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MAN2A1–FER Fusion Gene is Expressed by Human Liver and Other Tumor Types and has Oncogenic Activity in Mice

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Abstract

Background & Aims—Human tumors and liver cancer cell lines express the product of a fusion between the first 13 exons in the mannosidase alpha class 2A member 1 gene (*MAN2A1*) and the last 6 exons in the FER tyrosine kinase gene (*FER*), called *MAN2A1–FER*. We investigated whether *MAN2A1–FER* is expressed by human liver tumors and its role in liver carcinogenesis.

Methods—We performed reverse transcription PCR analyses of 102 non-small cell lung tumors, 61 ovarian tumors, 70 liver tumors, 156 glioblastoma multiform samples, 27 esophageal adenocarcinomas, and 269 prostate cancer samples, as well as 10 non-tumor liver tissues and 20 non-tumor prostate tissues, collected at the University of Pittsburgh. We also measured expression by 15 human cancer cell lines. We expressed a tagged form of *MAN2A1–FER* in NIH3T3 and HEP3B (liver cancer) cells; Golgi were isolated for analysis. *MAN2A1–FER* was also overexpressed in PC3 or DU145 (prostate cancer), NIH3T3 (fibroblast), H23 (lung cancer) and A-172 (glioblastoma multiforme) cell lines and knocked out in HUH7 (liver cancer) cells. Cells were analyzed for proliferation and in invasion assays, and/or injected into flanks of SCID mice; xenograft tumor growth and metastasis were assessed. Mice with hepatic deletion of PTEN were given tail-vein injections of *MAN2A1–FER*.

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Contribution: ZHC and JYT conducted experiments. YY and JHL conceived the idea and guided the experiments. GKM, SM and JBN provided expertise consultation. SL and GT conducted statistical analyses. RH, MN, RB JDL and AP selected and provided the specimens.

Results—We detected *MAN2A1–FER* mRNA and fusion protein (114 kD) in the hepatocellular carcinoma cell line HUH7, as well as in liver tumors, esophageal adenocarcinoma, glioblastoma multiforme, prostate tumors, non-small cell lung tumors, and ovarian tumors, but not non-tumor prostate or liver tissues. MAN2A1–FER protein retained the signal peptide for Golgi localization from MAN2A1 and translocated from the cytoplasm to Golgi in cancer cell lines. MAN2A1–FER had tyrosine kinase activity almost 4-fold higher than that of wild-type FER, and phosphorylated the epidermal growth factor receptor (EGFR) at tyrosine 88 in its N-terminus. Expression of *MAN2A1–FER* in 4 cell lines led to EGFR activation of BRAF, MEK, and AKT; HUH7 cells with *MAN2A1–FER* knockout had significant decreases in phosphorylation of these proteins. Cell lines that expressed *MAN2A1–FER* had increased proliferation, colony formation, and invasiveness and formed larger (more than 2-fold) xenograft tumors in mice, with more metastases, than cells not expressing the fusion protein. HUH7 cells with *MAN2A1–FER* knockout formed smaller xenograft tumors, with fewer metastases, than control HUH7 cells. HUH7, A-172, and PC3 cells that expressed MAN2A1–FER were about 2-fold more sensitive to the FER kinase inhibitor crizotinib and the EGFR kinase inhibitor canertinib; these drugs slowed growth of xenograft tumors from MAN2A1–FER cells and prevented their metastasis in mice. Hydrodynamic tail-vein injection of *MAN2A1–FER* resulted in rapid development of liver cancer in mice with hepatic disruption of *Pten*.

Conclusion—Many human tumor types and cancer cell lines express the *MAN2A1–FER* fusion, which increases proliferation and invasiveness of cancer cell lines and has liver oncogenic activity in mice.

Keywords

fusion gene; driver; biomarker; ectopic activation

Introduction

Cancer is one of the most lethal diseases for human. In the US, cancer related death reached 595,690 in 2015[1]. A mortality rate is only second to that of cardiovascular diseases. Liver cancer is one of leading causes of cancer related death for both men and women worldwide[2]. Even though significant progress has been made in the past several decades, much remains to be understood regarding the mechanisms of cancer development and progression. In our previous study, we discovered a panel of fusion genes that are expressed in highly aggressive prostate cancer[3]. One of these fusion genes is mannosidase alpha class 2A member 1 - FER tyrosine kinase gene (*MAN2A1-FER*). Recent screening of liver cancer cell lines indicates that HUH7, a highly aggressive liver cancer cell line, expresses high level of *MAN2A1-FER*. This suggests that MAN2A1-FER fusion may play a significant role in liver cancer development.

MAN2A1 is a Golgi enzyme required for conversion of high mannose to complex type structure of N-glycan for mature glycosylation of a membrane protein[4, 5]. Little is known about its relation with human malignancies. On the other hand, *FER*, a tyrosine kinase, is a well-documented oncogene[6]. *MAN2A1-FER* fusion occurs between the first 13 exons in the 5′end of mannosidase alpha class 2A member 1 (*MAN2A1*) and the last 6 exons in the 3′ end of FER tyrosine kinase (*FER*). The chimera MAN2A1-FER protein contains 703 amino

acids from the N-terminus of MAN2A1 and 251 amino acids from the C-terminus of *FER*. The resulting chimera protein lost glycoside-hydrolase domain in the C-terminus of *MAN2A1* and SH2 domain from the N-terminus of FER, while leaving the tyrosine kinase domain in *FER* largely intact. Previous analysis showed that about 80% patients with *MAN2A1-FER* positive prostate cancer experienced poor clinical outcomes. However, it is unclear whether *MAN2A1-FER* is the driver of the aggressive behavior of human cancers. In this report, we found that *MAN2A1-FER* fusion occurred in significant number of liver cancers and 5 other types of human malignancies. The expression *MAN2A1-FER* protein led to carcinogenesis *in vitro* and *in vivo*. Treatment of cancers positive for *MAN2A1-FER* with tyrosine kinase inhibitors resulted in dramatic improvement of survival of animals xenografted with cancers positive for the fusion gene.

Materials and methods

Tissue samples

Ten liver donor samples and 20 organ donor prostates, 102 non-small cell lung cancers, 61 ovarian cancers, 70 liver cancers, 156 glioblastoma multiforme, 27 esophageal adenocarcinoma and 269 prostate cancer samples were obtained through Institution Review Board (IRB) of University of Pittsburgh approved protocols in compliance with institutional regulatory guidelines (Supplemental Tables 1 through 6).

RNA extraction, cDNA synthesis and Taqman RT-PCR

microdissection was performed on slides of FFPE samples to obtain at least 50% cancer cells. Total RNA was extracted from epithelial cells with the Trizol method (InVitrogen, CA). The extraction procedure was performed according to manufacturer's recommendation. Random hexamer was used in the first strand cDNA synthesis with 1 ug of total RNA and Superscript II TM (InVitrogen, inc, CA). This was followed by Taqman PCR (94°C for 2 min, then 94°C for 30 seconds, 61°C for 30 second, 72°C for 30 second for 50 cycles) in Eppendorf Realplex™ cycler using primers TGGAAGTTCAAGTCAGCGCAG/ GAAGTTTTATCCTTTAATGTGCCC and Taqman probe 5′-/56-FAM/TCA GAA ACA/ZEN/GCC TAT GAG GGA AAT T/3IABkFQ/-3′. β-actin Taqman RT-PCR was used quantity and quality controls using primers GCATGGGTCAGAAGGATTCCT/ GTGCTCGATGGGGTACTTCAG and Taqman probe 5′-/56-FAM/CGA CGA GGC /ZEN/CCA GAG CAA GAG/3IABkFQ/-3′. Samples with Ct less than 36 were called positive for *MAN2A1-FER*. When samples showed β-actin Ct higher than 35, nested Taqman RT-PCR for *MAN2A1-FER* was performed under this condition: 94°C for 2 min, then 94°C for 30 seconds, 61°C for 30 second, 72°C for 30 second for 25 cycles using primers CTGCTTCAGGAAAACCTGTGG/TACATGTTTTAACAGCAACAGAAG. This was followed with a nested PCR using primers TGGAAGTTCAAGTCAGCGCAG/ GAAGTTTTATCCTTTAATGTGCCC and Taqman probe: 5′-/56-FAM/TCA GAA ACA/ZEN/GCC TAT GAG GGA AAT T/3IABkFQ/-3′ under the this condition: 94°C for 2 min, then 94°C for 30 seconds, 61°C for 30 second, 72°C for 30 second for 40 cycles. Ct threshold of MAN2A1-FER detection: 30 cycles for formalin-fixed paraffin-embedded tissues, 25 cycles for frozen tissues. No template negative control and *MAN2A1-FER* cDNA templates were used as negative and positive controls in each batch, respectively.

Sucrose gradient centrifugation to isolate Golgi apparatus

HEP3B cells (2×10^8) induced to express *MAN2A1-FER-FLAG* were re-suspended in 3 ml HM buffer (0.25 M sucrose, 10 mM Tris Cl, pH7.4), and homogenized in Dounce homogenizer. This is followed by addition of 6 ml of 2.0 M sucrose gradient solution (0.8, 1.2, 1.6, 2.0 M). The sample was then overlaid with 6 ml of 1.2 M sucrose gradient solution and 3 ml of 0.8 M sucrose solution. The tube was subsequently underlaid with 2 ml of 1.6 M sucrose gradient solution by a syringe and metal cannula. The sample was then centrifuged at 110,000×g for 2 hours at 4°C. The factions of sample solution were then collected from the bottom of the tube. For Golgi isolation experiment, a Golgi isolation kit (Sigma-Aldrich, St. Louise, MO) was used. The procedure followed the manual provided by the manufacturer.

Tumor Growth and Spontaneous Metastasis

The xenografting procedure was described previously[7-13]. Briefly, Approximately 5×10^6 viable PMF, DMF, HEPMF, GMF, HMF and HUH7 and HUH7/ko cells suspended in 0.2 mL of Hanks' balanced salt solution (Krackeler Scientific, Inc., Albany, NY) were subcutaneously implanted in the abdominal flanks of 71 SCID mice to generate one tumor per mouse. The breakdown of the treated groups is the following: 12 for PMF cells; 12 for DMF cells; 11 for HEPMF cells; 12 for GMF cells, 12 for HMF cells, 6 for HUH7 cells; And 6 for HUH7/ko cells. Six mice from each of PMF, DMF, HEPMF, GMF and HMF groups were fed with 5 μg/ml tetracycline water daily. Mice were observed daily, and their body weight and tumor size were recorded weekly. Tumor sizes were measured on weekly until the end of sixth week after the xenografting. Mortality and metastases were recorded.

For details of cell lines and tissue microdissection, vector construction, colony formation and matrigel traverse assays, cell cycle analysis, in vitro kinase assays, cell cycle analysis, immunoblotting, treatment of mice xenografted with tumor cells and mouse tail vein hydrodynamic injection, please see Supplemental Materials and Methods.

Results

MAN2A1-FER fusion expression in human malignancies

Fusion between MAN2A1 and FER resulted in loss of 441 amino acids from the C-terminus of MAN2A1 and 571 amino acids from the N-terminus of FER. This produces major structural alterations in the protein (figure 1A). To examine whether MAN2A1-FER fusion gene occurs in human cancer cell line, we screened 15 human cancer cell lines derived from several different types of cancers (data not shown). HUH7, a hepatocellular carcinoma cell line, was found to express significant level of *MAN2A1-FER* transcript. A *MAN2A1-FER* chromosome breakpoint was found located between intron 13 of *MAN2A1* and intron 14 of FER (figure 1B). Separately, through whole genome sequencing of a prostate cancer sample positive for *MAN2A1-FER* (PRCA159T), another chromosome breakpoint was found (figure 1B). Only 32 bps separate this breakpoint from that found in HUH7 cell line. To investigate what type of human cancers harbors *MAN2A1-FER* fusion, we examined glioblastoma multiforme, liver cancer, prostate cancer, non-small cell lung cancer, ovarian cancer and esophagus adenocarcinoma, using Taqman quantitative RT-PCR. All 6 types of

these cancers were found positive for *MAN2A1-FER* fusion, ranging from 2-25.9% (figure 1C and supplemental tables 1-6). This includes 15.7% (11/70) liver cancer (supplemental figure 1), 16.8% (17/101) non-small cell lung cancer, 7.1% (11/156) GBM, 25.9% (7/27) esophagus adenocarcinoma, 5.2% (14/269) prostate cancer, and 1.7% (1/60) ovarian cancer (figure 1C). All 20 organ donor prostate samples and 10 liver donor samples were negative for *MAN2A1-FER* fusion. The chimeric *MAN2A1-FER* protein contains 954 amino acids, while *MAN2A1* has 1144 and *FER* 822 amino acids, respectively. Thus, the protein molecular weight of *MAN2A1-FER* chimera protein is projected to be 114 kd, while those of *MAN2A1* and *FER* are 137 kd and 99 kd, respectively. When immunoblots using antibodies specific for MAN2A1 or FER were performed, a protein band with a molecular weight 114 kd was recognized by both antibodies specific for *MAN2A1* and *FER* in HUH7 cells. This protein band is absent in *MAN2A1-FER* transcript negative HCC cell line HEP3B (figure 1D). This suggests that *MAN2A1-FER* fusion gene produces a stable chimeric protein. To test whether *MAN2A1-FER* protein was also expressed in primary cancer samples, similar immunoblotting was performed on prostate cancer positive for *MAN2A1-FER* fusion. As shown in figure 1D, samples (PRCA159T, PRCA23T, HCC#1, HCC#2 and HCC#5) that were positive for *MAN2A1-FER* transcript contained an extra protein band of molecular weight 114 kd recognized by antibodies specific for *MAN2A1* and *FER*, while samples (PRCA25T, PRCA20T PRCA119T, HCC#10, HCC#37, Normalliver#71 and Normalliver#72) that were negative for *MAN2A1-FER* contained no such protein. These experiments indicate that *MAN2A1-FER* protein is stably expressed when fusion transcript is present.

MAN2A1-FER is located in Golgi apparatus

FER protein contains FCH domain, which is the binding site for microtubule for *FER* protein. In *MAN2A1-FER* fusion, this domain is lost. Instead, *MAN2A1-FER* retains the signal peptide for Golgi localization from *MAN2A1.* As a result, the kinase domain of *FER* is likely translocated to Golgi apparatus. To test this hypothesis, NIH3T3 and HEP3B cells were transfected with pCDNA4-MAN2A1-FER-FLAG/pCDNA6. Stable tetracycline inducible *MAN2A1-FER-FLAG* expression clones were selected (NMF and HEPMF, figure 2A). Co-immunostaining using antibodies specific for FLAG and resident Golgi protein N-acetylgalactosaminyltransferase showed that *MAN2A1-FER-FLAG* and N-acetylgalactosaminyltransferase were co-localized in Golgi apparatus (figure 2B). Sucrose gradient centrifugation separation of factions of HEP3B cells transformed with *MAN2A1-FER-FLAG* showed that *MAN2A1-FER-FLAG* was located in the Golgi faction, distinctly different from the wild type *FER* protein, which was found in the cytoplasm faction (figure 2C). Finally, Golgi localization of *MAN2A1-FER* from HUH7 and NMF cells were also confirmed by Golgi isolation method (figure 2D).

MAN2A1-FER kinase activates *EGFR*

FER is a tyrosine kinase playing significant role in signal transduction. To test whether MAN2A1-FER retains its tyrosine kinase activity, *MAN2A1-FER* was ligated into pGEX-5×-3 vector to create pGST-MAN2A1-FER so that the fusion protein was expressed as GST-MAN2A1-FER chimera protein in *E.coli.* As shown in figure 3A, *GST-MAN2A1-FER* showed high level of tyrosine kinase activity when poly (EY 4:1), an essay substrate for

FER kinase, was used as a substrate. Interestingly, *GST-MAN2A1-FER* showed 3.75 fold higher kinase activities than *GST-FER*, suggesting that removal of SH2 domain from *FER* increased the tyrosine kinase activity of the fusion protein. In addition, the kinase activity of *MAN2A1-FER* is sensitive to crizotinib, a tyrosine kinase inhibitor originally developed for *ALK* and *FEZ*[14], with an IC50 ~29 nM (figure 3B).

Since *MAN2A1-FER* fusion results in translocation of *FER* kinase to Golgi apparatus, the targets of *FER* kinase may be altered as a result. Epidermal growth factor receptor (*EGFR*) signaling activation has been shown to be one of the major mechanisms that drive the progression of human cancers[15-17], and only the N-terminus of *EGFR* is exposed in the lumens of Golgi apparatus. We hypothesized that *EGFR* N-terminus is the substrate of *MAN2A1-FER* kinase. To test this hypothesis, we used the N-terminus of *EGFR* (HisTAG-EGFR$^{aa1-650}$) as the substrate in our kinase assay. As shown in figure 3C, *GST-MAN2A1-FER* phosphorylated the recombinant HisTAG-EGFR$^{aa1-650}$ in an ATP dosage-dependent manner. To investigate which tyrosine residue was phosphorylated by *GST-MAN2A1-FER*, synthetic peptides corresponding to each tyrosine residue in the N-terminus of *EGFR* was chemically synthesized, and assayed for the phosphorylation by *GST-MAN2A1-FER*. The results showed that only the sequence corresponding to FLKTIQEVAGYVLIALNTVER (peptide 3) in *EGFR* was phosphorylated by *GST-MAN2A1-FER*. This peptide contains a tyrosine residue at aa88 of *EGFR*. To validate whether Y^{88} in *EGFR* is indeed phosphorylated by *GST-MAN2A1-FER*, a peptide containing a mutation of this tyrosine (Y88A) was assayed for the protein kinase activity of *GST-MAN2A1-FER*. The result indicated that mutant peptide was not phosphorylated by the fusion protein. To verify whether the phosphorylation of HisTAG-EGFR$^{aa1-650}$ by *GST-MAN2A1-FER* fusion is the result of Y88 phosphorylation, HisTAG-ΔEGFR$^{aa1-650}$ phosphorylation was performed by excessive amount of peptide 3. As shown in figure 3C (lanes 7-8), peptide 3, but not the mutant counterpart, partially blocked the phosphorylation of *EGFR* N-terminus by *GST-MAN2A1-FER*. No phosphorylation was found when Y88 was mutated to alanine in *EGFR* N-terminus (figure 3C). Thus, Y88 is the only *GST-MAN3A1-FER* phosphorylation site in the EGFR N-terminus. MAN2A1-FER bound *EGFR* N-terminus *in vitro* and *in vivo* (supplemental figure 2).

To investigate whether phosphorylation of the N-terminus of *EGFR* by *MAN2A1-FER* kinase occurs *in vivo*, HEPMF cells were induced with tetracycline to express *MAN2A1-FER-FLAG*. *EGFR* was partially fragmented with thrombin or hydroxylamine. The digested peptides were then immunoprecipitated with antibodies specific for the N-terminus of *EGFR*. The results showed that *MAN2A1-FER* expression resulted in phosphorylation of *EGFR* N-terminus, while uninduced controls were negative for the N-terminus *EGFR* phosphorylation (figure 3D). Expression of *MAN2A1-FER* led to activation of tyrosine kinase activity of *EGFR*, as evidenced by dramatic increase of autophosphorylation of Y^{1068} and Y^{848} in the kinase domain of *EGFR* when *MAN2A1-FER* was present (figure 3E). To investigate whether N-terminus phosphorylation of *EGFR* is essential for *MAN2A1-FER* mediated *EGFR* activation, a mutant with a point mutation in amino acid 88 (Y88A) of EGFR was created. This mutant was transfected into HEPMF cells, and these cells were subsequently induced to express *MAN2A1-FER-FLAG*. As shown in figure 3F, mutation of tyrosine 88 of EGFR abrogated the tyrosine kinase activation of *EGFR* as evidenced by

negative autophosphorylation and dimerization when *MAN2A1-FER* was induced to express. The activation of *EGFR* was also identified in prostate cancer sample that was found positive for *MAN2A1-FER* fusion (figure 3G), but not in sample that was negative, suggesting a significant clinical relevance for the activation of *EGFR* induced by *MAN2A1-FER*.

Activation of the kinase activity of *EGFR* by *MAN2A1-FER* may lead to cascade of pro-growth signaling in cancer cells. To examine whether expression of *MAN2A1-FER* activates *EGFR* signaling pathways, *EGFR-B-raf-MEK* and *EGFR-Akt* pathways were examined. As shown in figure 4, expression of *MAN2A1-FER* in 4 cell lines of different origins (PC3-prostate cancer, HEP3B-liver cancer, NIH3T3-mouse immortalized fibroblast and A-172-glioblastoma multiforme) led to activation B-raf, MEK and Akt kinases. To examine whether *EGFR* is the main activator of these kinases, a dominant negative mutant of *EGFR* ($\Delta EGFR^{aa1\text{-}650}$) that has its kinase domain deleted was transfected into these cell lines. The results indicated that the presence of $\Delta EGFR^{aa1\text{-}650}$ largely eliminated the activations of these kinases (figure 4B. The kinase negative mutant of *MAN2A1-FER* failed to activate any of these kinases (figure 4C). Using CRISPR system, genome of *MAN2A1-FER* in HUH7 cells was interrupted such that no *MAN2A1-FER* expression is present in the cell line (figure 4, lanes 25-26). HUH7 cells with knockout of *MAN2A1-FER* showed significant decrease of phosphorylation of *EGFR*, *B-raf*, *MEK*, and *Akt*, suggesting that hyper-activation of these kinases in HUH7 cells are largely dependent on the *MAN2A1-FER* fusion.

MAN2A1-FER accelerates cell growth and invasion

To examine the consequence of *MAN2A1-FER* induced activation of pro-growth signaling pathways, cell cycle analysis was performed on cell lines of 4 different origins (HEP3B-liver cancer, PC3 and DU145-prostate cancer, A-172-glioblastoma multiforme, H23-lung cancer) and NIH3T3 cells that were transformed with *MAN2A1-FER-FLAG*. As shown in figure 5A, induction of expression of *MAN2A1-FER* by tetracycline increased the S-phase of these cell lines dramatically: 42 fold for HEP3B, 2.1 fold for PC3, 52% for DU145, 40 fold for H23, 3.9 fold for A-172 and 2 fold for NIH3T3 cells. However, these pro-growth impacts completely disappeared when kinase negative mutant of *MAN2A1-FER-FLAG* was introduced into these cells (figure 5A). Expression of dominant negative mutant $\Delta EGFR^{aa1\text{-}650}$ also significantly attenuated the increase of S-phase induced by *MAN2A1-FER-FLAG* in HEP3B cells. Genomic interruption of *MAN2A1-FER* in HUH7 cells resulted in significant reduction of S-phase and M-phase cells.

Parallel to these cell cycle analyses, colony formation analysis of cells transformed with *MAN2A1-FER-FLAG* showed 90% to 2.7 fold increase of colonies when *MAN2A1-FER-FLAG* was induced (figure 5B). The impact of *MAN2A1-FER-FLAG* on colony formation disappeared when *MAN2A1-FER* kinase mutant or dominant negative *EGFR* mutant was used. Interruption of *MAN2A1-FER* in the genome HUH7 cells resulted in 55% decrease in colony formation of these cells. Expression of *MAN2A1-FER* also resulted in higher levels of invasiveness (figure 5C). To examine the impact of *MAN2A1-FER* expression *in vivo*, HEP3B, PC3, DU145, A-172, H23 cells transformed with *MAN2A1-FER* were xenografted

into subcutaneous tissues of severe combined immunodeficiency (SCID) mice. *MAN2A1-FER-FLAG* expression was induced through tetracycline water (5 μg/ml). As shown in figure 5D-F, expression of *MAN2A1-FER-FLAG* produced 2.3 fold larger volume of tumors for PC3 and DU145, 2.6 fold for HEP3B, and 2.4 fold for A-172 tumors. There was a 55% drop in tumor volume when *MAN2A1-FER* was interrupted in HUH7 cells. Higher level of metastases was also seen in tumor with *MAN2A1-FER* expression: 3/36 for tumor negative for *MAN2A1-FER* versus 19/35 for *MAN2A1-FER* positive ($p<0.0001$, supplemental table 7 and supplemental figure 3). Eighty-three percent (30/36) of SCID mice without *MAN2A1-FER* survived through 6 weeks while only 28.6% (10/35) mice with *MAN2A1-FER* did so ($p=4.5 \times 10^{-7}$).

MAN2A1-FER positive cancers are sensitive to crizotinib and canertinib

Since the kinase activities of *MAN2A1-FER* and *EGFR* activation are the drivers of MAN2A1-FER oncogenic activity, it is of interest to examine whether inhibition of the kinase activity of *MAN2A1-FER* or *EGFR* interrupts the oncogenic activity of *MAN2A1-FER*, and thus the growth of the tumors. To investigate this hypothesis, crizotinib, a *MAN2A1-FER* kinase inhibitor, and canertinib, an *EGFR* kinase inhibitor, were chosen to treat HUH7, A-172 or PC3 with or without *MAN2A1-FER*. Our results indicate that the presence of *MAN2A1-FER* increased the sensitivity of cancer cells to crizotinib, ranging from 1.8 to 2.1 fold, depending on the cell lines (supplemental figure 4 and supplemental table 8). Interestingly, these cancer cell lines appear more sensitive to canertinib: All showed IC50s below 15 nM. The presence of *MAN2A1-FER* increased the sensitivity by 2.1 to 2.5 fold. To investigate whether the increased sensitivity of malignant cell lines with *MAN2A1-FER* to these kinase inhibitors translates into better outcomes for the cancers treated with these drugs, HUH7, PC3 and A-172 cells with or without MAN2A1-FER were xenografted into the subcutaneous region of SCID mice. The tumors were allowed to grow for 2 weeks to an average size of 183 mm^3. Then, these mice were treated with crizotinib (12.5 mg/kg), canertinib (10 mg/kg) or the combination of these 2 drugs, 3 times a week through peritoneal injection. When *MAN2A1-FER* was present, treatment of crizotinib reduced the final tumor sizes by an average of 80.7% ($p<0.001$) in comparison with DMSO controls for HUH7 cells, 67.6% ($p<0.001$) for A-172 and 78.6% ($p<0.001$) for PC3 cells. When *MAN2A1-FER* was not present, crizotinib was much less effective: An average 27.6% reduction of tumor sizes for HUH7/ko, 4% increase for A-172 and 27.1% reduction for PC3 cells. When SCID mice were treated with canertinib, similar results were obtained: 85% ($p<0.001$) reduction of tumor size for PC3 cells, 73.2% ($p<0.001$) for A-172 and 81.7% ($p<0.001$) for HUH7, when *MAN2A1-FER* was present (figure 6A). This compared favorably against cells without *MAN2A1-FER*: 32.0% reduction for PC3, 19.2% for A-172 and 40.2% for HUH7 cells. The best results came from the combination of crizotinib and canertinib: 92.2% ($p<0.001$) reduction of tumor size of PC3, 89.0% ($p<0.001$) for A-172 and 86.0% ($p<0.001$) for HUH7 cells, when *MAN2A1-FER* was present. Without *MAN2A1-FER*, the effectiveness was reduced to 46.5% for PC3 and 43.2% for HUH7 cells. The combination of crizotinib and canertinib was ineffective for A-172 tumor if *MAN2A1-FER* was not present (19.4% increase of tumor size). Treatment of either crizotinib or canertinib or the combination of these 2 drugs completely eliminated the metastasis of all tumors (PC3, A-172 and HUH7, 0%, 0/18) versus controls (61%, 11/18)($p<0.001$), when *MAN2A1-FER*

was present (figure 6B). The effects of these drugs on *MAN2A1-FER* negative cells, however, were largely insignificant: 2/18 for crizotinib and/or canertinib treated versus 1/18 for control. These results may reflect pervasive low metastasis rate of these malignant cells after *MAN2A1-FER* was removed. Treatment of crizotinib, cancertinib or the combination of these 2 drugs completely eliminated the mortality of the mice xenografted with tumors positive for *MAN2A1-FER* (figure 6C). The mortality improvement effect by these drugs, however, was not observed when they were applied to tumors negative for *MAN2A1-FER*. These results clearly indicate that MAN2A1-FER is the driver of cancer progression. The presence of *MAN2A1-FER* drives *EGFR* signaling that sensitizes the cancer cells to drugs specific for this pathway.

MAN2A1-FER produces spontaneous liver cancer

Among the 10 human liver cancer samples that were positive for MAN2A1-FER, 6 showed Pten deletions, suggesting a potential association between the 2 events for the cancer development. To investigate whether Pten deletion and *MAN2A1-FER* fusion are sufficient to generate cancers, a mouse somatic cancer model was developed to mimic somatic mutations in human cancers. In such model, $Pten^{tm1Hwu/J}$ mice of which exon 5 of Pten gene was flanked by loxP sites was treated with adeno-associated virus-cre (1×10^{10} PFU) through intra-peritoneal injection to create Pten deletion in most hepatocytes. This was followed by tail vein hydrodynamic injection[18, 19] of pT3-*MAN2A1-FER-FLAG* such that 1-2% hepatocytes were transfected with the vector (figure 7A). These mice was allowed to live up to 14 weeks or died from liver cancer naturally. All mice injected with pT3-MAN2A1-FER-FLAG developed hepatocellular carcinoma (figure 7B). These mice all had enlarged livers, with liver to body ratio more than 172% of the controls (figure 7C). Hyper activations of *EGFR*/*Akt* pathways were found in these mice (figure 7D). Clear morphological cancer cells were observed in as early as 4 weeks after introduction of pT3-MAN2A1-FER-FLAG through the tail vein (figure 7E). The cancer islets expanded rapidly in 8 weeks such that most hepatic parenchyma was replaced with cancer cells at the end of 12 weeks. These cancer cells contained large nuclei and nucleoli, were rich in fatty vacuoles and had significant higher number of *Ki-67* positive cells (supplemental figure 5). The glutamine synthetase staining pattern was lost in the cancers (supplemental figure 5). These features are characteristic of high grade liver cancer. Indeed, no mice survived through 14 weeks after introduction of pT3-MAN2A1-FER-FLAG. In contrast, AAV-cre/pT3 treated mice displayed no detectable pathological phenotype in the same period. Taken together, these analyses suggest that *MAN2A1-FER* is one of the key drivers for human cancer progression.

Discussion

Fusion gene is one of the key features in human malignancies. *MAN2A1-FER* fusion is the result of recombination in the long arm of chromosome 5. The significant presence of *MAN2A1-FER* in liver cancers, esophageal adenocarcinoma and 4 other types of human malignancies suggests that this fusion may play significant roles in the development of human cancers. Search of TCGA transcriptome sequencing data of 17 types of human cancers failed to detect MAN2A1-FER fusion. Such discrepancy may result from significant

less proportion of mapped read located at the 5′ ends of both *MAN2A1* and *FER* genes in the TCGA data (supplemental figure 6), since high numbers of reads in the 5′ end of mRNA of MAN2A1 and FER are required for MAN2A1-FER detection. To our knowledge, *MAN2A1-FER* is the first example of translocation of a constitutively activated tyrosine kinase to Golgi apparatus in a fashion that leads to oncogenic activation of *EGFR* signaling pathways. The crucial link in this activation process is the phosphorylation of tyrosine residue 88 in the extracellular domain of EGFR. It appears that tyrosine 88 is the only tyrosine residue of *EGFR* phosphorylated by *MAN2A1-FER*, and phosphorylation of this residue is essential for *MAN2A1-FER* mediated activation and dimerization of *EGFR*. Tyrosine 88 is located in the interface of domain I/II of *EGFR*. This region presumably involves in initiation of *EGFR* dimerization. Mutations in this region had been reported and all shown to be oncogenic[20], probably due to alteration in the domain conformation for dimerization[21]. It is possible that phosphorylation of tyrosine 88 similarly alters the conformation of *EGFR* dimerization domain and leads to oncogenic activation of the receptor. Despite the apparent enhancement of *EGFR* activation and dimerization, *MAN2A1-FER* does not appear to impact on the speed of *EGFR* transportation from Golgi apparatus to plasma membrane (Supplemental figure 7).

FER, a tyrosine kinase, is a well-documented oncogene[6, 22]. Several studies showed that *FER* activates androgen receptor (*AR*) by phosphorylating Tyr223 in *AR*[23]. Some studies indicate that *FER* is an essential component of stem cell tyrosine kinase 1 (*STK1*)[24] and mast cell growth factor receptor (*kit*)[25, 26] and c-met[27] signaling. Over-expression of *FER* is associated with poor clinical outcomes of breast cancer[28], renal cell carcinoma[29, 30], non-small cell lung cancer[31, 32] and hepatocellular carcinoma[33]. The kinase activity of *FER* is clearly maintained, and perhaps hyper-activated in *MAN2A1-FER* fusion due to removal SH2 domain in *FER* protein. The loss of FCH domain that is required for microtubule binding may make the *MAN2A1-FER* kinase unavailable for most of its physiological substrates. Thus, total alteration of substrate pattern may occur *in vivo*. It is possible that some of the over-expressions of *FER* detected in human cancers are in fact fusion gene associated with *FER*.

Our analysis suggest that constitutive activation and translocation of *FER* kinase to Golgi apparatus lead to hyper-activation of *EGFR* signaling. The consequence of such activation not only leads to hyper-growth of cancers but also makes cancer cells susceptible to kinase inhibitors along the *EGFR* signaling pathways. Indeed, by targeting at the kinases of *FER* and *EGFR*, we showed that this approach is consistently effective against different types of human cancers positive for *MAN2A1-FER* both *in vitro* and *in vivo*. The effectiveness of these inhibitors was largely abrogated if *MAN2A1-FER* protein is removed from the cancer cells, even though the native *FER* protein is still present. These findings clearly argue that *MAN2A1-FER* fusion, rather than the native *FER*, is the driver of human cancers. The effectiveness of crizotinib and canertinib on *MAN2A1-FER* positive human malignancies has significant clinical implications: The presence of *MAN2A1-FER* fusion can be screened in these human cancers. Patients with positive *MAN2A1-FER* fusion can be treated with these drugs to achieve better clinical outcomes, particularly for those patients with metastasis and who are not suitable for surgery or radiation. The current study only provides examples of drug treatment for *MAN2A1-FER* fusion gene positive cancers. Likely, other

kinase inhibitors targeting at *MAN2A1-FER* or *EGFR* signaling pathway are effective against human cancers positive for this fusion gene. Thus, *MAN2A1-FER/EGFR* targeting may hold promise as an effective treatment for significant subsets of liver cancer and other malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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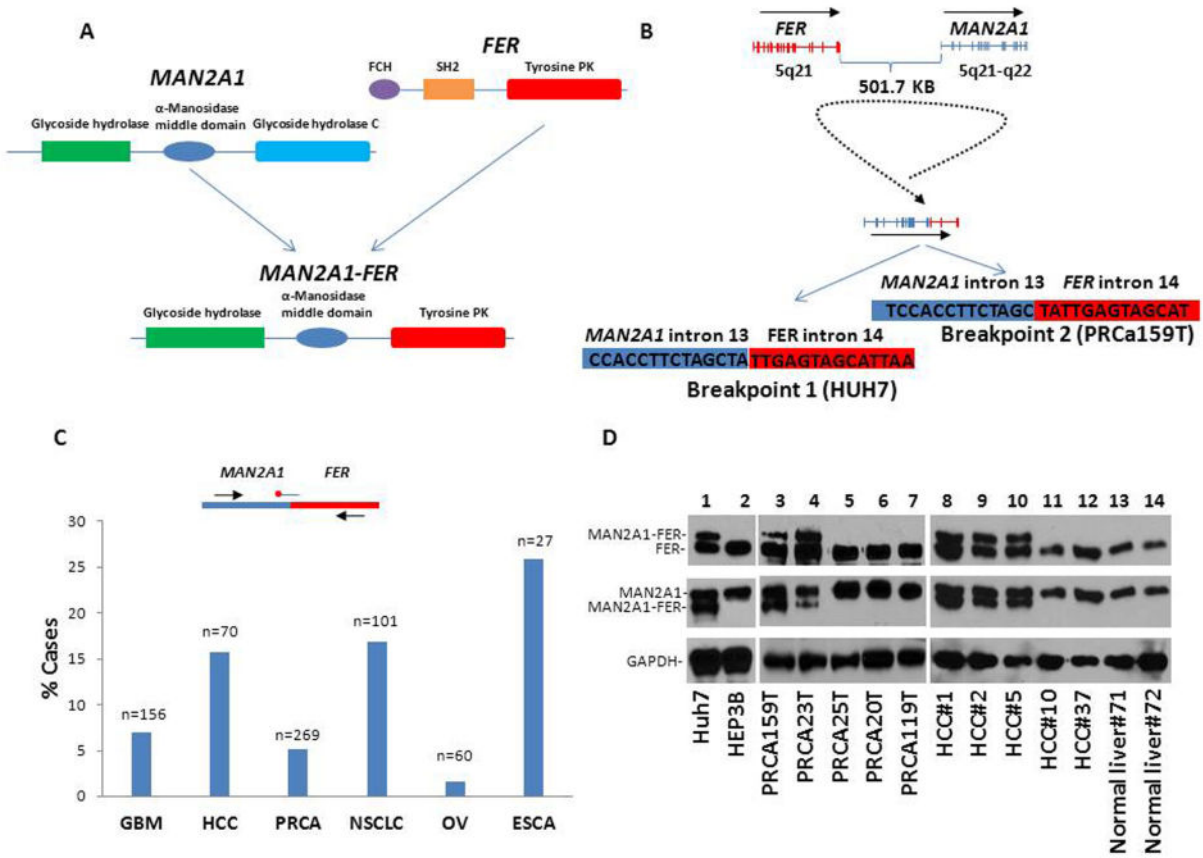

Figure 1. *MAN2A1-FER* in human malignancies

(A) Schematic diagram of *MAN2A1*, *FER* and *MAN2A1-FER* proteins. FCH denotes FER-CP4 homologous region. PK denotes protein kinase. SH2 denotes Src Homology 2. (B) Chromosome breakpoints of MAN2A1-FER is located in intron 13 of MAN2A1 and intron 14 of FER. One breakpoint was identified in liver cancer cell line HUH7 through nested PCRs. Another breakpoint was identified in prostate cancer sample PRCa159T through whole genome sequencing. (C) Frequency of *MAN2A1-FER* fusion transcript in glioblastoma multiforme (GBM), Hepatocellular carcinoma (HCC), prostate cancer (PRCA), non-small cell lung cancer (NSCLC), ovarian cancer (OV) and esophageal adenocarcinoma (ESCA). The numbers of samples examined are indicated. Inset: primer and probe positions in *MAN2A1-FER* fusion. (D) Expression of *MAN2A1-FER* protein in human cancers. Immunoblotting was performed on protein extracts from liver cancer cell lines HUH7 and HEP3B, prostate cancer samples positive (PRCA159T and PRCA23T) or negative (PRCA25T, PRCA20T and PRCA119T), liver cancer samples positive (HCC#1, HCC#2 and HCC#5) or negative (HCC#10 and HCC#37) samples for MAN2A1-FER transcript, using antibodies specific for *MAN2A1* or *FER*. *MAN2A1*, *FER* and *MAN2A1-FER* proteins are indicated. Immunoblotting using antibodies for *GAPDH* was used as control. Two normal liver organ donor samples (#71 and #72) were also included as negative controls.

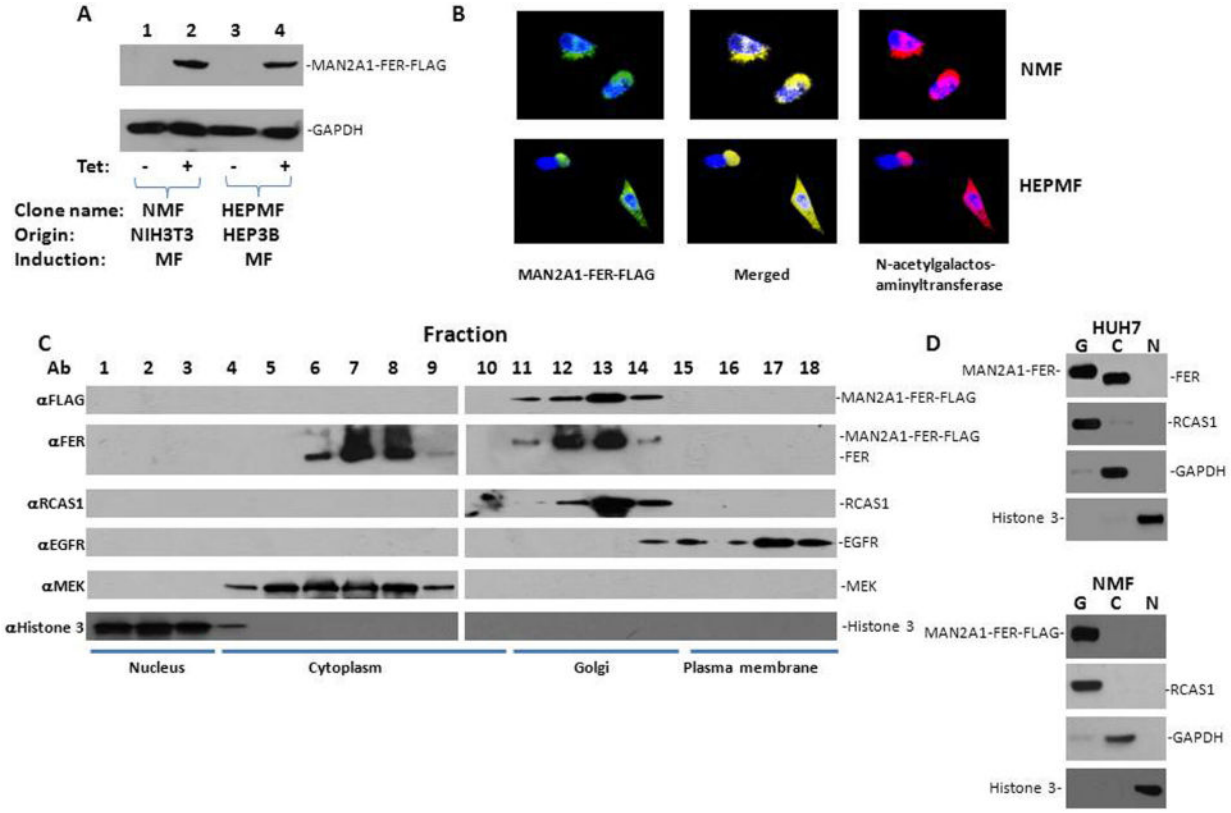

Figure 2. MAN2A1-FER fusion protein is located in Golgi apparatus

(A) Expression of *MAN2A1-FER-FLAG* in pCDNA4-MA2A1-FER-FLAG/pCDNA6-TO transformed NIH3T3 (NMF) and HEP3B (HEPMF) cells. Immunoblotting using antibodies specific for *FLAG* or *GAPDH* was performed. (B) *MAN2A1-FER-FLAG* is colocalized with Golgi resident protein N-acetylgalactosaminyltransferase. Immunofluorescence staining of FLAG and N-acetylgalactosaminyltransferase. Pictures were taken with con-focal microscopy. (C) Sucrose gradient ultracentrifugation of HEPMF cells induced to express *MAN2A1-FER-FLAG.* Faction numbers are indicated at the top. Subcellular locations are indicated at the bottom. Immunoblotting was performed using the indicated antibodies (right). Receptor-binding cancer-associated surface antigen (RCAS1) was used as a marker for Golgi apparatus. (D) *MAN2A1-FER* in Golgi was confirmed by Golgi isolation method. Golgi factions of HUH7 (top) and NMF (bottom) cells expressing *MAN2A1-FER* were isolated, and immunoblotted with antibodies specific for *FER* (HUH7) or *FLAG* (NMF). Antibodies specific for *RCAS1, GAPDH* and Histone 3 were used as purity controls. G-golgi; C-cytoplasm; N-nucleus.

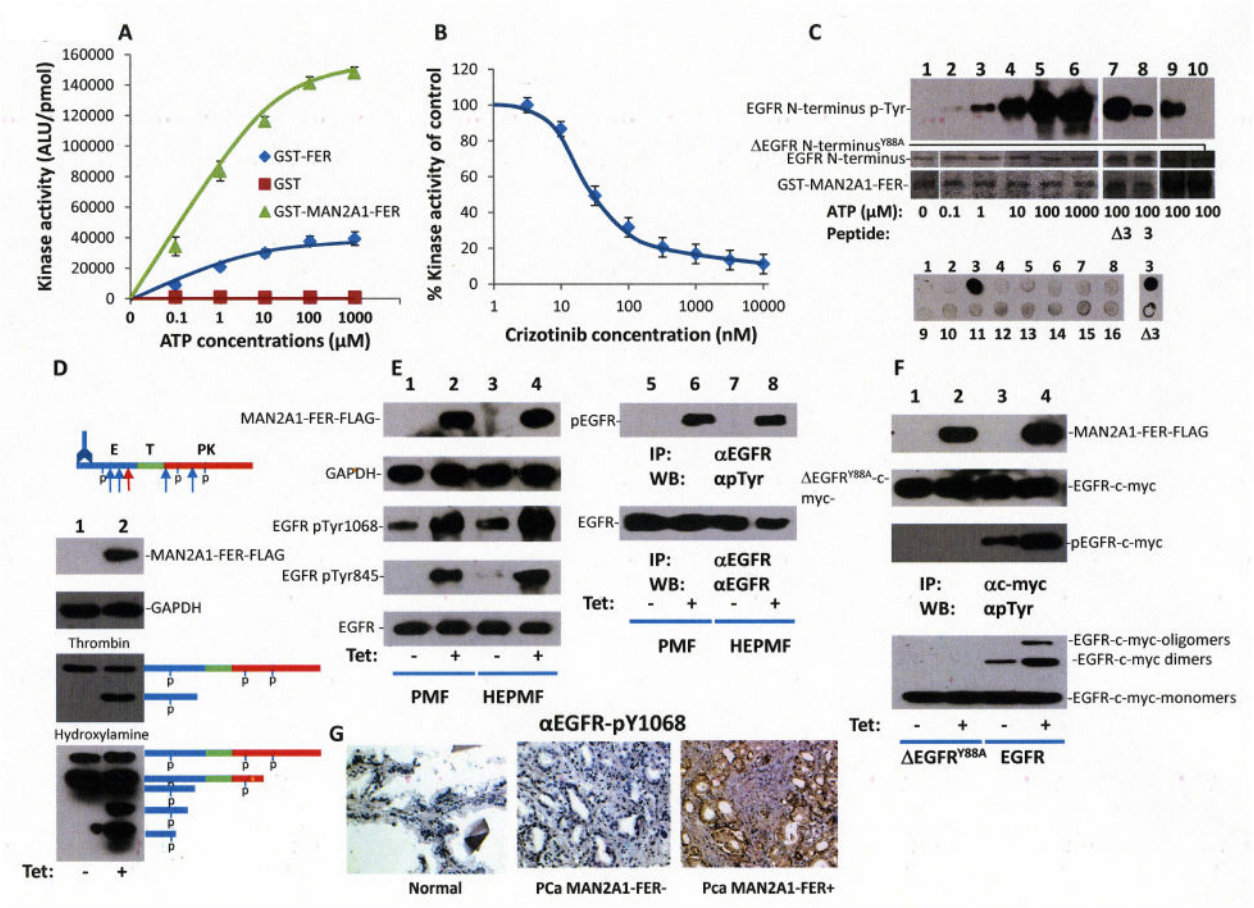

Figure 3. Tyrosine kinase activity of *MAN2A1-FER*

(A) *In vitro* kinase assay using poly (EY 4:1) as substrate. *GST-FER*, *GST-MAN2A1-FER* and *GST* proteins were expressed in *E.coli.* BL21, extracted and purified through glutathione column. Kinase activity was quantified and normalized to pmol per protein. (B) Crizotinib inhibits kinase activity of *GST-MAN2A1-FER*. (C) *GST-MAN2A1-FER* phosphorylates tyrosine 88 of *EGFR*. Top panel: ATP dosage dependent phosphorylation of HisTAG-EGFR$^{aa1-650}$ (lanes 1-9) or HisTAG-ΔEGFR N-terminus^{Y88A} (lane 10). Phosphorylation of HisTAG-ΔEGFR$^{aa1-650}$ by *GST-MAN2A1-FER* was performed with excessive peptide 3 (3, FLKTIQEVAGYVLIALNTVER) or mutant peptide 3 (Δ3, FLKTIQEVAGAVLIALNTVER) (lanes 7-8). Bottom panel: Phosphorylation assay on synthetic peptide listed in supplemental table 9 by *GST-MAN2A1-FER*. (D) Immunoblot analyses of partial digested EGFR products from HEPMF cells treated with fetal bovine serum with or without *MAN2A1-FER-FLAG*. The protein extracts from HEPMF cells were exposed to thrombin or hydroxylamine. This was followed by immunoprecipitated by antibodies specific for the N-terminus of *EGFR*. The immunoprecipitates were resolved in 15% SDS-PAGE and immunoblotted with antibodies specific phospho-tyrosine. Red arrow-thrombin cutting site; Blue arrows-hydroxylamine cutting sites; Blue line-*EGFR* extracellular domain; Green line-*EGFR* transmembrane domain; Red line-*EGFR* intracellular domain. (E) *MAN2A1-FER-FLAG* activates *EGFR*. Serum starved PC3 with

inducible *MAN2A21-FER-FLAG* (PMF) or HEPMF cells were induced to express *MAN2A1-FER-FLAG*. Immunoblotting was performed using antibodies specific for pY1068 or pY845 (lanes 1-4). Separately, *EGFR* was immunoprecipitated with antibodies specific for *EGFR*, and then immunoblotted with antibodies specific for pY (lanes 5-8). *EGFR* and *GAPDH* were used as controls. (F) Y88 of *EGFR* is required for *MAN2A1-FER-FLAG* induced activation of *EGFR*. Top panel: Serum starved HEPMF cells were transfected with pCMV-ΔEGFR^{Y88A}-c-myc (lanes 1-2) or pCMV-EGFR-c-myc (lanes 3-4). These cells were induced to express *MAN2A1-FER-FLAG* and immunoprecipitated with anti-c-myc antibodies. The immunoprecipitates were then blotted with anti-phosphotyrosine antibodies. Bottom panel: Induction of MAN2A1-FER-FLAG expression as the top panel. This is followed by cross-linking of *EGFR-c-myc*, and then by blotting with anti-c-myc antibodies. (G) Immunostaining of *EGFR* pY1068 in prostate cancer samples positive (right) or negative (middle) for *MAN2A1-FER*. Normal prostate (left) was used as negative control.

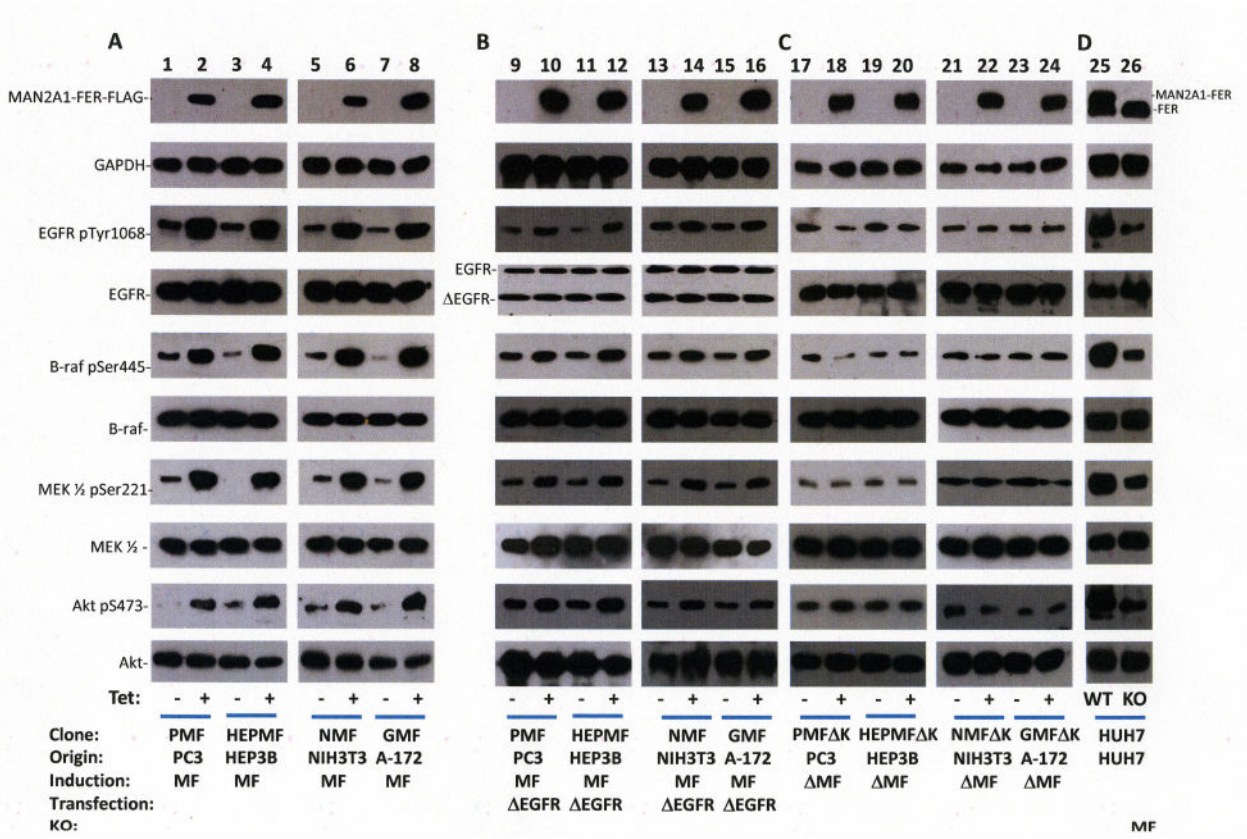

Figure 4. Activation of EGFR signaling pathways by *MAN2A1-FER*

PC3 (PMF), HEP3B (HEPMF), NIH3T3 (NMF), A-172 (GMF) cells transformed with pCDNA4-MAN2A1-FER-FLAG/pCDNA6-TO were serum starved for 24 hours and treated with tetracycline to induce the expression of MAN2A1-FER-FLAG (lanes 1-8). EGFR pY1068, B-raf pS445, MEK1/2 pS221 and Akt pS473 were examined for their phosphorylation status by immunoblotting. GAPDH, EGFR, B-raf, MEK1/2 and Akt were also immunoblotted as normalization controls. (B) Dominant negative mutant $\Delta EGFR^{aa1\text{-}650}$ was transfected into the cells of (A) (lanes 9-16). Similar immunoblotting was performed as (A). (C) PC3 (PMFΔK), HEP3B (HEPMFΔK), NIH3T3 (NMFΔK), A-172 (GMFΔK) cells transformed with pCDNA4-ΔMAN2A1-FER-FLAG^{K723A}/pCDNA6-TO were treated with tetracycline to induce the expression of ΔMAN2A1-FER^{K723A}-FLAG (lanes 17-24). Similar immunoblotting was performed as (A). (D) Interruption of MAN2A1-FER in HUH7 cells decreased hyper-phosphorylation of *EGFR, B-raf, MEK* and *Akt.*

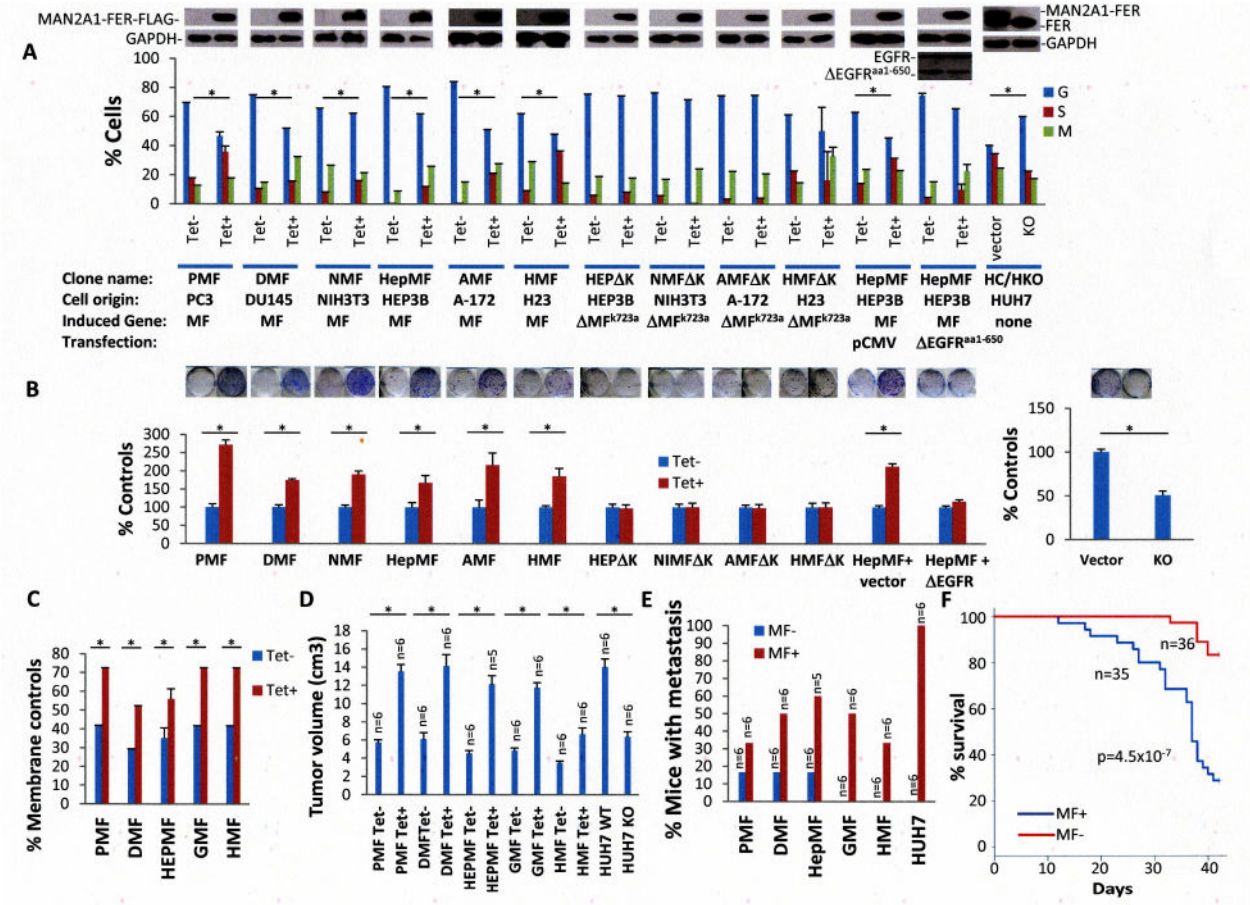

Figure 5. Pro-growth and invasion activity of MAN2A1-FER

(A) Cell cycle analyses of cells transformed with pCDNA4-ΔMAN2A1-FER-FLAG^{K723A}/pCDNA6-TO or its wild type counterpart as indicated. Dominant negative mutant ΔEGFR$^{aa1-650}$ was used in HEPMF cells to examine whether *MAN2A1-FER* pro-growth activity was dependent on EGFR activation. HUH7 and its MAN2A1-FER knockout counterpart (KO) were used to examine whether cell growth was dependent on MAN2A1-FER. *-p<0.05. (B) Colony formation assays of the duplicates from (A). *-p<0.05. (C) Matrigel traverse analysis of PMF, DMF, HEPMF GMF and HMF cells with or without *MAN2A1-FER-FLAG*. *-p<0.05. (D) *MAN2A1-FER* increased tumor volumes of xenografted human cancers. *-p<0.05. (E) *MAN2A1-FER* increased the rate of metastasis of xenografted human cancers. (F) *MAN2A1-FER* increased the mortality of mice xenografted with human cancers.

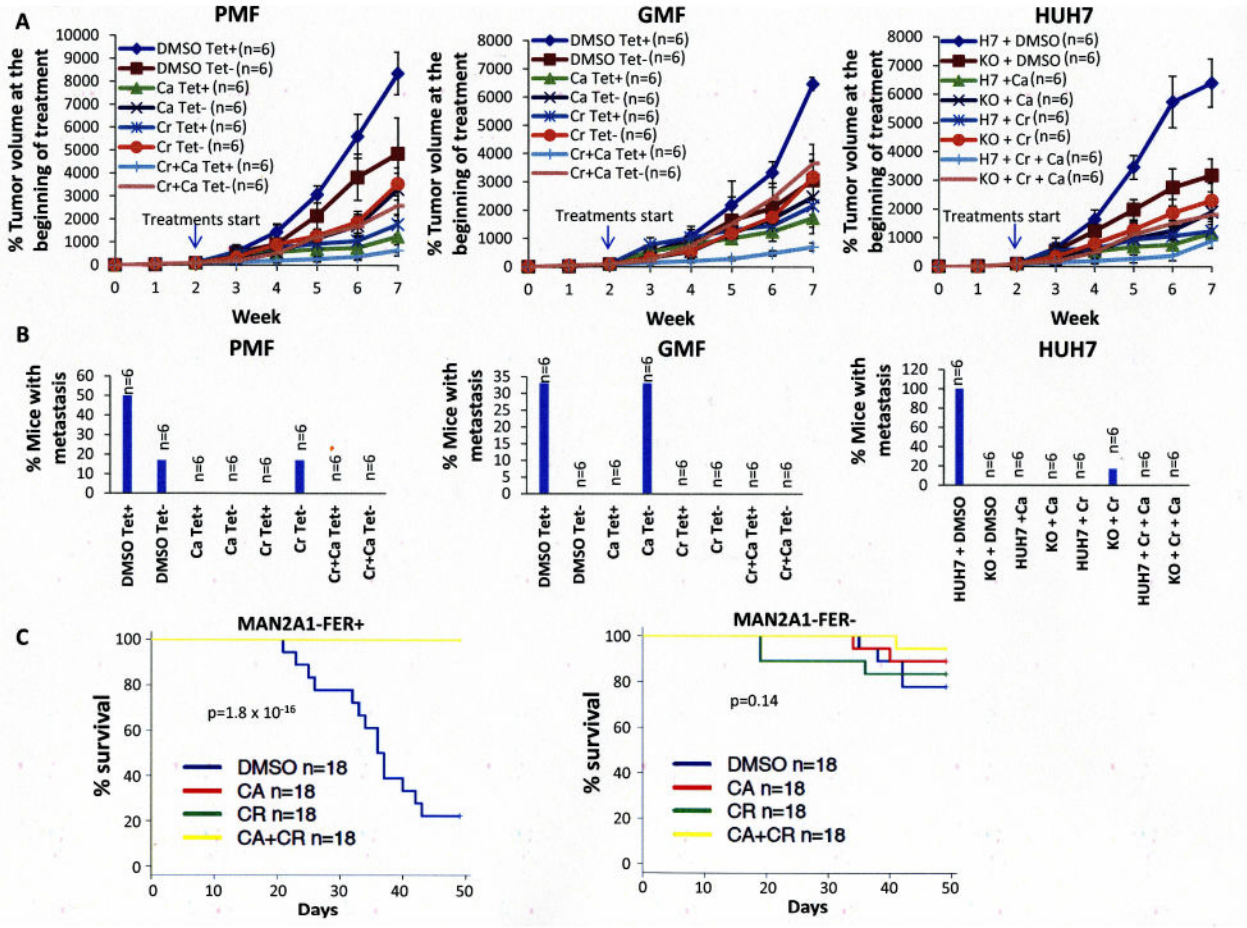

Figure 6. *MAN2A1-FER* increased cancer sensitivity to kinase inhibitors crizotinib and canertinib

(A) Treatment of crizotinib and/or canertinib decreased the volumes of xenografted cancers positive for MAN2A1-FER. CR-crizotinib; CA-canertinib; DMSO-dimethylsulfoxide. (B) Treatment of crizotinib and/or canertinib decreased the metastasis incidents of xenografted cancers positive for *MAN2A1-FER*, but not those negative for *MAN2A1-FER*. CR-crizotinib; CA-canertinib; DMSO-dimethylsulfoxide. (C) Treatment of crizotinib and/or canertinib decreased the mortality of SCID mice xenografted with cancers positive for MAN2A1-FER, but not those negative for MAN2A1-FER. CR-crizotinib; CA-canertinib; DMSO-dimethylsulfoxide.

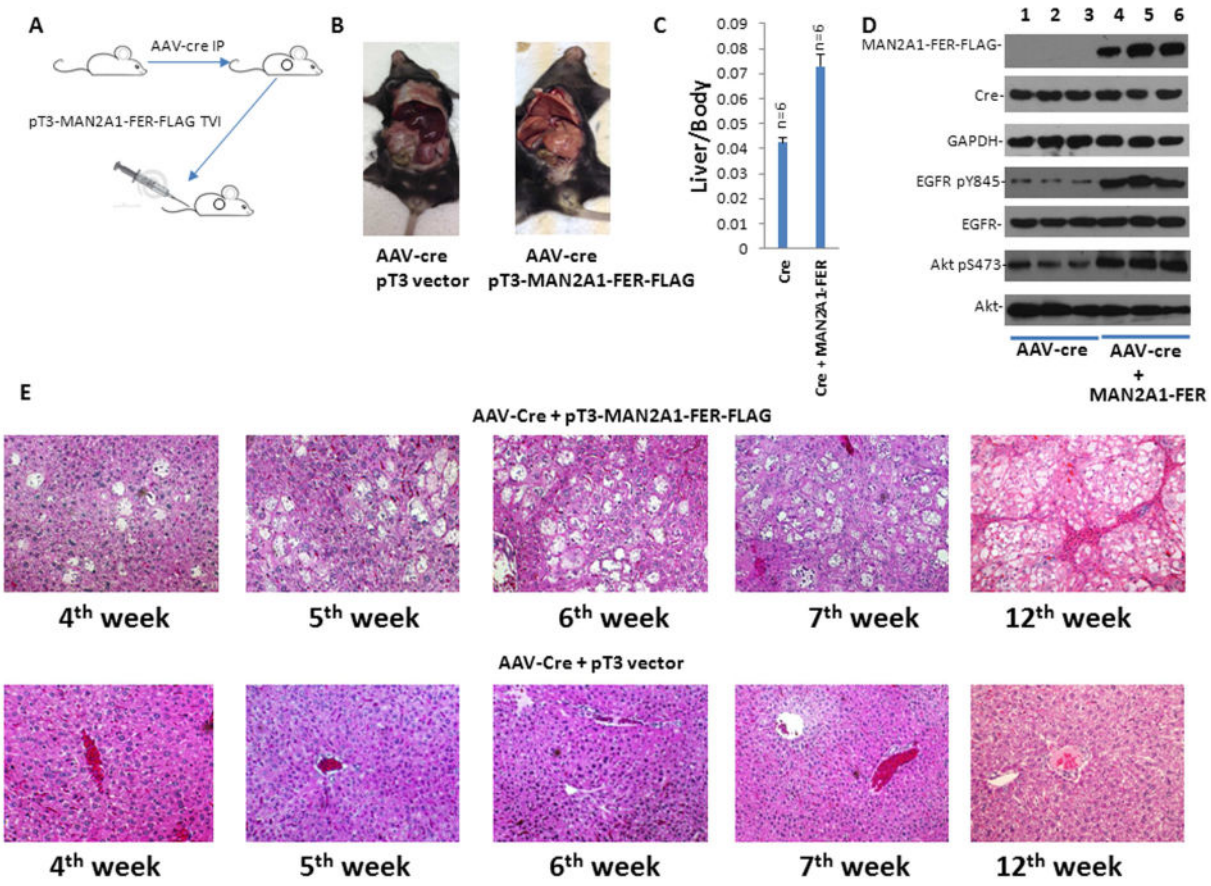

Figure 7. *MAN2A1-FER-FLAG* induced spontaneous liver cancer in mice with somatic Pten deletion

(A) Schematic diagram of the procedure. (B) Representative pictures of mice with gross liver cancer (right) and negative control (left). AAV-CRE plus control vector pT3 were used as controls. (C) Tail vein hydrodynamic injection of pT3-MAN2A1-FER-FLAG increased liver size. AAV-CRE plus control vector pT3 were used as controls. (D) Tail vein hydrodynamic injection of pT3-MAN2A1-FER-FLAG induced activation of *EGFR* and *Akt.* AAV-CRE plus control vector pT3 were used as controls. (E) Progression of liver cancer produced by tail vein hydrodynamic injection of pT3-MAN2A1-FER-FLAG. AAV-CRE plus control vector pT3 were used as controls.