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ORIGINAL ARTICLE

Clinical Significance of Trk Receptor Expression as a New Therapeutic Target in Hepatocellular Carcinoma

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Abstract

Oncogenic fusion of the tropomyosin receptor kinase (Trk) receptor family encoded by the *NTRK* gene has been found in several carcinomas. About ten targeted therapies have been developed and clinical trials are in progress. However, the results of studies on expression of the Trk receptor in HCC have not yet been published. Immunohistochemical staining was performed using anti-TrkA+B+C antibody (ab181560, Abcam) in 288 curatively resected primary HCC samples, and the correlation between Trk expression and *NTRK* copy number was assessed. Targeted next generation sequencing was performed in cases with Trk overexpression to detect *NTRK* fusion genes. Overexpression of Trk protein was observed in 21 (7.3%) of 288 cases. The Trk overexpression group showed a trend toward shorter recurrence-free survival (RFS) ($p = 0.092$) and overall survival (OS) ($p = 0.079$) than the low expression group, with frequent multicentric occurrence. Differences in RFS and OS were statistically significant in specific sub-populations including AJCC T1 stage HCCs, tumors less than 5 cm, patients without cirrhosis, tumors without vascular invasion, or Edmondson grades I and II. Trk expression was also an independent prognostic factor in both RFS and OS. Trk expression was not associated with copy number of each *NTRK* gene, and *NTRK* fusion was not detected in HCCs with Trk overexpression. Trk expression might play an important role in the development and progression of HCC, and emerging target therapy against the Trk protein could be applicable in patients with Trk-overexpressing HCC.

Keywords Hepatocellular carcinoma · Prognosis · *NTRK* · Trk · Target

Introduction

Hepatocellular carcinoma (HCC) has poor prognosis because of the high incidence of tumor recurrence and metastasis. Sorafenib has been recognized as the most effective treatment for advanced HCC, but new molecular targeted therapies such as regorafenib and lenvatinib, or a programmed cell death protein-1 immune checkpoint inhibitors (nivolumab and pembrolizumab) have recently been approved by the FDA due to their demonstrated efficacy and safety in clinical trials [3, 10, 15, 28]. In the era of precision medicine, molecular profiling of cancer will be essential for therapeutic decisions.

The neurotrophic tyrosine kinase receptor (*NTRK*) family consists of three proto-oncogenes, *NTRK1*, *NTRK2*, and *NTRK3*, which encode receptor tyrosine kinase proteins, TrkA, TrkB, and TrkC, respectively. Trk proteins are involved in cell survival, proliferation, and differentiation in physiologic conditions. Oncogenic fusions of the *NTRK* gene have been found in various cancers and clinical trials using Trk inhibitors are in progress [1]. Although *NTRK* gene rearrangement is rarely found in tumors, its clinical significance is increasing as target therapy has proved its efficacy and safety in several clinical trials. However, a study about Trk receptor expression in HCC has not yet been published.

In this study, we investigated Trk protein expression by immunohistochemistry and its association with prognosis or tumor aggressiveness and suggest that the *NTRK* oncogene could be a new molecular target in HCC. We also performed a targeted next generation sequencing (NGS) test to determine

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whether Trk overexpression is associated with *NTRK* fusion in HCC.

Materials and Methods

Tissue Samples

HCC tissues were collected from 291 patients who underwent curative surgical resection from July 2000 to May 2006 at Samsung Medical Center, Seoul, Korea. Three cases were excluded due to lack of available tissue. Curative resection was defined as complete resection of all tumor nodules with clear microscopic resection margins and no residual tumor, as indicated by a computed tomography (CT) scan one month after operation. All tumor tissues were histologically confirmed. The Institutional Review Board of Samsung Medical Center approved this study and waived informed consent. Tumor stages were determined by both the American Joint Committee on Cancer (AJCC) 8th staging system and Barcelona Clinic Liver Cancer (BCLC) staging classification [2, 17]. Tumor differentiation was graded according to Edmondson and Steiner criteria [9]. Microvascular invasion was considered present when at least one or more endothelial cells or the tunica media of the vessel surrounded tumor cells. Intrahepatic metastasis and multicentric occurrence were defined according to the criteria of the Liver Cancer Study Group of Japan [16]. Tumor recurrence was classified as either early recurrence or late recurrence, using two years as the cut off [13]. Tumor tissue microarrays (TMA) were produced as described previously [12]. Two 2-mm cores were taken from formalin-fixed paraffin-embedded (FFPE) blocks of each specimen. All patients were followed up every three months by monitoring serum alpha-fetoprotein (AFP) levels and three-phase dynamic CT scans, and patients with suspicious imaging findings and/or continuously elevated AFP levels were further evaluated with magnetic resonance imaging and/or positron emission tomography-CT. The median follow-up period was 120 months (range 14–151 months) for survivors. Recurrence-free survival (RFS) was defined as the time from the date of surgical resection to the date of tumor recurrence, metastasis or last follow-up. Overall survival (OS) was defined as the time from the date of resection until the date of death or last follow-up.

Immunohistochemical Analysis

Immunohistochemical staining (IHC) was performed on 4-μm-thick tissue sections from TMA blocks, using a Bondmax automated immunostainer (Leica Biosystems, Melbourne, Australia) and a Bond™ Polymer refine detection system, DS9800 (Vision Biosystems, Melbourne, Australia). The primary antibody was anti-panTrk (TrkA+TrkB+TrkC) antibody (ab181560, clone EPR17341, 1:100, Abcam, Cambridge, MA, USA). Briefly, antigen retrieval was performed at 97 °C for 20 min in ER2 buffer (Leica Biosystems, Melbourne, Australia). After blocking endogenous peroxidase activity with 3% hydrogen peroxide for 5 min, samples were incubated with primary antibody for 15 min at room temperature, and antigen-antibody chromogenic reactions were detected for 10 min. For a positive control, we used the KM12 cell line, which is known to have a *TPM3-NTRK1* rearrangement.

We used the Remmele scoring system, which is a 12-point scoring system that accounts for both the intensity and proportion of the stain [20]. We evaluated two TMA cores for every patient and calculated the mean value. Neural tissue was considered an internal positive control (Fig. 1c, arrow). We regarded cytoplasmic Trk expression as high when the IHC Remmele score was greater than 5.5.

Correlation between Trk Immunohistochemistry and *NTRK* Copy Number

We performed correlation analysis using copy number data from a study previously published by our group to evaluate the association between Trk protein expression by IHC and *NTRK* copy number [26].

NTRK Fusion Evaluation Using Targeted Next-Generation Sequencing

Genomic DNA were extracted from FFPE blocks that were cut into 5-μm-thick sections and microdissected. Targeted NGS was performed to identify *NTRK* gene rearrangement as previously described, using the Oncomine Focus Assay panel (Thermo Fisher Scientific, San Francisco, CA) [6]. Briefly, Targeted DNA amplification was used for library preparation followed by sequencing on an Ion Torrent PGM Machine system. Ion Torrent software (Ion Reporter™ 5.2) and Oncomine Knowledgebase Reporter were used for automated data analysis. Examined partner genes of *NTRK1, NTRK2,* and *NTRK3* genes were as follows: *CD74-NTRK1*, *CEL-NTRK1*, *IRF2BP2-NTRK1, LMNA-NTRK1, MPRIP-NTRK1, NFASC-NTRK1, RNF213-NTRK1, SSBP2-NTRK1, SQSTM1-NTRK1, TPR-NTRK1, TFG-NTRK1, TPM3-NTRK1, AFAP1-NTRK2, AGBL4-NTRK2, NACC2-NTRK2, QKI-NTRK2, SQSTM1-NTRK2, TRIM24-NTRK2, VCL-NTRK2, BTBD1-NTRK3, COX5A-NTRK3, ETV6-NTRK3.*

Statistical Analysis

We used the X-tile statistical software program to determine the optimal cutoff of Remmele IHC score of Trk or *NTRK* gene expression with the most significant prognostic effect [4]. Correlations between clinicopathologic characteristics

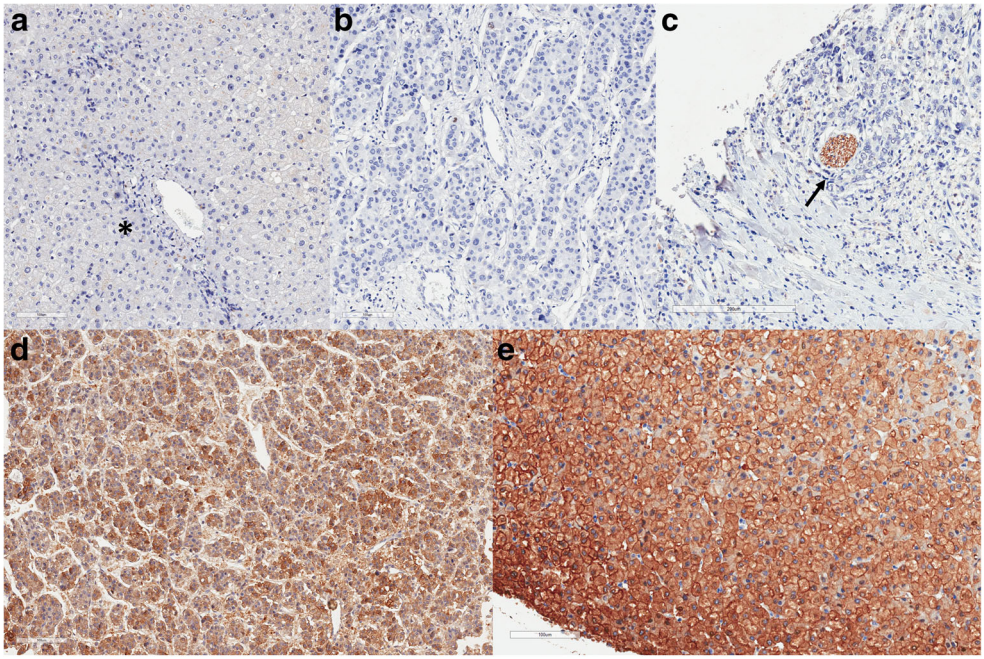

Fig. 1 Representative figures of Trk immunohistochemistry. **a** non-tumor liver parenchyma including a portal tract (asterisk) shows no expression of Trk. **b**-**c** Representative figures of no expression of Trk in hepatocellular carcinoma. Neural tissue served as an internal positive controls (arrow). **d**-**e** Representative figures of high expression of Trk in hepatocellular carcinoma. Tumor cells show cytoplasmic and occasional membranous staining

and Trk expression or *NTRK* gene expression were analyzed by chi-square test, Fisher's exact test or Cochan Armitage test, as appropriate. Kaplan–Meier survival curves and log-rank statistics were used for survival analysis, and the Cox proportional hazards model was used for multivariate analysis. The correlation between Trk expression and *NTRK* copy number was assessed using Pearson's correlation analysis. Statistical analyses were performed using R and SPSS statistical packages (IBM, NY, USA) and a p value <0.05 (two sided) was considered statistically significant.

Results

Clinicopathologic Patient Characteristics

The study included 239 men and 49 women with a median age of 53 years (range, 17 to 76 years). Chronic hepatitis B virus (HBV) infection was detected in 218 patients (75.7%), chronic hepatitis C virus (HCV) infection in 26 patients (9.0%), and combined HBV and HCV infection in 4 patients (1.4%). There was no viral marker detected in 40 patients (13.9%). Tumor recurrence was detected in 192 patients (66.7%), early recurrence in 142 patients (49.3%), and late recurrence in 50 patients (17.4%). A total of 82.6% of patients had AJCC T stage 1 or 2 disease. Median tumor size was 3.7 cm, and one-third of patients had a tumor larger than 5 cm. Microvascular invasion, major portal invasion, intrahepatic metastasis, and multicentric occurrence was observed in 54.9%, 4.5%, 23.2%, and 6.6% of patients, respectively. About 50% of HCCs occurred in the background of cirrhosis.

Trk Protein Expression in HCC and its Association with Prognosis

High Trk expression was defined as cytoplasmic staining corresponding to a Remmele score of 5.5 or higher, with maximum statistical significance related to RFS determined by X-tile software and was observed in 21 of the 288 HCC cases (7.3%) (Fig. 1). The associations between Trk expression and clinicopathologic parameters are summarized in Table 1. High Trk expression was associated with younger age ($p = 0.016$). Multicentric occurrence was more frequent in the high Trk expression group than in the low expression group (31.6% vs. 5.6%, $p = 0.001$).

The Trk overexpression group showed a trend toward shorter RFS ($p = 0.092$) and OS ($p = 0.079$) than the low expression group on survival analysis with a mean follow-up period of 106 months (Fig. 2). Differences in RFS and OS were statistically significant in specific sub-populations including HCCs with AJCC T1 stage, size less than 5 cm, no cirrhosis, no vascular invasion, or Edmondson grades I and II (Fig. 3). Trk expression was an independent prognostic factor for both RFS ($p = 0.010$, hazard ratio 1.998) and OS ($p = 0.008$, hazard ratio 2.299) in multivariate analysis, although it was not statistically significant in univariate analysis (Tables 2 and 3).

Correlation between Trk Expression and *NTRK* Copy Number

Correlations between Trk expression and *NTRK* copy number are depicted in Fig. 4. There was a weak negative correlation between Trk IHC score and *NTRK1* copy number ($r = -0.149$,

Table 1 The association between Trk expression and clinicopathologic parameters

		TrkA+B+C expression		
		Low	High	
	Total	*n* = 267 (92.7%)	21 (7.3%)	p value
Age, year				
≤55	168	161 (95.8)	7 (4.2)	0.016
>55	120	106 (88.3)	14 (11.7)	
Gender				
Female	49	47 (95.9)	2 (4.1)	0.546[c)]
Male	239	220 (92.1)	19 (7.9)	
Tumor size, cm				
≤5.0	191	175 (91.6)	16 (8.4)	0.320
>5.0	97	92 (94.8)	5 (5.2)	
Edmondson grade				
I	31	28 (90.3)	3 (9.7)	0.386[c)]
II	233	215 (92.3)	18 (7.7)	
III	24	24 (100)	0 (0)	
Microvascular invasion				
(−)	130	118 (90.8)	12 (9.2)	0.251
(+)	158	149 (94.3)	9 (5.7)	
Major portal vein invasion				
(−)	275	255 (92.7)	20 (7.3)	1.000[c)]
(+)	13	12 (92.3)	1 (7.7)	
Intrahepatic metastasis				
(−)	221	203 (91.9)	18 (8.1)	0.425[c)]
(+)	67	64 (95.5)	3 (4.5)	
Multicentric occurrence				
(−)	269	254 (94.4)	15 (5.6)	0.001[c)]
(+)	19	13 (68.4)	6 (31.6)	
AJCC T-stage				
1	122	113 (92.6)	9 (7.4)	0.525[d)]
2	116	106 (91.4)	10 (8.6)	
3	44	42 (95.5)	2 (4.5)	
4	6	6 (100)	0 (0)	
BCLC stage				
0-A	165	153 (92.7)	12 (7.3)	0.981[d)]
B	108	100 (92.6)	8 (7.4)	
C	15	14 (93.3)	1 (6.7)	
Albumin level, g/dL				
>3.5	259	240 (92.7)	19 (7.3)	1.000[c)]
≤3.5	29	27 (93.1)	2 (6.9)	
AFP level, ng/mL[a)]				
≤200	174	159 (91.4)	15 (8.6)	0.127
>200	104	100 (96.2)	4 (3.8)	
Etiology				
Non-viral	40	34 (85.0)	6 (15.0)	0.116[c)]
HBV	218	206 (94.5)	12 (5.5)	
HCV	26	23 (88.5)	3 (11.5)	
HBV + HCV	4	4 (100)	0 (0)	
Liver cirrhosis				

Table 1 (continued)

		TrkA+B+C expression		
		Low	High	
(−)	143	135 (94.4)	8 (5.6)	0.271
(+)	145	132 (91.0)	13 (9.0)	
Early recurrence (≤2 years)				
(−)[b)]	146	138 (94.5)	8 (5.5)	0.230
(+)	142	129 (90.8)	13 (9.2)	
Late recurrence (>2 years)				
(−)[b)]	96	92 (95.8)	4 (4.2)	0.446[c)]
(+)	50	46 (92.0)	4 (8.0)	

AJCC American Joint Committee on Cancer, *BCLC* Barcelona Clinic Liver Cancer, *AFP* α-fetoprotein, *HBV* hepatitis B virus, *HCV* hepatitis C virus.
[a)] Partial data was not available, [b)] No early or late recurrence, [c)] by Fisher's exact test, [d)] Cochran-Armitage test, otherwise by chi-square test

$p = 0.018$). There was no significant correlation between Trk IHC and *NTRK*2 or *NTRK*3 copy number.

Investigation of *NTRK* Gene Rearrangement by Targeted NGS

NGS was performed in 21 HCC samples with Trk overexpression. Sequencing was failed in five cases because of poor tissue quality. An *NTRK* fusion gene was not detected in the remaining 16 samples that passed quality control (Supplementary Table 1).

Discussion

In this study, Trk overexpression was found in about 7% of HCC samples. It was an independent predictor of shorter RFS and OS, and was associated with multicentric occurrence. It was not correlated with *NTRK* copy number gain, and an *NTRK* fusion gene was not identified in cases with Trk-overexpression that were available for testing.

NTRK gene fusion is known to occur at low frequency in various tumors, including papillary thyroid carcinoma (12.1%, 4/33), cholangiocarcinoma (3.5%, 1/28), colorectal carcinoma (4%, 13/346), glioblastoma (3%, 3/115), lung

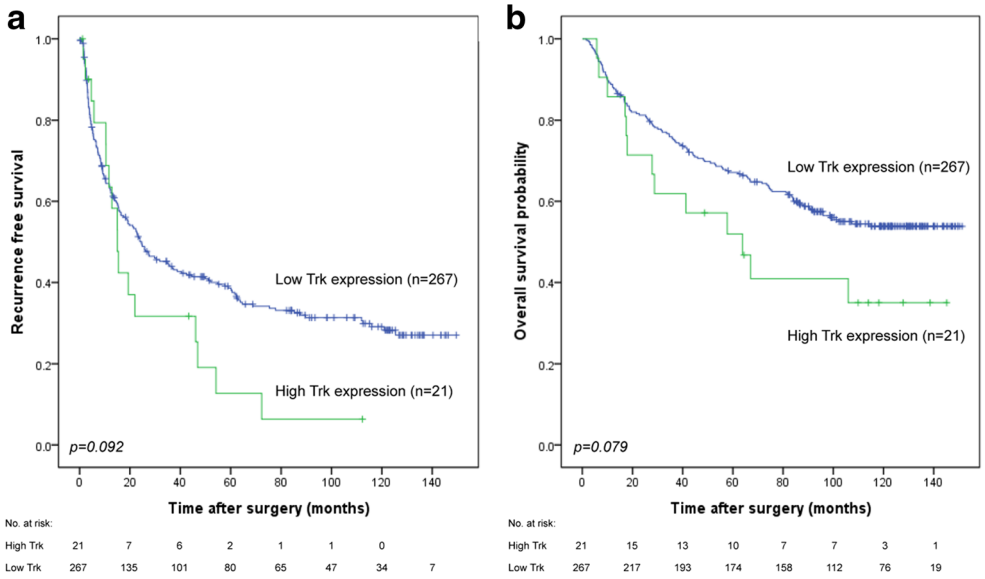

Fig. 2 Kaplan–Meier survival curves according to Trk expression by immunohistochemistry. (**a**) Recurrence free survival (**b**) Overall survival

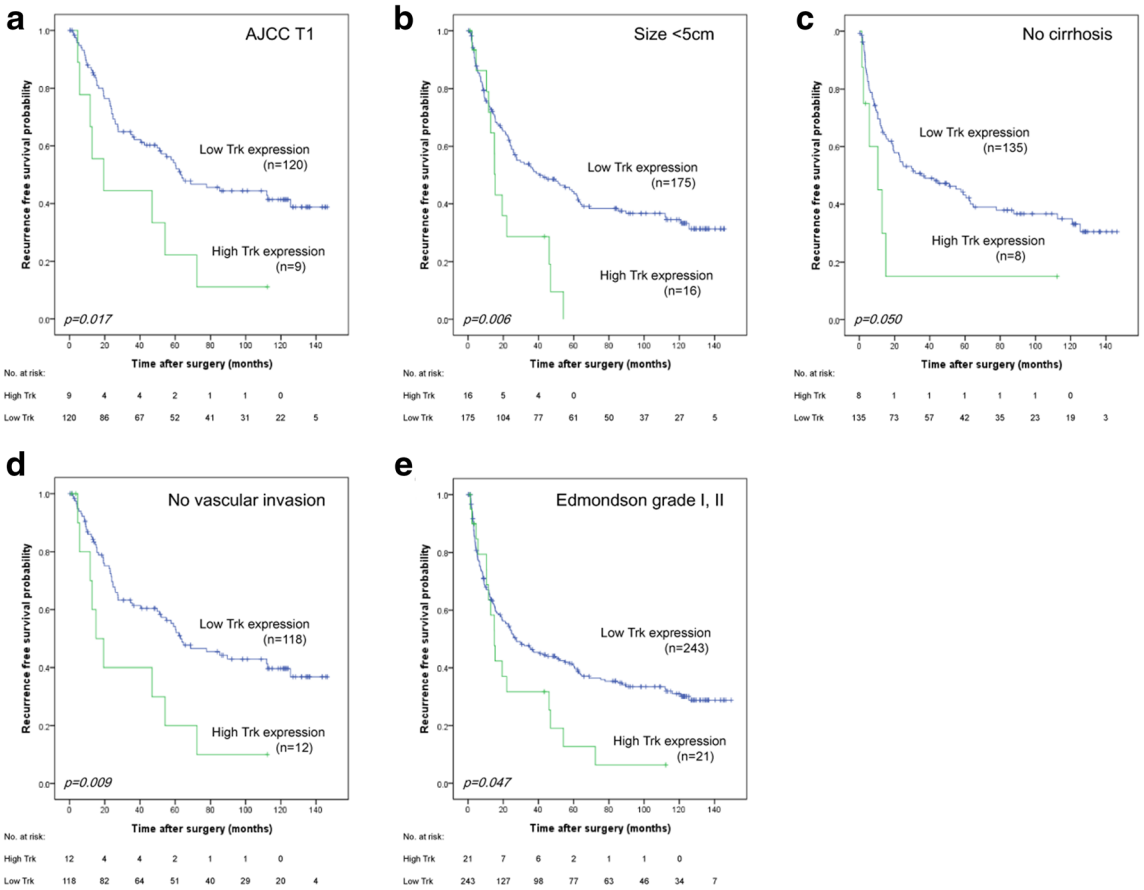

Fig. 3 Kaplan–Meier survival curves for recurrence-free survival according to Trk expression by immunohistochemistry in subgroups. (**a**) AJCC T1 stage (**b**) Size less than 5 cm (**c**) No cirrhosis (**d**) No vascular invasion (**e**) Edmonson grade I and II

adenocarcinoma (3.3% 3/91), and melanoma (0.3%, 1/374) [14, 25]. Despite its low frequency, the clinical significance of *NTRK* fusion is increasing since several tyrosine kinase inhibitors, especially larotrectinib and entrectinib, have demonstrated their efficacy and safety in clinical trials [7]. Larotrectinib was tried in 55 patients with 17 different types of *NTRK*-fusion positive tumors, and the overall response rate was 80% (95% confidence interval, 67 to 90). The response was identified regardless of tumor type or *NTRK* fusion characteristics [7]. Entrectinib was applied in four patients with *NTRK*-fusion tumors, including non-small cell carcinoma, metastatic colorectal cancer, glioneuronal tumor, and mammary analogue secretory carcinoma, and the objective response rate was 100% (95% confidence interval, 44 to 100)

Table 2 Univariate analyses of recurrence free survival and overall survival

		Recurrence free survival		Overall survival	
		HR (95% CI)	p value	HR (95% CI)	p value
Tumor size, cm	>5.0 vs ≤5.0	1.784 (1.333–2.386)	<0.001	2.334 (1.653–3.296)	<0.001
Edmondson grade	III vs I, II	2.200 (1.395–3.469)	0.001	2.289 (1.355–3.867)	0.002
Microvascular invasion	(+) vs (−)	2.226 (1.659–2.987)	<0.001	2.427 (1.680–3.507)	<0.001
Major portal vein invasion	(+) vs (−)	3.913 (2.170–7.057)	<0.001	4.571 (2.451–8.525)	<0.001
Intrahepatic metastasis	(+) vs (−)	5.005 (3.630–6.902)	<0.001	4.932 (3.454–7.043)	<0.001
Albumin level, g/dL	≤3.5 vs >3.5	1.884 (1.216–2.917)	0.005	2.789 (1.758–4.423)	<0.001
AFP level, ng/mL	>200 vs ≤200	1.651 (1.234–2.208)	0.001	1.392 (0.977–1.984)	0.067
Etiology	Viral vs Non-viral	2.016 (1.240–3.278)	0.005	1.129 (0.686–2.857)	0.633
Trk expression	High vs Low	1.533 (0.929–2.528)	0.094	1.662 (0.937–2.949)	0.083

Table 3 Multivariate analyses of recurrence free survival and overall survival

		Recurrence free survival		Overall survival	
		HR (95% CI)	p value	HR (95% CI)	p value
Tumor size, cm	>5.0 vs ≤5.0	1.043 (0.736–1.478)	0.813	1.315 (0.866–1.997)	0.200
Edmondson grade	III vs I, II	1.515 (0.921–2.492)	0.102	1.459 (0.820–2.598)	0.199
Microvascular invasion	(+) vs (−)	1.213 (0.834–1.765)	0.313	1.229 (0.756–1.995)	0.405
Major portal vein invasion	(+) vs (−)	0.947 (0.487–1.843)	0.874	1.203 (0.603–2.400)	0.600
Intrahepatic metastasis	(+) vs (−)	3.751 (2.457–5.726)	<0.001	3.919 (2.404–6.389)	<0.001
Albumin level, g/dL	≤3.5 vs >3.5	1.673 (1.047–2.673)	0.032	2.857 (1.753–4.654)	<0.001
AFP level, ng/mL	>200 vs ≤200	1.308 (0.959–1.783)	0.090	0.995 (0.678–1.460)	0.978
Etiology	Viral vs Non-viral	1.533 (0.920–2.554)	0.101	0.915 (0.528–1.587)	0.751
Trk expression	High vs Low	1.998 (1.176–3.394)	0.010	2.299 (1.245–4.245)	0.008

[8]. Since the clinical trials conducted thus far did not include HCC, the effect of Trk inhibitors in HCC patients is unclear. As Trk expression is associated with poor prognosis, applying targeted therapy to Trk-overexpressing HCC may help to improve survival rate in these patients.

Several studies have suggested that pan-Trk IHC can be used as an efficient screening tool to detect *NTRK* fusion in various types of tumors. Hechtman et al. performed pan-TrK IHC in 21 cases with various types of cancer, including colorectal carcinoma, glioblastoma, and others, and the sensitivity and specificity were 95.2% and 100%, respectively [11]. Rudzinski et al. also demonstrated high sensitivity and specificity (97% and 98%) with 22 cases of infantile fibrosarcomas and cellular mesoblastic nephroma [21]. In the present study, we found no *NTRK* fusion genes in 16 out of 21 HCCs with Trk overexpression. However, we cannot definitely conclude that there is no *NTRK* fusion in HCCs because testing for *NTRK* fusion detection failed in five cases, and *NTRK* fusion with a novel partner cannot be detected on targeted NGS testing. Consistent with our data, a recent study by Okamura et al. showed no *NTRK* fusion in 374 HCC samples [19]. We also demonstrated that *NTRK* copy number was not related to Trk expression score. Thus, in HCC, Trk overexpression is not likely to be associated with fusion or amplification, although this finding is not conclusive.

The effect of target therapy on tumors with Trk overexpression but without gene rearrangement has not been definitely established. In patients with lung adenocarcinoma harboring *ALK* translocation, *ALK* inhibitors, such as crizotinib, have been reported to be clinically effective in IHC-positive but fluorescence in situ hybridization-negative cases in a few studies [18, 24, 27]. In those studies, *ALK* gene arrangement was confirmed by other methods such as reverse transcription polymerase chain reaction or NGS in only a few cases with available tissue samples, so the possibility of clinical effect in ALK IHC-positive only cases without fusion could be still

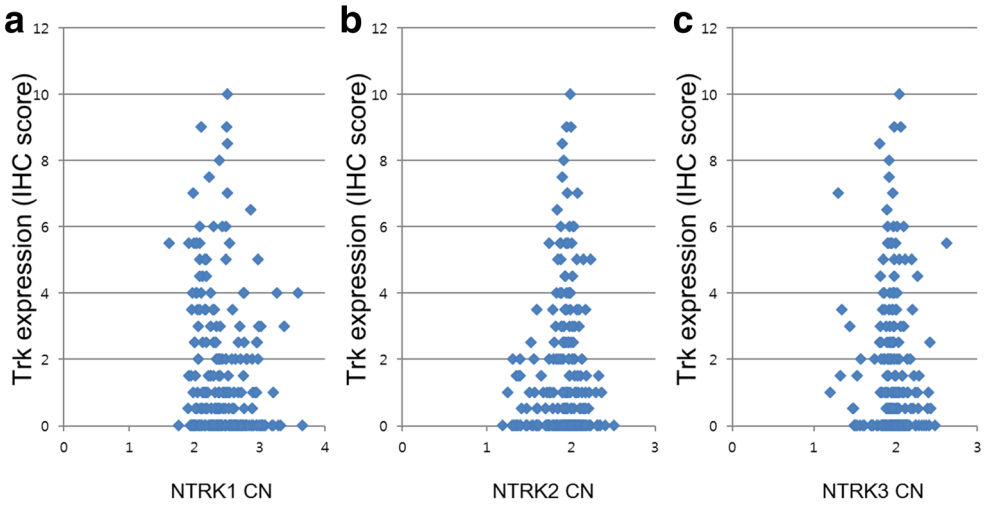

Fig. 4 Correlation between Trk expression and *NTRK* copy number (CN). (**a**) *NTRK1* (**b**) *NTRK2* (**c**) *NTRK3*

considered. Thus, in the absence of *NTRK* translocation as in this study, it is not clear whether there would be clinical response in HCC, but targeted therapy may be worth trying in these patients because many of these tumors exhibit Trk protein overexpression.

Meanwhile, the prognostic role of panTrk expression have been reported in a few studies, where overexpression of Trk were related to unfavorable outcome in oral cavity squamous cell carcinoma [5], and aggressive behavior in pancreatic cancer and adenoid cystic carcinoma [22, 23]. These results were in concordance with our study. Further validation study is necessary to clarify the prognostic role of panTrk expression in HCC.

Conclusion

We found that Trk overexpression was observed in about 7% of HCC samples. It was an independent predictor of shorter RFS and OS, and was associated with multicentric occurrence. Thus, we indirectly demonstrated that Trk expression plays an important role in the development and progression of HCC. Though *NTRK* translocation was not identified in this study, emerging target therapy against the Trk protein could be applicable in patients with Trk-overexpressing HCC.

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Compliance with Ethical Standards

Conflict of Interest All contributing authors have no financial support relevant to this article, and no conflict of interest to declare.

EHTICS Approval and CONSCENT to Participate The Institutional Review Board of Samsung Medical Center approved this study and waived informed consent for this retrospective study.

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