RESEARCH ARTICLE

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Bioinformatics Data Analyses Revealed Novel Prognostic Biomarker Candidates For Hepatocellular Carcinoma

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Objective: Hepatocellular carcinoma (HCC) is a frequently diagnosed cancer type with low overall survival (OS) rates. Known prognostic biomarkers of HCC are inefficient to monitor disease progression. Therefore, identification of novel patient OS time predictive biomarkers is needed.

Materials and Methods: Cbioportal, OncoLnc, and dbDEMC tools were utilized to analyse DNA-sequencing, mRNA-sequencing and miRNA-sequencing data of HCC patients in The Cancer Genome Atlas (TCGA) database. Integrated molecular interactions network of the novel biomarker candidates were generated using NetworkAnalyst and MiRNet tools.

Results: Next generation sequencing data analyses revealed expression profiles of 11 frequently mutated and differentially expressed genes as well as two differentially expressed miRNAs, which predict OS time. Transcriptional upregulation of GPATCH4 gene (P:0.009) and downregulation of four genes (PPARGC1A P:0.000013, PIK3R1 P:0.002, COL18A1 P:0.009, and A1BG P:0.01) were correlated with poor prognosis of HCC patients, for the first time. Integrated network of these molecules also revealed novel regulatory molecules and interactions associated with prognosis of HCC.

Conclusion: As a resul of this study, in silico data that can benefit the development of novel molecularly targeted diagnostic and therapeutic applications specific to HCC have been obtained.

Key words: *Bioinformatics, biomarkers, hepatocellular carcinoma, survival, transcriptional networks*

Hepatoselüler Kansere Özgü Yeni Prognostik Biyobelirteç Adaylarının Biyoenformatik Veri Analizleriyle Belirlenmesi

Amaç: Hepatoselüler kanser (HSK), en sık görülen ve sağkalım oranı düşük olan bir kanser tipidir. HSK'ya özgü bilinen prognostik biyobelirteçler hastalığın seyrinin izleminde yetersiz kalmaktadır. Bu nedenle, hastaların sağkalım sürelerinin tahmininde kullanılabilecek yeni biyobelirteçlerin belirlenmesine ihtiyaç duyulmaktadır.

Gereç ve Yöntem: HSK hastalarının, Kanser Genom Atlası (TCGA) veri tabanındaki yeni nesil DNA, mRNA ve miRNA dizileme verilerinin analizleri cbioportal, OncoLnc ve dbDEMC araçları kullanılarak gerçekleştirilmiştir. Belirlenen yeni biyobelirteç adaylarının yaptıkları entegre moleküler etkileşimler ağı NetworkAnalyst ve MiRNet araçları kullanılarak oluşturulmuştur.

Bulgular: Yeni nesil dizileme veri analizleriyle, HSK'da sıklıkla mutasyona uğrayan ve ekspresyon farklılığı gösteren 11 gen ile iki miRNA'nın ekspreyon örüntüleriyle hasta sağkalım süresinin tahmin edilebildiği belirlenmiştir. Bunlardan bir genin ifadesinin artışının HSK hastalarının kötü prognozuyla doğru orantılı (GPATCH4 P:0.009), dört genin ifadesindeki azalışın (PPARGC1A P:0.000013, PIK3R1 P:0.002, COL18A1 P:0.009 ve A1BG P: 0.01) ise ters orantılı olduğu ilk kez belirlenmiştir. Bu moleküllerin oluşturduğu entegre moleküler etkileşimler ağı da HSK'nın prognozuyla ilişkili yeni düzenleyici molekülleri ve etkileşimleri ortaya çıkarmıştır.

Sonuç: HSK'ya özgü moleküler hedefli yeni tanı ve tedavi uygulamalarının geliştirilmesine fayda sağlayabilecek in silico veriler elde edilmiştir.

Anahtar Kelimeler: *Biyobilişim, biyobelirteçler, hepatoselüler kanser, sağkalım, transkripsiyonel şebekeler*

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Introduction

Liver cancer, the most common type is hepatocellular carcinoma (HCC), is the sixth most frequently diagnosed cancer type and the second cause of cancer related deaths in the world. Risk factors, causing HCC are well-defined, but diagnostic and therapeutic applications of HCC remain poor. Currently, the most effective therapy for HCC is surgical resection, but late-stage tumors are mostly inoperable (1). Therefore, chemotherapeutic applications need to be applied for treatment of HCC because of the late-stage diagnosis issue. However, chemotherapeutic drugs provide modest benefit on patient survival because HCC cells are highly resistant to the currently available drugs (2). Because of these limitations, 5-year survival rates of HCC patients are very poor (1, 2). Currently, the most widely used biomarker for HCC diagnosis and monitoring is alpha-fetoprotein (AFP). However, patient prognosis cannot be effectively monitored through AFP levels due to its limited diagnostic sensitivity (3). Therefore, there is urgent need for identification of novel candidate diagnostic and prognostic biomarkers for HCC to improve patient survival.

Recent developments in the next generation sequencing technology and bioinformatics data analysis techniques provided a growing number of publicly available pre-processed and unprocessed data. Analyses of these data reveal previously unidentified disease associated markers and driver molecules to develop novel translational approaches. The aim of this study is to analyse publicly available deoxyribonucleic acid sequencing (DNA-Seq), messenger ribonucleic acid sequencing (mRNA-Seq) and micro RNA sequencing (miRNA-Seq) data of HCC patients in The Cancer Genome Atlas (TCGA) and to integrate the obtained data for the identification of novel prognostic biomarker candidates and their corresponding molecular network in the context of HCC. With this purpose, five genes, whose expression profiles significantly predict overall survival (OS) time of patients, have been determined. In addition, HCC prognosis-associated molecular interactions network of the determined genes and miRNAs has also been identified.

Materials and Methods

HCC Patient Mutation Data Analyses: TCGA liver hepatocellular carcinoma (LIHC)-coded whole-exome sequencing data of 363 HCC patients, were analysed by TCGA consortium, and made publicly available (4, 5). Top 370 genes, which were frequently mutated in HCC patients, with $P<0.05$ significance value, were selected and further analysed by using the cbioportal tool) (date of access: February, 2020).

mRNA Expression Based Patient Survival Data Analyses: Tier 3 mRNA sequencing V2 whole genome transcriptomic data of 360 HCC TCGA samples with patient survival data were pre-processed and made accessible in OncoLnc (6) (date of access: February, 2020). Firstly, the list of differentially expressed genes, with $P<0.05$ significance value, were determined using OncoLnc tool. Secondly, common genes that were listed both in frequently mutated and differentially expressed genes were determined. After that, for each gene, mean average gene expression values among data of 360 patients were calculated, and 180 samples, which have expression values below the mean value (low expression samples), and the other 180 samples with expression values higher than the mean (high expression samples) were calculated to use during the survival analyses. Genes, whose mean reads per kilobase million (RPKM) basal expression values are les than 0.7 in both groups, were discarded to eliminate less significant genes in the final list. Kaplan-Meier (KM) survival plots, log-rank P-values of survival analyses and cox prognostic coefficient values of the genes in the final list were determined by comparing low mRNA expressing and high mRNA expressing profiles of genes using default parameters of OncoLnc tool.

miRNA-Expression Based Patient Survival Data Analyses: The list of differentially expressed miRNAs between 370 HCC and 50 normal liver (NL) TCGA samples have been publicly available in the database of differentially expressed miRNAs in human cancers 2.0 (dbDEMC 2.0) (7) (date of access: February, 2020). Selected miRNAs with differential expression value more than 1.5 fold between NL and HCC samples were further analysed to test their prognostic efficiencies using OncoLnc tool that contains pre-processed TCGA miRNA-sequencing data of 362 HCC patients along with survival data. The list of low and high expressing sample groups (181 samples in each group) of total samples were determined for each miRNA. After that, differentially expressed miRNAs between two sets of samples were selected and common miRNAs were correlated with survival analysis data. KM survival plots, survival associated log-rank p-values and patient prognosis predictive cox survival coefficients of these miRNAs were determined using OncoLnc tool with default parameters.

Protein-Protein Interaction (PPI) and mRNA-miRNA Network Analyses: PPI network of the determined prognosis marker genes was generated using NetworkAnalyst tool (8) (date of access: February, 2020), which uses the Search Tool for Retrieval of Interacting Genes (STRING) data (9). Confidence score cutoff value of 900 (400 for minimum confidence and 1000 for maximum confidence) was used to construct a highly confident PPI network. MiRNA-mRNA interaction network of the determined miRNAs was generated using miRNet tool (date of access: February, 2020), which accesses 10 different miRNA databases for human miRNAs (TarBase, miRTarBase, miRecords, miR2Disease, HMDD, PhenomiR, SM2miR, PhamacomiR, EpimiR, and starBase), using the default parameters (10). Two networks were integrated through shared edges of both sub-networks to generate HCC patient prognosis biomarkers network.

Results

Both Frequently Mutated and Differentially Expressed Genes in HCC Patients Affect Patient OS Time by Their Gene Expression Patterns: In order to determine the effect of frequently mutated and differentially expressed genes, on HCC patients' OS, firstly TCGA HCC DNA-Seq data were analysed (4, 5). Upon analyses of whole-exome sequencing data of 363 HCC cases, 370 genes were found to be frequently mutated with high significance in HCC samples as indicated before (5). After that, RNA-sequencing transcriptomics data of 360 HCC cases in TCGA database were analysed using OncoLnc tool (4, 6). Among 15848 genes analysed in OncoLnc database, a list of 1112 genes, whose gene expression profiles.

Table 1. List of frequently mutated genes, whose gene expression profiles significantly predict HCC patient OS. The list of 11 genes whose differential expression between low expression and high expression HCC cases significantly correlated with patient OS. While seven of them have positive cox values, four of them have negative cox values indicating that gene expression profiles are positively or negatively correlated with poor prognosis, respectively. All genes in the list are among the ones that were previously identified as frequently mutated genes in HCC patients (5).

	Rank	Gene	Survival						Mutations		
			LM	HM	FC	P exp	P surv	Cox	N pat	% pat	P
Positive	1	CCT3	6816.92	13890.91	2.038	0.015	0.0002	0.297	7	1.93	0.001
	2	PYGO2	1064.89	2076.23	1.95	0.026	0.002	0.32	4	1.1	0.01
	3	SRRT	1442.43	2292.1	1.589	0.025	0.004	0.294	4	1.1	0.002
	4	NCL	7818.03	12395.74	1.586	0.006	0.007	0.393	4	1.1	0.01
	5	GPATCH4	646.79	1339.98	2.072	0.039	0.009	0.268	7	1.93	0.00001
	6	TNPO1	1212.04	2130.49	1.758	0.041	0.016	0.282	6	1.65	0.02
	7	PRKDC	1631.41	4132.75	2.533	0.037	0.022	0.309	19	5.23	0.04
Negative	8	PPARGC1A	364.4	2928.06	8.035	0.005	0.00003	-0.36	4	1.1	0.03
	9	PIK3R1	1244.37	3824.39	3.073	0.04	0.002	-0.268	5	1.38	0.03
	10	COL18A1	16789.52	38893.94	2.317	0.026	0.009	-0.311	6	1.65	0.0499
	11	A1BG	14217.45	74070.2	5.21	0.034	0.01	-0.301	5	1.38	0.04

LM: Mean expression value of low-expressing samples; HM: Mean expression value of high-expressing samples; FC: High vs. low mean fold change values; P exp: P-value of gene expression comparisons; P surv: Log-rank P value of high vs. low samples survival analysis; Cox: Cox coefficient value of survival analysis, N pat: Number of patients harboring mutations; % pat: Percentage of mutation bearing patients; P: value of significance of mutation status.

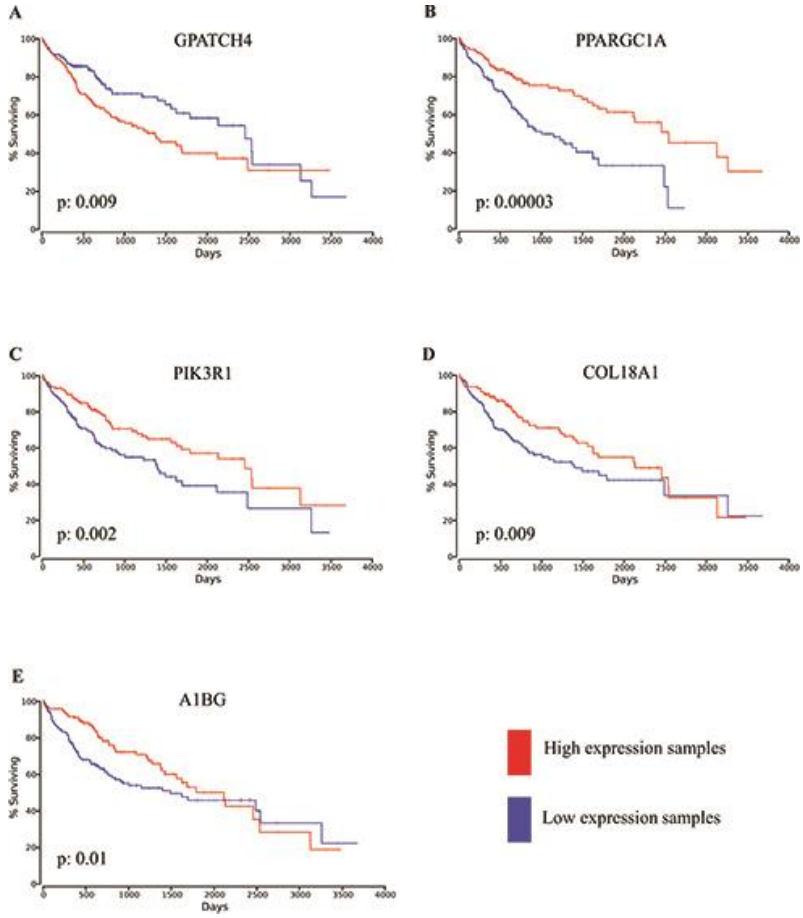

Figure 1. Kaplan-Meier survival plots of selected genes and miRNA. Survival plots of selected five genes in Table 1, which are associated with HCC patient OS for the first time. One of these genes has positive cox coefficient (a); while the remaining four genes (b-e) have negative cox coefficient. Survival-predictive (survival associated log-rank p-values are indicated in plots) between high-expressing (red) and low-expressing (blue) samples.

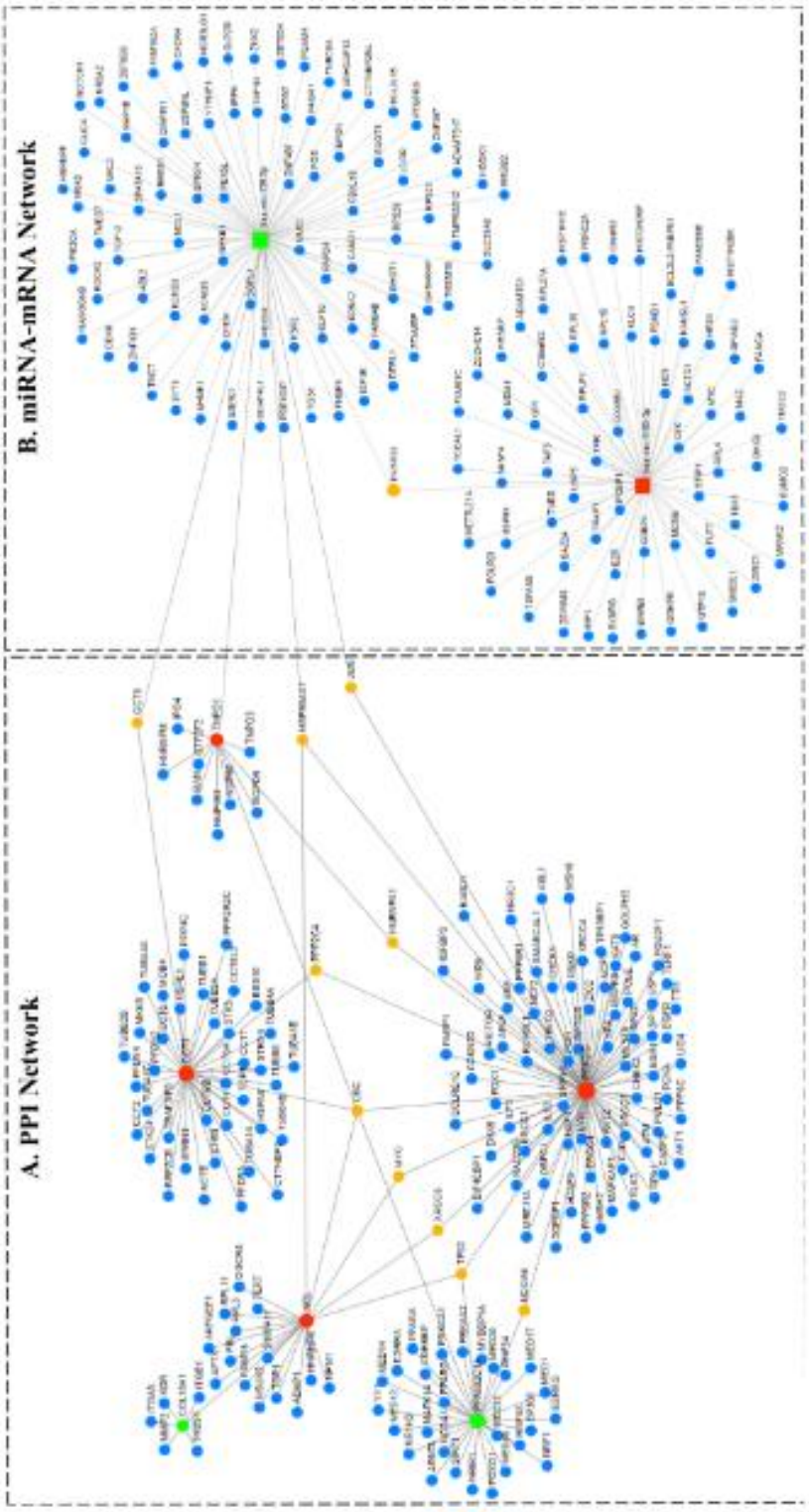

Figure 2. Integrated PPI and miRNA-mRNA interaction network of HCC patient prognosis-associated molecules. a. PPI network of genes described in Table 1 and b. miRNA-mRNA interaction network of the determined miRNAs were generated and integrated. Six out of 11 genes were able to construct highly confident PPI network. Red node: Input and positively correlated with poor prognosis when up-regulated; Green node: Input and negatively correlated with poor prognosis when up-regulated; Yellow node: Interactor node with at least two node connections in network; Blue node: Interactor node that only interacts with one input gene or miRNA (dark blue for proteins and light blue for mRNAs); Lines: Direct interaction edges.

showed significant correlation with HCC patient OS time, were determined. 26 of these genes were also present in the previously identified frequently mutated 370 genes list. In order to determine a list of bioinformatically more significant genes among identified 26 genes, average expression values for each gene were calculated. Following that, 15 genes with mean average values of gene expression less than 0.7 FPKM values were discarded from the list, in order to continue to work with high gene expression values. Gene expression, patient survival prediction and mutation data of the final 11 genes were determined and presented in Table 1. In addition, KM survival plots of the five genes, which are associated with HCC patient OS prediction for the first time with this study, were also depicted (Figure 1).

Determination of Differentially Expressed miRNAs Correlated with HCC Patient OS Time: In order to determine a list of HCC patient OS associated miRNAs, a list of miRNAs differentially expressed between 370 HCC samples and 50 NL samples of TCGA Research Network were determined through dbDEMC 2.0 database (7). 229 up-regulated and 173 down-regulated miRNAs (402 miRNAs in total) were identified via the dbDEMC2 database. Among these differentially expressed miRNAs, 25 of them showed more than 1.5-fold change between NL and HCC samples (11 up-regulated and 14 down-regulated). To determine significant correlation between HCC patient OS and differentially expressed miRNAs, OncoLnc tool was used (6). Pre-processed miRNA-Seq expression data of 362 HCC cases were retrieved from OncoLnc database and analysed. Among 485 miRNAs, 80 of them showed significant correlation with HCC patient survival rates. In order to define significant correlation between differentially expressed miRNAs and OS rates, common miRNAs in both dbDEMC and OncoLnc derived lists were determined and finally two miRNAs (miR-1180-3p and miR-139-5p) were identified. One of these miRNAs, miR-1180-3p, is 1.53 fold upregulated in HCC samples compared to normal liver samples; and overexpression of miR-1180-3p in HCC is associated with poor prognosis (P: 0.01). The other miRNA, miR-139-5p, is 1.65 fold downregulated in HCC samples compared to normal liver samples; and its downregulation is associated with poor prognosis for HCC patients (P: 2.56 E^{-06}).

Integrated Network of HCC Patient OS Effectors was Determined: Regulatory interactions network of the determined genes and miRNAs was constructed in two steps to identify candidate patient prognosis markers network for HCC. First, NetworkAnalyst tool (8) was applied to construct PPI network of 11 genes presented in Table 1 based on their interaction data in STRING database. Confidence cutoff value of 900 was selected (minimum 400 and maximum 1000) to construct highly confident PPI network. Thus, a PPI network containing 178 nodes and 185 edges with six seeds (six out of 11 input genes) was constructed for survival associated genes (Figure 2a). The PPI network revealed that two seed gene products (NCL and COL18A1) have direct interactions. In addition, UBC and TP53 were found to be main hub proteins connecting five and three seed genes, respectively. Secondly, miRNet tool, which retrieves and combines data from 10 different miRNA databases for human miRNAs (10), was used to depict the mRNA targets of the prognosis-predictive miRNAs. Furthermore, a miRNA-mRNA regulatory network comprising 151 nodes and 150 edges with two seeds (two input miRNAs) was constructed. miRNA-mRNA regulatory network showed that, DUSP22 is a direct target of both seeds (Figure 2b). Finally, both sub-networks were combined via four shared interaction nodes (CCT5, TNPO1, HSP90AA1 and JUN) to construct the main integrated interaction network (Figure 2).

Discussion

HCC is the second deadliest cancer type with approximately 850.000 annual new cases and poor survival rates (1). Known prognostic biomarkers of HCC are insufficient to monitor disease progression or develop novel molecularly targeted therapeutic applications (1). Thus, identification and characterization of novel patient survival affecting molecules are needed for HCC. Up to now, variable prognostic biomarkers such as differentially expressed protein coding or non-coding RNA's (especially miRNAs), mutation profiles, and epigenetic modifications such as DNA methylation patterns have been determined for HCC (3). Among these identified biomarkers, AFP and Glypican 3 (GPC3) expression display the highest patient OS time predictive efficiencies for HCC. However, these biomarkers display limitations in sensitivity and specificity for prognosis monitoring and novel biomarkers should be urgently identified for HCC (3). Thus, DNA-Seq, mRNA-Seq and miRNA-Seq data of HCC patient samples of TCGA were analysed and integrated to identify novel patient OS affecting biomarkers for HCC.

In this study, 11 genes and two miRNAs, whose predictive expression patterns showed correlation with patient OS time by using bioinformatics data of TCGA HCC patient samples were determined. Among them, differential expressions of A1BG, CCT3, COL18A1, NCL, PIK3R1, PRKDC, PYGO2, miR-1180, and miR-139-5p were previously reprted as poor prognosis biomarkers for cholangiosarcoma (11), multiple myeloma (12), non-small cell lung cancer (NSCLC) (13), acute myeloid leukemia (14), breast cancer (15), gall bladder cancer (16), lung cancer (17), pancreatic adenocarcinoma (18), and NSCLC (19), respectively.

Among identified genes and miRNAs, increased levels of CCT3 (20), PYGO2 (21), SRRT (22), NCL (23), PRKDC (24), TNPO1 (25), miR-1180 (26), and miR-139-5p (27) were previously reported to be correlated with poor prognosis of HCC patients (Table 1). Indeed, identification of these genes and miRNAs strengthens the credibility of identified novel candidates as prognostic biomarkers (Table 1). Among 11 genes in Table 1, five of them (GPATCH4, PPARGC1A,

PIK3R1, COL18A1, and A1BG) were correlated with HCC patients' OS estimation for the first time with this study (Table 1, Figure 1).

Upon literature search focusing on HCC, GPATCH4, which ranked five in the list (Table 1), comes out as a strong prognostic marker candidate as it locates in chromosome 1q21-24 region along with CCT3 and PYGO2 whose upregulations were previously correlated with poor prognosis of HCC (20, 21). Moreover, PIK3R1, whose expression negatively correlated with HCC patient OS time within this study, has the potential to be a strong candidate, since it was previously shown to be regulated by miRNAs involved in growth suppression of HCC (28). PPARGC1A, which was shown to mediate HCC cells migration through modulating E-cadherin expression (29), is a promising prognostic biomarker and therapeutic target candidate particularly for prevention of HCC metastasis. COL18A1 also comes out as an interesting target since its overexpression was shown to be associated with HCC previously (30). Lastly, there is no reported study in the literature about A1BG gene's functional role in HCC, which makes it an interesting candidate for further analysis.

The miRNA-Seq analyses identified that the differential expression of only two miRNAs miR-1180-3p and miR-139-5p are associated with poor prognosis of HCC. Altough these miRNAs have recently been experimentally associated with poor prognosis of HCC (26, 27), an integrated network of HCC prognosis-associated mRNAs and miRNAs has been generated for the first time. Interestingly, the mRNA sub-network revealed six novel candidate HCC prognosis-related proteins (Figure 2a). The other sub-network consists of two miRNAs, both targeting DUSP22 mRNA (Figure 2b). Both sub-networks are connected via miR-139-5p through CCT5, TNPO1, HSP90AA1, and JUN mRNAs that are direct targets of miR-139-5p (Figure 2). Thus, miR-139-5p, which is down-regulated in HCC patients with poor prognosis, may be an important hub regulator of HCC patients' survival because miR-139-5p has direct connection with TNPO1 and indirect connections with CCT3, NCL, and PRKDC genes that are also associated with poor prognosis of HCC (Table 1, Figure 2).

In conclusion, this study identified a network of novel biomarker candidates for prediction OS time of HCC patients. Identification of the roles of these molecules with future studies may provide novel information to better monitor disease prognosis and develop novel targeted therapeutic applications against HCC.

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