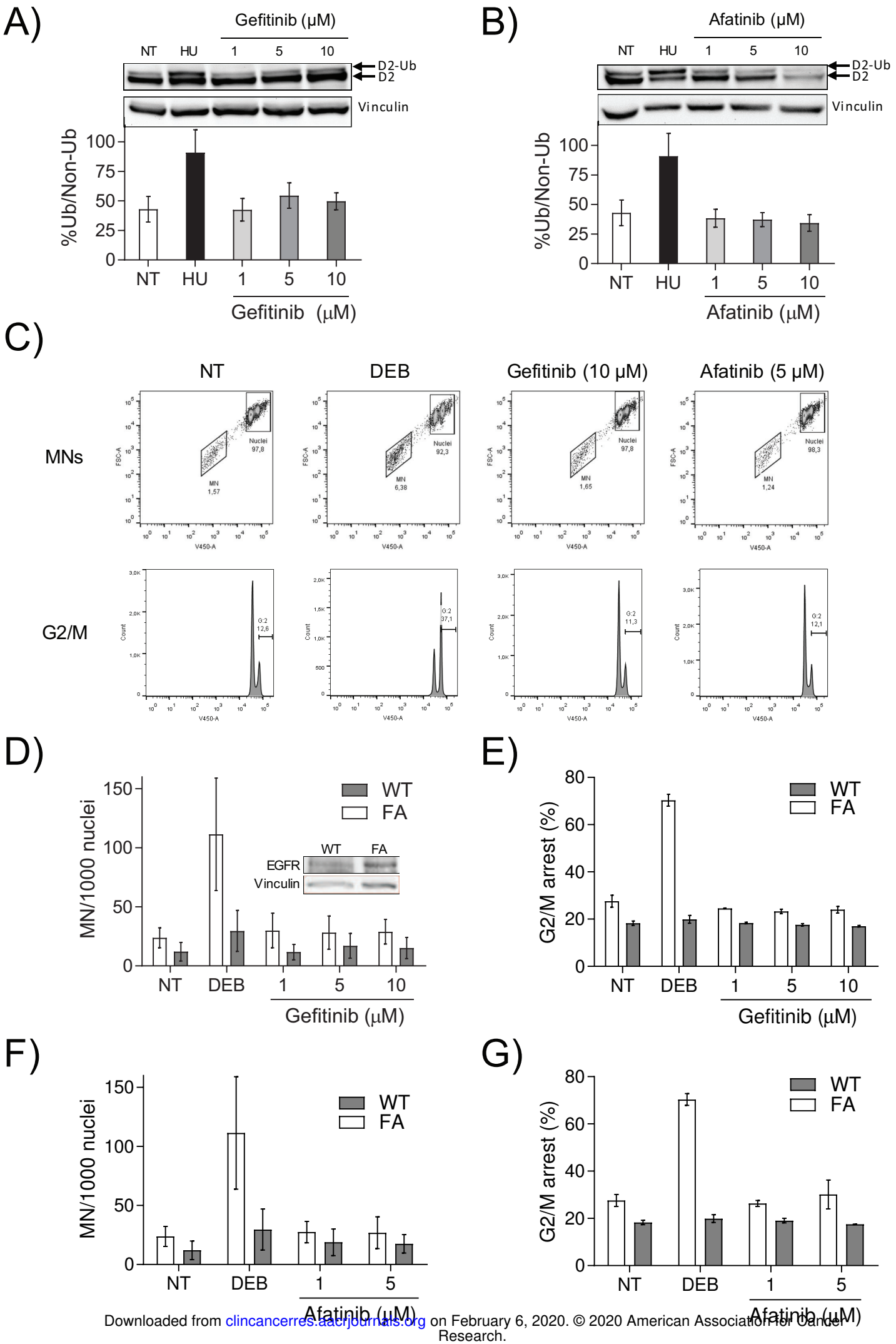
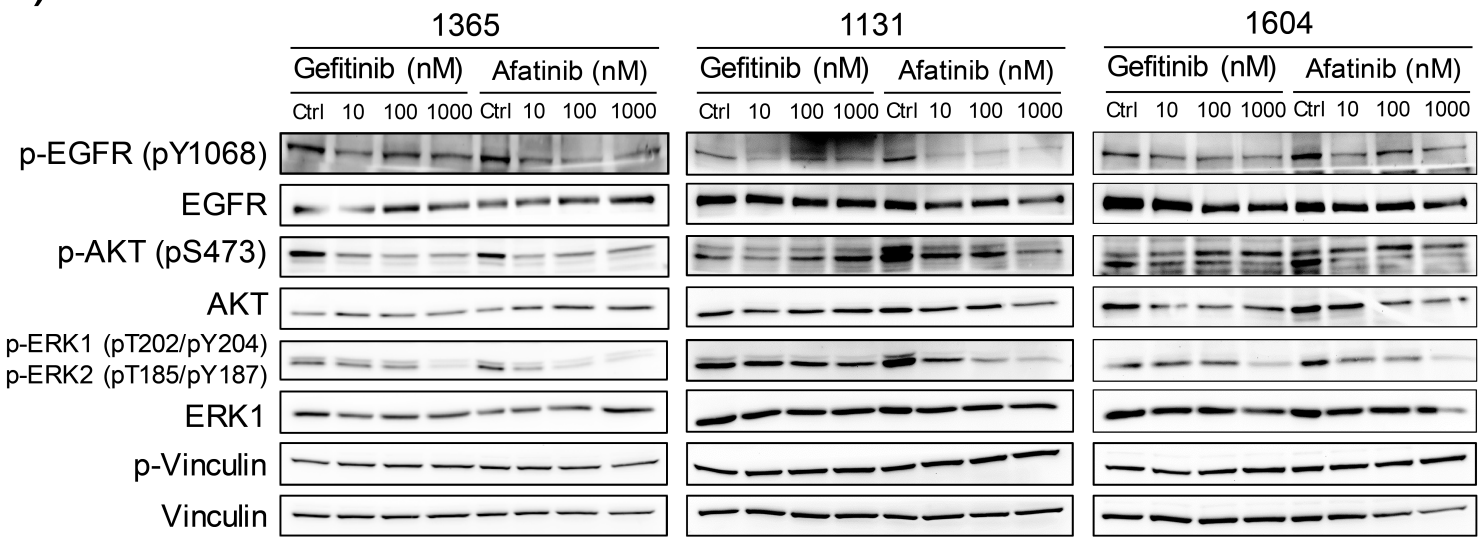


Figure 3

A)



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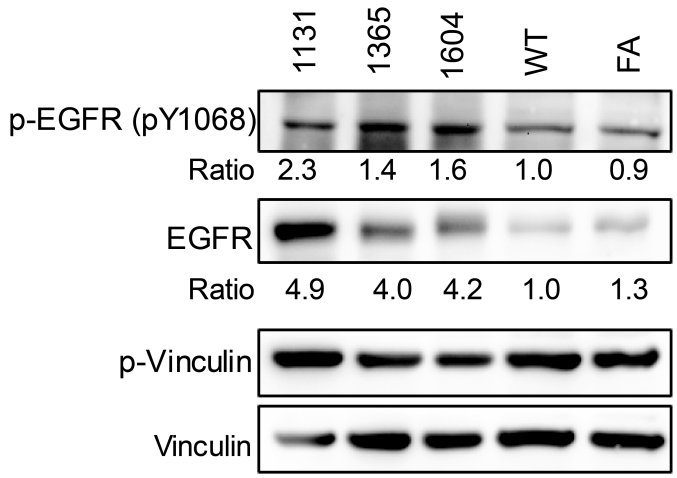

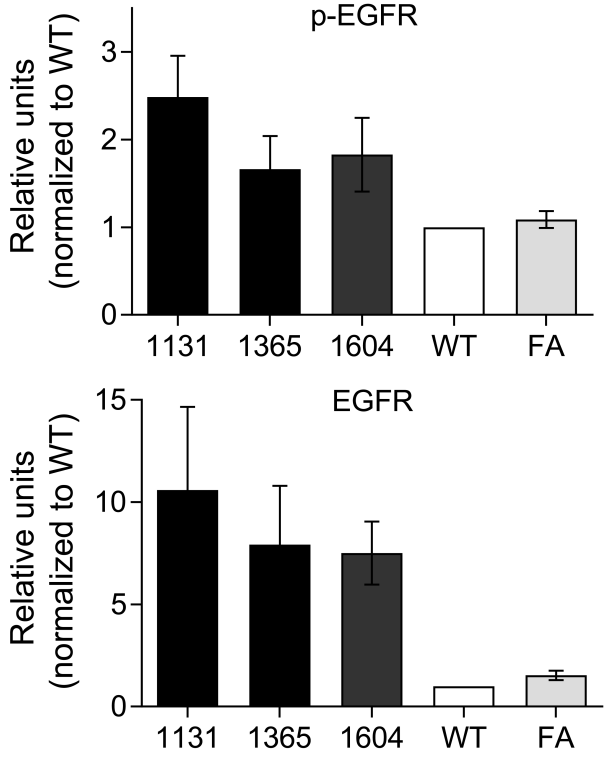

C)

Variants identified in FA HNSCCs

Cell line	Gene	AA change	Variant Type	Nucleotide Change	Variant Frequency
1131	TP53	p.Arg273Leu	Missense variant	c.818G>T	0.996
1604	TP53	p.Val216GlyfsTer5	Frasmeshift variant & feature truncation	c.647_648delTG	0.995
1365	TP53	p.Arg282Trp	Missense variant	c.844C>T	0.339

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 4

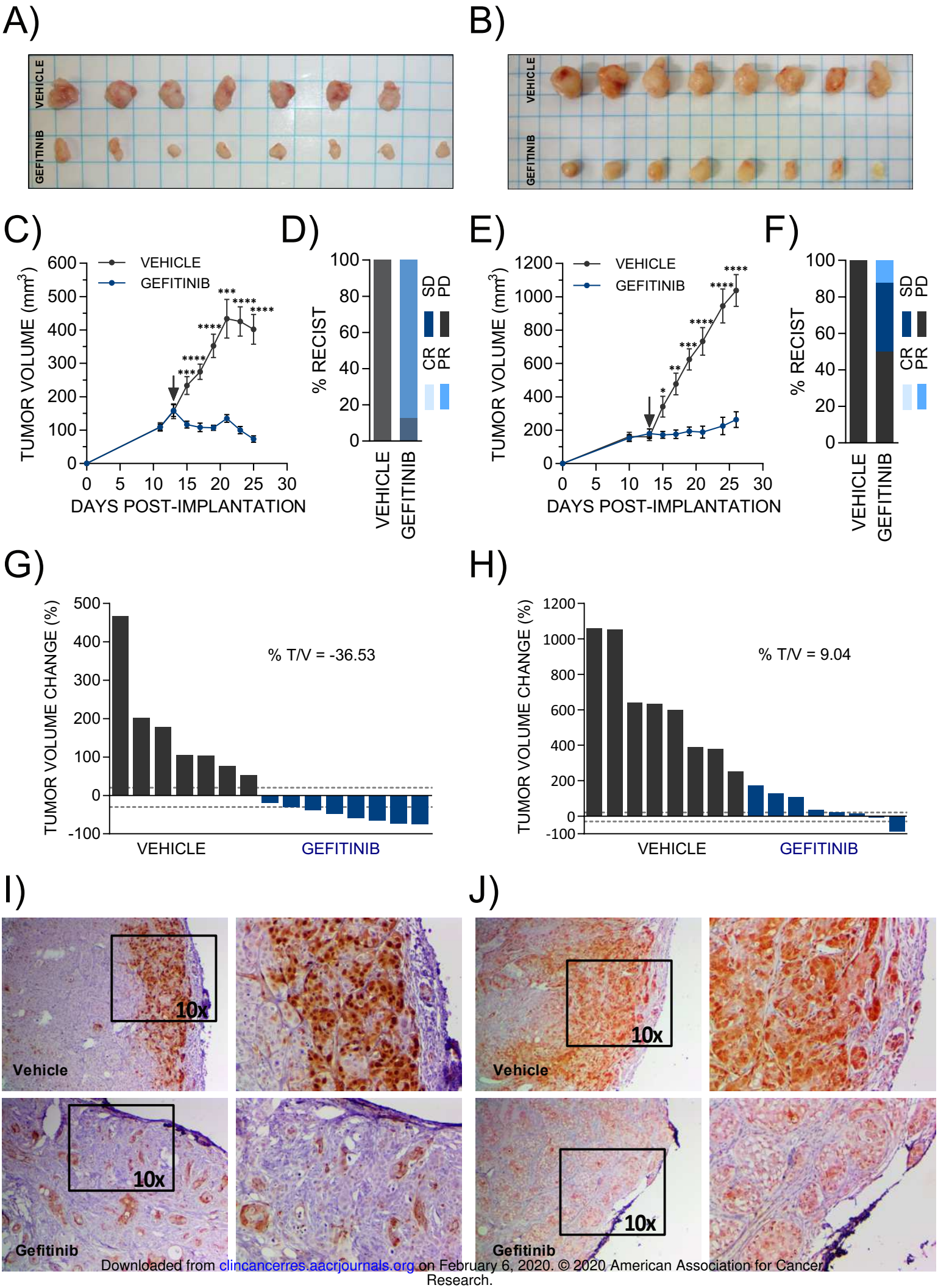

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Figure 5

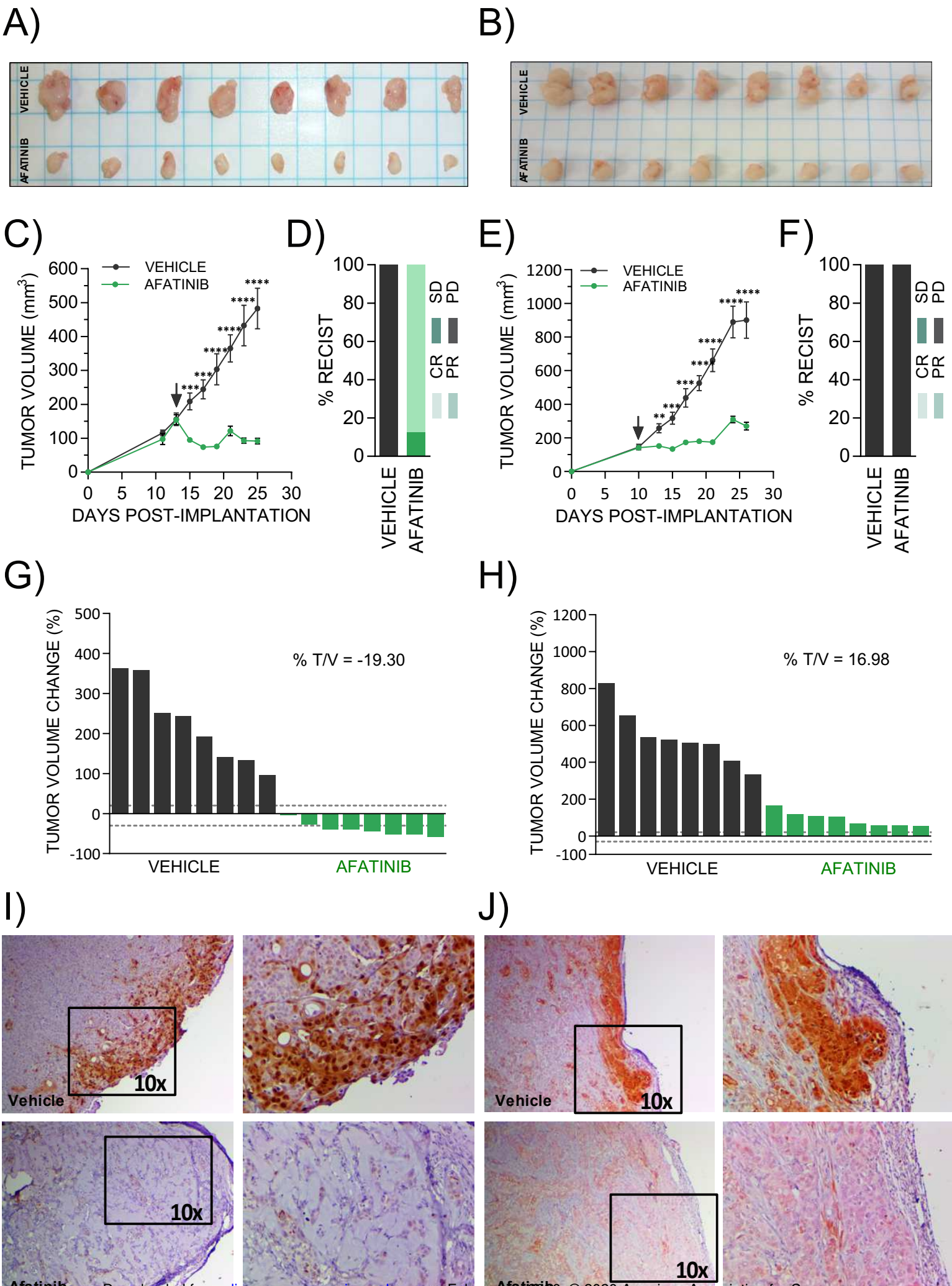


Figure 6

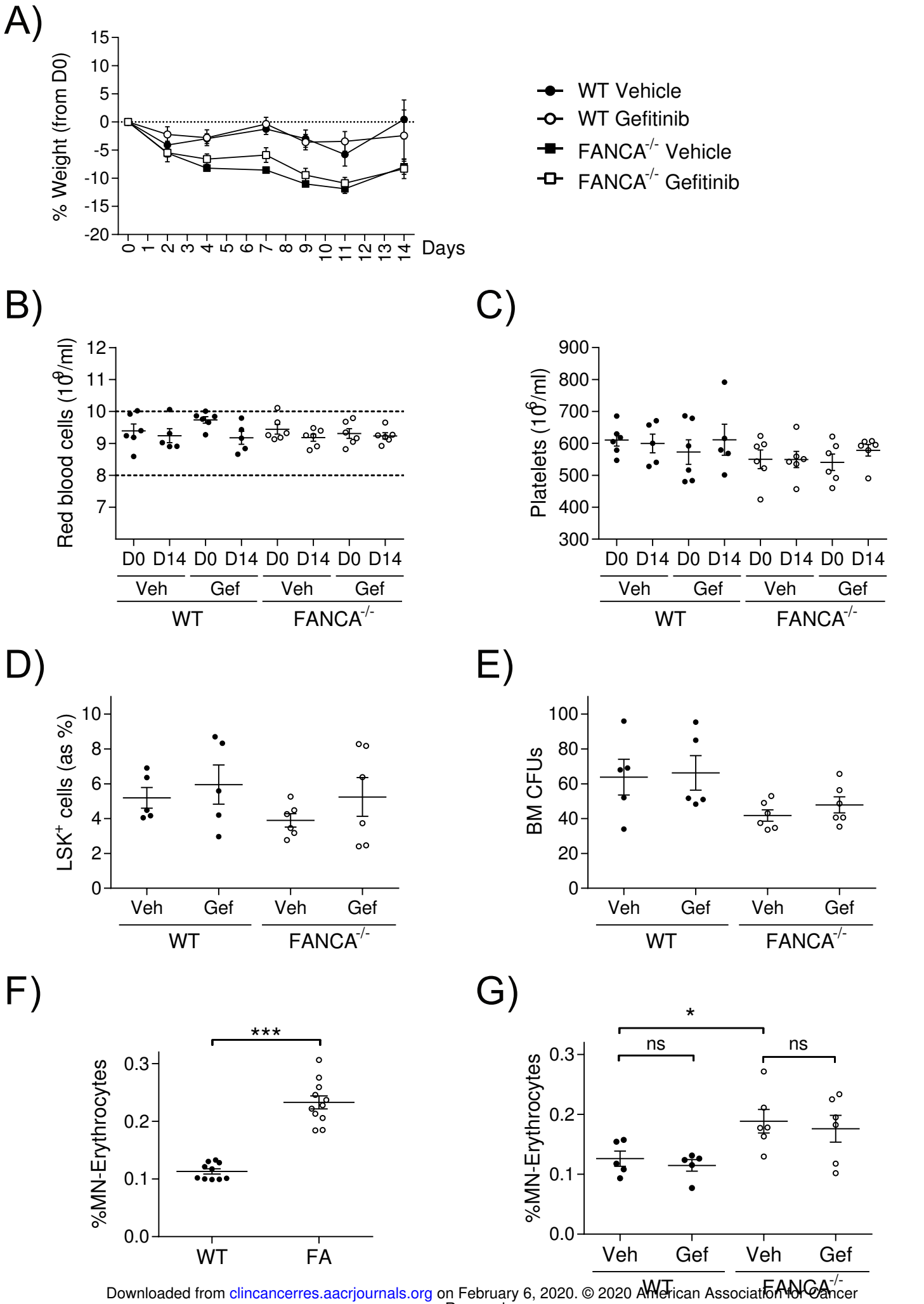
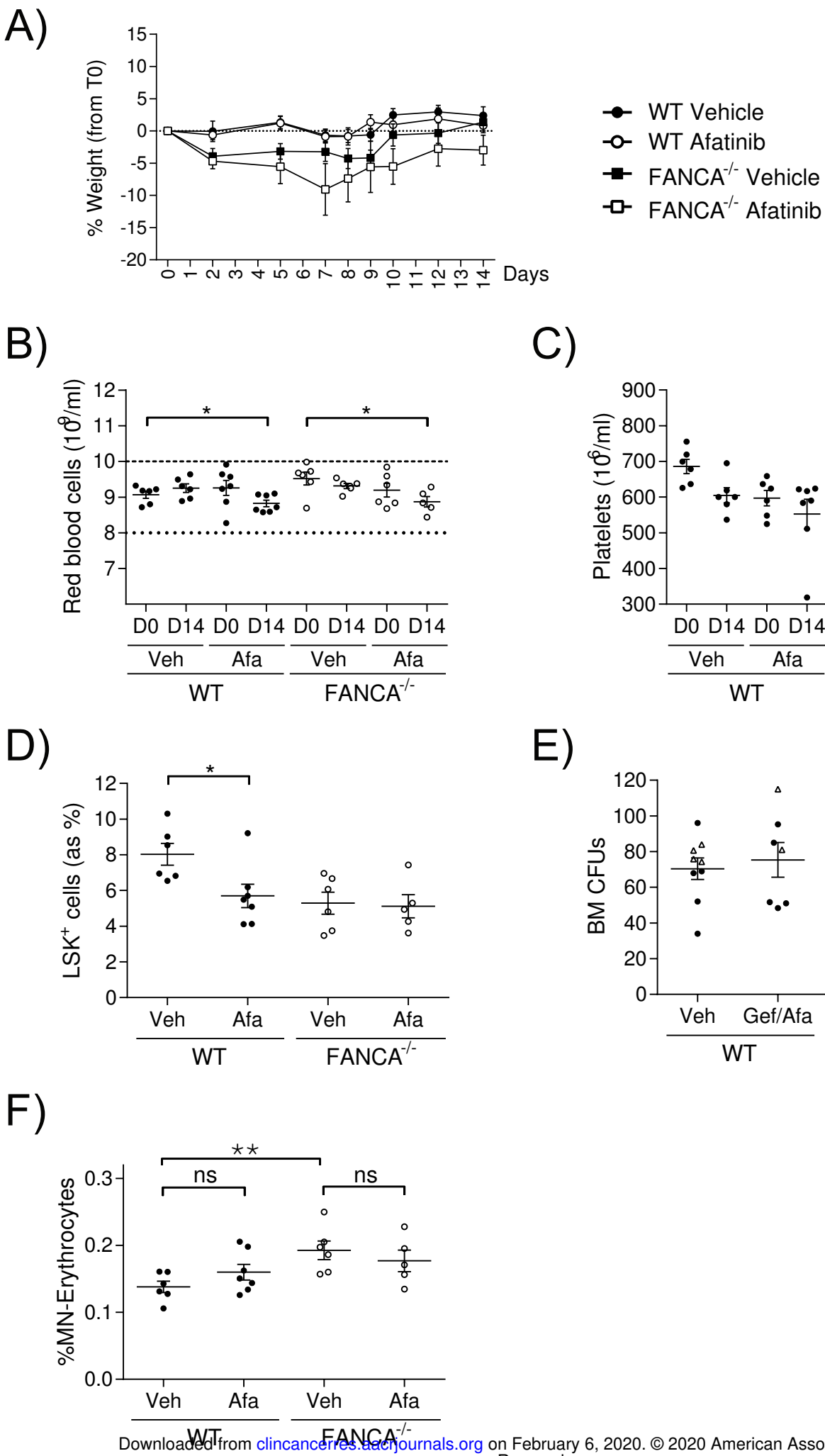


Figure 7



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Gefitinib and afatinib show potential efficacy for Fanconi anemia-related head and neck cancer

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