Integrated analysis of pseudogene *RP11-564D11.3* expression and its potential roles in hepatocellular carcinoma

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Aim: We aim to identify differentially expressed pseudogenes and investigate their functional roles in carcinogenesis. **Materials & methods:** Here, we identify dysregulated pseudogenes, analyze their prognostic values and investigate their potential functions through pseudogene–miRNA–mRNA network from public -omics repositories. **Results:** We identified 16 frequently upregulated pseudogenes among which high expression levels of *RP11-564D11.3* was significantly associated with poor overall survival in seven types of human cancers. *RP11-564D11.3* was strongly correlated with pathways in cancer, PI3K–Akt signaling pathway and the neurotrophin signaling pathway. Further studies revealed that *RP11-564D11.3* functions as a competitive endogenous RNA through targeting *VEGFA* in hepatocellular carcinoma. **Conclusion:** Our findings suggest *RP11-564D11.3* as a novel biomarker and therapeutic potential target against hepatocellular carcinoma.

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Keywords: hepatocellular carcinoma • pseudogene • *RP11-564D11.3* • *VEGFA*

Global transcriptomic analysis have shown that of the 70–90% of human genome transcribed into RNAs, only 1–2% is protein-coding genes and the remainder is noncoding RNA (ncRNA) [1]. To date, several ncRNAs have been identified, such as miRNA, long ncRNAs, and circular RNAs, as well as pseudogenes. Emerging evidence have established that dysregulated ncRNAs play crucial roles in physiological and pathophysiological processes, including control of chromosome modification, RNA processing, translation inhibition and mRNA degradation [2–4].

The competitive endogenous RNA (ceRNA) hypothesis may explain, at least in part, the complex biological functions of ncRNA in the pathogenesis of human cancer [5,6]. The ceRNA crosstalk is based on an ‘miRNA response element language’ in which any transcripts containing the same miRNA response element can co-regulate each other by competing for binding to shared miRNAs [6]. The ceRNA network was discovered in diverse species, including humans, mice, plants and viruses [7–10]. Therefore, the ceRNA hypothesis provides a basic framework to predict the potential functions of novel ncRNAs.

Pseudogenes are a class of ncRNAs and defunct copies of protein-coding genes arisen from random replication and mutation during evolutionary process [11,12]. There are two types of pseudogenes: processed and nonprocessed, which were generated from gene retrotransposition and duplication events, respectively [13,14]. Recent advances have shown that pseudogenes can regulate their parental genes and also unrelated genes in multiple ways in the pathogenesis of human cancer [15–17]. For example, pseudogene *PTENP1* functions as a ceRNA to regulate parental *PTEN* expression and affect tumorigenesis by sponging miR-19b, miR-21 and miR-499-5p [18–20]. Meanwhile,

PTENP1 can also act as ceRNA for *PTEN*-independent genes, such as *SOCS6*, *PHLPP* and *P62* [21,22]. These findings suggest that pseudogenes can exert biological functions through regulating not only their parental genes but also unrelated genes.

In this study, we first identified differentially expressed pseudogenes by browsing the pseudogene database dreamBase. Then we explored the prognostic values of the dysregulated pseudogenes based on the Cancer Genome Atlas (TCGA) databases and further investigated its potential functions and molecular mechanisms through the pseudogene–miRNA–mRNA network.

Materials & methods

Identification of differentially expressed pseudogenes

RNA-Seq expression data of pseudogenes in 32 types of human cancers were downloaded from the dreamBase database (<http://rna.sysu.edu.cn/dreamBase/index.php>) [23]. The cutoff for differentially expressed pseudogenes was set at $|\log_2 \text{FC}| > 2.0$. Their expression levels were further validated by expression landscape. Heat map of differentially expressed pseudogenes was visualized using MeV v4.8.1 [24].

Kaplan–Meier survival analysis

For pseudogenes, Kaplan–Meier overall survival analysis and log-rank test were used to evaluate the statistical significance of survival differences between the two groups using GEPIA [25]. The cut-off value between the two groups was ‘Median’. For mRNA, Kaplan–Meier overall survival analysis was performed using Kaplan–Meier Plotter [26].

Construction of pseudogene–miRNA–mRNA regulatory network

miRNAs binding to pseudogene were identified using starBase v2.0 [27]. Target genes for identified miRNA were retrieved from miRTarBase [28]. Pseudogene–miRNA–mRNA ceRNA network were visualized using Cytoscape v3.4.0 [29].

Gene ontology & KEGG pathway enrichment analysis

Pathway enrichment analysis for enriched target genes was conducted using ClueGO v2.3.5 plugin of Cytoscape v3.4.0 [30]. GO items/pathways with $p < 0.05$ were considered significant difference.

Protein–protein interaction network & hub genes analysis

Protein–protein interaction network for enriched target genes was analyzed using STRING v10.0 and visualized by Cytoscape v3.4.0 [31]. Genes with top ten degree were identified as hub genes using Centiscape 2.2 plugin of Cytoscape v3.4.0 [32].

Gene expression analysis

VEGFA mRNA and protein expression levels in hepatocellular carcinoma tissues were analyzed using ULACAN and HumanProteinAtlas, respectively [33,34].

Mutations & copy-number alterations analysis

DNA mutations and copy-number alterations of hub genes from 442 patients with liver hepatocellular carcinoma (TCGA, provisional) were analyzed using cBioPortal [35].

Results

Identification of dysregulated pseudogenes in human cancers

In China, lung adenocarcinoma, stomach adenocarcinoma and liver adenocarcinoma are the most common malignant tumors with high morbidity and mortality. To investigate the potential roles of pseudogenes in human cancers, we first identified frequently abnormally expressed pseudogenes using dreamBase. As shown in Figure 1A & B, 42 upregulated and two downregulated pseudogenes were identified in lung adenocarcinoma, stomach adenocarcinoma and hepatocellular carcinoma. After screening by pseudogene names and expression levels, 16 frequently upregulated pseudogenes in 32 types of human cancers were selected for further investigation (Table 1). Their expression levels in 32 types of human cancers were shown in Figure 1C. These results indicate that these 16 upregulated pseudogenes may play key roles in carcinogenesis.

Figure 1. Identification of differentially expressed pseudogenes in human cancers. RNA-Seq expression data of pseudogenes in 32 types of human cancers were downloaded from dreamBase (<http://rna.sysu.edu.cn/dreamBase/index.php>). The cutoff for differentially expressed pseudogenes was set at $|\log_2 \text{FC}| > 2.0$. Their expression levels were further validated by expression landscape. **(A)** Upregulated pseudogenes in lung adenocarcinoma, stomach adenocarcinoma and hepatocellular carcinoma. **(B)** Downregulated pseudogenes in lung adenocarcinoma, stomach adenocarcinoma and hepatocellular carcinoma. **(C)** Heat map of 16 frequently upregulated pseudogenes in 32 types of human cancers visualized by MeV v4.8.1. Red represents upregulated genes and green represents downregulated genes.

Prognostic values of differentially expressed pseudogenes

Next, we explored the prognostic values of 16 dysregulated pseudogenes in human cancers based on TCGA data using GEPIA. As shown in Figure 2, high expression levels of *RP11-424C20.2* were identified to be most significantly correlated with overall survival, worse prognosis in seven human cancers and better prognosis in thymoma. High expression levels of *RP11-564D11.3* was associated with worse outcomes in seven human cancers. High mRNA expression levels of *SDHDP6* was significantly associated with better outcome in patients with breast invasive carcinoma ($p = 0.032$). Increased *RP11-146E13.5*, *ZBTB45P1* and *HMGB2P1* were significantly associated with poor outcomes in pheochromocytoma and paraganglioma ($p = 0.0094$), cervical and endocervical cancer ($p = 0.028$) and kidney clear cell carcinoma ($p = 0.0053$), respectively. These results indicate that *RP11-564D11.3* may have high prognostic values, and function as an oncogene in tumorigenesis and tumor progression.

Table 1. Information of 16 frequently upregulated pseudogenes in human cancers identified by dreamBase.

Pseudogene name	Ensembl ID	Genome location	Gene type	Parent gene name
<i>SDHDP6</i>	ENST00000414693	chr1:25294164-25294643	processed_pseudogene	<i>SDHD</i>
<i>RP11-565J7.1</i>	ENST00000416056	chr1:211936249-211936634	processed_pseudogene	<i>PIN4</i>
<i>RP11-146E13.5</i>	ENST00000619332	chr14:19131842-19132890	processed_pseudogene	<i>TOMM40</i>
<i>RP11-424C20.2</i>	ENST00000540175	chr12:20551590-20553012	processed_pseudogene	<i>UHRF1</i>
<i>RP11-247I13.3</i>	ENST00000445477	chr22:31559483-31559939	processed_pseudogene	<i>RPS18</i>
<i>MRPL40P1</i>	ENST00000542687	chr12:67351436-67352039	processed_pseudogene	<i>MRPL40</i>
<i>RP11-141C7.3</i>	ENST00000603917	chr3:195650146-195651472	processed_pseudogene	<i>PPP4R2</i>
<i>RP11-564D11.3</i>	ENST00000368895	chr10:122879851-122898024	transcribed_processed_pseudogene	<i>C10orf88</i>
<i>UBE2CP2</i>	ENST00000583290	chr18:22900486-22900995	processed_pseudogene	<i>UBE2C</i>
<i>ZBTB45P1</i>	ENST00000452761	chr2:109986939-109988394	processed_pseudogene	<i>ZBTB45</i>
<i>HMGB2P1</i>	ENST00000591146	chr19:1203209-1203842	transcribed_processed_pseudogene	<i>HMGB2</i>
<i>BMS1P22</i>	ENST00000414726	chr22:15805847-15806503	transcribed_unprocessed_pseudogene	<i>BMS1</i>
<i>RP11-173M1.5</i>	ENST00000582878	chr17:27351858-27352584	processed_pseudogene	<i>SYPL1</i>
<i>AURKAP51</i>	ENST00000451805	chr1:220266706-220267917	transcribed_processed_pseudogene	<i>AURKA</i>
<i>NCAPGP1</i>	ENST00000505112	chr8:120635558-120638204	processed_pseudogene	<i>NCAPG</i>
<i>HNRNPA1P21</i>	ENST00000424115	chr3:39334979-39335939	processed_pseudogene	<i>HNRNPA1</i>

Cancer type	SDHDP6	RP11-565J7.1	RP11-146E13.5	RP11-424C20.2	RP11-247I13.3	MRPL40P1	RP11-141C7.3	RP11-564D11.3	UBE2CP2	ZBTB45P1	HMGB2P1	BMS1P22	RP11-173M1.5	AURKAP51	NCAPGP1	HNRNPA1P21
Adrenocortical carcinoma	0.35	0.32	0.003	0.00025	0.0021	0.02	0.11	0.2	0.68	0.52	0.27	0.005	0.00000	0.16	0.28	0.35
Bladder urothelial carcinoma	0.79	0.58	0.34	0.062	0.27	0.97	0.21	0.74	0.31	0.053	0.094	0.8	0.21	0.25	0.5	0.92
Breast invasive carcinoma	0.032	0.8	0.27	0.19	0.044	0.73	0.44	0.93	0.26	0.72	0.3	0.58	0.47	0.5		0.16
Cervical squamous cell carcinoma and endocervical adenocarcinoma	0.46	0.53	0.39	0.78	0.17	0.19	0.92	0.91	0.1	0.028	0.074	0.17	0.94	0.12		0.16
Cholangio carcinoma	0.51	0.69	0.2	0.88	0.73	0.58	0.68	0.8	0.39	0.083	0.85	0.91	0.45	0.47	0.58	0.15
Colon adenocarcinoma	0.51	0.7	0.1	0.615	0.02	0.68	0.99	0.5	0.86	0.85	0.066	0.007	0.37	0.9088	0.49	
Lymphoid neoplasm diffuse large B-cell lymphoma	0.56	0.73		0.79	0.085	0.73	0.62	0.95	0.9	0.33	0.24	0.11	0.31	0.24		0.79
Esophageal carcinoma	0.51	0.013	0.82	0.61	0.52	0.5	0.89	0.19	0.25	0.29	0.61	0.36	0.37	0.027	0.87	0.17
Glioblastoma carcinoma	0.12	0.99	0.51	0.99		0.82	0.64	0.67	0.27	0.27		0.73	0.26	0.41		
Head and neck squamous cell carcinoma	0.84	0.38	0.7	0.4	0.38	0.96	0.4	0.93	0.51	0.46	0.91	0.068	0.21	0.64		0.0038
Kidney chromophobe	0.48	0.38		0.91	0.96		0.73	0.013	0.97	0.067	0.92	0.003	0.92	0.23	0.17	0.26
Kidney renal clear cell carcinoma	0.13	0.066	0.063	0.27	0.00018	0.0035	0.0002	0.023	0.0017	0.97	0.0033	0.0003	0.002	0.18	0.00027	0.0025
Kidney renal papillary cell carcinoma	0.57	0.73		0.554	0.00081	0.17	0.16	0.00031	0.56	0.31	0.13	0.0035	0.001	0.62		0.46
Acute myeloid leukemia	0.58	0.91		0.98	0.39	0.47	0.46	0.35	0.99	0.23	0.31		0.97	0.18	0.34	0.83
Brain lower grade glioma	0.065	0.48		0.36		0.036	0.34	0.85	0.022	0.48		0.27	0.12	0.11		0.83
Liver hepatocellular carcinoma	0.12	0.82	0.081	0.032	0.74	0.062	0.1	0.0087	0.7	0.74	0.08	0.047	0.017	0.018	0.038	0.37
Lung adenocarcinoma	0.81	0.82	0.22	0.0009	0.23	0.0078	0.81	0.035	0.48	0.45	0.97	0.014	0.3	0.6	0.032	0.83
Lung squamous cell carcinoma	0.55	0.066	0.59	0.29	0.086	0.71	0.96	0.19	0.74	0.19	0.87	0.75	0.49	0.76		0.49
Mesothelioma	0.08	0.92	0.73	0.000035	0.0015	0.61	0.81	0.013	0.089	0.18	0.092	0.24	0.64	0.28		0.43
Ovarian serous cystadenocarcinoma	0.12	0.87	0.75	0.4	0.1	0.47	0.63	0.96	0.5	0.37	0.56	0.76	0.15	0.95	0.51	0.15
Pancreatic adenocarcinoma	0.1	0.52	0.76	0.2	0.76	0.042	0.73	0.98	0.4	0.91	0.5	0.16	0.27	0.27	0.16	0.5
Pharyngeal carcinoma and paraganglioma	0.88	0.78	0.0004	0.23	0.61	0.47	0.19	0.00088	0.75	0.63	0.92	0.67	0.77	0.12		0.5
Prostate adenocarcinoma	0.83	0.45	0.76	0.016	0.99	0.52	0.035	0.74	0.068	0.44	0.49	0.81	0.24	0.55		0.49
Rectum adenocarcinoma		0.55		0.17	0.38	0.43	0.18	0.12	0.21	0.056	0.17	0.21	0.056	0.076	0.2	0.74
Sarcoma	0.54	1	0.44	0.000	0.98	0.89	0.75	0.77	0.037	0.16		0.18	0.72	0.68		0.4
Skin cutaneous melanoma	0.13	0.035	0.093	0.0004	0.72	0.85	0.85	0.18	0.57	1		0.98	0.53	0.74	0.4	0.000016
Stomach adenocarcinoma	0.82	0.74	0.068	0.27	0.45	0.43	0.86	0.33	0.91	0.14	0.8	0.81	0.35	0.87	0.53	0.006
Testicular germ cell tumors	0.052	0.52		0.48	0.98	0.78	0.95	0.55	0.57	0.96	0.38	0.55	0.2	0.75	0.46	0.064
Thyroid carcinoma	0.18	0.31	0.15	0.14	0.86	0.59	0.99	0.46	0.16	0.15		0.69	0.48	0.85		0.82
Thymoma	0.84	0.55	0.74	0.03	0.93	0.078	0.52	0.19	0.41	0.55	0.66	0.58	0.83	0.4		0.083
Uterine corpus endometrial carcinoma	0.74	0.88	0.1	0.86	0.33	0.97	0.88	0.27	0.68	0.72	0.54	0.078	0.85	0.77	0.78	0.64
Uterine carcinosarcoma	0.84	0.75	0.88	0.74	0.83	0.010	0.79	0.62	0.0068	0.72		0.18	0.88	0.86		0.17
Uveal melanoma	0.58	0.57		0.47	0.055	0.44	0.26		0.63				0.77	0.76		

Figure 2. Prognostic values of 16 differentially expressed pseudogenes in 33 types of human cancers. Kaplan–Meier overall survival analysis for 16 frequently upregulated pseudogenes were performed using GEPIA. The cut-off value between the two groups was ‘Median’. GEPIA uses log-rank test, a.k.a the Mantel-Cox test, for hypothesis test. p value < 0.05 was considered statistically significant. Red represents poor outcome, yellow represents neutral outcome, green represents good outcome and white means that the sample size is insufficient at custom thresholds.

GEPIA: Gene expression profiling interactive analysis.

Pseudogene–miRNA–mRNA regulatory network

ceRNA is an important regulatory mechanism for pseudogenes, in which pseudogenes function as RNA sponges for miRNAs and regulate target genes’ expression through competing for binding of common miRNAs. starBase was used to identify potential miRNAs which can bind to pseudogenes. For *RP11-564D11.3*, miR-9-5p, miR-101-3p and the miR-200 family (miR-141-3p, miR-200a-3p, miR-200b-3p, miR-200c-3p and miR-429) were identified to be candidate miRNAs (Table 2). miRNA target genes were validated by at least one strongly experimental method (reporter assay, Western blot or qRT-PCR) using miRTarBase. In total, 338 genes were identified to be direct targets of candidate miRNAs and the pseudogene–miRNA–mRNA regulatory network was then visualized using Cytoscape (Figure 3). Thus, *RP11-564D11.3* may exert biological functions through regulating these target genes.

Table 2. miRNAs targeting *RP11-564D11.3* were predicted by starBase v2.0.

miRNA names	mirAccession	TargetSites	BioComplex	ClipReadNum
miR-200b-3p	MIMAT0000318	1	2	458
miR-200a-3p	MIMAT0000682	1	6	709
miR-429	MIMAT0001536	1	2	458
miR-101-3p	MIMAT0000099	1	6	709
miR-9-5p	MIMAT0000441	1	1	3
miR-200c-3p	MIMAT0000617	1	2	458
miR-141-3p	MIMAT0000432	1	6	709

Table 3. Ten hub genes identified by Centiscape 2.2.

Gene names	Degree unDir	Closeness unDir	Betweenness unDir	Gene descriptions
<i>TP53</i>	150	0.002155	10,580.06	Tumor protein p53
<i>MYC</i>	123	0.002024	4377.777	MYC proto-oncogene, bHLH transcription factor
<i>VEGFA</i>	112	0.001992	4076.718	Vascular endothelial growth factor A
<i>EGFR</i>	109	0.001949	5191.221	Epidermal growth factor receptor
<i>JUN</i>	107	0.001953	2706.145	Jun proto-oncogene, AP-1 transcription factor subunit
<i>CTNNB1</i>	101	0.001938	3890.535	Catenin beta 1
<i>NOTCH1</i>	99	0.001942	2583.603	Notch 1
<i>IL6</i>	97	0.001894	2853.689	Interleukin 6
<i>CCND1</i>	96	0.001905	2476.921	Cyclin D1
<i>BCL2</i>	94	0.001887	1533.52	B cell leukemia/lymphoma 2

Functional enrichment analysis of miRNA target genes

To investigate the potential functions of *RP11-564D11.3* in cancer development, we performed GO and KEGG pathway enrichment analysis using analyzed ClueGo. For *RP11-564D11.3*, 81 GO terms were enriched and finally classified into 24 groups with KappaScore (Figure 4). The most significantly enriched gene sets were pathways in cancer (KEGG: 05200; $p = 8.84\text{E-}28$), the PI3K–Akt signaling pathway (KEGG: 04151; $p = 1.13\text{E-}17$) and neurotrophin signaling pathway (KEGG: 04722; $p = 9.01\text{E-}11$). Consistent with the prognostic value of *RP11-564D11.3* in human cancers, renal cell carcinoma, small-cell lung cancer, non-small-cell lung cancer, hepatitis C and hepatitis B were enriched. These findings confirm that upregulated *RP11-564D11.3* is involved in the initiation and progression of hepatocellular carcinoma, lung adenocarcinoma and renal cell carcinoma.

VEGFA was identified as target gene of *RP11-564D11.3*

To further analyze the functional roles of *RP11-564D11.3*, a protein–protein interaction network was generated using STRING and hub genes were selected using Centiscape 2.2 (Figure 5). Genes with top ten degree (*TP53*, *MYC*, *VEGFA*, *EGFR*, *JUN*, *CTNNB1*, *NOTCH1*, *IL6*, *CCND1* and *BCL2*) were identified as hub genes for *RP11-564D11.3* (Table 3). Pearson correlation analysis between *RP11-564D11.3* and ten hub genes in kidney renal clear cell carcinoma, hepatocellular carcinoma and lung adenocarcinoma results showed that six out of ten hubs (*TP53*, *VEGFA*, *JUN*, *CTNNB1*, *NOTCH1* and *BCL2*) in hepatocellular carcinoma, three out of ten hubs (*VEGFA*, *IL6* and *BCL2*) in lung adenocarcinoma and one out of ten hubs (*EGFR*) in kidney renal clear cell carcinoma were significantly correlated with *RP11-564D11.3* expression levels, respectively (Table 4). These results indicate that *RP11-564D11.3* may possess higher prognostic values or play more crucial roles in hepatocellular carcinoma compared with that of kidney renal clear cell carcinoma and lung adenocarcinoma.

Then we investigated the gene mutations and copy-number alterations of these ten hubs in hepatocellular carcinoma using cBioportal. The results demonstrated that *TP53* and *CTNNB1* were exclusively mutant, occurred in 111 (30.8%) of 360 patients and 96 (26.7%) of 360 patients, respectively. *MYC*, *VEGFA* and *CCND1* were significantly amplified, occurred in 63 (17.5%) of 360 patients, 30 (8.3%) of 360 patients and 26 (7.2%) of 360 patients, respectively (Figure 6). We also analyzed the prognostic values of ten hubs in hepatocellular carcinoma using Kaplan–Meier plotter from GEO datasets. We found that high mRNA expression levels of *TP53*, *EGFR* and *CCND1* were significantly associated with good overall survival ($p = 0.029$; $p = 0.0085$; $p = 0.03$,

Figure 3. Construction of pseudogene-miRNA-mRNA regulatory network. miR-141-3p, miR-200a-3p, miR-200b-3p, miR-200c-3p and miR-429 were identified as candidate miRNAs targeting *RP11-564D11.3* using starBase v2.0. Target genes for these miRNAs were retrieved by at least one of strongly experimental methods (reporter assay, Western blot or qRT-PCR) from miRtarbase. Pseudogene-miRNA-mRNA interaction network was visualized using Cytoscape v3.4.0.

respectively; Table 5). Only high mRNA expression level of *VEGFA* was significantly associated with poor overall survival ($p = 0.0025$). According to ceRNA hypothesis, *VEGFA* might be a potential target of *RP11-564D11.3* in hepatocellular carcinoma (Figure 7A). *VEGFA* mRNA and protein expression levels were further validated higher

Figure 4. Functional enrichment analysis of miRNA target genes. GO and KEGG pathway enrichment analysis of 338 *RP11-564D11.3*-related genes were performed using ClueGo v2.3.5 plugin of Cytoscape v3.4.0. Total 81 terms/pathways were enriched ($p < 0.05$). After merging redundant groups with $>50.0\%$ overlap, 24 terms/pathways were finally selected. Enrichment/depletion (two-sided hypergeometric test) and Bonferroni step down were used for statistical test and correction method, respectively. Pathways in cancer (KEGG: 05200; $p = 8.84E-28$), PI3K-Akt signaling pathway (KEGG: 04151; $p = 1.13E-17$) and Neurotrophin signaling pathway (KEGG: 04722; $p = 9.01E-11$) were the top three significantly enriched gene sets.

in hepatocellular carcinoma tissues compared that of normal tissues (Figure 7B & C). These finding indicate that *RP11-564D11.3* functions as a ceRNA through targeting *VEGFA* in hepatocellular carcinoma.

Discussion

Pseudogenes have long been dismissed as ‘junk’ or ‘fossil’ DNA. Recent studies have characterized several functional pseudogenes, such as *PDIA3P1*, *NMRAL2P* and *HMGA1P5* [36–38]. *HMGA1P6* and *HMGA1P7* are two processed pseudogenes, and overexpression of *HMGA1P6* and *HMGA1P7* could increase the levels of HMGA1 and other

Figure 5. Identification of hub genes. The protein–protein interaction network of 338 genes was constructed using STRING v10.0. Confidence score ≥ 0.4 was set as the cutoff criterion. Then the gene degree was further analyzed by Centiscape 2.2 plugin of Cytoscape v3.4.0. Genes with top 10 degree value were identified as hub genes.

Table 4. Pearson correlation analysis between <i>RP11-564D11.3</i> and ten hub genes expressions in hepatocellular carcinoma, lung adenocarcinoma and kidney renal clear cell carcinoma using GEPIA.						
Gene names	Hepatocellular carcinoma		Lung adenocarcinoma		Kidney renal clear cell carcinoma	
	p-value	R	p-value	R	p-value	R
<i>TP53</i>	0.015	0.13	0.059	0.2	0.58	0.025
<i>MYC</i>	0.39	0.045	0.067	0.084	0.35	0.041
<i>VEGFA</i>	1.90E-07	0.27	0.0031	0.13	0.22	-0.053
<i>EGFR</i>	0.089	0.089	0.76	0.014	0.033	0.093
<i>JUN</i>	0.011	0.13	0.055	-0.087	0.83	-0.0097
<i>CTNNB1</i>	0.0012	0.17	0.096	0.076	0.48	-0.031
<i>NOTCH1</i>	5.10E-08	0.28	0.23	-0.054	0.37	-0.039
<i>IL6</i>	0.91	0.0056	0.01	0.12	0.46	0.032
<i>CCND1</i>	0.77	0.015	0.13	0.07	0.19	-0.057
<i>BCL2</i>	0.00018	0.19	0.03	0.099	0.1	-0.071
GEPIA: Gene expression profiling interactive analysis.

Figure 6. Gene mutations and copy-number alterations analysis of ten hub genes in 442 patients (only 360 patients' data available) with liver hepatocellular carcinoma (TCGA, provisional) were analyzed using cBioPortal. Tumor samples are shown in columns. TCGA: The Cancer Genome Atlas.

Table 5. Prognostic values of ten hub genes in hepatocellular carcinoma identified by Kaplan–Meier plotter.				
Gene names	RNAseq IDs	p-value	HRs with 95% CIs	Prognostic outcomes
TP53	7157	0.029	0.65 (0.45–0.96)	Good
MYC	4609	0.088	1.38 (0.95–1.99)	Poor
VEGFA	7422	0.0025	1.74 (1.21–2.5)	Poor
EGFR	1956	0.0085	0.61 (0.43–0.89)	Good
JUN	3725	0.36	1.18 (0.83–1.66)	Poor
CTNNB1	1499	0.37	1.17 (0.83–1.65)	Poor
NOTCH1	4851	0.10	0.75 (0.53–1.06)	Good
IL6	3569	0.42	1.16 (0.81–1.65)	Poor
CCND1	595	0.03	0.68 (0.48–0.97)	Good
BCL2	596	0.072	0.72 (0.51–1.03)	Good

CI: Confidence interval; HR: Hazard ratio.

cancer-related proteins through a ceRNA mechanism, and contribute to carcinogenesis [38–41]. However, pseudogenes and their underlying regulatory mechanisms remain largely unknown. Identifying differentially expressed pseudogenes and exploring their potential functions will facilitate the prognostic prediction and targeted therapy of human cancers.

In this study, we explored the abnormally expressed pseudogenes in hepatocellular carcinoma, lung adenocarcinoma and stomach adenocarcinoma by utilizing the dreamBase database and identified 16 frequently upregulated pseudogenes in 32 types of human cancers. Kaplan–Meier survival analysis revealed that all the 16 pseudogenes were significantly associated with overall survival in at least one type of human cancer. Among them, high expression levels of *RP11-564D11.3* were obviously correlated with poor overall survival of patients with kidney diseases, hepatocellular carcinoma, lung adenocarcinoma, mesothelioma and pheochromocytoma and paraganglioma. These findings suggest that *RP11-564D11.3* may function as an oncogene involved in carcinogenesis.

ceRNAs hypothesis provides an important predictive pattern for unidentified ncRNA. In this pattern, pseudogenes act as decoys for miRNAs to affect cognate or noncognate target genes. To predict the functional roles of *RP11-564D11.3*, we constructed a pseudogene–miRNA–mRNA interaction network. *RP11-564D11.3* was identified to be a direct target gene for miR-9-5p, miR-101-3p and the miR-200 family, and 334 genes may be potential regulatory targets of *RP11-564D11.3*.

Figure 7. *VEGFA* was identified as the target gene of *RP11-564D11.3*. (A) Pearson correlation analysis and prognostic outcomes analysis of 10 hub genes revealed *VEGFA* was a target gene of *RP11-564D11.3*. (B) *VEGFA* mRNA expression level in 371 hepatocellular carcinoma tissues and 50 normal hepatocellular tissues was analyzed using UALCAN. (C) *VEGFA* protein expression level in hepatocellular carcinoma tissues and normal hepatocellular tissues was analyzed using immunohistochemical staining from HumanProteinAtlas.

** p value < 0.01.

TCGA: The Cancer Genome Atlas.

Several studies demonstrated that miR-9-5p has a dual role in carcinogenesis as either a tumor suppressor or a tumor promoter. For example, upregulated miR-9-5p promotes cell growth and metastasis of non-small-cell lung cancer and hepatocellular carcinoma [42,43]. On the other hand, miR-9-5p inhibited cell proliferation and progression of gastric cancer and ovarian cancer [44,45]. miR-101-3p was frequently downregulated and could regulate growth, metastasis and tumor metabolism in a variety of human cancers, including bladder cancer, hepatocellular carcinoma, lung squamous carcinoma and breast cancer [46–49]. The miR-200 family was reported as tumor suppressor miRNA and has been implicated in epithelial-to-mesenchymal transition, metastasis, angiogenesis and chemoresistance in many different cancers [50–53].

Functional enrichment analysis of 334 target genes revealed multiple *RP11-564D11.3*-associated GO items/pathways. We also observed that pathways in cancer (KEGG: 05200), PI3K–Akt signaling pathway (KEGG: 04151) and neurotrophin signaling pathway (KEGG: 04722) were the top three significantly enriched gene sets.

The PI3K–Akt signaling pathway is one of the most frequently activated signal transduction pathways in human cancer. Activation of the PI3K–Akt signaling pathway triggers a cascade of responses, from cell proliferation and survival to chemoresistance [54–56]. Interestingly, we found that *RP11-564D11.3* was also significantly associated with neurotrophin signaling pathway. The neurotrophins family comprises four related proteins; NGFB, BDNF, NTF3 and NTF4/5. They play important roles in proliferation, differentiation and survival of neural cells [54]. Recent studies revealed neurotrophins and their receptors are also expressed in cancer cells and regulate cell proliferation, aggressiveness and metastasis [58–60].

In this study, we also performed hub genes identification and person correlation analysis between *RP11-564D11.3* and hub genes. We observed mRNA expression levels of six out of ten hubs (*TP53*, *VEGFA*, *JUN*, *CTNNB1*, *NOTCH1* and *BCL2*) were significant positively correlated with *RP11-564D11.3* expression level in hepatocellular carcinoma, indicating that *RP11-564D11.3* may play more crucial roles in the hepatocellular carcinoma pathogenesis. Prognostic values analysis of hepatocellular carcinoma patients from GEO datasets showed *TP53*, *VEGFA*, *EGFR* and *CCND1* were significantly associated with overall survival of hepatocellular carcinoma patients. Mutations and copy-number alterations analysis revealed that *TP53*, *CTNNB1*, *MYC*, *VEGFA* and *CCND1* were exclusively mutant or amplified in hepatocellular carcinoma. These findings suggest that *VEGFA* was more likely to be potential target gene for *RP11-564D11.3*. Several studies demonstrated *VEGFA* is a well-known angiogenesis inducer, and overexpression of *VEGFA* can inhibit hepatocellular carcinoma cell proliferation, invasion and angiogenesis [61–63]. Further investigations are needed to confirm the *RP11-564D11.3*-mediated ceRNA regulatory network and explore the functional roles of *RP11-564D11.3* in the tumorigenesis of hepatocellular carcinoma.

Conclusion

In summary, the present study for the first time systematically revealed that pseudogene *RP11-564D11.3* was frequently upregulated and associated with poor prognosis in hepatocellular carcinoma. Our findings also indicated that *RP11-564D11.3* may function as an oncogene or ceRNA through targeting *VEGFA* and its potential roles in carcinogenesis is worthy of further study. More importantly, we here provide a reliable prediction method for unidentified genes based on a series of analysis, including prognostic values evaluation, gene-miRNA-mRNA ceRNA network, hub genes recognition and expression correlation analysis.

Future perspective

Pseudogenes play a pivotal and multifaceted role in human cancer and *RP11-564D11.3* can serve as a useful biomarker and novel therapeutic target against hepatocellular carcinoma.

Summary points

- We identified 16 frequently upregulated pseudogenes in human cancers using dreamBase.
- We found that high expression level of *RP11-564D11.3* was significantly associated with poor overall survival in seven types of human cancers.
- Through pseudogene–miRNA–mRNA network, we identified seven candidate miRNAs and 338 *RP11-564D11.3*-related target genes.
- Functional enrichment analysis revealed that *RP11-564D11.3* was strongly correlated with pathways in cancer, such as the PI3K–Akt signaling pathway and the neurotrophin signaling pathway.
- We demonstrated that *RP11-564D11.3* functions as a competitive endogenous RNA or oncogene through targeting *VEGFA* in hepatocellular carcinoma.
- We provided a reliable prediction method for unidentified genes based on a series of bioinformatics analysis.

Author contributions

J Zhou, Y Li and X Hao conceived and designed the study; H Song, J Yang and Y Zhang obtained and analyzed data; H Song and J Yang drafted the manuscript; J Zhou and Y Li collected funds. All authors reviewed the manuscript before submission. All the authors approved the final version of the manuscript.

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