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Research Letter

**The recurrent liver MAN2A1-FER oncoprotein lacks kinase activity:
Implications for the use of tyrosine kinase inhibitors.**

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Data Availability Data and supporting materials will be made available to other researchers upon reasonable request and the approval from the corresponding author.

The MAN2A1–FER Fusion Gene is frequently found in esophageal adenocarcinoma (ESCA) and hepatocellular carcinoma¹. FER is a ubiquitously expressed non-receptor tyrosine kinase, closely related to FES and to the product of the *v-fes* and *v-fps* viral oncogenes, responsible for feline and avian sarcomas, respectively. These two oncogenes have the property to transform fibroblasts and induce tumor formation in mice^{2,3}. However, until recently, there was a lack of evidence supporting their role as oncogenes in humans⁴. The recent discovery of the recurrent chromosomal rearrangement leading to the MAN2A1-FER fusion is a major finding⁵. While only a limited number of fusion genes have been characterized in liver cancers, MAN2A1-FER was identified in 15.7% of hepatocellular carcinoma. Additionally, it is present in 26% of esophagus adenocarcinoma, 16.8% of non-small cell lung cancer, 7.1% of glioblastoma multiforme, 5.2% of prostate cancer, and 1.7% ovarian cancer¹.

MAN2A1-FER oncoprotein increased the proliferation and invasiveness of cancer cell lines, and induce spontaneous liver cancer in a PTEN-deficient mouse model¹. In addition, the study suggested that the oncogenic mechanism of action of the fusion protein was associated with increased FER catalytic activity. Given the sensitivity of FER to clinical-grade ALK and MET inhibitors, MAN2A1-FER fusion emerged as an attractive therapeutic target for the treatment of liver cancer and other malignancies^{6,7}.

MAN2A1-FER arises from chromosomal rearrangements on chromosome 5, bringing together the first 13 exons of alpha-mannosidase 2 (MAN2A1) gene with the 3' portion of FER encoding for the tyrosine kinase catalytic domain. The rearrangements always occur within the same introns; hence, all resulting MAN2A1-FER fusion proteins share the same amino-acid composition. At the protein level, the oncoprotein merges the N-terminus of α -mannosidase 2A (MAN2A1) with the C-terminal amino-acids 572-822 of FER (Figure 1A). Intriguingly, MAN2A1-FER lacks the first

portion of the kinase domain that structures the ATP binding lobe, including the first two residues of the conserved GXGXXG glycine-rich loop, one of the most highly conserved sequence motifs in protein kinases (Figure 1B and Supplementary Figure 1). Indeed, all protein kinases share invariable residues required for ATP binding⁸. Therefore, it was surprising that MAN2A1-FER exhibited increased kinase activity compared to the FER protein. To clarify this issue, we obtained the expression construct of *MAN2A1-FER* from the authors of the original publication¹ and we analyzed the kinase activity of this fusion protein. The autophosphorylation of MAN2A1-FER (Figure 1C), and the phosphorylation of cortactin, a FER substrate (Figure 1D), were assessed. In both assays, there was no evidence for kinase activity of MAN2A1-FER oncoprotein in contrast to the wild-type (WT) FER. Additionally, we generated a kinase-dead (KD) variant of MAN2A1-FER through *in vitro* mutagenesis of the conserved K591 residue in the ATP-binding domain of FER, which is essential for ATP binding and, consequently, kinase activity⁹. A comparative analysis of the WT MAN2A1-FER and KD version of MAN2A1-FER through immunoblotting revealed the same absence of phosphorylation signals (Figure 1C and 1D). This strongly suggested that MAN2A1-FER is a catalytically inactive kinase.

To definitively conclude, we next performed a quantitative *in vitro* kinase-assay using poly-EY peptides as substrate, as done in the previous study. WT FER was used as a positive control, while the kinase-deficient KD forms of FER and MAN2A1-FER were evaluated as negative controls. As depicted in Figure 1E, MAN2A1-FER did not exhibit kinase activity, mirroring the kinase-deficient controls.

Finally, we also repeated the colony-formation assay used by Chen et al (2017) to assess the enhanced clonogenicity induced by MAN2A1-FER oncoprotein. Consistent with their findings, MAN2A1-FER oncoprotein conferred a two-fold increased colony formation capacity to the transfected cells (Figure 1F). Remarkably, this feature was not dependent on the kinase activity as

demonstrated by the KD mutant (Figure 1F). We concluded that MAN2A-FER is a catalytically deficient tyrosine kinase.

The initial published characterization of MAN2A1-FER oncoprotein proposed that the oncogenic activity of the fusion protein was linked to FER catalytic activity. As a consequence, MAN2A1-FER is now considered as a target for tyrosine kinase inhibitors-based therapy^{7,8}. This conclusion was supported by *in vitro* kinase assays and the use of Crizotinib, an inhibitor of ALK, MET, ROS1 and RON receptor, which also targets many other protein kinases including FER. This mechanism was however surprising given the absence of the first nine amino-acids of the NH₂-terminal lobe of FER catalytic domain in the fusion protein, which included a conserved amino-acid residue, part of an invariant ATP-binding motif. Our results demonstrate that the MAN2A1-FER oncoprotein is devoid of kinase activity. We thus argue that the oncogenic feature of MAN2A1-FER fusion is independent of the kinase activity. This issue is of crucial importance for basic, preclinical and clinical research, because small molecules which target FER are available, but those would have no direct inhibitory effects on the oncoprotein. It should be noted, however, that kinase inhibitors may still have anti-tumor activity owing to the off-target effects of these molecules on other protein kinases. Alternatively, PROTAC methods aiming at targeting FER have been successfully developed¹⁰. Finally, our findings also imply that the MAN2A1 portion, responsible for localization of the fusion protein to the Golgi apparatus, may be responsible for the oncogenic properties of the fusion protein rather than the FER catalytic activity.

In conclusion, our data strongly support that MAN2A1-FER lacks kinase activity and, therefore, it cannot be targeted by small molecule kinase inhibitors.

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Figure legends

Figure 1: MAN2A1-FER oncoprotein is devoid of kinase activity.

(A) Schematic representation of MAN2A1 and FER domains are shown above and the MAN2A1-FER fusion protein below. Amino acids VILGELLGK of FER kinase domain are excluded in the MAN2A1-FER fusion.

(B) Alignment of protein sequences showing the conserved GXGXXG motif (in red) and invariant lysine (K in bold) of the ATP binding lobe of tyrosine kinases.

(C) Autophosphorylation of WT FER, MAN2A1-FER and MAN2A1-FER-KD proteins. Immunoprecipitated FER proteins were probed using an anti-phosphotyrosine antibody (p-Tyr). FER and MAN2A1-FER protein expression are shown (Flag) and Tubulin was used as a control.

(D) Phosphorylation of cortactin substrate. Flag-tagged cortactin was co-expressed with FER, MAN2A1-FER or MAN2A1-FER-KD proteins as indicated. Following immunoprecipitation, phosphorylated proteins were detected using an anti-phosphotyrosine antibody (top panel), and all transfected proteins using an anti-Flag antibody (bottom panel).

(E) *In vitro* kinase assay on MAN2A1-FER. Immunoprecipitated FER and MAN2A1-FER variants were incubated with poly-Glu-Tyr peptide as a substrate. Kinase activity was quantified using the ADP-Glo Kinase Assay kit. Statistical analysis was done using ANOVA.

(F) Colony formation assay using 3T3 cells transfected with control plasmid (pcDNA3), MAN2A1-FER or MAN2A1-FER-KD. Colonies were monitored after 10 days. Left panel: representative pictures of the plates showing colonies. Right panel: quantification of the number of colonies. Statistical analysis was done using ANOVA.