Research Article

Detection of Novel Fusion Transcript VTI1A-CFAP46 in Hepatocellular Carcinoma

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Keywords

Fusion transcript · RNA sequencing · Hepatocellular carcinoma · Hepatoma · Liver transplantation

Abstract

Background: Hepatocellular carcinoma (HCC) is now the second-highest cause of cancer death worldwide. Recent studies have discovered a wide range of somatic mutations in HCC. These mutations involve various vital signaling pathways such as: Wnt/ β -Catenin, p53, telomerase reverse transcriptase (TERT), chromatin remodeling, RAS/MAPK signaling, and oxidative stress. However, fusion transcripts have not been broadly explored in HCC. **Methods:** To identify novel fusion transcripts in HCC, in the first phase of our study, we performed targeted RNA sequencing (in HCC and paired non-HCC tissues) on 6 patients with a diagnosis of HCC

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undergoing liver transplantation. **Results:** As a result of these studies, we discovered the novel fusion transcript, VTI1A-CFAP46. In the second phase of our study, we measured the expression of wild-type VTI1A in 21 HCC specimens, which showed that 10 of 21 exhibited upregulation of wild-type VTI1A in their tumors. VTI1A (Vesicle Transport via Interaction with t-SNARE homolog 1A) is a member of the Soluble N-ethylmaleimide-Sensitive Factor (NSF) attachment protein receptor (SNARE) gene family, which is essential for membrane trafficking and function in endocytosis, autophagy, and Golgi transport. Notably, it is known that autophagy is involved in HCC. **Conclusions:** The link between novel fusion transcript VTI1A-CFAP46 and autophagy as a potential therapeutic target in HCC patients deserves further investigation. Moreover, this study shows that fusion transcripts are worthy of additional exploration in HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the ninth most common in women, as well as the second most frequent cause of cancer death in both sexes overall [1]. The largest burden of HCC occurs in eastern and southeastern Asia and central and western Africa, which together account for more than 80% of cases. 50% of all cases of HCC occur in China alone [1, 2]. Unlike many other cancers, the principal risk factors associated with HCC have, in fact, been elucidated. The key etiologic causes for HCC development include hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholic liver disease, and nonalcoholic fatty liver disease [3, 4]. With recent advances in genomic sequencing technologies, molecular genetic factors have now also been implicated in HCC development. For example, inflammation mediated by TNF- α , IL1- β , or TGF- β , cell cycle control by p53, growth factor pathways, and telomerase reactivation through telomerase reverse transcriptase (TERT) have all been recognized as contributory. Additionally, genetic factors are involved in invasion, metastasis, metabolism, and drug resistance of HCC [5].

Fusion transcripts are chimeric RNA molecules resulting from the fusion or rearrangement of two separate genes. One well-described example is the Philadelphia chromosome that occurs in chronic myelogenous leukemia (CML). This is a reciprocal translocation that generates the BCR-ABL fusion gene. This fusion gene, which encodes the BCR-ABL tyrosine kinase, results in abnormal cell proliferation [6]. Recently, chromosomal rearrangements and fusion transcripts have been increasingly described in solid tumors. For example, a fusion between EML4 (Echinoderm Microtubule-associated protein-Like 4) and ALK (Anaplastic Lymphoma receptor tyrosine Kinase) is reported in non-small lung cancer, and ALK inhibitors are currently being evaluated in trials in patients harboring this alteration [7]. The TMPRSS2-ERG fusion transcript is used in the diagnosis and prognostication of prostate cancer [8]. A reciprocal translocation between PLAG1, a developmentally regulated zinc finger gene, and CTNNB1 (beta-catenin) has been reported in a pleiomorphic salivary gland tumor [9]. These fusions illuminated a key mechanism of tumorigenesis and have become a target of increasing research into both diagnostic markers and cancer therapeutics [10–12]. However, unlike leukemias or lymphomas, as well as certain other types of solid cancer, fusion transcripts have not been extensively studied in advanced hepatic fibrosis or HCC.

Herein, we used targeted-transcriptome RNA sequencing (RNA-seq) to identify novel fusion transcripts in HCC, which led us to discover a novel fusion transcript, VTI1A-CFAP46. Vesicle Transport via Interaction with t-SNARE homolog 1A (VTI1A) is a member of the Soluble N-ethylmaleimide-Sensitive Factor (NSF) attachment protein receptor (SNARE) family that is found in complexes with either syntaxin-13 or syntaxin-16 and synaptotagmin-6 and

Fig. 1. Algorithm showing the number of patients in phases 1 and 2 of the study. In phase 2 of the study, 15 samples were obtained from HCC lesions, from a total of 13 patients.

VAMP-4. SNAREs are essential for membrane trafficking and function in endocytosis, autophagy, and Golgi transport [13, 14]. Notably, these processes have been implicated in hepatocellular carcinogenesis [15, 16]. Cilia and Flagella Associated Protein 46 (CFAP46), previously known as Tetratricopeptide Repeat Domain 40 (TTC40), is a recently described gene whose function is not well known [17]. The identification and characterization of fusion events in HCC promises to deepen our understanding of its underlying pathology, while simultaneously generating promising early diagnostic markers and therapeutic targets, which should help improve outcomes in this deadly malignancy.

Materials and Methods

Patient Samples and Cell Lines

In the first phase of this study (Fig. 1), HCC specimens and adjacent nonmalignant tissue from 6 patients were obtained at the time of deceased donor liver transplant at the Johns Hopkins Hospital under an Institutional Review Board (IRB)-approved protocol. We extracted RNA from these 6 HCC and 6 paired non-HCC samples. We then performed targeted RNA-seq on these 12 samples. Under the same IRB protocol, in the second phase of the study, we obtained paired HCC and non-HCC tissues from an additional 13 patients (12 patients following liver transplant and 1 patient following liver resection for HCC). Among these 13 patients, 2 had two discrete HCC lesions; thus, in the second phase we added another 15 HCC samples and 13 matched nonmalignant controls. Therefore, in both phases of the study we evaluated a total of 19 patients (total of 40 specimens: 21 HCC and 19 non-HCC samples) (Fig. 1). We also collected and analyzed RNA from two HCC-derived cell lines (97 L, 97 H) [18].

cDNA Library Preparation and Sequencing

Total RNA from specimens was extracted using an RNeasy Mini Kit (QIAGEN). Total RNA was converted into a cDNA library, then selectively enriched for 1,385 cancer-associated genes using the TruSight RNA Pan-Cancer Panel kit according to the manufacturer's instructions (Illumina). The TruSight RNA Pan-Cancer panel is a targeted sequencing strategy that enables measurement of gene expression, variant calling, and fusion detection of cancer-related genes with limited sample quantity. This approach has the ability to detect fusions between any of the 1,385 genes in the panel, but also between any of these genes and other novel fusion partners, for instance FGFR1-X fusions, where X represents any possible gene. All RNA-seq was performed on MiSeq instruments with V3 reagents according to the manufacturer's instructions (Illumina).

Table 1. Clinical characteristics of HCC patients

	Age/ sex	Etiology	HCV RNA	Fibrosis stage	MELD score	Tumor recurrence	Pre-LTx AFP	Death	Follow- up, days
<i>First phase of study: 6 patients</i>									
Patient 1	64/M	HCV/HIV	Detectable	3	12	No	2.4	No	532
Patient 2	61/M	HCV	Undetectable	4	15	No	2.6	Yes	318
Patient 3	68/M	HCV	Undetectable	4	10	No	4.5	No	517
Patient 4	64/M	HCV/ETOH	Undetectable	4	11	No	4.9	No	442
Patient 5	48/M	HCV	Undetectable	4	10	No	8.4	No	445
Patient 6	63/M	HCV/HBV/HIV	Undetectable	4	9	No	4.2	No	437
<i>Second phase of study: additional 13 patients</i>									
Patient 7	69/F	NASH	N/A	4	8	No	3.8	No	442
Patient 8	51/M	ETOH	N/A	4	7	Yes	7.2	No	427
Patient 9	61/F	HCV	Detectable	4	13	No	43.8	No	405
Patient 10	55/M	HCV	Undetectable	4	6	No	3.8	No	363
Patient 11	66/M	HCV	Detectable	4	10	No	179.3	No	387
Patient 12	68/M	HCV	Detectable	4	8	No	41.2	No	383
Patient 13	62/F	NASH	N/A	4	8	No	3.7	No	339
Patient 14	58/F	HCV	Detectable	3	6	No	10.1	No	293
Patient 15	54/F	HCV	Detectable	4	10	No	60.7	No	285
Patient 16	56/M	Cryptogenic	N/A	4	10	Yes	3.5	No	160
Patient 17	60/M	HCV	Yes	3	7	No	8.4	No	125
Patient 18 ^a	59/M	HCV	No	3	14	No	1.8	No	125
Patient 19	54/M	HBV	N/A	4	9	No	71.5	No	149

HCV, hepatitis C virus; HIV, human immunodeficiency virus; HBV, hepatitis B virus; ETOH, alcoholic liver disease; NASH, non-alcoholic steatohepatitis; MELD, model for end-stage liver disease; LTx, liver transplant; AFP, alpha fetoprotein; N/A, not applicable. ^a This patient underwent liver resection rather than liver transplant.

Bioinformatics Analysis

We used The BaseSpace® RNA-Seq Alignment v1.0 suite (Illumina) to analyze the sequence data. This package provides a wrapper for the alignment, summarization, variant calling, and fusion detection methods like STAR, Manta, Cufflinks, Isaac Variant Caller, and Tuxedo Suite (including Bowtie, Bowtie2, and TopHat). As a reference for alignment, we use the UCSC hg19 version of the human genome. First, the BaseSpace RNA-Seq Alignment workflow uses Bowtie to identify and prefilter the input reads against abundant sequences, such as mitochondrial or ribosomal sequences, as defined by iGenomes prebuilt indexes [19]. After filtering, STAR was used for the final mapping prior to fusion detection. STAR supports both splice junction and fusion read detection. STAR aligns reads by finding the Maximal Mappable Prefix (MMP) hits between reads (or read pairs) and the reference genome. Manta was used in combination with STAR to identify fusion transcripts. This workflow is followed by fusion filtering and scoring in BaseSpace RNA-Seq Alignment package based on the following criteria: (1) read counts across fusion and alignment; (2) genome-wide realignment of fusion contigs to filter candidates that could be explained by a local alignment elsewhere in the genome; and (3) length of coverage around breakpoints, indicating the presence of stable fusion transcripts. Only high-confidence candidate fusion transcripts passing these filters were selected for downstream analysis [20–22].

Reverse Transcriptase PCR

Total RNA was extracted from tissues or cell lines using an RNeasy Mini kit (QIAGEN, Aarhus, Denmark). 0.5 µg of total RNA were reverse-transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, MA, USA), following the manufacturer's instructions. The resultant cDNA served as a template for PCR amplification using the following primers spanning the fusion transcript junctions:

VTI1A sense strand (Exon1) 5' CACCTTCGGGGTTCCTAAG 3'; VTI1A sense strand (Exon8) 5' CTTCGGGAAACAGATGCTAA 3'; CFAP46 sense strand (Exon30) 5' CAGCAGGAAAAATCAGTTCTGG 3'.

Table 2. Characteristics of tumors on pathology in HCC patients

	HCC lesions, <i>n</i>	Largest lesion, cm	Within MC	Tumor differentiation	MVI
<i>First phase of study: 6 patients</i>					
Patient 1	2	2.8	Yes	Moderate	No
Patient 2	1	2.8	Yes	Well	No
Patient 3	2	2.5	Yes	Moderate	No
Patient 4	1	1.2	Yes	Moderate	No
Patient 5	2	1.5	Yes	Well	No
Patient 6	1	2.1	Yes	Well	Yes
<i>Second phase of study: additional 13 patients</i>					
Patient 7	2	3	Yes	Well	No
Patient 8	1	3	Yes	Moderate	Yes
Patient 9	1	2.8	Yes	Moderate	No
Patient 10	1	2.5	Yes	Moderate	No
Patient 11	3	2.1	Yes	Moderate	No
Patient 12	2	1.9	Yes	Well	No
Patient 13	4	1.7	No	Moderate	No
Patient 14	5	3	No	Moderate	Yes
Patient 15	1	1.5	Yes	Moderate	No
Patient 16	4	1.7	No	Well	No
Patient 17	1	2	Yes	Moderate	No
Patient 18 ^a	1	3.6	Yes	Moderate	Yes
Patient 19	2	4.5	No	Moderate	Yes

HCC, hepatocellular carcinoma; MVI, microvascular invasion; MC, Milan Criteria. ^a This patient underwent liver resection rather than liver transplant.

We used Taq DNA polymerase (Invitrogen) for PCR amplification. PCR products were analyzed by agarose gel electrophoresis.

Sanger Sequencing

Amplified reverse transcriptase PCR (RT-PCR) products were cloned into vector pCR™4-TOPO TA Vector and transformed into *E. coli* according to the manufacturer's instructions (Thermo-Fisher Scientific). Then, the vector was extracted using a Plasmid Mini Kit (QIAGEN) and sequenced via Sanger sequencing according to the manufacturer's instructions.

Quantitative RT-PCR

Using the same cDNA referred above, we performed quantitative RT-PCR (qRT-PCR). cDNA was amplified using iQ™ SYBR® Green Supermix. The primer sequences for qRT-PCR were:

VTI1A sense strand (Exon 8) 5' CTTCGGGAAACAGATGCTAA 3'; CFAP46 sense strand (Exon 30) 5' CAGCAGGAAAATCAGTTCTGG 3'; VTI1A sense strand (Exon 1) 5' CACCTTCGGGGTTCCTAAG 3'; VTI1A anti-sense strand (Exon 2) 5'TCTTTCGCTTCTTCAAGCTG 3'; GAPDH sense strand 5'CAGCCTCAAGATCATCAGCA 3'; GAPDH antisense strand 5'TGTGGTCATGAGTCCTTCCA 3'.

We used GAPDH as an internal control. We measured both wild-type VTI1A transcript levels and VTI1A-CFAP46 fusion transcript expression levels using the $2^{-\Delta(\Delta C_t)}$ method.

Protein Isolation for Western Blotting and Antibodies Used

HCC and non-HCC liver tissues were collected and homogenized in H-medium (210 mM mannitol, 70 mM sucrose, 2 mM HEPES, 0.05% BSA, plus protease and phosphatase inhibitors). VTI1 antibody was obtained from BD Bio Sciences (San Jose, CA, USA). GAPDH antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Densitometry was performed using the NIH Image J program.

Table 3. Results of RNA sequencing of HCC and matched non-HCC tissues performed in 6 patients enrolled in the first phase of the study

		Chr 1	Pos 1	Strand 1	Chr 2	Pos 2	Strand 2	Gene 1	Gene 2
Patient 1	Non-tumor tissue	10	96541752	Positive	10	96493052	Positive	CYP2C19	CYP2C18
		10	96827000	Negative	10	96535204	Positive	CYP2C8	CYP2C19
	Tumor tissue	–	–	–	–	–	–	–	–
Patient 2	Non-tumor tissue	17	11984847	Positive	X	72745873	Negative	MAP2K4	MAP2K4P1
		1	155644801	Negative	1	155753880	Negative	YY1AP1	GON4L
		1	155646339	Negative	1	155753880	Negative	YY1AP1	GON4L
	Tumor tissue	10	96702075	Positive	10	96827033	Negative	CYP2C9	CYP2C8
		10	96535273	Positive	10	96827033	Negative	CYP2C19	CYP2C8
		16	89484692	Negative	15	93467550	Positive	ANKRD11	CHD2
		1	155631098	Negative	1	155741008	Negative	YY1AP1	GON4L
Patient 3	Non-tumor tissue	10	96826989	Negative	10	96535226	Positive	CYP2C8	CYP2C19
	Tumor tissue	–	–	–	–	–	–	–	–
Patient 4	Non-tumor tissue	10	96702075	Positive	10	96827033	Negative	CYP2C9	CYP2C8
		10	96826988	Negative	10	96535228	Positive	CYP2C8	CYP2C19
		10	96541752	Positive	10	96493052	Positive	CYP2C19	CYP2C18
	Tumor tissue	10	96826985	Negative	10	96535225	Positive	CYP2C8	CYP2C19
Patient 5	Non-tumor tissue	6	44218178	Positive	15	58984703	Negative	HSP90AB1	HSP90AB4P
		10	96702073	Positive	10	96827034	Negative	CYP2C9	CYP2C8
		10	96702073	Positive	10	96535226	Positive	CYP2C9	CYP2C19
		10	96702073	Positive	10	96447961	Positive	CYP2C9	CYP2C18
	Tumor tissue	–	–	–	–	–	–	–	–
Patient 6	Non-tumor tissue	10	96702075	Positive	10	96827033	Negative	CYP2C9	CYP2C8
		22	36696211	Negative	19	50774702	Positive	MYH9	MYH14
	Tumor tissue	10	114428755	Positive	10	134691514	Positive	VTI1A	TTC40
		10	96541752	Positive	10	96493052	Positive	CYP2C19	CYP2C18

Chr, chromosome; Pos, position; CYP2C, cytochrome P450 2C; MAPK, mitogen-activated protein kinase; YY1AP1, YY1-associated protein 1; GON4L, GON4-like protein; MYH, myosin heavy chain; ANKRD11, ankyrin repeat domain 11; CHD2, chromodomain-helicase-DNA-binding protein 2; HSP90, heat shock protein 90; TTC40, tetratricopeptide repeat domain 40.

Results

Clinical Data

Clinical information on the 6 patients enrolled in the first phase of the study (Fig. 1) as well as the additional 13 patients enrolled in the second phase of the study (Fig. 1) is summarized in Table 1 and 2. Patients with a diagnosis of HCC who underwent liver transplantation at the Johns Hopkins Hospital in 2015 and 2016 (with the exception of 1 patient who had liver resection), were identified under our approved IRB protocol, and consent was obtained prior to surgery. Patients were between 48 and 68 years of age. Biological MELD scores ranged from 6 to 15. Regarding etiology of underlying liver disease, 14 out of 19 (73.7%) patients had HCV cirrhosis (2 patients had concomitant HIV infection, 1 had concomitant hepatitis B infection, and 1 had concomitant alcoholic liver disease); 3 patients had NASH or cryptogenic cirrhosis, 1 patient had HBV cirrhosis, and 1 patient had alcoholic cirrhosis. Pretransplant

Fig. 2. Validation of the VTI1A-CFAP46 fusion transcript. **a** VTI1A and CFAP46 are both located in chromosome 10: VTI1A is located at position 112446988–112947204, while CFAP46 is located at position 132808392–132942585 (NCBI Reference GRCh38.p7 Primary Assembly). Transcription of these genes occurs in opposite directions; as a result, the fusion transcript consists of the sense strand of VTI1A exons 1–4 and 6–8 fused to the antisense strand of CFAP46 exons 1–30. RT-PCRs of paired tumor/nonmalignant cases using primers spanning each fusion junction confirmed that bands corresponding to the fusion transcript were present only in tumor. Sanger sequencing confirmed the fusion junction sequences of VTI1A exon 8 and CFAP46 exon 30. **b** The protein encoded by the fusion transcript has lost 29 amino acids and changed 1 amino acid (I→Q) at the C-terminus of VTI1A due to a stop codon caused by the fusion.

AFP (alpha fetoprotein) levels ranged from 1.8 to 179.3. The duration of posttransplant follow-up was between 125 and 532 days. During the follow-up period, 1 patient required retransplantation and subsequently died due to complications from hepatic artery thrombosis and frequent infections. Two (10.5%) patients had HCC recurrence following surgery that was diagnosed during the follow-up period.

Pathology

Characteristics of tumors on explant pathology were reviewed by an expert pathologist and are summarized in Table 2. All patients had between 1 and 5 HCC lesions. 4 of 19 (21%) were discovered to be outside of the Milan Criteria on explant pathology. Five (26%) patients had well-differentiated tumors on explant histopathological exam, while the remainder had moderately differentiated HCCs. Overall, 5 (26.3%) of 19 patients had microvascular invasion on explant.

Targeted RNA-seq of 6 HCC and Paired Non-HCC Liver Tissues with Advanced Fibrosis or Cirrhosis, and Discovery of Fusion Transcripts

Six HCC samples and their matched nonmalignant tissues were first analyzed by targeted RNA-seq, as described above. The mean number of reads was $4,910,333 \pm 4801945$ (SD), and the length of each read was 76 nucleotides. The mean ratio of aligned reads was 97%; among aligned reads, 69.6% were matched to coding regions, 21.8% to UTR regions, 5.8% to intron regions, and 2.8% to nontargeted regions. We analyzed paired-end sequencing reads using Manta according to the strategy mentioned above, which identified various translocations or rearrangements in HCC and cirrhotic tissue.

Results of analysis of RNA-seq data are shown in Table 3. In HCC tissues, various rearrangements were noted that were paralogs likely false positive. We evaluated one of the rearrangements involving chromosome 10 which occurred in an HCC, between VTI1A and TTC40, and will be discussed.

Validation of the VTI1A-CFAP46 Fusion Transcript

We chose to study VTI1A-CFAP46 fusion in HCC, based on the reported fusions of these genes in the literature in other types of cancers [12, 17]. To validate our initial finding of the VTI1A-CFAP46 fusion transcript, we performed RT-PCR using primer pairs spanning the junctions of this fusion transcript (Fig. 2a). We were able to detect a band of the correct

Fig. 3. a Expression of wild-type VTI1A and the VTI1A-CFAP46 fusion transcript, measured by qRT-PCR in sample 12. In patient 6, total VTI1A expression was 6-fold upregulated in tumor relative to nonmalignant tissue; but, expression of the fusion transcript was 16-fold lower than wild-type VTI1A in tumor tissue. In this graph, the y axis illustrates relative expression of mRNA, with the expression of mRNA in sample 11, nontumor samples being 1. **b** Expression of wild-type VTI1A RNA in 19 studied patients (21 HCC and 19 paired nonmalignant specimens: total of 40 specimens). Among 21 HCC samples studied, VTI1A was upregulated in 10, downregulated in 4, and unchanged in the other 7 samples, compared to their matched non-HCC tissue. **c** Expression in all HCC samples compared to all non-HCCs. In this graph, the y axis illustrates relative expression of mRNA, with the expression of mRNA in each matched nontumor samples being 1. Significance was calculated using the paired *t* test, with $p < 0.05$ considered statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. C, cirrhosis; H, HCC. **d** Wild-type VTI1A protein expression in HCC and matched cirrhotic tissues of 12 patients. Western blot analysis was performed using antisera against VTI1A and GAPDH. S, sample; C, cirrhosis; H, HCC. * Anti-VTI1A and GAPDH quantity was low in sample 8 due to lack of adequate sample. ** Proteins were degraded in sample 10, precluding measurement of VTI1A and GAPDH. **e** The Western blot illustrating protein levels of anti-VTI1A in reported patient with VTI1A-CFAP46 mutation in HCC and non-HCC tissues.

	Number of cases, total	Number of cases, deceased	Median months survival
Cases with alteration(s) in query gene(s)	6	2	NA
Cases without alteration(s) in query gene(s)	359	125	58.84

	Number of cases, total	Number of cases, deceased	Median months survival
Cases with alteration(s) in query gene(s)	13	6	83.51
Cases without alteration(s) in query gene(s)	352	121	58.84

c

predicted size, representing the fusion transcript, in one primary HCC tissue (patient 6, sample 12). Next, we performed Sanger sequencing to confirm the breakpoints of VTI1A and CFAP46. Sequencing results confirmed that this fusion transcript arose from the sense strand of VTI1A exons 1–4 and 6–8 fused to the antisense strand of CFAP46 exons 1–30 (Fig. 2a). We also confirmed that the VTI1A genomic DNA was not similar to any other genomic region on chromosome 10 by BLAST. VTI1A and CFAP46 are both located on chromosome 10; therefore, we concluded that this transcript was transcribed from a genomic DNA VTI1A-CFAP46 translocation event. VTI1A isoform B has two domains, which are called V_SNARE and SNARE_Vti1a. CFAP46 has 13 domains, which are known as tetratricopeptide repeats (TPR) 1–13 (Fig. 2b). Finally, we were unable to detect this VTI1A-CFAP46 fusion transcript in either of two HCC-derived cell lines (97L, 97H) using RT-PCR.

Measuring Expression of the VTI1A-CFAP46 Fusion Transcript and the Wild-Type VTI1A Transcript

To measure expression levels of the VTI1A-CFAP46 fusion transcript in HCC, we performed qRT-PCR. We also measured the expression of wild-type VTI1A, using paired nonmalignant tissues as controls. This comparison revealed that the expression of the VTI1A-CFAP46 fusion transcript was 16-fold lower than the expression of wild-type VTI1A in the same tumor (Fig. 3a). Additionally, we measured the expression of wild-type VTI1A, which showed that in 10/21 HCC samples analyzed, wild-type VTI1A was upregulated (Fig. 3b, c).

Measurement of VTI1A Protein Expression in HCC and Non-HCC Tissues

To evaluate potential downstream modulation in protein expression, Western blotting was performed in the original HCC sample in which the VTI1A-CFAP46 fusion transcript was detected and compared with paired non-HCC tissue. The wild-type VTI1A protein was detected at 29 kDa in both samples and was similar in expression level between HCC and non-HCC cirrhotic tissues (Fig. 3e). There was no evidence of a mutated protein product resulting from VTI1A-CFAP46 fusion, at least on the Western blot. Western blotting was also performed on the remaining 6 paired HCC and non-HCC samples for anti-VTI1A; this experiment revealed no differences between normal and tumor tissues (Fig. 3d).

VTI1A and CFAP46 Mutations from cBioPortal

We then performed a search in the cBioPortal database [23–27] to look for any similar mutations reported in the literature for two genes of interest in patients with HCC: VTI1A and CFAP46. We did not find any reported fusions between VTI1A and CFAP46 in HCC. However, there were reports of other somatic mutations in CFAP46 and VTI1A genes on chromosome 10 (Table 4). Overall, both genes were altered in 26 (3%) of 872 sequenced patients with HCC. VTI1A was altered in 2 (0.2%) of 872 sequenced cases, and CFAP46 was altered in 24 (2.8%) of 872 sequenced cases (Fig. 4a, Table 4). In terms of overall survival, there was no difference between patients who had VTI1A mutation when compared to patients with no VTI1A mutation in the TCGA database (log-rank test $p = 0.819$) (Fig. 4b). It is important to note that the number of patients with mutation was small. Similarly, the overall survival was not different between patients with or without CFAP46 mutation (log-rank test $p = 0.592$) (Fig. 4c) [23–27].

Fig. 4. a Frequency of the somatic mutations for CFAP46 and VTI1A in cBioportal [23–27]. Overall, both genes were altered in 26 (3%) of 872 sequenced patients with HCC [23–27]. **b** Comparison of overall survival for patients with or without VTI1A in TCGA and cBioportal database (cBioPortal.org). **c** Comparison of overall survival for patients with or without CFAP46 in TCGA and cBioportal database (cBioPortal.org).

Table 4. Reported VT11A and CFAP46 mutations in cBioPortal [23–27]

Study	Mutation type	Copy number	Protein change	Allele frequency (T)	Chromosome
A VT11A mutations					
TCGA, provisional	Missense	ShallowDel	H117L	0.15	10
TCGA, provisional	Missense	Gain	V41L	0.28	10
TCGA, provisional	Missense	Gain	E42D	0.24	10
TCGA, provisional	Non-sense	Gain	K43*	0.25	10
TCGA, provisional	Frameshift deletion	Gain	V41Nfs*32	0.18	10
B CFAP46 mutations					
Inserm	Missense	–	A1606	–	10
Inserm	Nonsense	–	E192*	–	10
Inserm	Missense	–	K502E	–	10
Inserm	Missense	–	V1568L	–	10
Inserm	Missense	–	G2198R	–	10
Inserm	Missense	–	T1805N	–	10
Inserm	Missense	–	G1318S	–	10
Inserm	Missense	–	A1040T	–	10
Inserm	Missense	–	R598Q	–	10
Inserm	Missense	–	A18T	–	10
AMC	Missense	–	I1248V	0.25	10
AMC	Missense	–	G1571W	0.17	10
AMC	Missense	–	A18S	0.22	10
TCGA, provisional	Missense	Diploid	V366F	0.35	10
TCGA, provisional	Missense	Diploid	D1088N	0.49	10
TCGA, provisional	Missense	Diploid	M1213V	0.10	10
TCGA, provisional	Frameshift insertion	ShallowDel	R756Afs*17	0.08	10
TCGA, provisional	Missense	ShallowDel	L181I	0.68	10
TCGA, provisional	Missense	Gain	C2125S	0.4	10
TCGA, provisional	Missense	Gain	K1053M	0.49	10
TCGA, provisional	Missense	Diploid	D648Y	0.23	10
TCGA, provisional	Missense	ShallowDel	P2421S	0.08	10
TCGA, provisional	Missense	Diploid	L1006Q	0.28	10
TCGA, provisional	Missense	Diploid	V587A	0.05	10
TCGA, provisional	Missense	Diploid	I1772T	0.05	10
TCGA, provisional	Missense	Diploid	L1298Q	0.54	10
TCGA, provisional	Missense	ShallowDel	E1673G	0.05	10

Discussion

Newer genome sequencing technologies have now enabled us to explore the genomic profile of HCC more deeply to better identify its complex molecular pathophysiology. Several studies applying deep sequencing have provided information on the mutational profile of HCC [28–30]. These studies have revealed involvement of various critical signaling pathways. The most common mutations described have occurred in the TERT promoter, Wnt/ β -Catenin pathway genes, p53, chromatin remodeling genes, RAS/MAPK signaling components, and oxidative stress genes [31]. In addition, HCC mutations have been implicated in crucial cellular processes such as homeostasis, proliferation, and cell-cycle control. Hepatocellular tumors have been classified into two main groups based on their mutational profiles: proliferative and nonproliferative, each representing approximately 50% of patients. Overall, the proliferative subclass is defined by classic activation of RAS, mTOR and/or IGF signaling and is associated with a poor outcome relative to the

growth factor receptor 2 (FGFR2) have been discovered in patients with cholangiocarcinoma. Studies evaluating small molecular inhibitors targeting FGFR2 are under investigation [40].

It is now apparent that fusion transcripts are more common in solid tumors than was previously believed. Fusion transcripts offer several advantages as potential clinical tools. For example, they can be used in diagnosis, screening, and monitoring of disease recurrence [36, 41, 42]. Additional significance is conferred by the possibility of discovering downstream fusion proteins resulting from chimeric RNAs, which may exert cancer-promoting pathogenic effects. One evident example concerns imatinib, which was discovered in 2001 and targets the BCR-ABL fusion protein in patients with CML, with substantial therapeutic benefit [41].

In the current work, we explored fusion transcripts in HCC and discovered a novel transcript of particular note: VTI1A-CFAP46. Although the VTI1A-CFAP46 fusion transcript was detected only in one HCC sample, wild-type VTI1A RNA was upregulated in many of the HCC specimens. VTI1A wild-type protein, however, was not upregulated in HCC. This apparent discrepancy between RNA and protein findings could be due to differences in posttranscriptional processing or protein stability. We did not detect any mutant or truncated VTI1A-CFAP46 protein using a VTI1A antibody. At the present time, the precise role of this VTI1A-CFAP46 fusion transcript is unclear. VTI1A is known to be required for maturation of the autophagosome and mediates autophagy flux. Autophagy has both positive and negative functions in cancer. In normal tissues or cells, it is essential for the turnover of cellular components and degrades damaged organelles and proteins. In this setting, it relieves cell stress and genomic damage and suppresses tumorigenesis. In the liver, decreased autophagy can result in tumorigenesis [16]. This may occur because the liver is more active metabolically compared to other organs. In contrast, autophagy promotes tumor growth and angiogenesis in pancreatic and gastric cancers [43, 44]. Therefore, we believe that future studies are required to better define the relationship between autophagy and VTI1A or other SNARE proteins in advanced fibrosis, cirrhosis, and HCC that will help illuminate the pathogenesis of HCC, the link between fibrosis and HCC, and development of future hepatocellular cancer therapies.

Fusion transcripts involving VTI1A have been described in colorectal cancers. In one study, VTI1A was fused with TCF7L2 in 3% of colorectal cancers. TCF7L2 encodes TCF4, which cooperates with β -catenin in colorectal carcinogenesis, but the fusion protein lacked the TCF4 β -catenin-binding domain [12]. Functions of the CFAP46 gene are not known and open to question. The CFAP46 protein contains a TPR that is a structural motif, consisting of a degenerate 34-amino acid sequence motif that has been identified in a wide variety of proteins. The TPR domain is found in tandem arrays of 3–16 motifs, which form scaffolds to mediate protein–protein interactions and often the assembly of multiprotein complexes. The consequences of CFAP46 dysregulation in cancer remain an unanswered question deserving further investigation [17]. In one study, the 5'-TTC40 gene (the old name for CFAP46) was frequently hypermethylated and associated with loss of mRNA expression in nasopharyngeal cancer [17]. It was therefore proposed that TTC40 (the old name for CFAP46) might play a role in the development of this cancer [17].

Our findings in the current study establish the importance of further fusion transcript research in HCC. Several critical questions remain, such as: (1) How frequent are fusion transcripts in HCC? (2) What causes fusion transcripts to occur in HCC? and (3) What is the significance of these types of genomic alteration in HCC? In the future, the frequency of these alterations can be determined by performing RNA-seq in much larger cohorts of HCC and paired nonmalignant tissues, since the occurrence rates of fusion transcripts may be very low [45]. Recently, chromothripsis and chromoplexy have been discovered as causes of fusion transcripts [46, 47]. Retrotransposition is also well known to cause fusion genes or insertions. This fusion transcript may also have been generated by cryptic splicing. In this context, the antisense strand of CFAP46 exon 30 has a splice acceptor site and polypyrimidine tract; moreover, cryptic splicing would explain why the breakpoint matched the middle sequence of the CFAP46 exon

(Fig. 5). Cryptic splicing is known to cause certain diseases, such as Hutchinson-Gilford progeria [48]. Although speculative, we hypothesize that cryptic splicing may also be involved in the pathogenesis of HCC but requires further studies.

Herein, we have described a novel fusion transcript, VTI1A-CFAP46, whose function merits further investigation in future studies. As discussed above, the link between this alteration and autophagy deserves further exploration. It is well known that autophagy is involved in HCC as well as benign liver disease. Our study has also revealed several fusions and rearrangement transcripts in HCC and cirrhosis that warrant further investigation. It is well known that advanced liver fibrosis and cirrhosis are strongly associated with HCC [48]. Although several potential mechanisms associating liver fibrosis with hepatocarcinogenesis have been described [49, 50], the precise mechanism underlying this connection has not yet been firmly elucidated [48]. Studying fusion transcripts in this context may help us to better understand the link between fibrosis and HCC.

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Statement of Ethics

These studies were performed in compliance with approved Johns Hopkins human subjects IRB Protocol NA_00004336.

Disclosure Statement

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Author Contributions

S.T.: study concept and design, acquisition of data, analysis and interpretation of data, statistical analysis, drafting of the manuscript; B.S.: study concept and design, acquisition of data, analysis and interpretation of data, statistical analysis, drafting of the manuscript; Y.C.: study concept and design, acquisition of data, analysis and interpretation of data, statistical analysis; Z.W.: acquisition of data, analysis and interpretation of data, statistical analysis; A.K.: acquisition of data, drafting of the manuscript; H.L.: acquisition of data; J.M.A.: analysis and interpretation of data, statistical analysis; M.D.Y.: acquisition of data; J.P.H.: acquisition of data, funding; F.M.S.: study concept and design, analysis and interpretation of data; C.V.-M.: acquisition of data, drafting of the manuscript, analysis and interpretation of data, statistical analysis; F.S.: acquisition of data, analysis and interpretation of data; B.P.: acquisition of data; A.M.C.: acquisition of data; Q.Z.: acquisition of data; R.A.: acquisition of data, analysis and interpretation of data; A.G.: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, study supervision, funding; S.J.M.: study concept and design, acquisition of data, drafting of the manuscript, analysis and interpretation of data, statistical analysis, critical revision of the manuscript for important intellectual content, study supervision, funding.

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