Characterization of Tropomyosin Receptor Kinase Signaling in intrahepatic Cholangiocarcinoma

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Introduction

Tropomyosin receptor kinases (TRK) TRKA, TRKB and TRKC (encoded by *NTRK1*, *NTRK2* and *NTRK3*, respectively), regulate survival, proliferation and differentiation of neurons through ligand-dependent activation of downstream signaling pathways including MAPK, PI3K and PKC[1-4]. Neurotrophin-3 (NTF3) is a member of the nerve growth factor facmily, the neurotrophins. NTF3 is the high-affinity ligand of TRKC, but can also bind to TRKA and TRKB[5]. We recently demonstrated that hepatic stellate cells (HSCs), which are precursors of cancer-associated fibroblasts (CAFs) in liver, release NTF3, promoting hepatocyte proliferation through paracrine TRKB signaling[6] *[Figure 1]*. The high-affinity ligand of TRKB is brain-derived neurotrophic factor (BDNF). As we uncovered TRK signaling as a novel growth signaling pathway in liver, for which FDA-approved antagonists (TRK inhibitors) exist, our aim was to clarify if TRK receptors and their ligands are expresssed in intrahepatic cholangiocarcinoma (iCCA). We hypothesized that CAFs release neurotrophins to promote fibrosis and proliferation in CAFs and CCA tumor cells through autocrine and paracrine TRK-signaling, respectively *[Figure 2]*. Administration of FDA-approved TRK inhibitors could inhibit both cancer cell-CAF and CAF-CAF crosstalk.

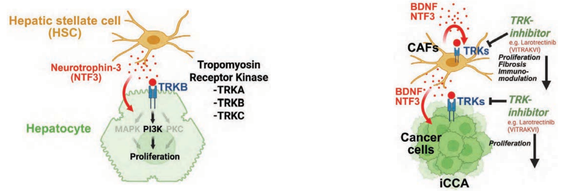

Figure 1. Tropomyosin receptor kinase (TRK) signaling. Hepatic stellate cells (HSCs) release neurotrophin-3 (NTF3) that promotes hepatocyte proliferation via paracrine TRKB signaling and activation of downstream signaling pathways including PI3K.

Figure 2. Working model. Cancer associated fibroblasts (CAFs) release neurotrophin-3 (NTF3) and brain-derived neurotrophic factor (BDNF), which promote a fibrotic tumor microenvironment and cancer cell proliferation through autocrine and paracrine TRK signaling, respectively. Administration of TRK inhibitors could interfere with both cancer cell-CAF and CAF-CAF crosstalk.

Methods

- Gene expression of *BDNF, NTF3*, *NTRK1*, *NTRK2* and *NTRK3* was assessed in publicly available iCCA datasets.
- Human iCCA specimens were assessed by western blotting and immunohistochemistry for BDNF, NTF3 and TRK expression.
- To assess growth proliferative effects of BDNF and NTF3 on cells, we incubated human CCA cell lines HuCCT1, Huh28 and HSCs cell line LX2 with BDNF and NTF3, or co-incubated with Larotrectinib and Taletrectinib, FDA-approved TRK inhibitors, to evaluate potential anti-proliferative effects.
- EdU-labeling was performed in patient-derived iCCA organoids (PDOs) to assess TRK-ligand dependent proliferation.

Results

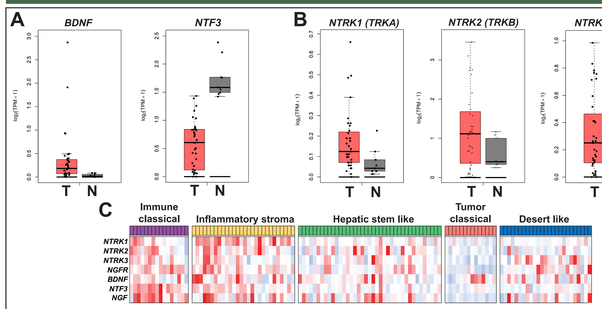

Figure 3. *TRKA*, *TRKB*, *TRKC*, *NGFR* and their ligands are overexpressed in CCA. A. Gene expression analysis for *BDNF* and *NTF3* in human CCA and normal liver tissue in the TCGA dataset (n=36 for Tu, n=9 for normal). **B.** Gene expression analysis for *NTRK1*, *NTRK2* and *NTRK3* in CCA and normal liver tissue in the TCGA dataset. **C.** Gene expression analysis for *NTRKs, NGFR, BDNF, NTF3 and NGF* in human iCCA stratified according to their subclassification[7].

Results

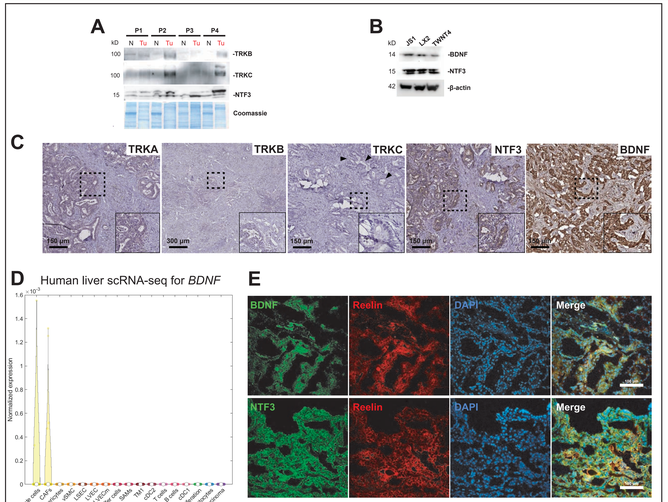

Figure 4. TRKB, TRKC and their ligands are overexpressed in human iCCA. A. Immunoblot analysis of human iCCA lysates and adjacent non-tumorigenic lysates for TRKB, TRKC and NTF3. Coomassie staining indicates equal loading across lanes. **B.** Immunoblot analysis of whole cell lysates of mouse and human HSC lines JS1, LX2 and TWNT4 for BDNF, NTF3 and β-actin. **C.** Immunostaining for TRKA, TRKB, TRKC, NTF3 and BDNF in human iCCA tissue. Inserts are enalrgements for indicated areas. Arrows indicate positive staining in CCA tumor cells. **D.** ScRNA-seq analysis for *BDNF* in a human liver scRNA-seq dataset[8]. **E.** Immunostaining for BDNF/Reelin and NTF3/Reelin in human iCCAs. Reelin: HSC marker.

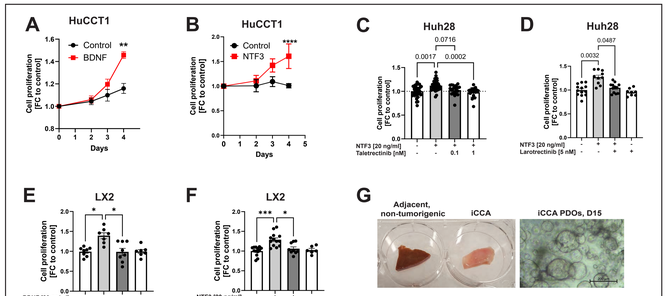

Figure 5. Pan-TRK inhibitors decrease NTF3- and BDNF-dependent proliferation in iCCA and HSC cell lines. A. BDNF induces proliferation in CCA cell line HuCCT1. **B.** NTF3 induces proliferation in CCA cell line HuCCT1. **C.** Incubation of CCA cell line Huh28 with NTF3 leads to increased proliferation that is inhibited by Taletrectinib. **D.** Incubation of CCA cell line Huh28 with NTF3 leads to increased proliferation that is inhibited by Larotrectinib. **E.** Incubation of HSC cell line LX2 with BDNF leads to increased proliferation that is inhibited by Taletrectinib. **F.** Incubation of HSC cell line LX2 with NTF3 leads to increased proliferation that is inhibited by Taletrectinib. **G.** Adjacent, non-tumorigenic liver tissue and human iCCA tissue; patient derived iCCA organoids on day 15 after initial generation. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

Results

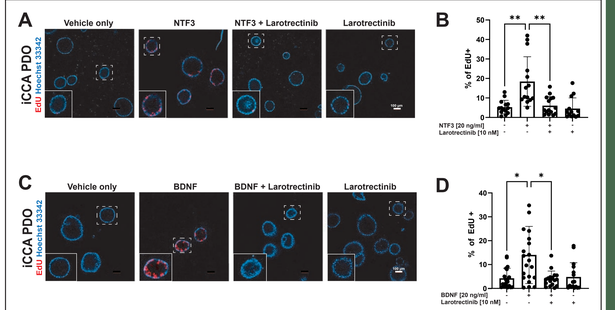

Figure 6. Pan-TRK inhibitors decrease NTF3- and BDNF-dependent proliferation in iCCA PDOs. A. Immunostaining for EdU in iCCA PDOs that were treated with NTF3 and/or Larotrectinib. **B.** Quantification of %EdU positive nuclei. Analysis with Kruskal-Wallis, posthoc Dunn's test or two-sided MWU test. **C.** Immunostaining for EdU in iCCA PDOs that were treated with BDNF and/or Larotrectinib. **D.** Quantification of %EdU positive nuclei. Analysis with Kruskal-Wallis, posthoc Dunn's test or two-sided MWU test. (*p<0.05, **p<0.01)

Conclusions

- TRKA, -B and -C and their ligands NTF3 and BDNF are overexpressed in a subset of human iCCA.
- NTF3 and BDNF induce TRK-dependent proliferation in iCCA cell lines, HSC cell lines and iCCA PDOs, which is inhibited by pan-TRK inhibitors.
- Pan-TRK inhibitors Larotrectinib and Taletrectinib inhibit BDNF/NTF3-TRK signaling in iCCA.
- Pan-TRK inhibitors merit further investigation as potential novel therapeutic approach for iCCA.

References

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Patient-Friendly Lay Summary Panel

Background:
Intrahepatic cholangiocarcinomas (iCCA) are often much denser and harder to the touch than the surrounding healthy liver tissue. This is because up to 70% of the tumor is made up of very dense fibrotic tissue, which is produced by cancer-associated fibroblasts (CAFs). By producing this dense tissue, the CAFs protect the cancer cells from e.g. immune cells and help them grow by releasing growth factors. Our lab and others found that liver fibroblasts unexpectedly release nerve growth factors (neurotrophins). In the nervous system, these neurotrophins promote proliferation and survival of nerve cells. In the liver, however, neurotrophins promote cell growth in fibroblasts themselves, but act also on other liver cells by activating tropomyosin receptor kinases (TRKs). Three different TRKs are known, TRKA, TRKB and TRKC. Among these three TRKs, neurotrophin-3 (NTF3) can activate all three, while brain-derived neurotrophic factor (BDNF) can only bind to TRKB. We hypothesized that this signaling pathway might also be active in iCCA and that by repurposing FDA-approved drugs (TRK inhibitors) that inhibit this pathway, we would be able to target both CAFs and cancer cells in a dual-targeted approach. Repurposing of drugs has the advantage that their safety profile is well known which decreases risks for patients and can accelerate their clinical implementation. In this study, we investigated if TRK signaling is activated in iCCA, and whether blocking the TRK signaling with existing drugs could be a possible treatment approach.

Methods:
We analyzed if the messenger RNA (mRNA) of TRKs and their growth factors were increased in iCCA patient samples. We also assessed the individual mRNA profiles of 122 iCCA patients that were classified into different groups depending on their profile. Our aim was to test which groups and how many patients might have increased TRK signaling. Next, we assessed the protein levels of iCCA patient samples, and tested if the proteins were detectable in human iCCA cell lines and liver fibroblast cell lines. In culture, we tested if iCCA cell lines and liver fibroblasts increase proliferation after stimulation with the nerve growth factors and if the proliferation can be inhibited by administration of two different TRK inhibitors, Larotrectinib and Taletrectinib. We then generated 3D iCCA tumor models from iCCA patient samples and tested if we observed increased proliferation after incubation with nerve growth factors NTF3 and BDNF, and if it can be decreased by co-administration of TRK inhibitors.

Results:
Public iCCA datasets showed increased mRNA levels of TRKA, TRKB and TRKC as well as NTF3 and BDNF. In another mRNA dataset, which allowed assessment of individual mRNA profiles of 122 patients, increased levels of TRK, NTF3 and BDNF were found in approximately one-third of patients which belonged to the subgroup 'immune classical' and 'inflammatory stroma'. These two subgroups have more fibrosis than the other subgroups. Analysis of iCCA patient samples verified increased expression of TRKB and TRKC protein, and also increased protein levels of the growth factors NTF3 and BDNF. Our culture experiments of human iCCA cell lines, liver fibroblasts and 3D iCCA models demonstrated that growth factors BDNF and NTF3 induced proliferation, which was decreased by co-administration of TRK inhibitors Larotrectinib or Taletrectinib.

Conclusion:
Our findings suggest that approximately one-third of iCCA patients have increased TRK singaling. iCCA cell culture experiments demonstrate that TRK signaling induces tumor growth that is inhibited by TRK inhibitors. Our studies demonstrate that repurposing of FDA-approved TRK inhibitors for iCCA might be a novel therapeutic strategy e.g. in combination with chemo/immunotherapies that merits further investigation.