

Original Article

Cross-cohort prioritization of TTC36, NPY1R and VIPR1 and characterization of VIPR1-associated phenotypes in hepatocellular carcinoma

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Abstract: Portal vein tumor thrombosis (PVTT) is a clinically important form of vascular extension in hepatocellular carcinoma (HCC). We reanalyzed two independent paired transcriptomic cohorts, GSE77509 and GSE69164, to identify reproducible HCC-associated and PVTT-associated candidate signals, and then examined VIPR1 in a separate cell-based follow-up. TTC36, NPY1R and VIPR1 were consistently lower in primary HCC than in paired adjacent liver. The HCC-to-PVTT contrast was smaller and less uniform; NPY1R showed the most consistent decrease across both cohorts, whereas principal-component analysis showed substantial overlap between HCC and PVTT. In the independent VIPR1 arm, restoration of VIPR1 in PVTT-derived CSQT-2 cells was accompanied by fewer colonies, reduced wound closure and lower Matrigel passage, together with a shifted single-plate lenvatinib curve, altered lipid-associated measurements and a larger reactive oxygen species (ROS)-associated high-fluorescence fraction. These cell experiments describe a VIPR1-associated phenotype in one model and do not establish that VIPR1 drives PVTT or that the tissue, lipid and pharmacologic observations form a causal sequence. The study therefore separates PVTT candidate discovery from VIPR1-focused functional hypothesis generation.

Keywords: Hepatocellular carcinoma, portal vein tumor thrombosis, tetratricopeptide repeat domain 36 (TTC36), neuropeptide Y receptor Y1 (NPY1R), vasoactive intestinal peptide receptor 1 (VIPR1), lipid metabolism, lenvatinib

Introduction

Hepatocellular carcinoma (HCC) remains a major cause of cancer mortality worldwide and is expected to impose an increasing clinical burden in the coming decade [1]. Curative-intent resection, ablation and transplantation have improved outcomes for selected patients, but recurrence and intrahepatic dissemination remain frequent. Portal vein tumor thrombosis (PVTT) is one of the most consequential manifestations of vascular invasion because it worsens portal flow, restricts local treatment options and is associated with rapid clinical deterioration [2-5]. A molecular framework that distinguishes reproducible HCC-associated signals from less consistent PVTT-associated changes could inform candidate prioritization and focused functional follow-up.

PVTT is biologically heterogeneous. Prior tissue and transcriptome studies have implicated extracellular-matrix remodeling, metabolic adaptation, cell adhesion, autophagy and vascular dissemination, but the consistency of individual gene-level signals across cohorts has been variable [4, 6-8]. Small paired datasets are particularly valuable because they reduce between-patient confounding, although they require careful interpretation of both effect size and multiplicity-adjusted evidence. In this setting, reproducibility across independently generated paired cohorts is more informative than an isolated ranked gene list.

TTC36, NPY1R and VIPR1 are plausible candidates for further study. TTC36 has been reported with context-dependent roles in HCC growth and treatment response [9, 13]. NPY1R participates in neuropeptide signaling and has diver-

gent associations across hepatic disease and malignancy [15-20]. VIPR1 is a G-protein-coupled receptor whose transcription can be constrained by epigenetic regulation in HCC, and experimental activation or restoration has been linked to reduced tumor-cell growth in prior studies [21-23]. These observations provide biological context but do not establish their role in PVTT.

Here, we used two linked but analytically separate stages. First, paired public cohorts were reanalyzed to describe reproducible HCC-associated signals and to identify which candidates showed the most consistent HCC-to-PVTT behavior. Second, the available CSQT-2 records were used for a focused VIPR1 perturbation study. The two stages were not treated as a causal chain: tissue composition, supra-physiologic receptor expression, changes in cell number and baseline growth can each influence the observed readouts. The study is therefore an evidence-grounded candidate framework plus a model-specific functional phenotype, rather than a mechanistic demonstration.

Materials and methods

Study design and public datasets

GSE77509 and GSE69164 were obtained from the Gene Expression Omnibus (GEO). GSE77509 contains 60 samples from 20 patients, with adjacent non-tumor liver, primary HCC and PVTT available as matched tissue classes. GSE69164 contains 33 samples from 11 patients with the same three tissue classes. The datasets were used as independent paired cohorts. No new human specimens were collected for this study.

Cross-cohort paired expression analysis

For both cohorts, expression values were transformed as $\log_2(\text{expression} + 1)$. Genes were retained when the mean normalized count was at least 10 in GSE77509 or the mean fragments per kilobase of transcript per million mapped reads (FPKM) was at least 1 in GSE69164. For each patient, paired contrasts were calculated for HCC versus adjacent tissue and PVTT versus primary HCC. Two-sided paired t-tests were applied to the transformed values. Nominally altered genes were defined

for visualization as $P < 0.05$ with absolute $\log_2$ fold change > 0.58 ; these thresholds were used for exploratory display and did not by themselves determine functional selection. Benjamini-Hochberg adjusted P values were exported for each contrast. Candidate-gene effect estimates, confidence intervals and cohort-specific P values were retained with the analysis output. The public analyses are exploratory and are not presented as prospective diagnostic validation.

Candidate evaluation, sample structure and retrospective displays

TTC36, NPY1R and VIPR1 were retained as HCC-associated candidates because each was lower in primary HCC than in paired adjacent tissue in both cohorts. NPY1R was the most consistent candidate for the HCC-to-PVTT contrast because its paired decrease was observed in both datasets. VIPR1 was selected for the separate functional arm because its HCC-associated decrease was reproducible, its PVTT point estimates were negative, retrospective normal-versus-tumor discrimination was observed, and an experimentally available PVTT-derived model was present. This selection does not imply that VIPR1 is a PVTT-specific marker or that it drives the public-tissue pattern. Candidate-level displays included paired trajectories, cohort-specific mean changes with 95% confidence intervals and principal-component analysis of all samples using the 1,000 most variable eligible features within each dataset. The principal components were used to examine global sample structure rather than to define a classifier.

Retrospective receiver operating characteristic curves and their reported 95% confidence intervals were reproduced from the supplied normal-versus-tumor analysis set. The supplied survival, clinicopathologic and three-cell-line qPCR panels were retained to show the context in which the candidates had originally been selected. Individual-level clinical records were not available in the current archive for recalculation of those panels; their original annotations were therefore preserved without adding new inferential claims. The ROC curves describe retrospective separation in the analyzed material and are not prospective diagnostic validation.

Exploratory immune and metabolic-context analyses

The supplied candidate-high versus candidate-low immune-transcript displays are retained as exploratory discovery context. They are analytically separate from the VIPR1 perturbation experiments and are not used to support a VIPR1 drug or pathway mechanism.

For a separate VIPR1-focused analysis, Spearman correlations were calculated across 22 GSE69164 tumor specimens between VIPR1 and 12 prespecified lipid-metabolism genes. The genome-wide rank included 12,862 eligible transcripts. The network display used an absolute rho threshold of 0.50, and Benjamini-Hochberg adjustment was applied to the pre-specified gene set. These correlations guided metabolic follow-up but did not establish direct regulation.

Data provenance and reproducibility safeguards

Computational and laboratory records were kept separately from the assembled manuscript figures. Recomputed panels retain their numerical source tables, sample-class structure and analysis settings. Supplied legacy survival, clinical, immune and baseline qPCR panels were retained as originally labeled when their individual-level source was not present in the current archive; they were not used to create additional numerical claims. Experimental figures were rebuilt from the available plate workbooks, image files, densitometry values and instrument exports. This distinction between recalculated and retained panels defines the reporting boundary of the study.

This provenance-first approach is especially relevant to PVTT studies, where a limited number of tissue pairs, heterogeneous platforms and diverse experimental systems can otherwise encourage overinterpretation. The two GEO datasets differ in sample number and expression platform, but both retain matched tissue classes. We therefore report the cohort-specific effects in parallel and do not merge them into a single inferred patient-level population. The public figures also retain effect estimates and confidence intervals so that significance is not evaluated as a binary label alone.

Cell model and perturbation strategy

CSQT-2 cells were used because the archived experiments tested VIPR1 restoration in this PVTT-derived line. Cells received a human VIPR1 expression construct or the corresponding empty vector. Sequence verification showed that the construct matched the expected coding sequence. This design tested the consequences of increased VIPR1 expression but did not assess whether endogenous VIPR1 was required.

Image and immunoblot processing

Representative colony, scratch, Transwell, confocal and immunoblot images came from the archived experimental records. A pre-stained molecular-weight marker (10-200 kDa; ServoBio, G2058) was run alongside the samples, and cropped bands were aligned according to marker positions. Full-length original membranes are retained in the project archive. For VIPR1 immunoblotting, VIPR1 intensity in three Vector and three VIPR1-OE lanes was normalized to β -actin. Lipid-metabolism lanes remained in the recorded order (V1, OE1, V2, OE2, V3 and OE3). Because lanes did not represent independent experiments, densitometry was summarized descriptively.

Boron-dipyrromethene (BODIPY) 493/503 fluorescence was quantified at the culture level from the available within-experiment observations; the images were not normalized to DAPI-positive nuclei, cell number, total protein or DNA. The flow cytometric panel reports the fraction within the M4-2 high-fluorescence region recorded in the source export. The original histogram exports consistently label this region M4-2; the processed spreadsheet contains a discrepant M2-2 column label. The original export label was therefore used. Because flow-cytometry standard (FCS) files were not present in the archive, the endpoint is described operationally as ROS-associated fluorescence and was not re-gated or assigned to a specific reactive oxygen species.

Drug-response and biochemical calculations

The lenvatinib experiment used a concentration series from 0 to 30 micromolar and CCK-8 absorbance after 48 h. The upper concentrations were selected to capture the complete

inhibitory profile in this cellular model and were not intended to mimic clinical steady-state exposure. The dose-response display uses technical-well means and a constrained Hill curve. The reported IC₅₀ values are nominal fitted parameters from the recorded plate. Such parameters are useful for describing the location of each observed curve, but a single plate cannot provide a valid estimate of inter-experiment variability. Accordingly, no *P* value, confidence interval for the difference, or claim of proven sensitization is reported.

Free fatty acid (FFA), total cholesterol (TC) and triglyceride (TG) values were interpolated from the available calibration standards and reported in the resulting units. The source record did not document normalization to viable-cell number, total protein or DNA. These values therefore describe the recorded cultures, not absolute intracellular concentrations. Variation in cell density or recovery may have affected the bulk measurements.

CSQT-2 cells were maintained in MEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in 5% CO₂. The archived experimental record identifies CSQT-2 as a PVTT-derived human HCC model; supplier, STR-authentication and mycoplasma-testing records were not included in the transferred archive. Cells were transfected with the human VIPR1 construct ZF-KL-04563 (NM_004624.4; 1,374-bp coding sequence) in vector ZBFV-023 (CMV-MCS-SV40-Neomycin; BamHI/EcoRI) or the corresponding empty vector using SignaGen transfection reagent (catalogue no. 61776). Sequence files showed a complete match to the expected coding sequence.

VIPR1 transcript abundance was assessed using an All-in-One first-strand synthesis kit, Taq SYBR premix and a QuantReady K9600 instrument. Primer sequences were VIPR1 forward 5'-TCATCCGAATCCTGCTTCAGA-3' and reverse 5'-AGGCGAACATGATGTAGTGTACT-3', and β -actin forward 5'-ACCCAGATCATGTTTGAGACC-3' and reverse 5'-GAGGGCATACCCCTCGTAGA-3'. Cycling comprised 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 30 s. Relative expression was calculated using the 2^{- $\Delta\Delta$ Ct} method. The three values per group were technical wells from one qPCR run and were summarized descriptively.

For immunoblotting, 30 μ g total protein was separated by SDS-PAGE and transferred to PVDF membranes. Membranes were probed with anti-VIPR1 (Proteintech, 14878-1-AP; 1:1,000) and anti- β -actin (GenuIN, 3784; 1:5,000), followed by appropriate HRP-conjugated secondary antibodies. Bands were detected by enhanced chemiluminescence and quantified using Fusion software. VIPR1 intensity was normalized to the β -actin signal in the same lane. The three recorded lane values per condition were summarized descriptively.

Approximately 200 transfected CSQT-2 cells were seeded in each well of a 12-well plate. After 10 days, colonies were fixed with paraformaldehyde, stained with 0.1% crystal violet for 10 min and counted. The resulting colony counts were summarized descriptively as within-experiment observations.

For scratch-wound assays, confluent monolayers were scratched, washed and maintained in serum-free medium. The same wound regions were imaged at 0, 24 and 48 h. Fractional wound closure was calculated separately for each well using that well's own 0-h width as the denominator: (width at 0 h - width at time *t*)/width at 0 h. Because serum-free conditions may affect viability and proliferation and neither was independently controlled, this endpoint is reported as wound closure rather than migration rate. The three within-experiment observations per condition were summarized descriptively.

For invasion assays, 24-well Transwell inserts (Corning, 3422) were coated with 100 μ L Matrigel at 1 mg/mL for 4-5 h at 37°C. A 100- μ L aliquot of a 2×10^5 cells/mL suspension was placed in the upper chamber and 600 μ L complete medium containing 20% fetal bovine serum in the lower chamber. After 24 h, invaded cells were fixed with paraformaldehyde, stained with 0.1% crystal violet and imaged at 100 $\times$. Three replicate-well values per condition were summarized descriptively.

CSQT-2 cells were seeded in 96-well plates at 5,000 cells per well and exposed for 48 h to 0, 0.5, 1, 2.5, 5, 10, 20 or 30 μ M lenvatinib. Cell viability was measured with Cell Counting Kit-8 (CCK-8) reagent (Servicebio, G4103-5mL) at 450 nm. Each concentration was measured in

three technical wells on one plate and analyzed descriptively.

Dose-response curves were fitted by nonlinear least squares to $Y = 100/[1 + (X/IC_{50})^h]$, with the upper and lower asymptotes constrained to 100% and 0% and $\log_{10}(IC_{50})$ and the Hill slope estimated. The vehicle-control point was offset only for display on a symmetric-logarithmic axis. IC50 values are descriptive nominal estimates from a single experiment; no inferential between-group comparison was performed.

For lipid-droplet imaging, cells were fixed with 4% paraformaldehyde for 15 min, stained for 15 min with BODIPY 493/503 from a lipid-droplet fluorescence kit (Beyotime, C2053S), counterstained with DAPI and imaged by confocal microscopy. Three culture-level fluorescence observations per condition were summarized descriptively.

For the oxidative-stress-associated readout, cells were incubated with the DCFH-DA-based ROS assay kit (Solarbio, CA1410) and analyzed by flow cytometry. The percentage of events within the source-exported M4-2 high-fluorescence region was recorded and reported operationally as ROS-associated fluorescence. Three within-experiment percentage values per condition were summarized descriptively. The archived source comprised histogram exports and the processed percentage table, but not FCS files; consequently, the gate could not be re-applied or re-analyzed.

Proteins were extracted in RIPA buffer containing PMSF, quantified using a BCA assay and analyzed by immunoblotting as above. Primary antibodies were FASN (Proteintech, 10624-2-AP; 1:5,000), SCD1 (Proteintech, 28678-1-AP; 1:5,000), CPT1A (Proteintech, 15184-1-AP; 1:5,000) and β -actin (GenuIN, 3784; 1:5,000). Band intensities were normalized to β -actin. The three displayed lane values per condition were summarized descriptively.

FFA, TC and TG were measured with Amplex Red kits (Beyotime, S0215S, S0211M and S0219M), and absorbance was read at 570 nm using an ALLSHENG AMR-100 reader. Readings were interpolated from calibration standards spanning 0-100 μ M for FFA, 0-500 μ M for TC and 0-10 mM for TG. Calibration-derived readings were reported as culture-level values with-

out normalization to viable-cell number, total protein or DNA and were summarized descriptively.

Statistical analysis

Paired t tests were two-sided with $\alpha = 0.05$, and Benjamini-Hochberg adjustment was applied where specified. Separate paired contrasts were used because the prespecified biological questions concerned the adjacent-liver-to-HCC and HCC-to-PVTT transitions; an omnibus repeated-measures ANOVA would not replace these direction-specific contrasts and was not used for candidate prioritization. R 4.3.0 was used with DESeq2 1.36.0, ggplot2 3.4.4, pROC 1.18.0 and the other packages specified in the analysis record; Python 3 with NumPy, pandas, SciPy and Matplotlib was used for descriptive calculations and figure preparation. Retrospective ROC values and legacy panel annotations were retained from the supplied analyses; no new P values were calculated without the underlying individual-level record. Experimental values are shown as mean $\pm$ standard deviation (SD) when several within-experiment observations were available. Technical wells, image fields and immunoblot lanes were not treated as independent biological replicates. The VIPR1 qPCR validation represents one run, and the lenvatinib response represents one plate with technical triplicates per concentration. Laboratory endpoints without documented independent biological replication were summarized descriptively and are displayed without confirmatory significance stars.

Results

Cross-cohort analysis separated reproducible HCC-associated signals from less consistent PVTT changes

The paired analysis comprised 20 patients in GSE77509 and 11 in GSE69164, each with adjacent liver, primary HCC and PVTT samples. After filtering, 17,054 and 12,862 genes were eligible, respectively. For the PVTT-versus-HCC comparison, 237 genes in GSE77509 and 281 in GSE69164 met the nominal display rule of $P < 0.05$ and absolute $\log_2$ fold change > 0.58 (**Figure 1A** and **1B**). The cohort heatmaps are feature-selected descriptive displays of the paired contrasts and are not used as transcrip-

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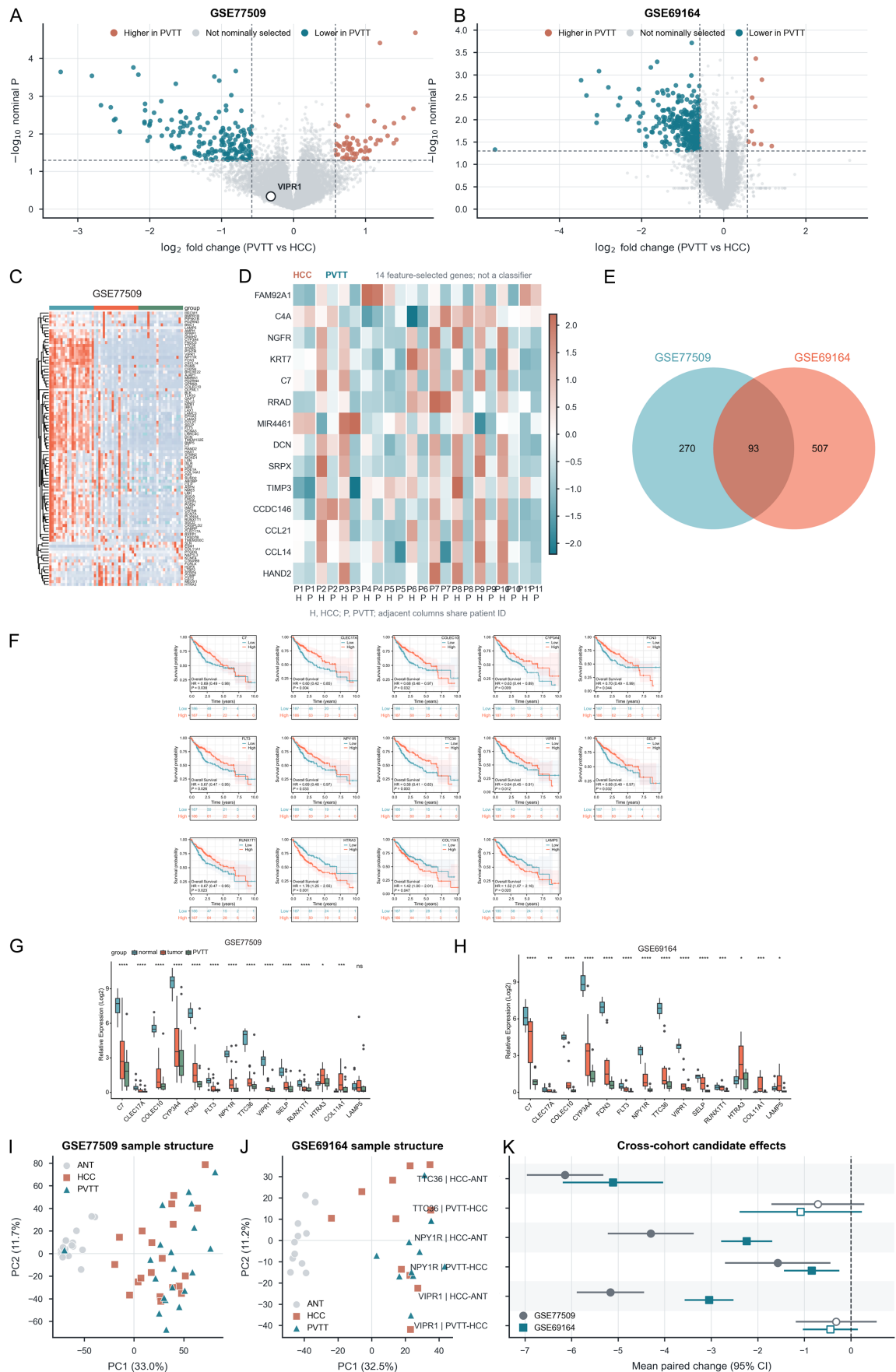


Figure 1. Cross-cohort discovery of reproducible HCC-associated signals and less uniform PVTT-associated changes. A, B. Paired PVTT-versus-HCC differential displays; genes meeting the nominal display rule of $P < 0.05$ and $|\log_2 \text{fold change}| > 0.58$ are highlighted. C. Supplied GSE77509 cohort heatmap. D. GSE69164 paired display of 14 feature-selected genes across 11 matched HCC/PVTT pairs, shown as row z-scores and ordered by patient; this descriptive panel is not a transcriptome-wide classifier. E, F. Supplied overlap and survival panels. In the supplied panels, group comparisons and survival differences are annotated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ and ns, not significant. G, H. Supplied tissue-expression panels; two-group comparisons are annotated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ and ns, not significant. I, J. Principal-component analysis (PCA) using the 1,000 most variable eligible features. K. Cross-cohort candidate effects with 95% confidence intervals.

tome-wide classification evidence (**Figure 1C** and **1D**). In particular, **Figure 1D** presents the supplied GSE69164 row z-scores for 14 selected genes across 11 patient-matched HCC/PVTT pairs, ordered by patient. The supplied cross-cohort overlap analysis identified 93 shared entries between GSE77509 and GSE69164 (**Figure 1E**). Retrospective overall survival curves for selected genes were also retained as exploratory clinical context (**Figure 1F**). These survival analyses were not recalculated because individual-level clinical data were unavailable.

TTC36, NPY1R and VIPR1 were markedly lower in primary HCC than in paired adjacent tissue in both datasets. Mean paired changes for TTC36 were -6.14 in GSE77509 and -5.11 in GSE69164; corresponding values were -4.30 and -2.24 for NPY1R and -5.16 and -3.05 for VIPR1. All six HCC-versus-adjacent contrasts remained significant after multiple-test adjustment. The tissue-expression panels and the effect-size summary show the same direction across platforms (**Figure 1G, 1H** and **1K**).

Changes between primary HCC and PVTT were smaller. NPY1R decreased in PVTT in both cohorts (mean paired changes, -1.57 and -0.84; both nominal $P < 0.05$), whereas the TTC36 and VIPR1 intervals crossed zero. VIPR1 nevertheless retained negative point estimates in GSE77509 (-0.32) and GSE69164 (-0.44). Principal-component analysis separated adjacent liver from most tumor samples, while HCC and PVTT occupied overlapping regions (**Figure 1I** and **1J**). This overlap is negative evidence against a universal transcriptome-wide separation of primary HCC from PVTT in these datasets. The dominant reproducible signal was the adjacent-liver-to-HCC transition, with a gene-specific and less uniform HCC-to-PVTT component.

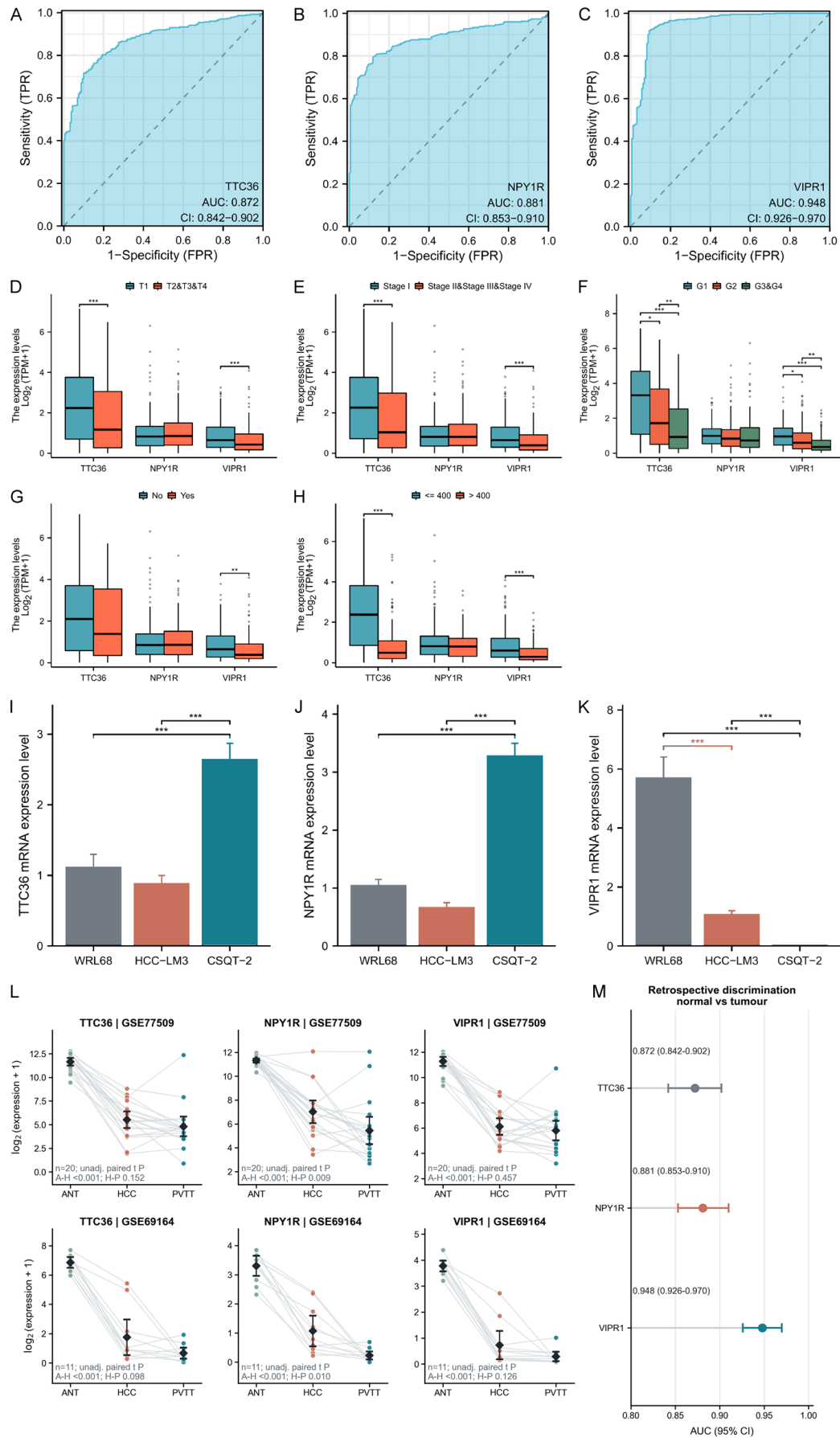
Candidate context motivated a separate VIPR1 functional arm

The supplied normal-versus-tumor ROC curves yielded AUC values of 0.872 for TTC36, 0.881 for NPY1R and 0.948 for VIPR1, with the displayed confidence intervals remaining above 0.80 (**Figure 2A-C** and **2M**). These estimates show retrospective separation in the analyzed data and do not establish a deployable diagnostic test. The supplied clinical subgroup panels are presented without recalculation because the individual-level table was not present in the current archive (**Figure 2D-H**).

The retained baseline qPCR panels placed TTC36 and NPY1R relatively high in CSQT-2, whereas VIPR1 was lowest in this PVTT-derived line compared with the other displayed cell models (**Figure 2I-K**). Patient-paired trajectories confirmed the large decrease from adjacent liver to HCC for all three candidates and the less consistent HCC-to-PVTT step (**Figure 2L**). Taken together, the tissue and cell-line context favored VIPR1 restoration as a focused perturbation rather than treating all three candidates as experimentally validated targets.

Figure 3A-C retain supplied candidate-context immune-transcript displays for TTC36, NPY1R and VIPR1. **Figure 3D-F** present a separate VIPR1 metabolic-context analysis of 22 GSE69164 tumor specimens: a genome-wide Spearman correlation rank, a network of gene pairs with $|\rho| \geq 0.50$, and correlations for 12 prespecified lipid-metabolism genes. The genome-wide rank is descriptive; Benjamini-Hochberg adjustment was restricted to the prespecified 12-gene set. These panels are hypothesis-generating and do not establish direct regulation or experimental drug sensitivity.

VIPR1-associated phenotypes in HCC



VIPR1-associated phenotypes in HCC

Figure 2. Retrospective candidate evaluation and supplied validation context. (A-C) Supplied normal-versus-tumor receiver operating characteristic (ROC) curves for TTC36, NPY1R and VIPR1. (D-H) Supplied clinicopathologic subgroup plots. (I-K) Supplied baseline quantitative polymerase chain reaction (qPCR) comparisons across WRL68, HCC-LM3 and CSQT-2 cells. The supplied annotations in (D-K) use the original thresholds: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (L) Patient-paired adjacent non-tumor liver (ANT), hepatocellular carcinoma (HCC) and portal vein tumor thrombosis (PVTT) trajectories for all three candidates in GSE77509 ($n = 20$) and GSE69164 ($n = 11$); gray lines connect tissues from the same patient, colored points are individual observations and black diamonds show means with 95% confidence intervals. Displayed P values are two-sided unadjusted paired t tests. (M) Area under the ROC curve (AUC) and supplied 95% confidence intervals. ROC results describe retrospective discrimination and are not prospective diagnostic validation.

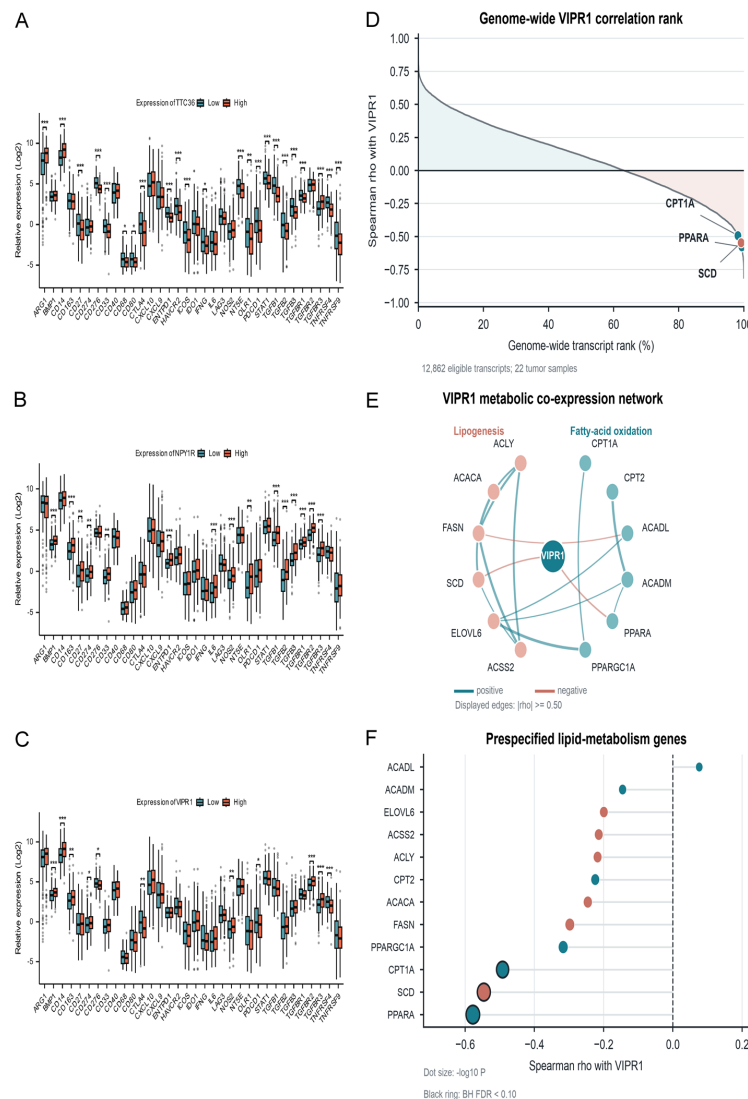


Figure 3. Exploratory candidate context and separate VIPR1 metabolic co-expression. (A-C) Supplied candidate-high versus candidate-low immune-transcript distributions for TTC36, NPY1R and VIPR1, respectively; the supplied group comparisons are annotated as follows: * $P < 0.1$, ** $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ and ns, not significant. (D) Descriptive genome-wide Spearman correlation rank between VIPR1 and each of the 12,862 eligible GSE69164 transcripts; rho denotes the Spearman rank correlation coefficient, and the rank is expressed as the percentile posi-

tion of each transcript within the genome-wide list. (E) VIPR1 metabolic co-expression network in which nodes denote genes and edges denote Spearman correlations with VIPR1; only edges with $|\rho| \geq 0.50$ are displayed, and edge colour distinguishes positive from negative correlations as indicated in the panel key. (F) Spearman rho between VIPR1 and each of 12 prespecified lipid-metabolism genes across 22 GSE69164 tumor specimens; point size indicates $-\log_{10}$ of the nominal P value, and a black ring indicates FDR (false discovery rate, Benjamini-Hochberg adjusted) < 0.10 , with the adjustment restricted to the prespecified 12-gene set. (D-F) are exploratory and do not establish direct regulation or experimental drug sensitivity.

VIPR1 transcript and protein signals increased after VIPR1 overexpression

Amplification and melt profiles were consistent with the recorded VIPR1 and β -actin assays (Figure 4A and 4B). In one qPCR run, the VIPR1-OE condition produced a 4.75-fold higher normalized VIPR1 signal than Vector cells (4.763 ± 0.843 versus 1.002 ± 0.073 relative units; three technical wells per condition; Figure 4C). Across the three displayed lanes, the VIPR1/ β -actin ratio was 0.883 ± 0.051 in VIPR1-OE and 0.621 ± 0.024 in Vector cells (Figure 4D and 4E). These observations confirm higher

VIPR1-associated phenotypes in HCC

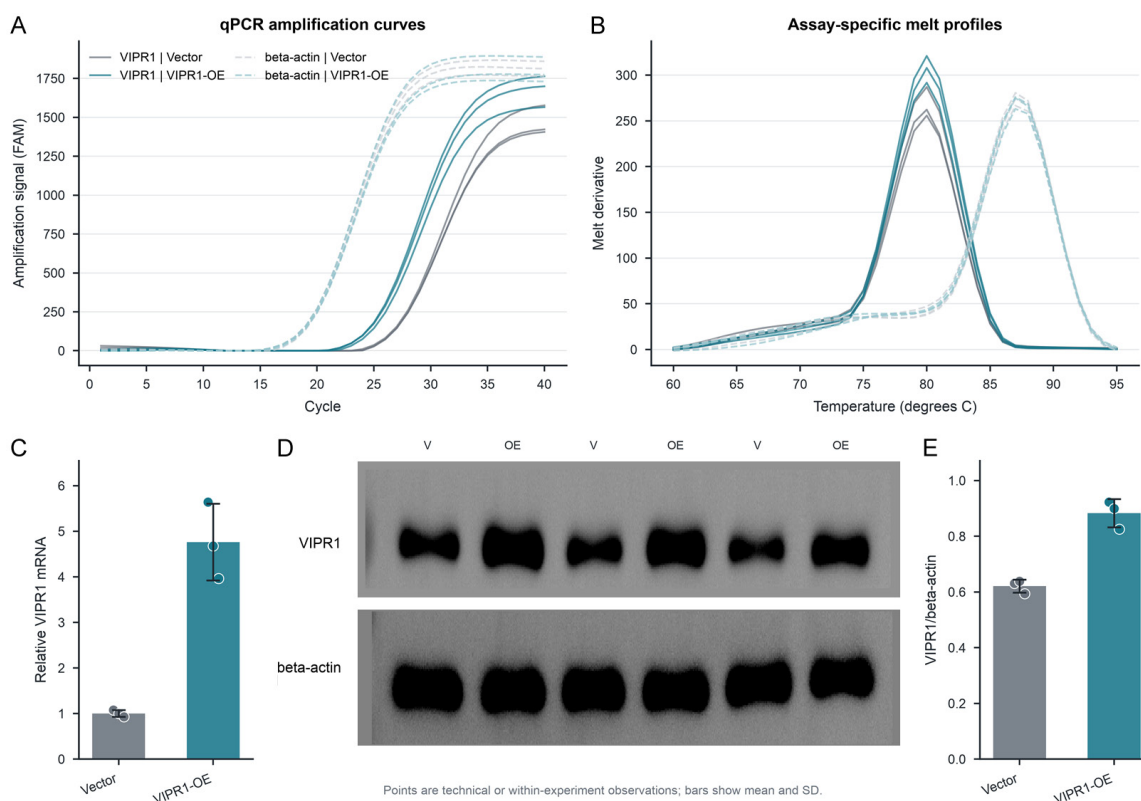


Figure 4. Molecular validation of VIPR1 overexpression in CSQT-2 cells. A. Recorded quantitative polymerase chain reaction (qPCR) amplification curves for VIPR1 and beta-actin. B. Assay-specific melt profiles. C. Relative VIPR1 messenger RNA in Vector and VIPR1-overexpression (VIPR1-OE) cells; points are technical wells from one qPCR run. D. Cropped VIPR1 and beta-actin immunoblots with the displayed lane order Vector (V), VIPR1-OE (OE), V, OE, V, OE; pre-stained 10-200-kDa markers were run alongside the samples and full-length membranes are archived. E. VIPR1/beta-actin densitometry for the three displayed lanes per condition. Bars show mean and standard deviation (SD). The points are technical or within-experiment observations; no experiment-level inferential comparison was performed.

VIPR1 signals in the recorded overexpression experiment without being treated as three independent biological replicates.

VIPR1 overexpression was accompanied by lower malignant-phenotype readouts

Representative colony wells, scratch fields and Transwell fields are shown in **Figure 5**; quantitative summaries use three within-experiment observations per condition. Colony counts were lower in VIPR1-OE than in Vector wells (524.7 ± 189.1 versus $1,255.3 \pm 93.9$; **Figure 5A** and **5B**). Fractional wound closure calculated from each well's own 0-h width was also lower at 24 h (0.069 ± 0.007 versus 0.137 ± 0.016) and 48 h (0.345 ± 0.029 versus 0.515 ± 0.073 ; **Figure 5C** and **5D**). Invaded cells per 100 $\times$ field decreased from $2,212.0 \pm 261.3$ under Vector conditions to 917.3 ± 226.6 after VIPR1 overex-

pression (**Figure 5E** and **5F**). Because the scratch assay used serum-free medium without independent viability or proliferation control, its endpoint is interpreted as wound closure. The concordant directions across the three assays support a less aggressive within-experiment phenotype, while the displayed wells and fields remain descriptive observations.

VIPR1 overexpression shifted the recorded lenvatinib response

Lenvatinib reduced CCK-8 viability with increasing concentration in both conditions after 48 h (**Figure 6A** and **6B**). The constrained Hill curves produced nominal IC₅₀ values of 7.442 μ M for Vector and 2.777 μ M for VIPR1-OE cells, corresponding to a VIPR1-OE/Vector ratio of 0.373 (**Figure 6C**). These values describe one plate with three technical wells at each dose; the

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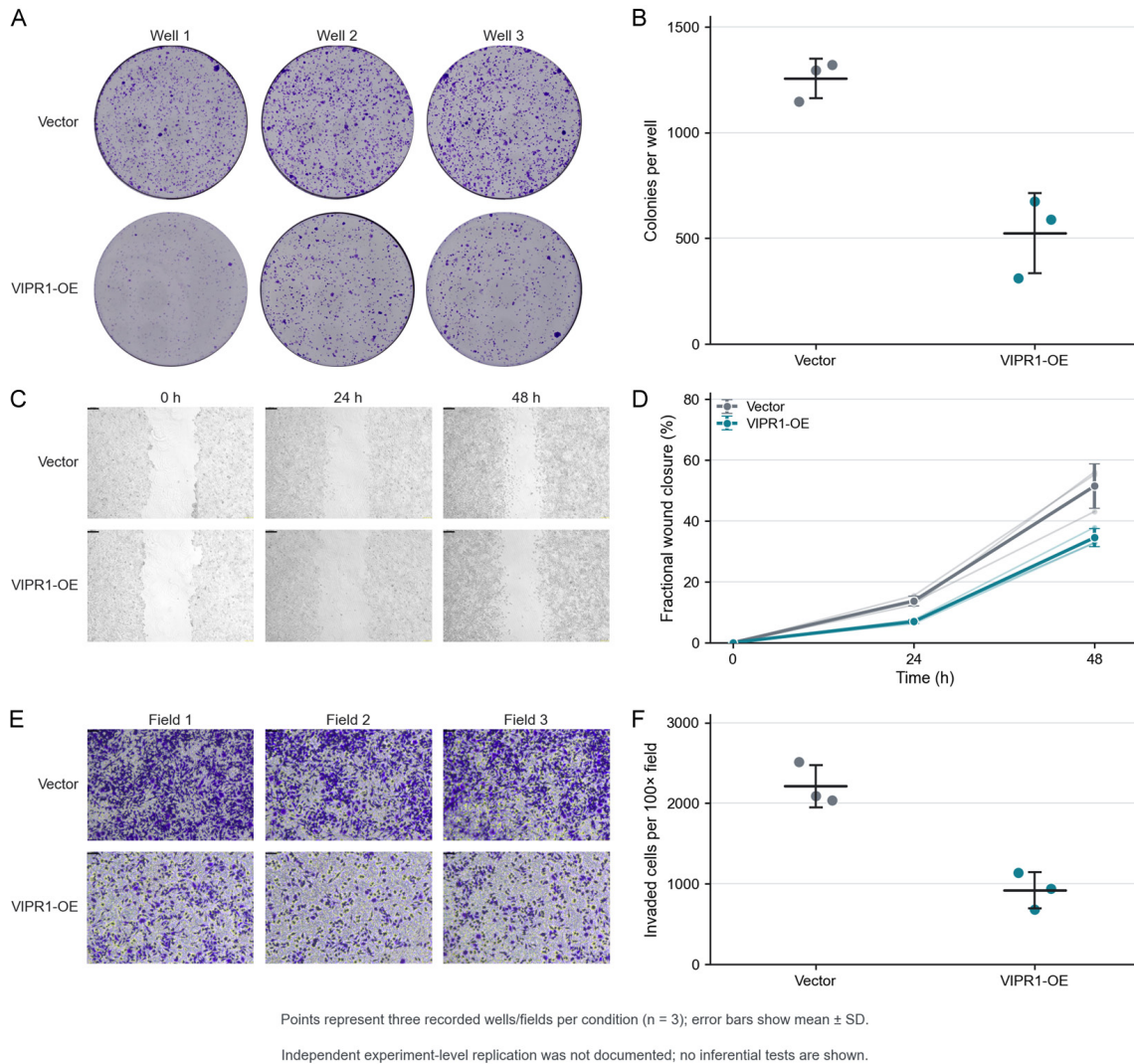


Figure 5. Growth- and invasion-associated readouts after VIPR1 overexpression in CSQT-2 cells. Colony formation (A, B), wound closure calculated from each well's own 0-h width (C, D) and Matrigel-Transwell invasion (E, F) are shown. Panel A contains representative colony wells and panel C representative scratch fields; quantitative summaries use three within-experiment observations per condition. Scratch images were acquired at 4 $\times$ and use the 200 μ m calibration shown in the source image. Transwell images were acquired at 100 $\times$ and use the 57 μ m calibration shown in the source image. Data are mean $\pm$ SD; no experiment-level inferential comparison was performed.

single plate did not permit an experiment-level inference of sensitization.

VIPR1 overexpression was associated with lipid and oxidative-stress changes

Culture-level BODIPY 493/503 fluorescence was lower in VIPR1-OE cultures than in Vector cultures (18.562 ± 1.711 versus 66.656 ± 6.123 arbitrary units; **Figure 7A** and **7B**). The fraction of events in the source-defined high-fluorescence gate was higher after VIPR1 overexpression ($18.410\% \pm 1.538\%$ versus 6.923%

$\pm 0.383\%$; **Figure 7C** and **7D**). The latter endpoint is described operationally as ROS-associated fluorescence because the available record did not support assignment to a specific reactive oxygen species.

Densitometry showed lower FASN (0.576 ± 0.077 versus 0.930 ± 0.121) and SCD1 (0.580 ± 0.079 versus 1.016 ± 0.029), with higher CPT1A (0.932 ± 0.094 versus 0.509 ± 0.096) in VIPR1-OE cells (**Figure 7E-H**). Culture-level calibration-derived readings were also lower for FFA (17.056 ± 1.832 versus 39.305 ± 2.785

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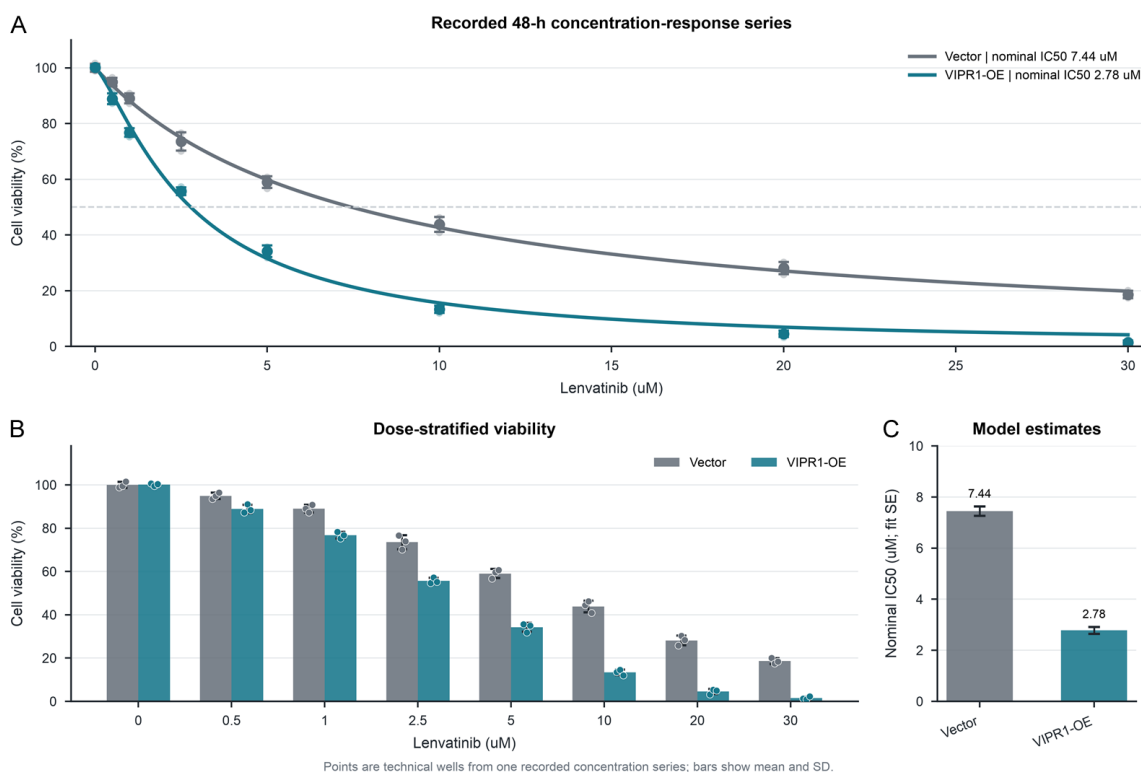


Figure 6. Recorded 48-h lenvatinib concentration-response series in Vector and VIPR1-overexpression (VIPR1-OE) CSQT-2 cells. Technical-well means and constrained Hill curves are shown across 0-30 micromolar lenvatinib (A, B), with nominal half-maximal inhibitory concentration (IC50) estimates from the fitted curves (C). Data are mean and standard deviation (SD; n = 3 technical wells per concentration from one plate); IC50 values are descriptive parameters and no experiment-level inferential comparison was performed.

μM), total cholesterol (105.561 ± 7.795 versus $150.740 \pm 12.076 \mu\text{M}$) and triglyceride (5.173 ± 0.650 versus $8.465 \pm 1.245 \text{ mM}$; **Figure 7I-K**). The biochemical values were not normalized to cell number or protein and therefore describe the recorded cultures, not absolute intracellular pools. Their shared direction with the imaging and protein readouts supports a VIPR1-associated metabolic phenotype without establishing pathway order.

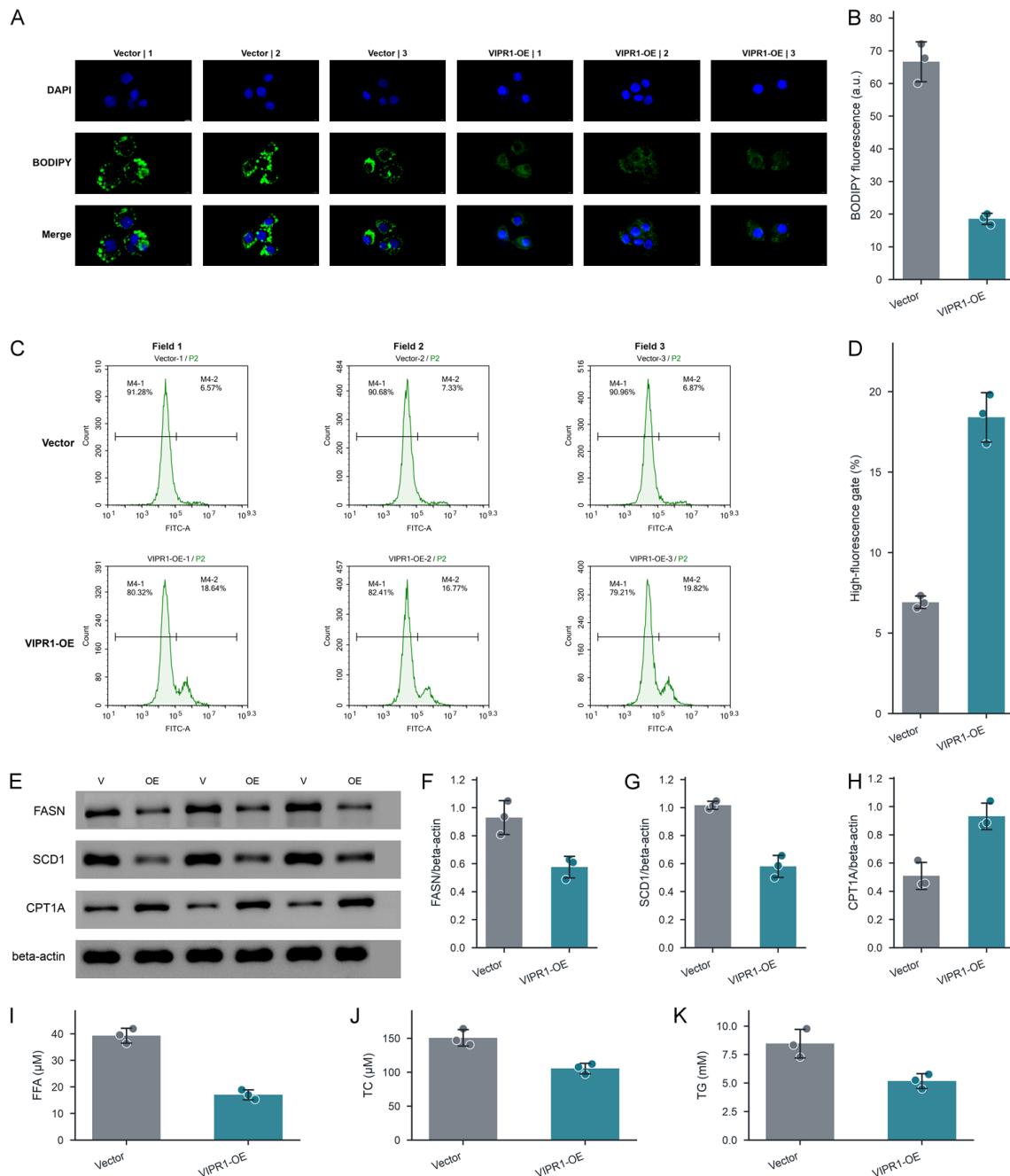
Discussion

PVTT is among the most consequential forms of HCC vascular extension, yet candidate genes drawn from a single small cohort can be difficult to distinguish from platform- or sample-specific effects. We addressed this problem by retaining patient pairing in two public datasets and testing VIPR1 in a PVTT-derived cell line. Three findings defined the main pattern across datasets and experiments. TTC36, NPY1R and VIPR1 decreased consistently from adjacent

liver to HCC, whereas the HCC-to-PVTT changes were smaller. In CSQT-2 cells, VIPR1 restoration coincided with lower colony formation, wound closure, Matrigel passage and lipid-associated readouts. The public cohorts showed the expression pattern, and the cell experiments examined one candidate without conflating the two evidence sources.

Patient pairing reduced the inter-individual variation that can dominate small transcriptomic cohorts. All three genes showed large HCC-versus-adjacent decreases despite differences in platform and cohort size. Principal-component plots, however, separated adjacent liver from tumor more clearly than HCC from PVTT. Candidate-level effects followed the same pattern: the adjacent-liver-to-HCC transition was reproducible, whereas vascular extension produced smaller, gene-dependent changes. TTC36, NPY1R and VIPR1 should therefore be considered HCC-associated candidates, not established markers of PVTT.

VIPR1-associated phenotypes in HCC



Points are technical or within-experiment observations; bars show mean and SD.

Figure 7. Lipid-associated and oxidative-stress readouts after VIPR1 overexpression. Confocal images show 4',6-diamidino-2-phenylindole (DAPI), boron-dipyrromethene (BODIPY) 493/503 and merged channels (A; 100× acquisition; 10 μm scale bar); culture-level BODIPY fluorescence is quantified in (B) without normalization to DAPI-positive nuclei, cell number, total protein or DNA. Flow-cytometric histograms and the source-exported M4-2 high-fluorescence fraction are reported operationally as reactive oxygen species (ROS)-associated fluorescence (C, D). Cropped fatty acid synthase (FASN), stearoyl-CoA desaturase 1 (SCD1), carnitine palmitoyltransferase 1A (CPT1A) and beta-actin immunoblots with beta-actin-normalized densitometry are shown in (E-H); lane order is Vector (V), overexpression (OE), V, OE, V, OE. Calibration-derived free fatty acid (FFA), total cholesterol (TC) and triglyceride (TG) readings are shown in (I-K). Data are mean and standard deviation (SD; n = 3 technical or within-experiment observations); biochemical readings were not normalized to cell number or protein and no experiment-level inferential comparison was performed.

We kept the cohorts separate because GS-E77509 reports normalized counts and GSE-69164 reports FPKM values. Direct pooling of these unlike scales could obscure genuine disagreement between cohorts. Only NPY1R decreased nominally from HCC to PVTT in both cohorts; the TTC36 and VIPR1 intervals crossed zero. VIPR1 was selected because its HCC-associated decrease was reproducible, its PVTT point estimates were negative, and suitable perturbation records were available. Selection for follow-up does not imply that VIPR1 drives portal-vein invasion.

The ROC, survival and clinicopathologic panels provide context but no prospective clinical validation. VIPR1 showed the highest retrospective normal-versus-tumor AUC, although the analyzed datasets were not diagnostic cohorts. We could not recalculate the retained subgroup or survival panels because individual-level clinical data were unavailable. Previous HCC studies have used multigene prognostic and lipid-related biomarker frameworks [10, 14]. Clinical use would still require external validation, prespecified thresholds and evidence beyond established staging variables.

The supplied immune-transcript displays provide limited candidate context and remain separate from the VIPR1 functional arm. The retained VIPR1 correlation panels focus on lipid-metabolism genes in GSE69164 tumor specimens; even these associations can reflect tumor composition or shared regulation and are hypothesis-generating rather than mechanistic.

TTC36 and NPY1R remain biologically relevant alongside the focused VIPR1 arm. TTC36 has been linked to immune features, growth control and treatment response in HCC, with context-dependent effects [9, 11-13]. NPY1R signaling also varies with tissue and disease context [15-20]. In the present design, these candidates establish a reproducible discovery framework, whereas VIPR1 provides the tractable functional follow-up. Cell-resolved or histology-guided analyses in matched tissues would further refine the cellular source of the bulk expression pattern.

VIPR1 was selected for a focused CSQT-2 perturbation study after the candidate-discovery analysis; this pairing is a study-design choice,

not proof that the public tissue signal causes the cell phenotype.

VIPR1-OE wells produced fewer colonies, less scratch closure and lower Matrigel passage. These assays capture complementary aspects of cell behavior: colony formation reflects growth and survival, wound closure integrates closure-associated cell behavior, and Transwell counts reflect matrix passage under the recorded conditions. The concordant directions support a focused VIPR1-associated phenotype in this model.

The lenvatinib curve shifted toward lower concentrations after VIPR1 overexpression, while viability declined with dose in both conditions. The concentration series was selected to define the inhibitory profile in this cellular model and is not intended to represent clinical exposure. The fitted IC50 values are therefore reported as model-specific descriptive parameters, and the observed shift is interpreted as an association accompanying VIPR1 restoration rather than as a clinical sensitization estimate.

VIPR1 restoration was followed by lower culture-level BODIPY fluorescence, FASN and SCD1 abundance, and calibration-derived FFA, cholesterol and triglyceride readings, whereas CPT1A increased. The combined pattern is consistent with altered lipid handling. The biochemical values represent total culture readings under the recorded conditions, whereas the imaging and immunoblot results provide complementary cellular and protein-level context. Together, these measurements support an associated lipid phenotype without assigning direct control of metabolic flux to VIPR1.

Fu et al. reported that VIPR1 activation suppressed progression in other hepatocellular carcinoma cell lines while altering arginine and pyrimidine metabolism [23]. That observation is compatible with the present lipid- and reactive oxygen species-associated phenotype and may reflect pleiotropic, context-dependent effects of VIPR1 rather than a single metabolic program. The CSQT-2 model is PVTT-derived, but the current comparison does not establish that PVTT origin explains the difference between metabolic readouts; cell background, culture conditions and assay design could also contribute. Moreover, the available experiments cannot distinguish whether the lipid phe-

notype is a driver of altered growth or drug response, a consequence of those changes, or a parallel correlate. Temporal perturbation, rescue and flux measurements will be needed to resolve these alternatives.

The larger reactive oxygen species (ROS)-associated high-fluorescence fraction is reported operationally from the source-defined M4-2 gate using the DCFH-DA-based assay. This endpoint provides a standardized fluorescence readout for the recorded experiment; it is not assigned to a single reactive oxygen species and is interpreted alongside the lipid-associated measurements.

Mechanistic follow-up should focus on the timing of events and functional rescue. Controlled VIPR1 induction could show whether signaling changes precede FASN, SCD1 or CPT1A changes. Stable-isotope tracing, acylcarnitine profiling and extracellular-flux assays could then separate lipid synthesis, storage and oxidation. A rescue experiment targeting a downstream metabolic component would provide the clearest test of pathway causality.

Cellular composition may also account for part of the public-tissue signal. Adjacent liver, HCC and PVTT contain different proportions of malignant, immune, stromal, endothelial and residual liver cells. Bulk VIPR1 and NPY1R expression may therefore reflect both tumor-cell regulation and shifts in cell composition. The inverse bulk VIPR1-CPT1A correlation and the increased CPT1A signal after VIPR1 overexpression are not contradictory: bulk correlation across tumor specimens can reflect cellular composition or shared regulation, whereas the CSQT-2 experiment measures a cell-autonomous association under culture conditions and cannot identify the source of the bulk-tissue difference. Paired single-cell, spatial or histology-guided studies are needed to connect these observations.

Laboratory observations were summarized at the recorded within-experiment level, whereas the public analyses used patients as paired biological units. Treating wells, fields or lanes as independent experiments would change the estimand, so these panels are presented descriptively and the replicate structure is stated in the Methods and legends. This distinction keeps the interpretation aligned with the available experimental design.

Clinical application would require independent matched tissues and a clinically annotated cohort relating candidate expression to vascular invasion and outcome, with adjustment for liver function, tumor burden, stage and treatment. Any classifier would then require a locked threshold and external evaluation. Accordingly, the term candidate biomarker is used here in a discovery context.

The study combines paired public cohorts with a focused VIPR1 perturbation in a PVTT-derived model and reports effect estimates together with the available wells, fields and lanes. The resulting evidence is intentionally framed as candidate prioritization and an associated cellular phenotype. Mechanistic attribution, cellular-source resolution in bulk tissue and clinical translation remain questions for follow-up studies, while the present analyses define the scope and reproducible direction of the recorded findings.

Together, the paired cohorts identify a reproducible HCC-associated expression pattern and a gene-specific, less uniform PVTT component, while the separate CSQT-2 experiments describe a VIPR1-associated cell phenotype.

Conclusion

Paired transcriptomic analysis identified reproducible decreases in TTC36, NPY1R and VIPR1 between adjacent liver and primary HCC across two cohorts. HCC-to-PVTT effects were smaller and candidate dependent, with NPY1R showing the most consistent decrease and PCA showing substantial HCC/PVTT overlap. In a separate CSQT-2 experiment, restored VIPR1 expression was accompanied by lower colony formation, wound closure and Matrigel passage, a lower nominal single-plate lenvatinib IC50, and lipid- and ROS-associated changes. These observations define a model-specific VIPR1-associated phenotype and do not establish a causal link to PVTT, lipid flux or drug sensitization.

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Disclosure of conflict of interest

None.

Abbreviations

ANT, adjacent non-tumor liver; AUC, area under the receiver operating characteristic curve; BODIPY, boron-dipyrromethene; CCK-8, Cell Counting Kit-8; CPT1A, carnitine palmitoyl-transferase 1A; DAPI, 4',6-diamidino-2-phenyl-indole; FASN, fatty acid synthase; FCS, flow-cytometry standard; FDR, false discovery rate; FFA, free fatty acid; GEO, Gene Expression Omnibus; HCC, hepatocellular carcinoma; IC50, half-maximal inhibitory concentration; NPY1R, neuropeptide Y receptor Y1; OE, overexpression; PCA, principal-component analysis; PVTT, portal vein tumor thrombosis; qPCR, quantitative polymerase chain reaction; ROC, receiver operating characteristic; ROS, reactive oxygen species; SCD1, stearoyl-CoA desaturase 1; SD, standard deviation; TC, total cholesterol; TG, triglyceride; TTC36, tetratricopeptide repeat domain 36; VIPR1, vasoactive intestinal peptide receptor 1.

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