

PTPN9-Mediated Dephosphorylation of IGF1R Y1165/1166 Reverses IGF1R-Driven Tyrosine Kinase Inhibitor Resistance in Cholangiocarcinoma

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Abstract

Cholangiocarcinoma (CCA) represents a highly aggressive cancer with few effective treatment choices. Emerging data indicate that some CCA patients may respond to multi-target tyrosine kinase inhibitors (mTKIs), such as surufatinib. The discovery of biomarkers that predict response to mTKI treatment is essential for optimizing clinical outcomes. This research investigated protein tyrosine phosphatase non-receptor type 9 (PTPN9) levels in CCA patients receiving surufatinib and evaluated its association with therapeutic outcomes. Immunoprecipitation combined with mass spectrometry (IP-MS) was used to discover new PTPN9 substrates. Orthotopic mouse models, biochemical tests, structural analyses, and functional assays were performed to confirm these discoveries. Single-cell RNA sequencing, ELISA, and immunohistochemistry were used to explore tumor-promoting factors in the microenvironment. Patients with CCA who did not respond to surufatinib showed markedly lower PTPN9 expression than those who responded. Reducing PTPN9 in orthotopic models decreased sensitivity to surufatinib. IGF1R was identified as a novel substrate of PTPN9, which selectively dephosphorylates IGF1R at Y1166. In patient samples, PTPN9 levels showed an inverse relationship with IGF1R Y1165/1166 phosphorylation. Creation of a surufatinib-resistant CCA cell line additionally verified reduced PTPN9 and heightened IGF1R pathway activity. Blocking IGF1R signaling in vivo substantially improved sensitivity to surufatinib. From a mechanistic standpoint, crystal structure examination identified Tyr333 and Asp335 as critical PTPN9 amino acids that interact with IGF1R; altering these sites reactivated IGF1R signaling and eliminated PTPN9's tumor-suppressing activity. Moreover, cancer-associated fibroblasts (CAFs) emerged as the primary producers of IGF1 in the CCA microenvironment, playing a key part in IGF1R-mediated tumor advancement. In conclusion, the PTPN9-IGF1R pathway is central to regulating mTKI responsiveness and cancer development in CCA. This pathway offers a valuable biomarker for selecting patients likely to benefit from mTKIs and provides a potential target for boosting mTKI effectiveness while addressing resistance.

Keywords: Cholangiocarcinoma, Surufatinib, IGF1R, PTPN9, CAF

Introduction

Cholangiocarcinoma (CCA) is a particularly invasive biliary tract cancer known for its restricted treatment

possibilities and unfavorable patient prognosis [1]. Although its worldwide occurrence and death rates are increasing, viable targeted therapies are still limited [2, 3]. Standard chemotherapy protocols, primarily gemcitabine plus cisplatin, yield unsatisfactory long-term survival, underscoring the urgent need for innovative approaches [4]. Genomic analyses have identified potential targets, including FGFR2 fusions and IDH1/2 mutations, yet these occur at relatively low frequencies [5]. While no multi-target tyrosine kinase inhibitors (mTKIs) have been approved by the FDA to

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date, limited clinical reports suggest that drugs such as surufatinib, lenvatinib, and anlotinib can benefit CCA patients, either alone or in combination with other therapies [6-10].

Nevertheless, some cases of cholangiocarcinoma demonstrate intrinsic resistance to mTKIs [9, 10]. In ongoing mTKI plus immunotherapy combinations, shifts in the tumor immune environment are associated with treatment success [11]. Importantly, studies examining inherent changes in CCA tumor cells that drive mTKI resistance or serve as predictive markers remain scarce. Both primary and adaptive resistance pathways substantially limit the effectiveness and durability of tyrosine kinase inhibitors (TKIs). Primary resistance often stems from existing or rapidly developing RTK pathway changes that reduce reliance on the inhibited receptor. For instance, in EGFR-mutant non-small cell lung cancer, third-generation EGFR-TKIs commonly lead to on-target mutations (e.g., C797S) and to alternative signaling (e.g., MET/ERBB), hindering sustained disease control [12, 13]. In renal cell carcinoma, resistance to VEGFR TKIs such as sunitinib involves mesenchymal/AXL pathways and integrin–focal adhesion signaling that foster drug tolerance and disease progression [14, 15]. In FGFR2-mutated cholangiocarcinoma, sequencing at progression often detects multiple secondary FGFR2 mutations and pathway adaptations, reducing the effectiveness of FGFR inhibitors [16]. In hepatocellular carcinoma (HCC), alternative activation of EGFR, VEGFR, and FGFR contributes to resistance against lenvatinib [17, 18]. Additionally, IGF1R bypass signaling is recognized as a resistance mechanism to mTKIs in various malignancies [19-23]. The IGF1R pathway is gaining attention in CCA biology [24, 25]. IGF1R, a receptor tyrosine kinase, becomes activated by IGF-1 or IGF-2, initiating downstream cascades including IRS-PI3K-AKT and RAS-RAF-ERK that drive cell growth, survival, and movement [26]. Although IGF1R normally controls tissue development, its abnormal activation frequently supports cancer formation, making it a potential prognostic indicator and treatment target in CCA [25].

Tyrosine phosphorylation is balanced by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Thus, mTKI resistance may involve impaired PTP function. Notably, earlier research found PTP mutations in 51.6% of intrahepatic cholangiocarcinoma (iCCA) cases [27]. Several studies link PTP functional changes to CCA patient outcomes,

cellular behavior, and resistance to chemotherapy [28, 29]. Our prior work showed that PTPN9 exhibits variable expression in CCA, with elevated levels associated with better prognosis [30]. Mechanistically, PTPN9 suppresses FGFR2 phosphorylation by binding to sec14p-APCP1-FGFR2, thereby affecting responses to Pemigatinib. However, the role of PTPN9 in broader mTKI resistance in CCA remains unclear.

In the present investigation, we found that elevated PTPN9 levels are associated with better responses to mTKI treatment in iCCA. We also established IGF1R as a key mediator of PTPN9-associated mTKI resistance. PTPN9 functions as a phosphatase that removes phosphate from p-IGF1R Y1166. Crystal structure analysis and enzymatic studies were conducted to clarify the molecular details, and the clinical significance of the PTPN9-IGF1R relationship was examined. Single-cell RNA sequencing and co-culture experiments were performed to study interactions between CAFs and tumor cells.

Materials and Methods

Crystallization and data collection

For crystallization experiments, His-PTPN9-C515S/D470A protein (at 15 mg/ml) was combined with the p-IGF1R Y1165/1166 peptide (IYETDpYpYRKGGK) at a 1:3 molar ratio in buffer A (pH 7.2, containing 20 mM HEPES, 350 mM NaCl, and 1 mM DTT). One microliter of the protein mixture was combined with 1 μ l of buffer B (pH 6.4, 20% PEG 4000, 0.2 M KSCN, 10% ethylene glycol, 0.1 M bis-tris propane) and incubated at 4 °C for 3 days until crystals formed. The cubic crystals were cryo-protected in storage buffer (buffer B plus 10% glycerol) before being flash-frozen in liquid nitrogen. Diffraction data were gathered at the Shanghai Synchrotron Radiation Facility beamline BL17U1 with 0.98 Å wavelength X-rays and processed using HKL2000.

Structural determination and refinement

The PTPN9-p-IGF1R Y1165/1166 peptide crystals belonged to space group P1. Molecular replacement for the PTPN9-peptide complex was performed with Phaser in the CCP4 suite, using the PTPN9 catalytic domain (PDB ID: 2PA5, excluding water molecules) as the search model. Subsequent refinement was performed with PHENIX, accompanied by manual model building in COOT.

Protein expression and purification

Wild-type and mutant GST-PTPN9 proteins were produced in BL21-DE3 *E. coli* cells induced with 0.4 mM IPTG (Sangon Biotech, B541007) for 16 h at 25 °C. Following cell disruption and centrifugation with a Sorvall LYNX 4000 high-speed centrifuge (Thermo Fisher Scientific, 46910, US), proteins were purified via binding to GST-Sepharose for 2 h and elution with GSH. All purified proteins were snap-frozen in liquid nitrogen and stored at -80 °C.

Results and Discussion

PTPN9-IGF1R axis mediates TKI resistance in CCA

Our earlier research indicated that higher PTPN9 expression is associated with improved prognosis in CCA, although the sample size of iCCA patients was modest and multivariate prognostic analysis was not conducted. To strengthen validation of PTPN9's prognostic value in CCA, we assembled a new group comprising 161 iCCA, 128 pCCA, and 138 dCCA patients. Due to some chip detachment, PTPN9 expression could be assessed in 153 iCCA, 117 pCCA, and 121 dCCA samples. In line with previous observations, elevated PTPN9 was connected to better survival across iCCA, pCCA, and dCCA. High PTPN9 was also related to smaller tumors in iCCA, better differentiation and lower T stage in pCCA, and improved differentiation in dCCA. Multivariate analysis established intratumoral PTPN9 as an independent prognostic marker for iCCA, pCCA, and dCCA, underscoring its importance in CCA progression.

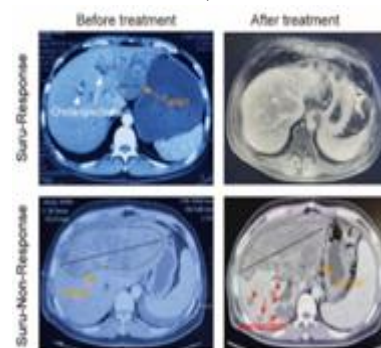
Recently, we observed primary resistance to surufatinib, an mTKI. To explore underlying resistance mechanisms, we assessed PTPN9 levels in tumor sections from CCA cases that showed either response or non-response to surufatinib (**Figures 1a and 1b**). Results revealed substantially reduced PTPN9 in non-responding tumors, suggesting PTPN9's involvement in surufatinib resistance (**Figures 1c and 1d**). To validate this, we generated an orthotopic CCA model in C57BL/6 mice using LD1 murine CCA cells with PTPN9 overexpression or knockdown, followed by oral surufatinib administration. Surufatinib suppressed tumor growth overall; PTPN9 overexpression enhanced this inhibition, while PTPN9 knockout significantly promoted tumor progression (**Figures 1e and 1f**). Since surufatinib targets VEGFR, FGFR, and CSF1R, and PTPN9's known effect on FGFR2 does not fully explain

the resistance, we suspected additional PTPN9 substrates—potentially tyrosine kinases unaffected by surufatinib. To test this, we conducted IP-MS with two PTPN9 substrate-trapping mutants (Flag-PTPN9 C515S and Flag-PTPN9 D470A) in HEK293T cells. Out of 148 proteins enriched in both mutants but absent in the Flag-vector control, IGF1R stood out as a key receptor, ranking fifth by peptide count (**Figure 1g**).

Analysis of TCGA-CHOL data showed IGF1R was prominently upregulated among RTKs in tumor versus normal tissues, with extracted expression levels displayed in a bar graph. We then screened IGF1R and PTPN9 protein levels in various CCA cell lines and a normal biliary epithelial line. QBC939 and RBE cells displayed relatively high IGF1R and low PTPN9, originating from intrahepatic and extrahepatic CCA, respectively, making them suitable for further studies. We verified PTPN9-IGF1R binding through exogenous (**Figures 1h and 1i**) and endogenous (**Figure 1j**) co-immunoprecipitation. Immunofluorescence in patient tissues demonstrated colocalization of PTPN9 and IGF1R in responding cases, supporting PTPN9's dephosphorylation role. In non-responding tissues, colocalization was notably weaker, consistent with reduced PTPN9, which limits its interaction with IGF1R and allows persistent IGF1R activation (**Figure 1k**).



a)



b)

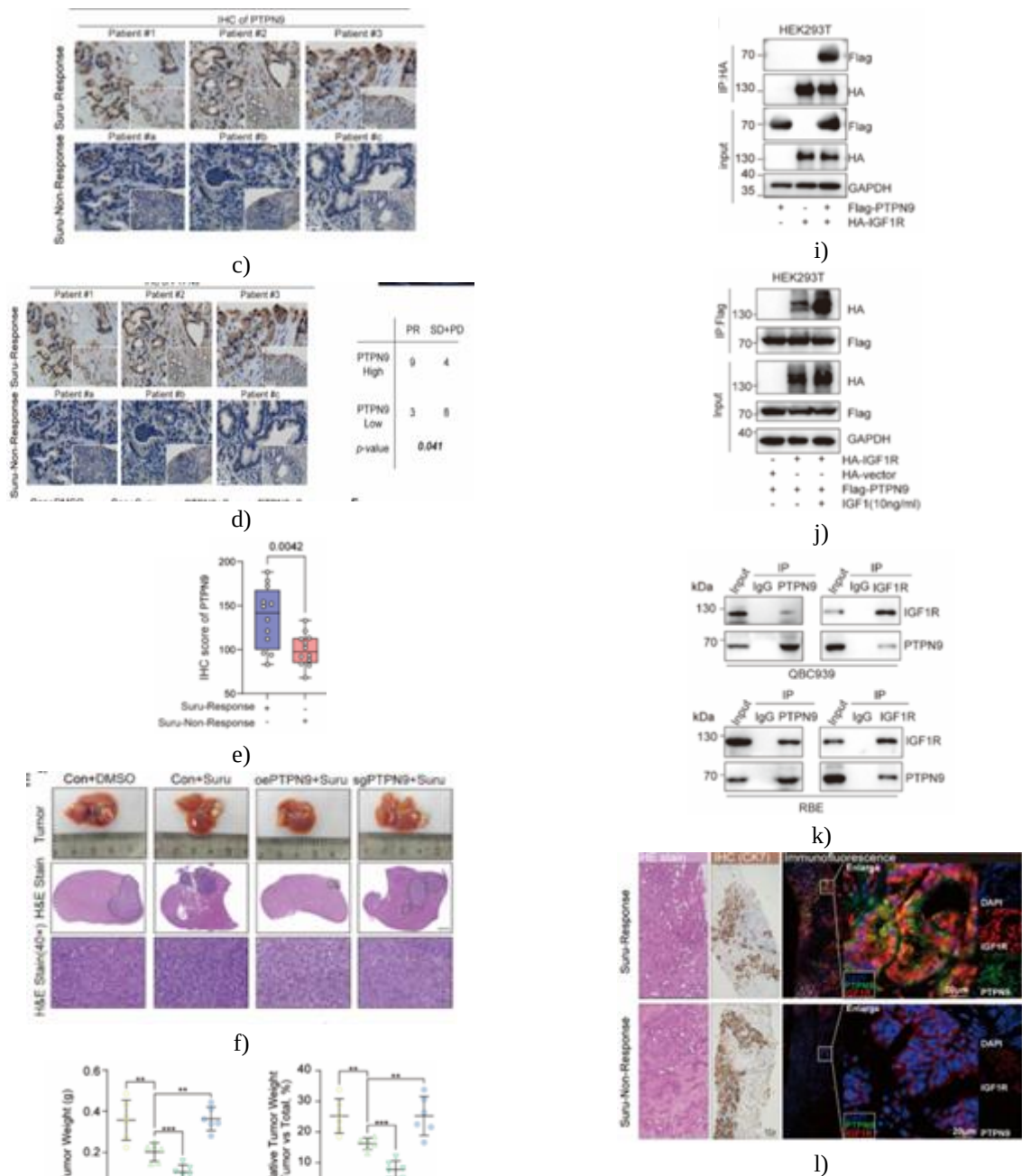


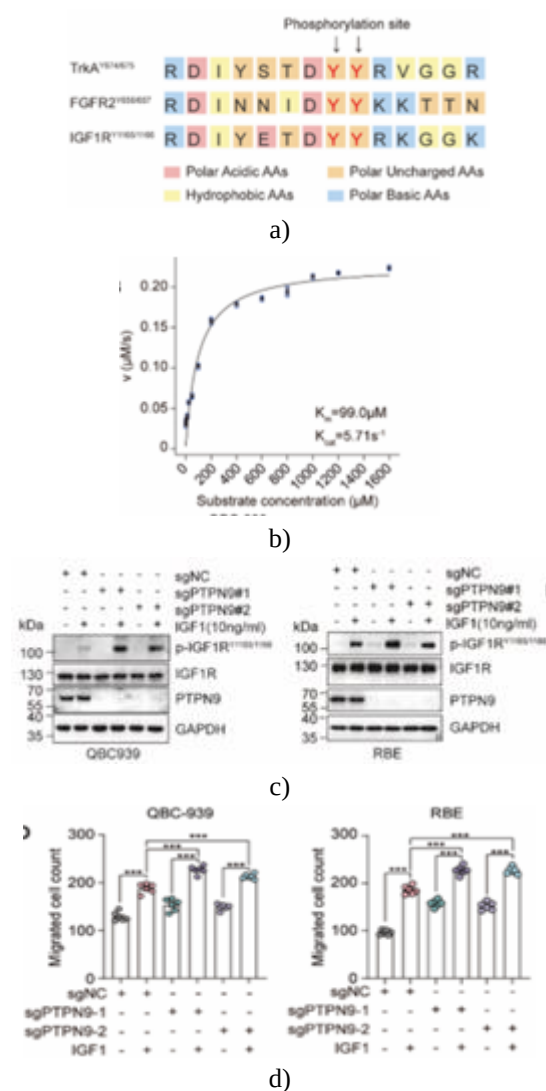
Figure 1. PTPN9 enhances responsiveness to surufatinib by engaging IGF1R in cholangiocarcinoma

(a) Overview diagram illustrating the clinical course of individuals with advanced-stage CCA undergoing oral surufatinib administration at 250 mg per day for repeated 4-week treatment cycles, (b) Typical examples of pre- and post-treatment CT and MRI scans from patients classified as responders versus non-responders to surufatinib, (c) Left panel: Characteristic IHC images depicting PTPN9 protein in tumor specimens obtained

from patients who responded or failed to respond to surufatinib. Right panel: Summary contingency table displaying the distribution of treatment outcomes according to low versus high PTPN9 expression groups. Scale bar: 50 μ m. (d) Statistical comparison of IHC scoring values for PTPN9 between surufatinib-responsive and non-responsive tumor samples. (e) Gross tumor photographs alongside corresponding H&E-stained sections of liver tissue harvested from orthotopic implantation models (n = 6 mice per experimental arm) after treatment with DMSO control, surufatinib monotherapy, PTPN9-overexpressing cells plus surufatinib, or PTPN9-knockdown (sgPTPN9-1) cells plus surufatinib. Dashed outlines indicate tumor margins. Scale bars: 2 mm (central images), 25 μ m (bottom images), (f) Quantification of absolute tumor mass (left) and relative tumor burden (right) across the orthotopic mouse cohorts, (g) Left: Venn diagram summarizing the overlap of candidate proteins recovered by LC-MS/MS analysis following immunoprecipitation performed on HEK293T cells transfected with empty vector control, Flag-tagged PTPN9C515S mutant, or Flag-tagged PTPN9D470A mutant. Right: List of PTPN9C515S-specific interactors ranked by prominence together with the top interactors shared with PTPN9D470A, (h) Co-IP assay confirming physical association between Flag-PTPN9 and HA-IGF1R proteins co-expressed in HEK293T cells, (i) Immunoprecipitation with anti-Flag antibody followed by immunoblotting analysis in HEK293T cells overexpressing Flag-PTPN9 in combination with either HA-IGF1R or empty vector, (j) Bidirectional endogenous co-immunoprecipitation experiments examining PTPN9-IGF1R complex formation in the QBC-939 (upper panels) and RBE (lower panels) human CCA cell lines, and (k) Representative micrographs of H&E histology, CK7 immunostaining, and dual immunofluorescence detection of PTPN9 (green channel) and IGF1R (red channel) performed on tissue sections from surufatinib-responsive (upper set) and non-responsive (lower set) CCA cases. Cell nuclei were visualized with DAPI counterstain (blue). Scale bars, 20 μ m. Results are derived from a minimum of three separate experiments and displayed either as mean values $\pm$ standard deviation or as illustrative images. Statistical evaluations employed the Chi-square test for panel c and unpaired two-tailed t-tests for panels (d) and (f). Asterisks denote statistical significance: **P < 0.01, ***P < 0.001.

PTPN9 directly associates with and removes phosphate groups from IGF1R at residues Y1165/1166 within CCA cells

Sequence alignment using bioinformatics tools demonstrated notable similarity in the amino acid context flanking the IGF1R autophosphorylation sites Y1165/1166 relative to previously validated PTPN9 target sites, including TrkA at Y674/675 and FGFR2 at Y656/657 (**Figure 2a**). The functional enzymatic engagement of PTPN9 with the phosphorylated IGF1R Y1165/1166 peptide was verified through Michaelis-Menten enzyme kinetics experiments, revealing a distinct substrate-enzyme interaction profile (**Figure 2b**).



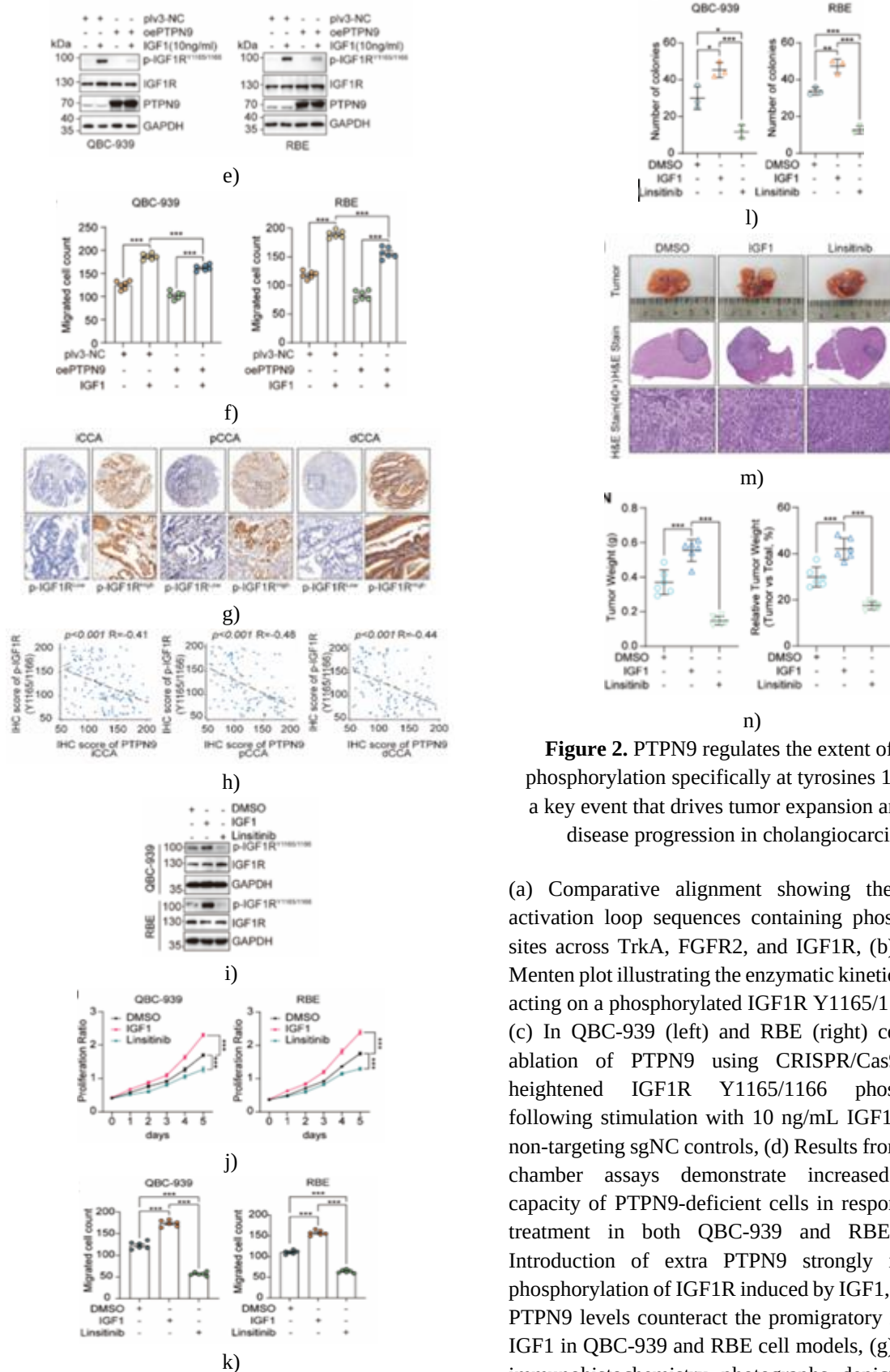


Figure 2. PTPN9 regulates the extent of IGF1R phosphorylation specifically at tyrosines 1165/1166, a key event that drives tumor expansion and overall disease progression in cholangiocarcinoma

(a) Comparative alignment showing the conserved activation loop sequences containing phosphotyrosine sites across TrkA, FGFR2, and IGF1R, (b) Michaelis-Menten plot illustrating the enzymatic kinetics of PTPN9 acting on a phosphorylated IGF1R Y1165/1166 peptide, (c) In QBC-939 (left) and RBE (right) cells, genetic ablation of PTPN9 using CRISPR/Cas9 leads to heightened IGF1R Y1165/1166 phosphorylation following stimulation with 10 ng/mL IGF1, relative to non-targeting sgNC controls, (d) Results from Transwell chamber assays demonstrate increased migratory capacity of PTPN9-deficient cells in response to IGF1 treatment in both QBC-939 and RBE lines, (e) Introduction of extra PTPN9 strongly inhibits the phosphorylation of IGF1R induced by IGF1, (f) Elevated PTPN9 levels counteract the promigratory influence of IGF1 in QBC-939 and RBE cell models, (g) Exemplary immunohistochemistry photographs depicting tumors

with strong versus weak p-IGF1R staining in clinical samples of iCCA, pCCA, and dCCA. Scale bar: 25 μ m, (h) Graphical scatter plots illustrating the statistically significant negative relationship between PTPN9 IHC scores and p-IGF1R Y1165/1166 staining intensities in tissue samples from iCCA, pCCA, and dCCA patients, (i) Western blotting of lysates from QBC-939 and RBE cells following exposure to DMSO, 10 ng/mL IGF1, or 10 μ M Linsitinib, highlighting changes in IGF1R Y1165/1166 phosphorylation; GAPDH served as the internal loading reference, (j) Cell viability measured by CCK-8 assay reveals that IGF1 (10 ng/mL) augments proliferation rates in QBC-939 and RBE cells, while addition of Linsitinib (10 μ M) blocks this stimulation, (k, l) IGF1 (10 ng/mL) markedly increases cellular migration (k) and anchorage-independent colony growth (l) in the tested CCA lines, effects that are reversed upon co-administration of 10 μ M Linsitinib, (m) Orthotopic implantation of CCA cells into the livers of C57BL/6 mice (n = 6 animals per cohort) followed by systemic treatment with DMSO vehicle, IGF1 (15 mg/kg i.p.), or Linsitinib (30 mg/kg i.p.). Scale bars: 2 mm (central images), 25 μ m (histological insets), and (n) Assessment of harvested tumor weights (left graph) and tumor weight relative to total liver weight (right graph) from the orthotopic experiment. All quantitative data are from at least 3 replicate experiments and are expressed as means $\pm$ standard deviation, accompanied by representative micrographs where applicable. Statistical comparisons were conducted via two-way ANOVA (j), Pearson's correlation coefficient (h), and unpaired t-tests (d, f, k, l, n). Levels of significance are marked as: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Additional experiments were performed to confirm that PTPN9 suppresses phosphorylation of IGF1R at Y1165/1166. Stable cell lines featuring either CRISPR-mediated PTPN9 deletion (sgPTPN9) or enforced PTPN9 overexpression (oePTPN9) were created from QBC-939 and RBE parental cells and confirmed via immunoblot. While total IGF1R protein abundance remained unaffected by changes in PTPN9 status, absence of PTPN9 led to pronounced elevation, and surplus PTPN9 caused a clear reduction in IGF1-evoked receptor phosphorylation at the specified sites (**Figure 2c**). In agreement, cellular assays indicated that PTPN9 loss dramatically boosted IGF1-dependent proliferation, colony-forming potential, directed migration, and matrix invasion (**Figure 2d**). On the other hand, PTPN9 overexpression effectively dampened these IGF1-

stimulated malignant phenotypes in both cell backgrounds (**Figure 2f**). Analysis of patient tissue microarrays for activated IGF1R showed a robust inverse association between p-IGF1R Y1165/1166 intensity and PTPN9 expression (**Figures 2g and 2h**). Consistent with this, stratification by p-IGF1R levels identified markedly inferior survival for patients with high receptor phosphorylation.

To define the contribution of IGF1R pathway engagement, IGF1 served as the activating ligand and linsitinib as the pharmacological blocker. Immunoblot experiments verified that IGF1 upregulated and linsitinib downregulated phosphorylation at IGF1R Y1165/1166 without modifying overall receptor protein expression (**Figure 2i**). Parallel phenotypic tests confirmed that IGF1 stimulation robustly enhanced cell growth, clonogenicity, motility, and invasiveness across both lines (**Figures 2j-2l**), whereas linsitinib exerted clear suppressive actions.

Complementary gain- and loss-of-function models were generated by stable overexpression (oeIGF1R) or CRISPR knockout (sgIGF1R) of IGF1R in QBC-939 and RBE cells, with validation by western blot. Heightened IGF1R expression promoted multiple aggressive traits including proliferation, colony formation, migration, and invasion, and these were further amplified by IGF1 ligand. In contrast, IGF1R elimination weakened these properties, with partial restoration upon IGF1 supplementation.

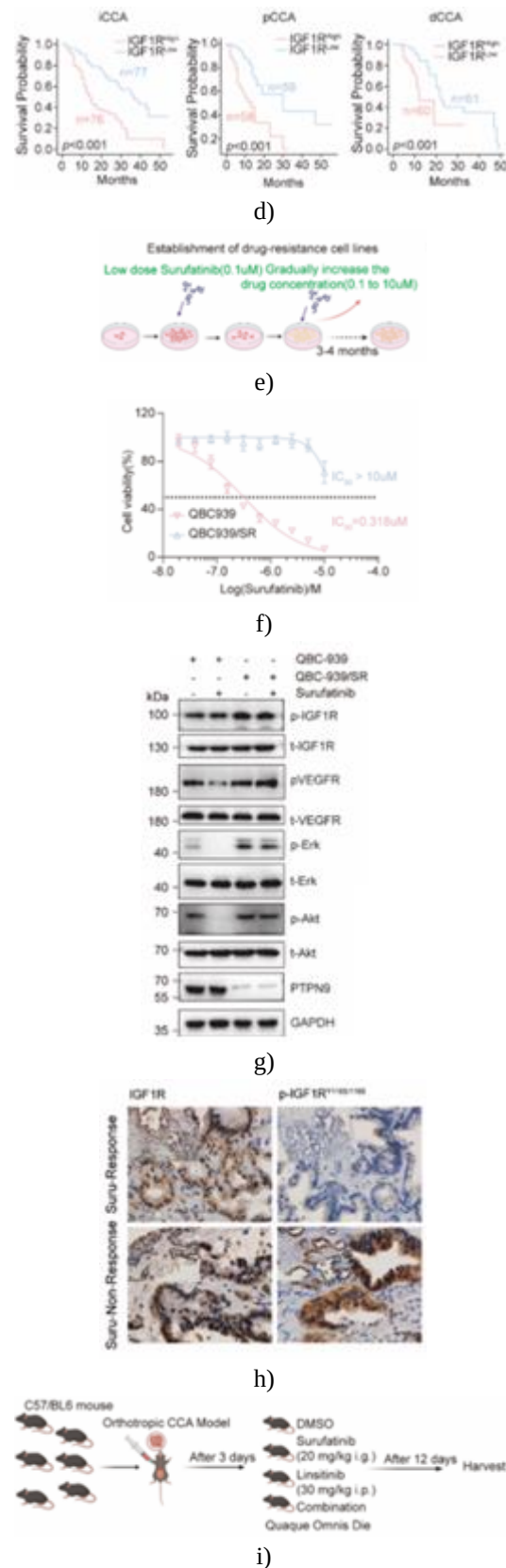
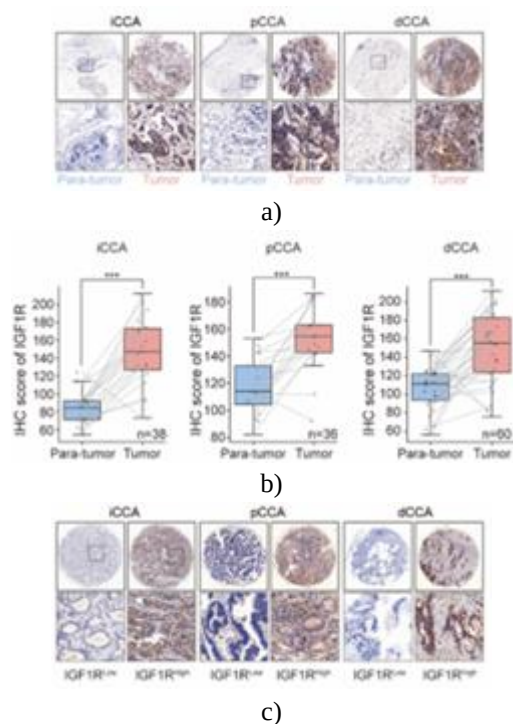
In vivo functional assessment was performed using an orthotopic liver tumor model generated by direct injection of LD1 cells into the livers of immunocompetent C57BL/6 mice. Following tumor establishment, systemic delivery of IGF1 or linsitinib was initiated. At the 12-day time point, IGF1 treatment accelerated intrahepatic tumor expansion, whereas linsitinib administration led to prominent tumor shrinkage compared with control animals (**Figure 2m**). Both raw tumor mass and the tumor-to-liver weight ratio were significantly augmented by IGF1 and attenuated by linsitinib (**Figure 2n**).

Taken together, the evidence shows that PTPN9 enzymatically dephosphorylates IGF1R at Y1165/1166, thereby restraining receptor activation. Manipulation of PTPN9 abundance directly affects downstream IGF1 signaling strength and, consequently, modulates the proliferative, migratory, and invasive behaviors of cholangiocarcinoma cells. Activation of IGF1R by its ligand, IGF1, accelerates tumor development in both

laboratory models and living organisms. In contrast, targeted IGF1R blockade with linsitinib potently opposes these oncogenic processes, underscoring the pivotal role of the PTPN9-IGF1R regulatory axis in cholangiocarcinoma pathobiology.

IGF1R compensatory activation contributes to TKI resistance in CCA

Immunohistochemical evaluation of tissue microarrays containing matched cancerous and adjacent normal tissues from iCCA, pCCA, and dCCA cases revealed markedly increased IGF1R protein expression within the tumor compartments of all three subtypes (**Figures 3a and 3b**). In a distinct patient TMA cohort, IGF1R staining intensities were quantified, and cases were classified as IGF1R^{High} or IGF1R^{Low} using the median score as the cutoff. Survival analysis by the Kaplan-Meier method demonstrated that patients with high tumoral IGF1R had considerably shorter overall survival than those with low IGF1R across iCCA, pCCA, and dCCA (**Figures 3c and 3d**). Supporting this, results from multivariate Cox regression modeling confirmed IGF1R overexpression as an independent marker of poor clinical outcome.



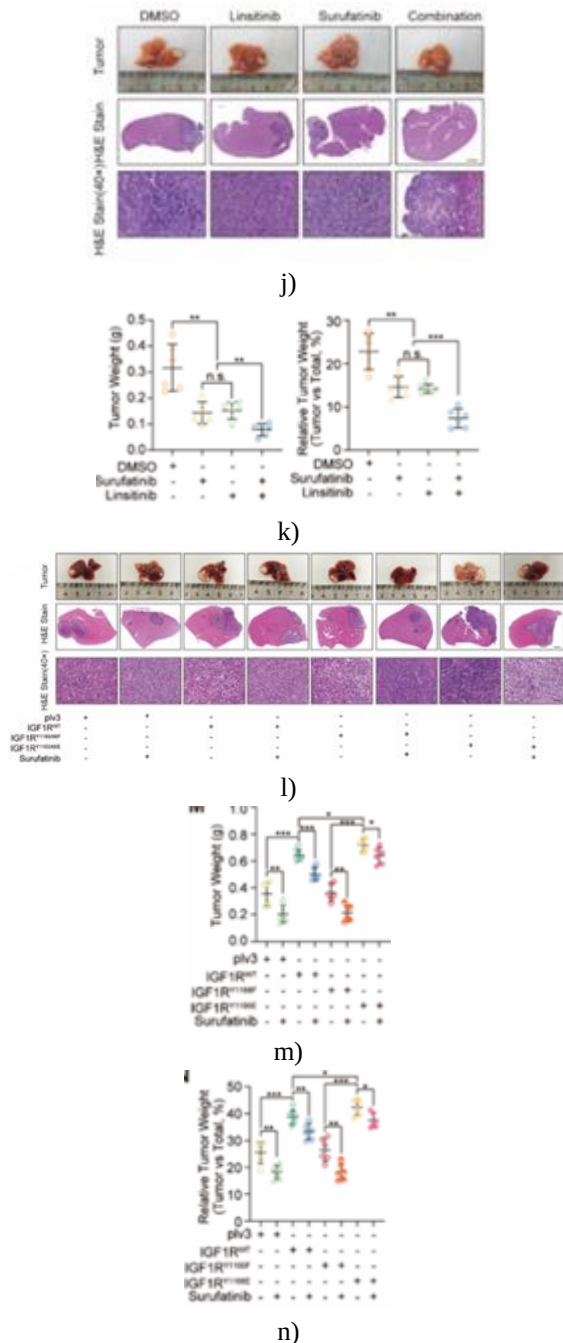


Figure 3. High IGF1R levels drive resistance to surufatinib and correlate with unfavorable outcomes in cholangiocarcinoma

(a) Illustrative immunohistochemistry micrographs displaying IGF1R staining patterns in adjacent non-tumoral tissue as well as tumors with high and low IGF1R expression from iCCA, pCCA, and dCCA cases. Scale bar: 25 μ m, (b) Box-and-whisker diagrams summarizing IGF1R IHC scoring results in matched

tumor versus para-tumor tissues across the iCCA, pCCA, and dCCA patient groups, (c) Representative IHC photographs of tumor sections classified as high- versus low-IGF1R expression in iCCA, pCCA, and dCCA. Scale bar: 25 μ m, (d) Kaplan–Meier curves for overall survival stratified by IGF1R expression status in patients with iCCA, pCCA, and dCCA, (e) Diagram outlining the stepwise incremental exposure protocol used to derive the surufatinib-resistant QBC-939/SR subline, (f) Dose-response viability curves showing markedly decreased sensitivity of QBC-939/SR cells to surufatinib relative to the parental line, (g) Immunoblot comparison of signaling molecules in parental QBC-939 versus QBC-939/SR cells under basal conditions and following surufatinib exposure, (h) Typical IHC staining for total IGF1R and phosphorylated IGF1R in tumor samples from surufatinib-responsive versus non-responsive cholangiocarcinoma patients. Scale bars: 50 μ m, (i) Experimental design for the orthotopic CCA model in C57BL/6 mice receiving DMSO, surufatinib (20 mg/kg oral gavage), linsitinib (30 mg/kg intraperitoneal), or the drug combination, (j) Gross tumor photographs and H&E histology from orthotopic models (n = 6 mice per arm) after treatment with DMSO, surufatinib monotherapy, linsitinib monotherapy, or combination therapy. Dashed lines delineate tumor boundaries. Scale bars: 2 mm (central panels), 25 μ m (histological panels). (k) Measured tumor weights (left) and tumor-to-liver weight ratios (right) in the orthotopic experiment. (l) Representative images of liver tumors and H&E-stained sections from orthotopic models (n = 6 per group) using LD1 cells expressing IGF1R^{WT}, IGF1R^{Y1165/66F}, or IGF1R^{Y1165/66E}, with or without surufatinib administration. Scale bars: 2 mm (middle panels), 25 μ m (lower panels), and (m, n) Quantitative analysis of tumor mass (m) and relative tumor burden (n). Data are derived from at least three independent experiments and are reported as mean $\pm$ SD or as representative images. Statistical methods included paired t-test (b), log-rank test (d), and unpaired t-test (k, m, n). Significance notations: n.s. = not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

Given the reduced PTPN9 abundance observed in surufatinib-nonresponsive CCA specimens and the established negative regulatory effect of PTPN9 on IGF1R phosphorylation at Y1165/1166, we examined whether sustained IGF1R pathway activation could represent a mechanism underlying surufatinib resistance. To this end, we generated a surufatinib-resistant variant

of QBC-939 cells (QBC-939/SR) through progressive dose escalation (**Figure 3e**). Viability testing confirmed substantially greater resistance to surufatinib in the resistant subline compared with parental cells (**Figure 3f**). To dissect the signaling basis of IGF1R-dependent resistance, we assessed receptor activation and downstream cascades in resistant versus sensitive cells in the presence or absence of surufatinib. Immunoblotting showed elevated phosphorylation of IGF1R at Y1165/1166 in QBC-939/SR cells, together with heightened activity of PI3K-Akt and MEK-Erk pathways. Notably, PTPN9 protein levels remained comparable regardless of surufatinib exposure (**Figure 3g**). In patient samples, total IGF1R expression showed no meaningful difference between responsive and non-responsive tumors, but phosphorylated IGF1R at Y1165/1166 was clearly higher in the non-responsive cohort (**Figure 3h**). Functional assays further demonstrated increased cellular proliferation, colony formation, and migratory/invasive abilities of the resistant cells even under surufatinib pressure.

Supplementary viability experiments indicated that linsitinib treatment reduced survival of QBC-939/SR cells. Moreover, dual administration of surufatinib plus linsitinib produced stronger suppression of colony formation than either monotherapy in parental QBC-939 cells. To test this strategy in a physiologically relevant setting, orthotopic CCA tumors were established in C57BL/6 mice and treated with surufatinib, linsitinib, or their combination (**Figure 3i**). After 12 days, combination therapy resulted in substantially smaller tumors than single-agent arms (**Figure 3j**), with correspondingly greater reductions in tumor weight and tumor-to-liver weight ratio (**Figure 3k**). Next, IGF1R-overexpressing LD1 cells were used for orthotopic implantation. IGF1R overexpression accelerated tumor progression, while surufatinib retained some inhibitory activity that was further potentiated by concurrent linsitinib. Finally, to directly assess the contribution of site-specific phosphorylation, LD1 cells expressing phosphorylation-mimetic or phosphorylation-deficient IGF1R Y1165/1166 mutants were implanted orthotopically. The phosphomimetic (Y1165/66E) variant conferred additional resistance to surufatinib's antitumor effect, whereas the non-phosphorylatable (Y1165/66F) mutant reduced baseline tumor growth and sensitized lesions to surufatinib (**Figures 3l–3n**). These data highlight the decisive influence of IGF1R

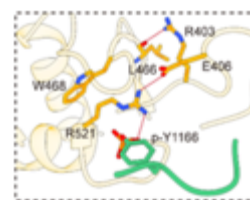
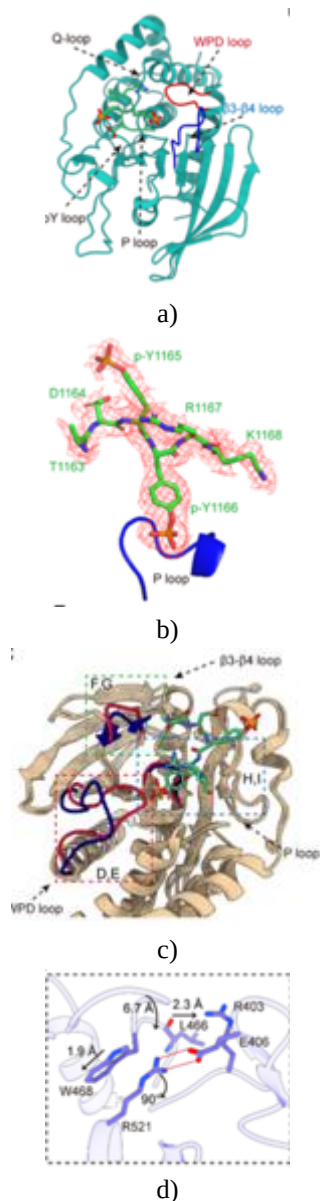
Y1165/1166 phosphorylation status on therapeutic responsiveness.

In aggregate, IGF1R is overexpressed in CCA and associated with inferior survival. Resistant cells and clinical non-responders exhibit prominent bypass activation of IGF1R, evidenced by increased p-IGF1R Y1165/1166 and consequent upregulation of PI3K–AKT and MEK–ERK cascades, without alterations in total receptor levels. Both genetic and pharmacological suppression of IGF1R signaling re-sensitize tumors to surufatinib. In vivo, the surufatinib plus linsitinib combination yields superior tumor control. Conversely, IGF1R overexpression or activating mutations at Y1165/1166 impair drug efficacy. Thus, phosphorylation of IGF1R, particularly at Y1165/1166, emerges as a critical modulator of TKI sensitivity, supporting combined IGF1R inhibition as a promising strategy to enhance surufatinib activity in CCA.

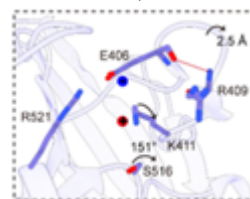
Structural mechanism underlying recognition and dephosphorylation of IGF1R Y1165/1166 by PTPN9

To elucidate the molecular details of how PTPN9 removes phosphate from IGF1R at Y1165/1166, we solved the co-crystal structure of PTPN9 in complex with a short IGF1R Y1165/1166 phosphopeptide using X-ray crystallography, achieving an overall resolution of 2.0 Å. Within the crystal lattice, the electron density corresponding to the IGF1R segment T1163DpYpYRK1168 clearly occupies the active-site cleft of PTPN9 (**Figures 4a and 4b**). Superposition of this complex onto the structure of ligand-free inactive PTPN9 revealed notable conformational rearrangements: the WPD loop shifts inward. In contrast, the β 3- β 4 loop moves outward (**Figure 4c**). Specifically, the phosphate group of pY1166 inserts deeply into the catalytic pocket, establishing a hydrogen bond with R521 of PTPN9 and inducing relocation of W468 toward R521. This motion brings the WPD loop backbone 6.7 Å closer to the catalytic center, where a hydrogen bond between L466 and R403 further stabilizes the closed conformation (**Figures 4d and 4e**). Within the β 3- β 4 loop, the side chain of S516 rotates by 151° upon engagement with pY1166 and forms a hydrogen bond with K411. Consequently, K411 reorients, disrupting its salt bridge with E406 and driving an outward displacement of the β 3- β 4 loop by 2.5 Å (**Figures 4f and 4g**). Additionally, R410 within the same loop shifts outward by 2.5 Å (**Figures 4f and 4g**). The P-loop undergoes flipping and establishes polar contacts with

P378 and C412, reinforcing the repositioned $\beta 3$ - $\beta 4$ loop (**Figures 4h and 4i**). Comparative analysis of the PTPN9 catalytic pocket interactions with the IGF1R peptide versus the previously reported PTPN9-C515S/D470A-NSF-pY83 complex identified both shared and unique contacts (**Figures 4j and 4k**). In the core pY region (pY1166 versus pY83), common interactions include hydrogen bonds with Y333, S516, I519, and R521, plus a cation- π stacking with Y471. Beyond the pY core, the threonine at position 1163 of the IGF1R peptide forms additional hydrogen bonds with R332 and the backbone of R410.



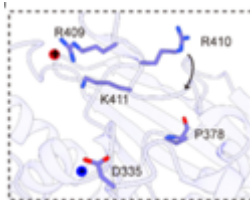
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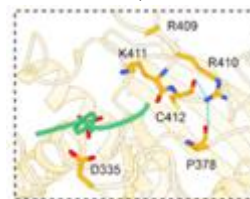
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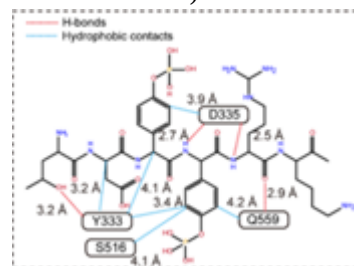
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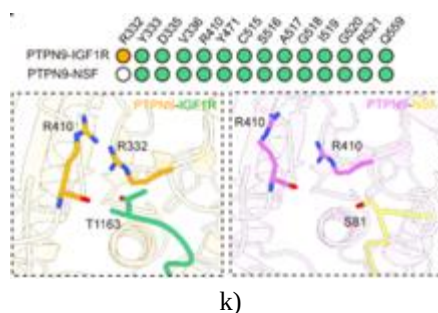


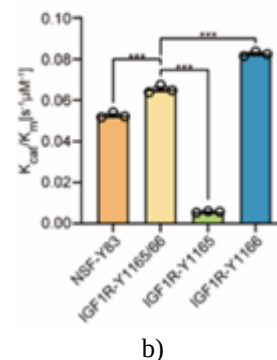
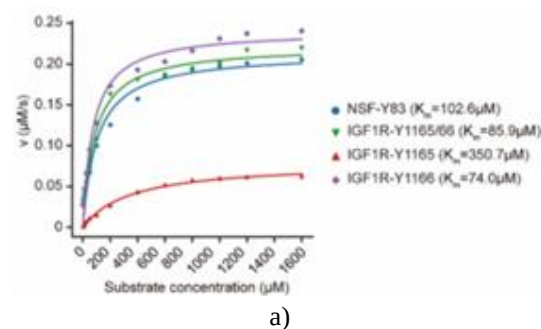
Figure 4. Structural basis for the molecular mechanism through which PTPN9 controls dephosphorylation of IGF1R at tyrosines 1165 and 1166

(a) Three-dimensional structural model depicting the complex formed between the PTPN9-C515S/D470A trapping mutant and the phosphorylated IGF1R Y1165/1166 peptide segment, (b) 2Fo-Fc annealed omit electron density map contoured around the p-IGF1R Y1165/1166 phospho-segment, with the P-loop highlighted in blue, (c) Three-dimensional overlay of the PTPN9-C515S/D470A-IGF1R complex structure (red) superimposed onto the inactive form of PTPN9 (PDB code 2PA5, blue). The WPD loop, $\beta 3$ - $\beta 4$ loop, and P-loop are emphasized in red for the complex and blue for the apo inactive structure. (d, e) Detailed three-dimensional views illustrating conformational rearrangements of the WPD loop in the PTPN9-C515S/D470A-IGF1R complex (d) compared with the inactive PTPN9 structure (e). Rotation of R521 induces relocation of W468, resulting in a 6.7 Å inward shift of the WPD loop accompanied by hydrogen bond formation between L466 and R403. (f-g) Detailed three-dimensional representations of $\beta 3$ - $\beta 4$ loop conformational shifts in the PTPN9-C515S/D470A-IGF1R complex (f) versus the inactive PTPN9 structure (g). Breakage of the salt bridge between E406 and K411, along with rotation of S516, drives the observed repositioning of the $\beta 3$ - $\beta 4$ loop. (h, i) Three-dimensional close-up views of P-loop conformational alterations in the PTPN9-C515S/D470A-IGF1R complex (h) and inactive PTPN9 (i). Rotation of R410 enables new polar contacts with P378 and C412, stabilizing the altered P-loop conformation. (j) Schematic diagram summarizing interactions between the p-IGF1R Y1165/1166 phospho-segment and residues lining the catalytic pocket of PTPN9-C515S/D470A. Blue dashed lines represent hydrogen bonds and red dashed lines indicate hydrophobic contacts. (k) Comparative analysis of

substrate recognition interfaces across different PTPN9 complexes. (Upper panel) Barcode-style illustration depicting the interaction patterns at the binding interface for PTPN9 engaged with the p-IGF1R Y1165/1166 segment versus the p-NSF Y83 segment. (Lower panel) Three-dimensional view highlighting specific differences in intermolecular contacts between the PTPN9-C515S/D470A-p-IGF1R Y1165/1166 complex and the PTPN9-C515S/D470A-p-NSF Y83 complex.

PTPN9 displays substrate selectivity and site preference in its phosphatase activity toward IGF1R Y1165/1166

To evaluate substrate specificity, enzymatic kinetic parameters were determined for wild-type PTPN9 using five distinct phosphotyrosine peptides: the dual-phosphorylated p-IGF1R Y1165/1166, singly phosphorylated p-IGF1R Y1165, p-IGF1R Y1166, and the reference p-NSF Y83 peptide. PTPN9 demonstrated robust catalytic efficiency against both the p-IGF1R Y1165/1166 and p-NSF Y83 substrates (**Figure 5a**). In contrast, activity toward the peptide phosphorylated exclusively at Y1165 was markedly reduced, as evidenced by a low k_{cat} and elevated K_m value. The peptide containing only pY1166 exhibited kinetic constants comparable to or slightly superior to those of the dual-phosphorylated substrate (**Figures 5b and 5c**). These results indicate that PTPN9 preferentially dephosphorylates Y1166.



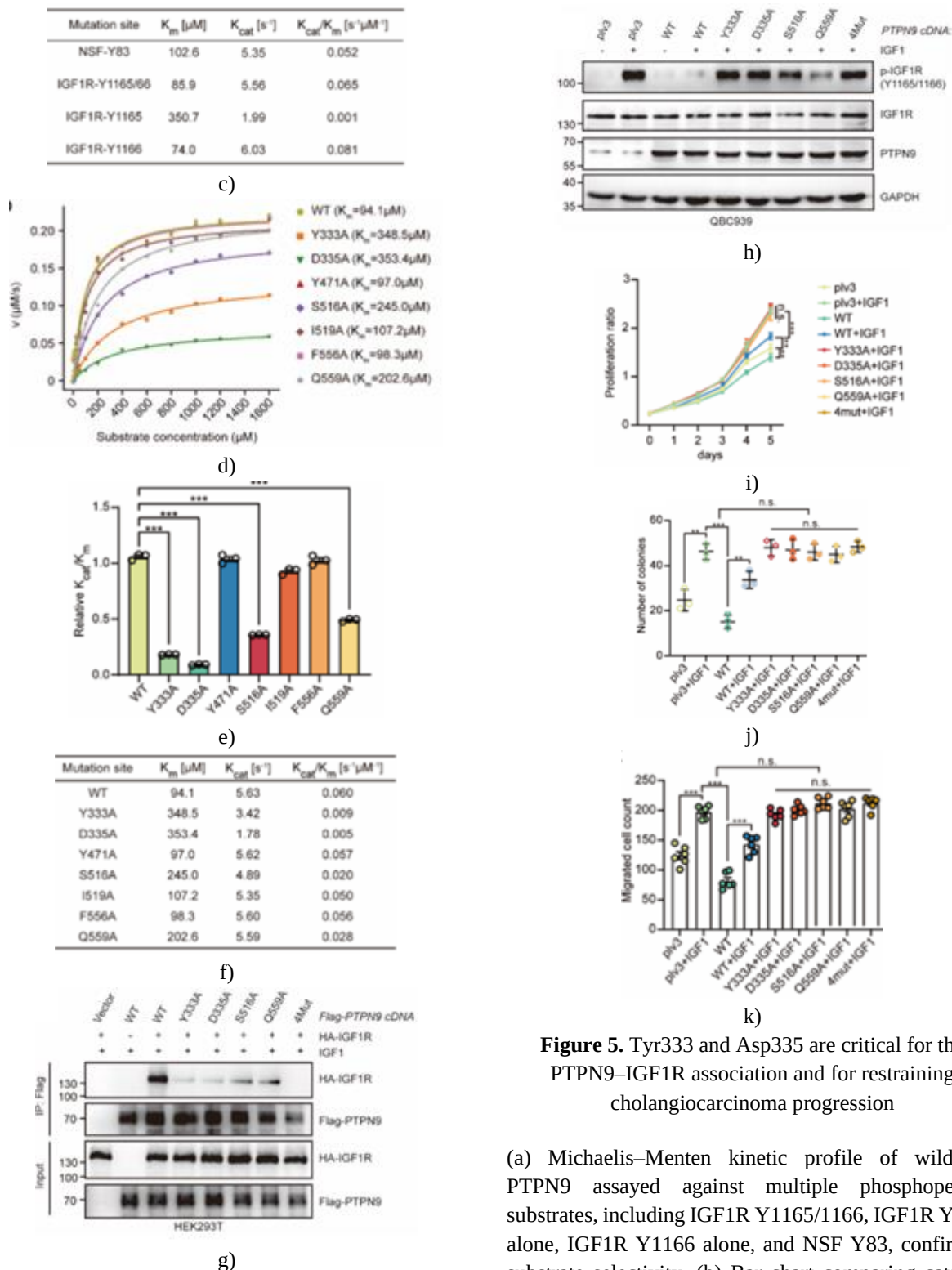


Figure 5. Tyr333 and Asp335 are critical for the PTPN9–IGF1R association and for restraining cholangiocarcinoma progression

(a) Michaelis–Menten kinetic profile of wild-type PTPN9 assayed against multiple phosphopeptide substrates, including IGF1R Y1165/1166, IGF1R Y1165 alone, IGF1R Y1166 alone, and NSF Y83, confirming substrate selectivity, (b) Bar chart comparing catalytic efficiency (k_{cat}/K_m) values of wild-type PTPN9 for each tested phosphosubstrate, revealing notably weaker performance toward the IGF1R Y1165 single-phospho peptide, (c) Summary table listing K_m , k_{cat} , and k_{cat}/K_m

parameters for wild-type PTPN9 acting on the panel of substrates, (d) Michaelis–Menten curves obtained for wild-type PTPN9 and various point mutants using the p-IGF1R Y1165/1166 peptide substrate (concentration range 0–1600 μM), (e) Quantitative comparison of catalytic efficiencies (k_{cat}/K_m) for wild-type PTPN9 versus its mutants; the Y333A and D335A variants display sharply diminished activity, (f) Detailed kinetic constants (K_m , k_{cat} , and k_{cat}/K_m) for wild-type and mutant PTPN9 proteins toward the IGF1R Y1165/1166 substrate, demonstrating that Y333A and D335A substitutions cause major losses in catalytic performance, (g) Co-immunoprecipitation experiments assessing PTPN9–IGF1R binding in HEK293T cells co-transfected with HA-IGF1R and either Flag-tagged wild-type PTPN9, single-point mutants (Y333A, D335A, S516A, Q559A), or the quadruple mutant (4Mut), following IGF1 stimulation, (h) Western blot detection of p-IGF1R Y1165/1166, total IGF1R, and PTPN9 in QBC939 cells expressing wild-type PTPN9 or the indicated mutants (Y333A, D335A, S516A, Q559A, or 4Mut) with or without 10 ng/mL IGF1 treatment. GAPDH served as a loading control; (i) Growth curves measuring proliferation rates over 5 days for the different PTPN9 variant groups; (j) Colony formation assay quantifying colony numbers under each experimental condition; and (k) Transwell migration assay counting migrated cells for the respective groups. Data represent at least three independent experiments and are shown as mean $\pm$ SD. Statistical analyses used unpaired t-tests (b, e, i–k). Significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

To corroborate the structural observations from the PTPN9–IGF1R co-crystal, a series of PTPN9 mutants targeting conserved residues in the catalytic domain were engineered. These recombinant proteins were purified and tested for enzymatic activity against the p-IGF1R Y1165/1166 peptide (**Figure 5d**). The Y333A substitution yielded a K_m of 348.5 μM and k_{cat} of 3.42 s^{-1} , while the D335A mutant showed a K_m of 353.4 μM and k_{cat} of 1.78 s^{-1} , both resulting in substantially decreased catalytic efficiencies (**Figure 5e**). Overall, the k_{cat}/K_m values for Y333A and D335A were markedly lower than those of wild-type PTPN9 and the other mutants examined (**Figure 5f**). These biochemical findings confirm the indispensable contributions of Tyr333 and Asp335 to substrate recognition and catalytic potency, aligning with the crystallographic data. Co-expression experiments in HEK293T cells further

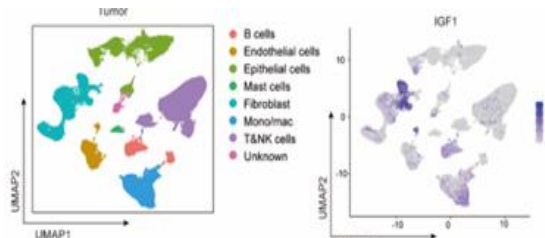
demonstrated that mutation of Tyr333 or Asp335 severely compromised the physical interaction between PTPN9 and IGF1R (**Figure 5g**). Stable QBC-939 cell lines overexpressing either wild-type PTPN9 or the selected mutants were then generated. Only wild-type PTPN9 strongly suppressed IGF1-stimulated phosphorylation of IGF1R at Y1165/1166, whereas the Y333A and D335A mutants largely failed to exert this dephosphorylating effect (**Figure 5h**). Consistent phenotypic outcomes were observed in subsequent functional tests (**Figures 5i–5k**).

In summary, these enzymatic and cellular studies establish IGF1R Y1166 as the preferred dephosphorylation target of PTPN9 and demonstrate that the catalytic residues Tyr333 and Asp335 are indispensable for effective PTPN9–IGF1R binding and downstream regulatory activity.

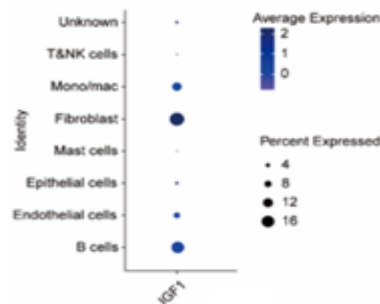
IGF1 secreted by cancer-associated fibroblasts activates IGF1R and accelerates cholangiocarcinoma progression

To pinpoint the cellular origin of IGF1 that drives IGF1R activation within the tumor microenvironment, single-cell RNA sequencing datasets were interrogated, revealing prominent IGF1 transcript expression in fibroblast populations (**Figures 6a and 6b**). Primary cancer-associated fibroblasts (CAFs) were subsequently isolated from fresh surgical specimens and representative lines corresponding to the dominant clusters in the scRNA-seq data were selected. Quantitative PCR analysis across multiple cell types corroborated elevated IGF1 mRNA in fibroblasts. Conditioned media harvested from these cultures were analyzed by ELISA, which confirmed significantly greater IGF1 protein secretion from CAFs compared with other cell populations (**Figure 6c**). Although THP-1 and SU-DHL-4 cells also produced relatively high IGF1 levels, functional co-culture experiments using conditioned media demonstrated that CAF-derived medium exerted the strongest stimulatory effects on tumor cell proliferation, migration, and invasion, indicating a predominant tumor-promoting role for CAFs. To directly test IGF1's contribution, CAF lines with IGF1 knockdown (CAF-sgIGF1) and overexpression (CAF-oeIGF1) were established and validated by western blot. ELISA measurements of their conditioned media showed correspondingly reduced IGF1 secretion in knockdown clones and increased secretion in overexpressing cells relative to controls

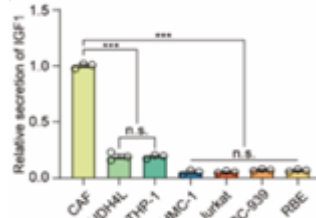
(Figure 6d). Functional assays revealed that CAF-conditioned medium robustly enhanced tumor cell proliferation, with this effect further amplified by medium from CAF-oeIGF1 cells and attenuated by medium from sgIGF1 cells (Figure 6e). Parallel results were obtained in migration and invasion assays.



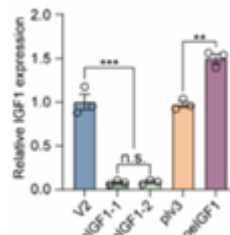
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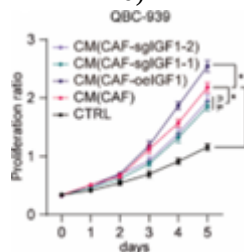
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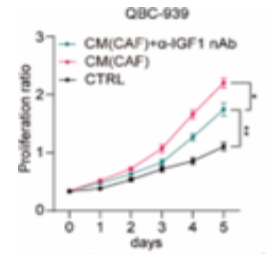
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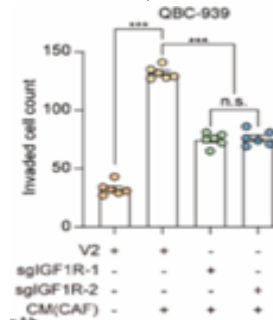
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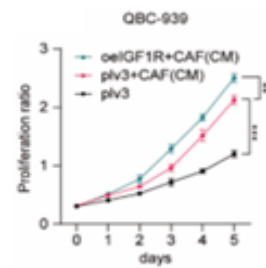
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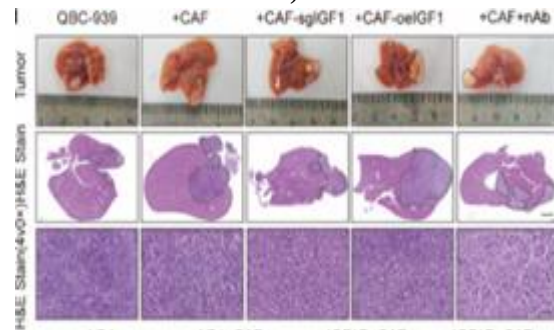
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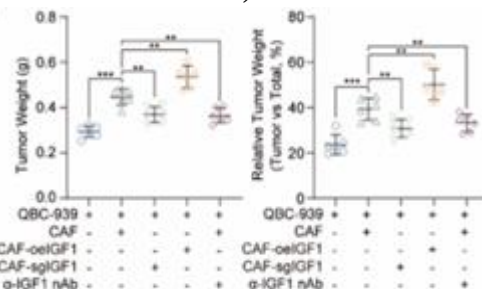
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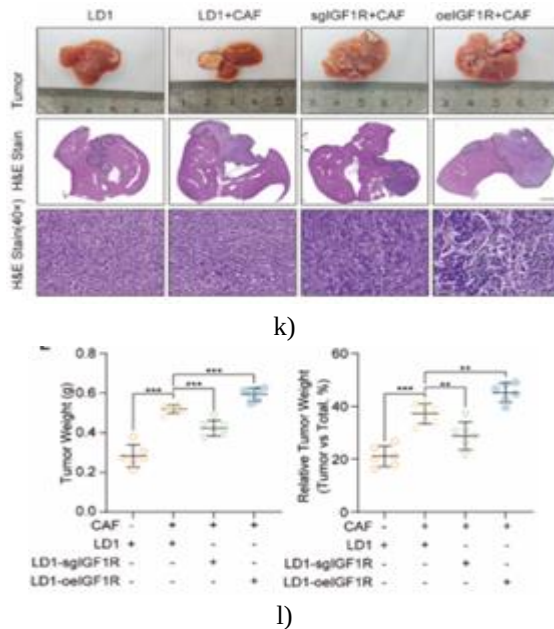


Figure 6. IGF1 produced by cancer-associated fibroblasts stimulates IGF1R-dependent signaling, thereby accelerating cholangiocarcinoma cell proliferation and invasive properties

(a) Left: UMAP projection illustrating the spatial distribution of major cell clusters, (a) Right: Visualization of IGF1 transcript levels across the identified cell populations, (b) Dot plot summarizing IGF1 expression patterns in distinct cell lineages, (c) Quantitative ELISA data comparing IGF1 amounts secreted by various cell types into culture supernatants, (d) ELISA results measuring IGF1 concentrations in conditioned media harvested from control (sgNC), IGF1-depleted (sgIGF1-1 and sgIGF1-2), empty-vector (plv3), and IGF1-overexpressing (oeIGF1) CAF populations, (e) Cell growth assay evaluating proliferation of QBC-939 cells incubated with conditioned media derived from unmodified CAFs, IGF1-knockdown CAFs, or IGF1-overexpressing CAFs, (f) Proliferation assay testing QBC-939 cells exposed to CAF-conditioned medium supplemented with or without an IGF1-specific neutralizing antibody (α -IGF1 nAb), (g) Transwell invasion assay revealing that IGF1R depletion (sgIGF1R) in QBC-939 cells limits the invasive response triggered by CAF-conditioned medium, (h) Proliferation assay demonstrating that IGF1R overexpression (oeIGF1R) in QBC-939 cells amplifies the growth-stimulatory influence of CAF-conditioned medium, (i) Orthotopic hepatic CCA model generated in NOD/SCID mice (n = 6 animals per cohort) through inoculation of

QBC-939 cells by themselves or together with CAFs, IGF1-overexpressing CAFs, IGF1-knockdown CAFs, or combined with IGF1-neutralizing antibody administration. Dashed outlines delineate tumor perimeters. Scale bars: 2 mm (central images), 25 μ m (histological sections), (j) Assessment of absolute tumor mass (left) and normalized tumor-to-liver weight ratio (right) obtained from the orthotopic experiment, (k) Orthotopic liver tumor model in C57BL/6 mice (n = 6 per group) created by implantation of LD1 cells alone or in combination with CAFs, sgIGF1R cells plus CAFs, or oeIGF1R cells plus CAFs. Tumor edges are indicated with dashed lines. Scale bars: 2 mm (central images), 25 μ m (histological sections), and (l) Measurement of tumor weights (left) and relative tumor burden (right) in the orthotopic setting. All results derive from at least 3 independent experiments and are expressed as mean $\pm$ SD or as representative images. Statistical evaluations employed unpaired t-tests (c, d, g, j, l) and two-way ANOVA (e, f, h). Significance markers: n.s. = not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

Further validation that IGF1 originating from CAFs supports malignant progression involved adding an IGF1-blocking antibody (α -IGF1 nAb) to CAF-derived conditioned medium before performing cellular assays. This neutralization substantially reduced CAF-CM's ability to boost tumor cell proliferation (**Figure 6f**), colony formation, and cell migration. Experiments with IGF1R-deficient tumor cells (sgIGF1R in QBC-939 and RBE backgrounds) confirmed that the pro-migratory and pro-invasive actions of CAF-CM required intact IGF1R, as knockdown cells displayed significantly weaker responses compared with control cells under identical CAF-CM exposure (**Figure 6g**). Colony formation results mirrored this pattern. In complementary gain-of-function studies, IGF1R-overexpressing cells showed stronger proliferative and migratory reactions to CAF-CM than matched vector controls (**Figure 6h**). When PTPN9 was overexpressed in tumor cells cultured with CAF-conditioned medium, it partially offset the CAF-induced enhancement of migration and invasion relative to control cells.

To examine these interactions in a living system, an orthotopic liver tumor model was developed in immunodeficient NOD/SCID mice. QBC-939 cancer cells were introduced either singly or alongside various CAF derivatives, with some groups also receiving IGF1-neutralizing antibody. Addition of CAFs led to noticeably larger tumors than cancer cells alone, as

judged by visual inspection and H&E staining of liver sections. This effect was exaggerated when IGF1-overexpressing CAFs were co-injected, while co-injection of IGF1-knockdown CAFs or treatment with IGF1-neutralizing antibody produced significantly smaller lesions (**Figures 6i and 6j**). To test dependence on tumor-cell IGF1R, LD1 cells engineered for IGF1R loss or gain were co-implanted with CAFs. Tumors derived from IGF1R-knockout cells were markedly smaller than those from wild-type controls, whereas IGF1R-overexpressing cells generated larger tumors (**Figures 6k and 6l**). These *in vivo* data strongly support the functional importance of the CAF-IGF1-IGF1R cascade in cholangiocarcinoma development. Additional tests indicated that exposure to CAF-conditioned medium blunted surufatinib's ability to suppress tumor cell behaviors, pointing to a stromal contribution to drug resistance.

Re-examination of single-cell RNA sequencing profiles revealed elevated IGF1 expression within CAFs from iCCA, pCCA, and dCCA specimens. Primary CAFs isolated separately from each subtype secreted high amounts of IGF1, with pCCA-derived CAFs showing the greatest output. All tumor-derived CAFs produced significantly more IGF1 than normal fibroblasts. When conditioned media from these subtype-specific CAFs were applied to cancer cells, pCCA-CAF medium provoked the most pronounced increases in proliferation, migration, and invasion, exceeding the effects of iCCA- or dCCA-CAF media, which were statistically similar to one another. This points to a particularly active role for pCCA-associated fibroblasts. In conclusion, the evidence presented establishes CAFs as the dominant source of IGF1 in the cholangiocarcinoma microenvironment. IGF1 released by these stromal cells engages IGF1R on malignant cells to promote key oncogenic processes including cell division, motility, tissue invasion, and *in vivo* tumor expansion. These actions are intensified by raising IGF1 levels in CAFs, weakened by lowering or blocking IGF1, diminished by removing IGF1R from tumor cells, and strengthened by increasing IGF1R. Overexpression of PTPN9 offers some resistance against the motility-enhancing influence of CAF secretions. CAF-derived factors also lessen surufatinib's therapeutic impact, implicating stromal IGF1 in the emergence of drug resistance. Subtype comparisons further reveal that pCCA CAFs are the most prolific IGF1 producers and exert the greatest tumor-supporting influence, identifying the CAF-IGF1-IGF1R

pathway—most notably in pCCA—as an actionable target for intervention.

This investigation reveals that a paracrine IGF1/IGF1R signaling loop originating from cancer-associated fibroblasts, normally restrained by the phosphatase PTPN9, serves as a major driver of cholangiocarcinoma progression and a key contributor to resistance to the multi-kinase inhibitor surufatinib. By combining proteomic screening, functional cellular studies, structural analyses, and animal models, we expand the understanding of adaptive bypass mechanisms in biliary tract malignancies and propose a biomarker-informed therapeutic combination capable of restoring drug responsiveness.

IGF1R was initially identified through our co-immunoprecipitation and mass spectrometry approach and subsequently confirmed as a prominently upregulated receptor tyrosine kinase in both TCGA-CHOL data and our own patient cohort, where elevated expression was strongly associated with reduced overall survival. These observations are consistent with established concepts that position the IGF pathway as a defining feature of CCA [2]. Experimental manipulation demonstrated that increasing IGF1R activity in QBC-939 and RBE cells promoted cell growth, invasiveness, and tumor formation in orthotopic implants. In contrast, inhibition with drugs or gene editing produced opposing effects, thereby establishing a direct causal role for IGF1R in disease progression. Although earlier clinical testing of IGF1R antagonists in other tumor types yielded only limited success [31], the clear association between IGF1R levels and patient outcomes in CCA suggests that patient stratification by IGF1R status could substantially improve response rates. Our study provides the first thorough documentation of IGF1R overexpression spanning all major CCA subtypes and its association with adverse prognosis, reinforcing the clinical relevance of the IGF system in biliary tract oncology.

Single-cell transcriptomic profiling of the tumor microenvironment identified CAFs as the primary producers of IGF1. Conditioned medium from IGF1-overexpressing CAFs strongly enhanced malignant traits in CCA cells, aligning with prior descriptions of growth factor-secreting inflammatory CAF subsets that actively support intrahepatic CCA [32]. Blocking secreted IGF1 with neutralizing antibodies or removing IGF1R from cancer cells abolished these stimulatory effects, confirming the existence of a critical paracrine circuit.

This work reinforces emerging views that the abundant desmoplastic stroma in CCA is not merely passive scaffolding but an active participant in tumor advancement [33]. Fibroblast-derived IGF1 can also trigger epithelial-to-mesenchymal transition, thereby fostering resistance to various therapies [34].

PTPN9 occupies a previously unrecognized position within this network: substrate-trapping proteomics and high-resolution crystallography established its direct engagement with the IGF1R activation loop. At the same time, enzymatic assays pinpointed Asp335 as a vital catalytic residue for selective dephosphorylation at Y1166. Although other phosphatases such as PTP1B and SHP2 are well-documented regulators of receptor tyrosine kinase activity [35-37], the tumor-suppressive functions of PTPN9 have mainly been studied in metabolic disorders [38]. Our results therefore identify reduced PTPN9 function as a novel mechanism that permits unchecked IGF1R signaling in cholangiocarcinoma.

Surufatinib targets VEGFR1-3, FGFR1, and CSF1R [39, 40] and has demonstrated sustained clinical benefit in neuroendocrine neoplasms [41]. Using gradual dose escalation, we generated the resistant QBC-939/SR line, which exhibited persistent hyperactivation of IGF1R and its downstream Akt and Erk effectors even under conditions that fully suppressed VEGFR signaling. Parallel immunohistochemical examination of patient samples obtained at disease progression on imaging confirmed this molecular pattern, establishing IGF1R reactivation as a relevant clinical escape pathway. Comparable IGF1R-mediated bypass has been reported following prolonged EGFR blockade in CCA [42] and after FGFR inhibition in fusion-driven models [43, 44], indicating shared adaptive strategies across different TKI treatments.

PTPN9 appears to intersect with multiple receptor tyrosine kinase pathways targeted by clinically used inhibitors. In breast carcinoma models, PTPN9 directly dephosphorylates EGFR and ErbB2, thereby dampening STAT3/STAT5 signaling and potentially modulating sensitivity to EGFR-directed agents [45, 46]. During HER2-targeted treatment, PTPN9 controls HER3 phosphorylation, and its downregulation has been linked to trastuzumab resistance, illustrating broader ErbB family interplay [47]. In the vascular endothelium, PTPN9 dephosphorylates VEGFR2 at Tyr1175, thereby limiting VEGF signaling, providing a mechanistic explanation for the efficacy of VEGFR inhibitors such as

sunitinib and sorafenib [48]. Of particular relevance to CCA, PTPN9 dephosphorylates FGFR2 at its activation loop (pY656/657) and cooperates with the FGFR inhibitor pemigatinib, suggesting a general role in FGFR-TKI responsiveness [30]. Additionally, EGFR blockade can suppress PTPN9 expression, leading to compensatory STAT3 activation; simultaneous inhibition of EGFR and STAT3 (for example, erlotinib combined with niclosamide) reverses resistance in preclinical systems, further implicating a PTPN9–STAT3 regulatory node that may apply across multiple TKI contexts [49].

From a therapeutic perspective, we show that the dual IR/IGF1R inhibitor linsitinib re-sensitized resistant cells in culture and acted synergistically with surufatinib to shrink tumors and lower proliferation markers in orthotopic models. Prior early-phase studies have documented acceptable safety for linsitinib in advanced solid cancers [50], making this combination clinically feasible. Reintroduction of PTPN9 similarly reduced IGF1R phosphorylation and countered the pro-invasive signals from CAF-derived IGF1 or surufatinib exposure, supporting its potential utility as both a predictive biomarker and a functional modulator.

Taken together, these results provide a refined model of TKI resistance in CCA. Instead of relying solely on “on-target” genetic alterations, our data emphasize the combined influence of stromal ligand production and phosphatase dysregulation in reactivating alternative survival pathways. This framework mirrors resistance patterns observed with FGFR2 inhibitors, in which feedback activation of EGFR or secondary mutations limit durability [43]. Strategically, simultaneous or sequential blockade of the IGF1R axis could therefore prevent multiple adaptive routes, an approach that is already showing promise in other malignancies.

Clinically, our observations advocate for individualized treatment strategies in cholangiocarcinoma. Baseline assessment of IGF1R phosphorylation and PTPN9 levels by immunohistochemistry on diagnostic biopsies could help identify patients most likely to respond to IGF1R-targeted combinations, thereby guiding biomarker-enriched trial enrollment. Given the generally tolerable safety profiles of both linsitinib and surufatinib in biliary cancers, prospective combination studies are justified. Such trials might also investigate concurrent inhibition of other stroma-derived factors, such as HGF or IL-6, to further augment efficacy. Non-invasive monitoring of circulating tumor DNA for IGF1R pathway reactivation,

combined with serial blood measurements of stromal IGF1, could enable early detection of resistance and prompt regimen adjustment.

Several limitations of the current work should be noted. Resistance mechanisms were primarily characterized in a single drug-adapted cell line, potentially underrepresenting the full spectrum of clinical escape pathways. Although linsitinib potently blocked IGF1R in our systems, its cross-reactivity with the insulin receptor raises concerns for metabolic toxicities in patients. Future development of more selective IGF1R degraders may mitigate these off-target effects and improve the therapeutic window. In conclusion, this research characterizes a previously underappreciated CAF-derived IGF1/PTPN9/IGF1R signaling network as an important contributor to cholangiocarcinoma progression and surufatinib resistance. Loss of PTPN9 removes critical negative regulation of IGF1R, sustaining oncogenic signaling and undermining TKI effectiveness. Crucially, simultaneous blockade of this axis through the combination of linsitinib and surufatinib produces marked antitumor synergy in preclinical models (**Figure 7**), offering a viable strategy to overcome resistance and achieve more durable responses. This dual-targeting approach addresses shortcomings of single-agent therapy while exploiting a key vulnerability in tumor-stroma interactions. Prospective biomarker-driven clinical trials that incorporate both cancer cell-intrinsic (IGF1R phosphorylation) and microenvironmental (PTPN9 expression) markers will be essential for patient selection and treatment optimization. Overall, these insights provide a strong foundation for evaluating the linsitinib–surufatinib regimen as a precision-medicine approach to improve outcomes in patients with biliary tract cancers.

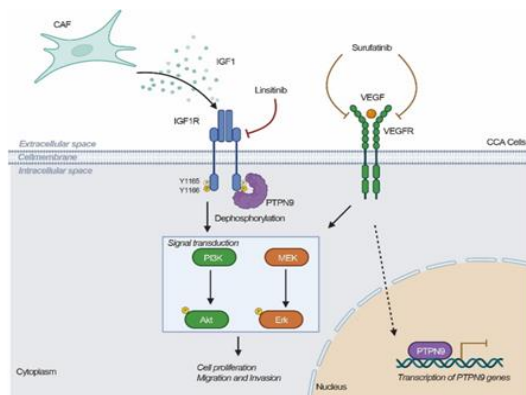


Figure 7. Schematic representation of the CAF-secreted IGF1/PTPN9-IGF1R regulatory network

and its involvement in cholangiocarcinoma (CCA) advancement and drug resistance.

In the tumor microenvironment, cancer-associated fibroblasts (CAFs) release substantial quantities of IGF1. This ligand binds to and activates IGF1R on the surface of CCA cells, leading to phosphorylation of tyrosine residues Y1165/1166. The activated receptor then triggers downstream PI3K/Akt and MEK/Erk cascades that enhance cell proliferation, motility, and invasive capacity. The protein tyrosine phosphatase PTPN9 physically associates with IGF1R and selectively dephosphorylates Y1165/1166, thereby acting as a brake on IGF1R signaling. Surufatinib inhibits VEGFR to suppress tumor growth, and emerging data indicate that VEGFR signaling may influence PTPN9 levels.

Conclusion

Nevertheless, alternative activation of IGF1R serves as a major route of therapeutic escape. Reduced PTPN9 expression further amplifies IGF1R-mediated oncogenic signals and promotes resistance to surufatinib. Combined inhibition of IGF1R using linsitinib reinstates sensitivity to the TKI.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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