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Metabolic Pathways Enhancement Confers Poor Prognosis in p53 Exon Mutant Hepatocellular Carcinoma

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ABSTRACT: RNA-Sequencing (RNA-Seq), the most commonly used sequencing application tool, is not only a method for measuring gene expression but also an excellent media to detect important structural variants such as single nucleotide variants (SNVs), insertion/deletion (Indels), or fusion transcripts. The Cancer Genome Atlas (TCGA) contains genomic data from a variety of cancer types and also provides the raw data generated by TCGA consortium. p53 is among the top 10 somatic mutations associated with hepatocellular carcinoma (HCC). The aim of the present study was to analyze concordant different gene profiles and the priori defined set of genes based on p53 mutation status in HCC using RNA-Seq data. In the study, expression profile of 11 799 genes on 42 paired tumor and adjacent normal tissues was collected, processed, and further stratified by the mutated versus normal p53 expression. Furthermore, we used a knowledge-based approach Gene Set Enrichment Analysis (GSEA) to compare between normal and p53 mutation gene expression profiles. The statistical significance (nominal *P* value) of the enrichment score (ES) genes was calculated. The ranked gene list that reflects differential expression between p53 wild-type and mutant genotypes was then mapped to metabolic process by KEGG, an encyclopedia of genes and genomes to assign functional meanings. These approaches enable us to identify pathways and potential target gene/pathways that are highly expressed in p53 mutated HCC. Our analysis revealed 2 genes, the hexokinase 2 (*HK2*) and Enolase 1 (*ENO1*), were conspicuous of red pixel in the heatmap. To further explore the role of these genes in HCC, the overall survival plots by Kaplan-Meier method were performed for HK2 and ENO1 that revealed high HK2 and ENO1 expression in patients with HCC have poor prognosis. These results suggested that these glycolysis genes are associated with mutated-p53 in HCC that may contribute to poor prognosis. In this proof-of-concept study, we proposed an approach for identifying novel potential therapeutic targets in human HCC with mutated p53. These approaches can take advantage of the massive next-generation sequencing (NGS) data generated worldwide and make more out of it by exploring new potential therapeutic targets.

KEYWORDS: RNA-Seq, HK2, ENO1, metabolism, p53, HCC

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Introduction

The major histological type of liver cancer is human hepatocellular carcinoma (HCC) that accounts for approximately 80% of liver cancer burden.[1] Hepatocellular carcinoma is one of the leading cancers worldwide, and the incidence and mortality rates for HCC are increasing rapidly by approximately 3% per year.[1] HCC incidence is high in Middle and Western African East and in South-East Asia, whereas rates are low in South-Central and Western Asia and Northern and Eastern Europe.[2] Chronic infection with hepatitis B and/or hepatitis C viruses, diabetes, obesity, and alcohol consumption are established risk factors for HCC that all contribute to the growing trend.[3,4] RNA-Sequencing (RNA-Seq) is a revolutionary tool based on next-generation sequencing technology that enables accurate quantifications of large number of genes; gene mutation status coupled with RNA-Seq data can also be identified.[5]

In the precision medicine era, data from RNA-Seq and other next-generation sequencing (NGS) tools may provide therapeutic strategy in treating HCC. Gene-expression databases generated by RNA-Seq, such as Gene Expression Omnibus (GEO), Sequence Read Archive (SRA), and the Cancer Genome Atlas (TCGA) databases on the human transcriptome, have been widely used to study the causes and seek for therapies of cancers, which have produced a large amount of expression data.[6] In transcriptomic experiments, Gene Set Enrichment Analysis (GSEA) is conventionally used to analyze and interpret coordinate pathway-level changes. The Molecular Signatures Database (MSigDB) is a collection of annotated gene sets for use with GSEA that is divided into 8 major collections, including H, C1, C2, C3, C4, C5, C6, and C7 and that accounts for gene expression patterns within a set of transcriptomic experiments to identify statistically significantly enriched gene sets.

The tumor suppressor p53 which has been found to be mutated in more than 50% of all human cancer was reported to play multiple roles in apoptosis, cell-cycle arrest, cellular senescence, and differentiation.[7] As a transcription factor, p53 activates the expression of many checkpoint and apoptotic genes, including *Puma, Noxa*, and *p21*; somatic p53 mutations occur in about half of all human cancers.[8] Most of these mutations are missense mutations within the DNA-binding core domain of exon5 to exon8, which are often more stable and correlated with poor prognosis of the cancer patient.[9] The p53 gain-of-function mutants promote tumorigenesis via a novel mechanism involving active disruption of critical DNA damage-response pathways, and p53 has been identified as high-risk mutations associated with increased distant metastases and decreased survival time.[10] Despite TP53 mutation contributes significantly to human cancer occurrence and progression, the role of mutant P53 in HCC development is not fully understood. Several lines of evidence revealed that p53 mutants act also as an oncoprotein that can gain activities in activating tumor progression by collaborating with Ras to cause primary rat cells transformation and promote tumorigenesis of Saos-2 cells in nude mice model, and some expressing p53 mutant cancer cells are resistant to wild-type p53-independent apoptosis induced by cytotoxic drugs.[10]

The present study aimed to explore gene expression profile and identify novel potential therapeutic targets in human HCC with mutated p53 using raw RNA-Seq data generated by TCGA consortium. We hope these approaches can take advantage of the massive NGS data generated worldwide and make more out of it by exploring new potential therapeutic target genes/pathways.

Methods

Data collection

We collected a total of 42 of paired tumor-adjacent normal HCC RNA-Seq datasets and 42 samples from the Cancer Genome Atlas (TCGA http://cancergenome.nih.gov/).

Gene Set Enrichment Analysis

GSEA is a method of analyzing and interpreting microarray and RNA-Seq data using biological knowledge. The data in question are analyzed in terms of their differential enrichment in a predefined biological set of genes that can publish information about biochemical pathway or co-expression in a previous experiment. GSEA was performed using javaGSEA Desktop Application from the Broad Institute at MIT. Parameters used for the analysis were as follows. The data set had 11 799 genes, c2.all.v6.2.symbols.gmt gene set from online pathway databases, publications in PubMed, and knowledge of domain experts was used for running GSEA.

Survival analysis

The survival analysis of genes expression of the study was performed in the Pan-cancer RNA-Seq Web server for generating Kaplan-Meier plots by auto selecting best cutoff values between lower and upper quartiles into high and low expression groups, which are computed in all stages, sex, race, and mutation burden (http://kmplot.com/analysis/index.php?p=service&cancer=pancancer_rnaseq).

Statistical analysis

All statistical analyses in this study were conducted using SPSS statistical software (SPSS Inc. PASW Statistics 18.0, Chicago). Independent-samples *t* test was, respectively, used for binary variables and continuous variables to compare tumor and adjacent normal tissue. The *P* value of the test was 2-tailed with a level of significance (α) = 0.05. A *P* value of $<.05$ indicated statistical significance.

Results

The top 10 somatic mutations in 42 human HCC

We explored and identified the top 10 genes with exon mutations associated with HCC from the TGCA RNA-Seq data. The most frequent exon mutations associated with HCC are from the following 10 genes: *LRP1B, DNAH10, ARID1A, SYNE1, ABCA13, NCAM1, MUC16, CTNNB1, TP53*, and *TTN* (Figure 1A). The *p53* gene is among the most frequently mutated genes and is the best characterized tumor suppressor in several human cancers including HCC. Twenty-nine percent (12/42) in these HCC had p53 exon mutations. Among them, there is no synonymous mutation detected, the nonsynonymous mutation substitution present in 11 samples produces the amino acid changes, and only 1 is the insertion type (Table 1). This amino acid altering substitutions are all heterozygotes in 12 p53 mutant HCC (Table 1). The analysis flow consists of the following components: HCC transcriptome expression and GSEA (Figure 1B). p53 mutations were detected in 12 of the 42 patients with HCC. In 2 cases (17%), p53 mutation and 6 gene mutations coexisted; in 2 cases (17%), p53 mutation and 4 gene mutations coexisted; in 2 cases (17%), p53 mutation and 3 gene mutations coexisted; in 3 cases (25%), p53 mutation and 2 gene mutations coexisted; in 3 cases (25%), p53 mutation and 1 gene mutation coexisted; neither mutation was found (Table 2). The survival data of top 10 gene expression have been completed by running Web server for Kaplan-Meier plots in Figure 1C to L. The results revealed that the expression of those top 10 genes is not associated with overall survival (OS) in HCC (Figure 1C to L).

Enrichment of the metastasis and poor prognosis terms in p53 mutant HCC by GSEA

Within the p53 mutant HCC, significantly upregulated gene sets with highest ranked at max metric suggest higher likelihood

Table 1. p53 mutation status in HCC.

HUGO_SYMBOL	VARIANT_CLASSIFICATION	REFERENCE_ALLELE	TUMOR_SEQ_ALLELE	EXON	AMINO ACID CHANGES
P53	Nonsense_Mutation	A	T, c.T831A	exon8	p.C277X
P53	Missense_Mutation	T	C, c.A578G	exon6	p.H193R
P53	Missense_Mutation	C	A, c.G481T	exon5	p.A161S
P53	Missense_Mutation	A	C, c.T376G	exon5	p.Y126D
P53	Missense_Mutation	T	C, c.A578G	exon6	p.H193R
P53	Missense_Mutation	G	T, c.C452A	exon5	p.P151H
P53	Missense_Mutation	G	A, c.C742T	exon7	p.R248W
P53	In_Frame_Ins	–	CGCGGA, c.476_477insTCCGCG	exon5	p.A159delinsVRA
P53	Nonsense_Mutation	G	A, c.C916T	exon8	p.R306X
P53	Missense_Mutation	C	A, c.G481T	exon5	p.A161S
P53	Missense_Mutation	C	A, c.G747T	exon7	p.R249S
P53	Nonsense_Mutation	C	A, c.G511T	exon5	p.E171X
P53	Missense_Mutation	A	C, c.T470G	exon5	p.V157G
P53	Missense_Mutation	A	G, c.T679C	exon7	p.S227P

Abbreviation: HCC, hepatocellular carcinoma.

of involvement in VANTVEER_BREAST_CANCER_METASTASIS_DN (Figure 2A and B) and VANTVEER_BREAST_CANCER_POOR_PROGNOSIS (Figure 3A and B) that genes whose expression is significantly and negatively correlated with poor breast cancer clinical outcome (defined as developing distant metastases in <5 years). Of these genes, there are 18 genes of core enrichment of breast cancer poor prognosis. We further examined whether these genes could serve as a powerful prognosis factor in HCC (Figure 4A). The Kaplan-Meier analysis revealed that patients with high expression of *NDC80, GNAZ, CCNE2, CENPA, NUSAP1, MELK, DTL, PRC1, MCM6, RUNDC1, DIAPH3, MMP9, ORC6, CDC42BPA*, and *DCK HCC* had significant poor survival compared with patients with lower expression of those genes (Figure 4B to S).

HK2 and ENO1 highly expressed in p53 mutant HCC than in p53 wild-type HCC

In the metastasis and poor prognosis genes of core enrichment expression, "metabolic pathway" expression appeared to rank at top, and "cell cycle" was on the second place by KEGG pathway clustering (Figure 5A). In the metabolic pathway, glycolysis/gluconeogenesis processing enzymes were analyzed by the ratio of enzyme expression of mean values of p53 mutant versus p53 wild-type and mean values of p53 wild-type versus p53 mutant. Two genes, *HK2* and *ENO1*, with a fold change (FC) >2 in the heatmap were then selected for further survival analyses (Figure 5B).

HK2 and ENO1 as the significant prognostic power

The cancer RNA-Seq database was searched to analyze the differential expression of HK2 and ENO1 mRNA between tumor and adjacent normal tissues. The average expression level of HK2 and ENO1 mRNA in HCC was significantly higher than that in the adjacent normal tissues ($P = .028$ and $P < .001$, respectively; Figure 6A and B). In addition, the levels of HK2 and ENO1 expression are higher in mutant p53 HCC than those in wild-type p53 HCC (Figure 6C and D; $P = .06$ and $P < .001$, respectively).

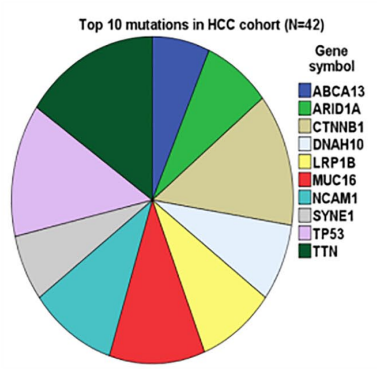
A Web tool was used to validate survival-associated mRNAs utilizing expression data from liver cancer (http://kmplot.com/analysis/index.php?p=service&cancer=pancancer_rnaseq). We found the high HK2 and ENO1 expression that split patients by the median has poor prognosis in OS and relapse-free survival (RFS) in patients with liver cancer ($P = .0082$ and .000055, respectively; Figure 6E and F).

Discussion

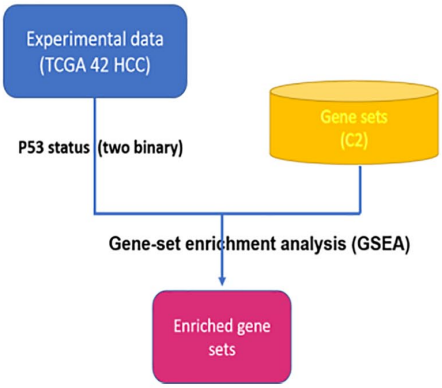
HCC is associated with persistent inflammation, and it is a clear example of inflammation-related cancer as more than 90% of HCCs arise in the cause of hepatic injury and inflammation,[11] and p53 can modulate the inflammatory microenvironment, tumor microenvironment, and tumor suppression.[12] Although previously the function of the p53 widely considered to induce cell-cycle arrest and apoptosis, an increasing number of evidence have indisputably served as the tumor suppressive

(A)

Top 10 mutations of RNA-seq in HCC cohort (N=42).	
Gene symbol	Frequency (%)
LRP1B	8 (19)
DNAH10	7 (17)
ARID1A	7 (17)
SYNE1	6 (14)
ABCA13	6 (14)
NCAM1	9 (21)
MUC16	10 (24)
CTNNB1	12 (29)
TP53	12 (29)
TTN	14 (33)



(B)



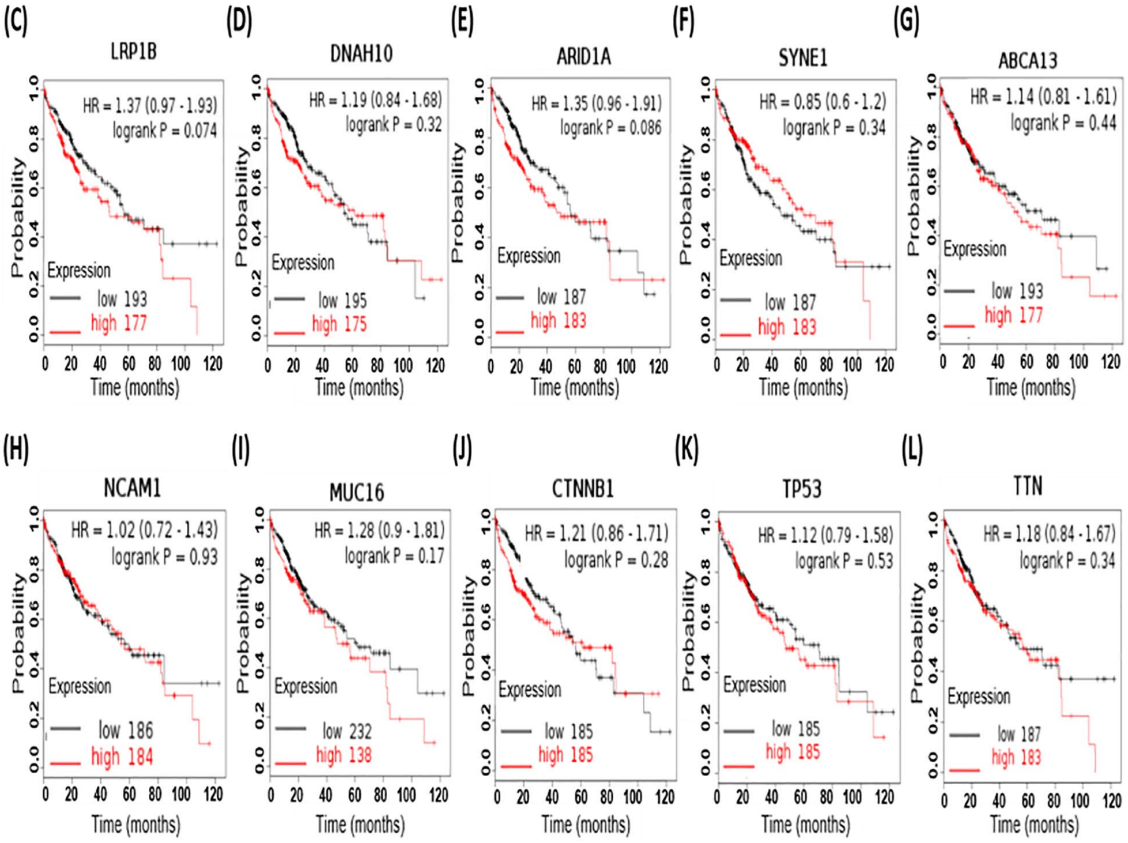


Figure 1. Mutations of RNA-Seq in HCC-cohort (N = 42). (A) Top 10 mutations frequency of RNA-Seq in HCC cohort in a pie chart. (B) A flowchart is a pictorial representation of the steps in a process where each step is represented by a block. (C-L) Kaplan-Meier curves of patients with HCC with low- versus high expression of top 10 mutated genes of HCC. HCC indicates hepatocellular carcinoma; RNA-Seq, RNA-sequencing.

Table 2. List of top 10 gene mutation distribution in 12 mutant p53 HCC.

PATIENT ID	TP53 MUTATION	*MUC16*	*TTN*	*DNAH10*	*ABCA13*	*NCAM1*	*SYNE1*	*LRP1B*	*CTNNB1*	*ARID1A*	TOTAL
TCGA-DD-A1EG-01A	TP53 mutation	V	V	V	V		V	V			6
TCGA-DD-A1EE-01A	TP53 mutation	V		V	V		V		V	V	6
TCGA-ES-A2HT-01A	TP53 mutation	V	V				V			V	4
TCGA-DD-A113-01A	TP53 mutation	V		V		V		V			4
TCGA-DD-A1EB-01A	TP53 mutation		V		V	V					3
TCGA-DD-A1EI-01A	TP53 mutation		V		V				V		3
TCGA-FV-A3R2-01A	TP53 mutation	V						V			2
TCGA-DD-A114-01A	TP53 mutation		V	V							2
TCGA-DD-A1EL-01A	TP53 mutation			V					V		2
TCGA-BC-A216-01A	TP53 mutation						V				1
TCGA-BD-A3EP-01A	TP53 mutation					V					1
TCGA-FV-A3I1-01A	TP53 mutation					V					1

Abbreviations: HCC, hepatocellular carcinoma; "V," mutation.

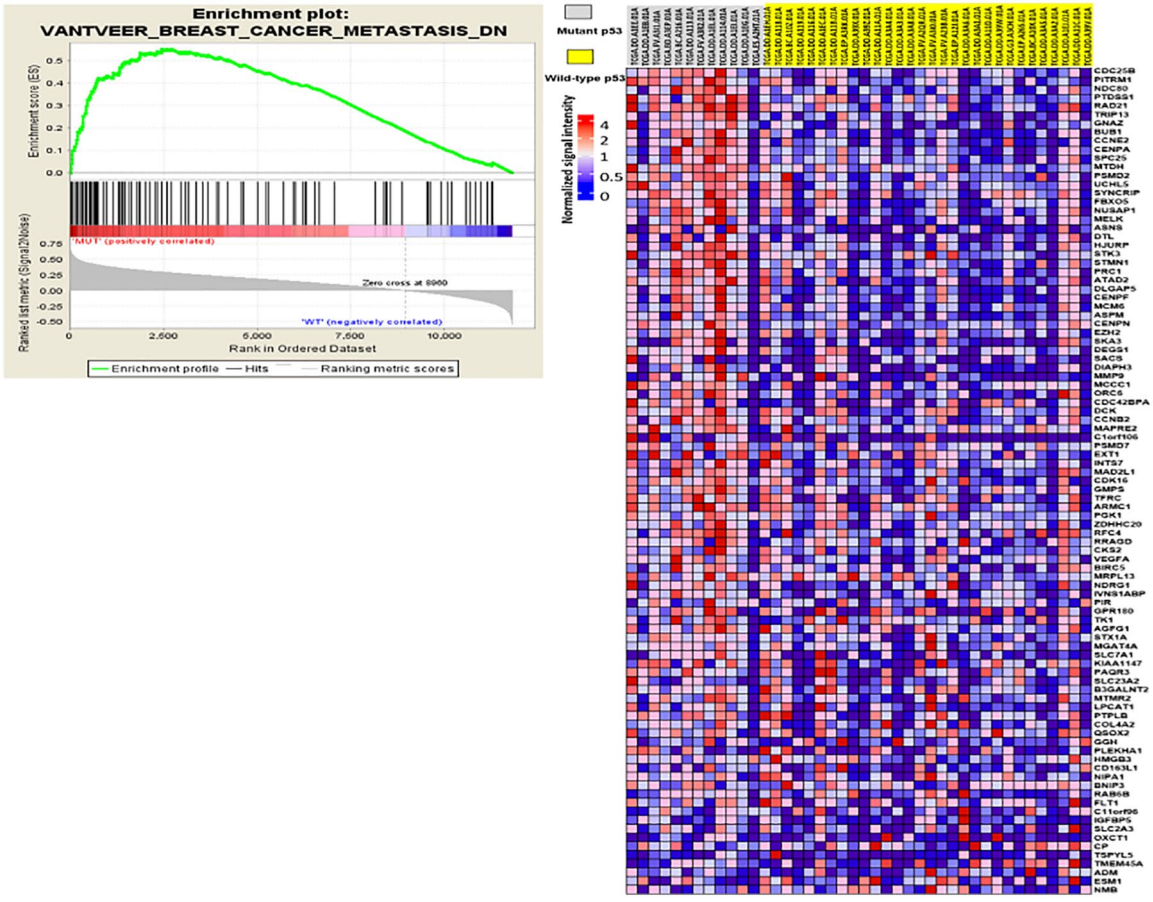


Figure 2. Metastasis genes mRNA expression of p53 status by GSEA. (A) An expression data set sorted by correlation with p53 status (gray: p53 mutated sample; yellow: p53 wild-type), and the corresponding heatmap of VANTVEER_BREAST_CANCER_METASTASIS_DN set within the sorted list from the C2 functional collection. (B) Plot of the running sum for VANTVEER_BREAST_CANCER_METASTASIS_DN, including the location of the maximum enrichment score (ES) and the leading-edge subset. GSEA indicates Gene Set Enrichment Analysis.

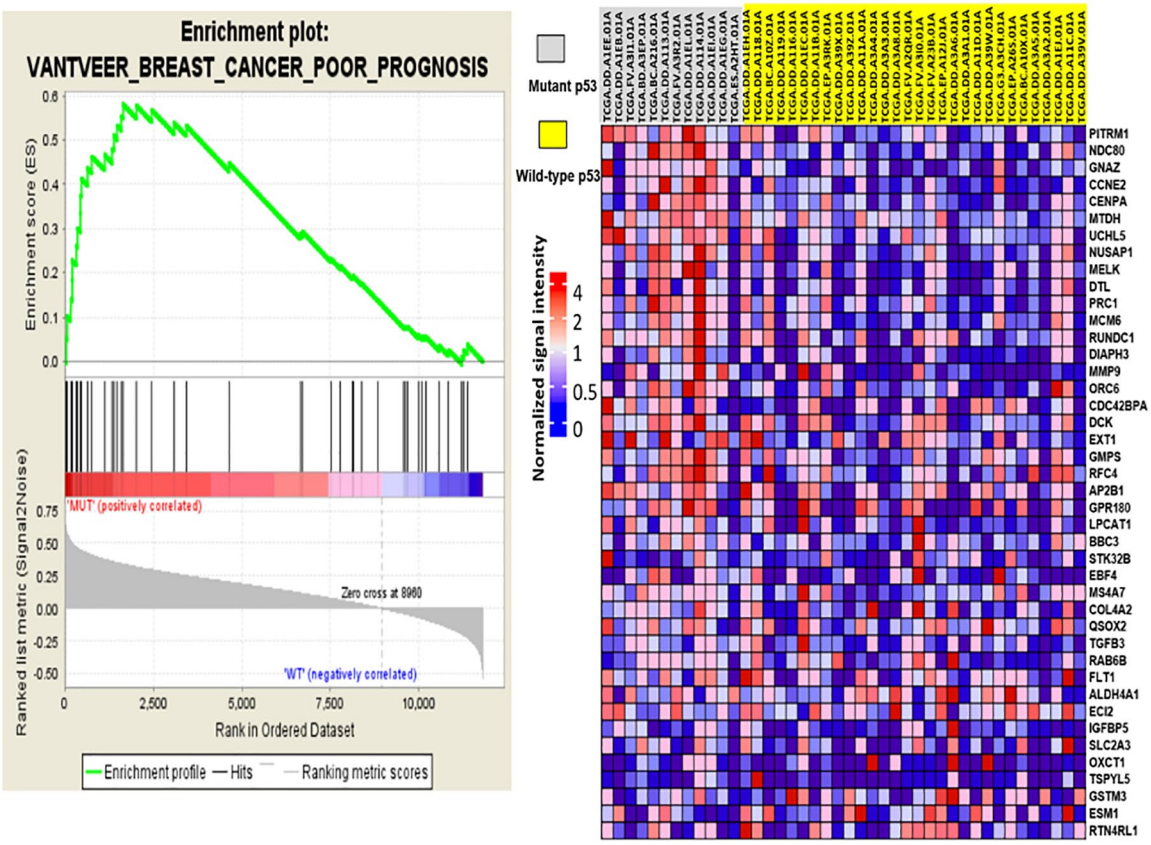


Figure 3. Poor prognosis gene mRNA expression of p53 status by GSEA. (A) An expression data set sorted by correlation with p53 status (gray: p53 mutated sample; yellow: p53 wild-type), and the corresponding heatmap of VANTVEER_BREAST_CANCER_POOR_PROGNOSIS set within the sorted list from the C2 functional collection. (B) Plot of the running sum for VANTVEER_BREAST_CANCER_POOR_PROGNOSIS, including the location of the maximum enrichment score (ES) and the leading-edge subset. GSEQ indicates Gene Set Enrichment Analysis.

capability of the p53 that also rely on its capability to control and modulate cellular metabolism and maintain cellular oxidative homeostasis.[13] We applied GSEA to identify the functional gene sets or biological pathways that enrich in differentially expressed wild-type p53 and mutant p53 groups. The output of results revealed that gene sets of "VANTVEER_BREAST_CANCER_METASTASIS_DN" and "VANTVEER_BREAST_CANCER_POOR_PROGNOSIS" were enriched in mutant p53 group of HCC. In addition, *TP53* is the most frequently mutated gene in invasive breast cancer of about 30% to 35% in all cases, and p53 is mutated in approximately 80% of triple-negative (TN) tumors (tumors negative for ER, PR, and HER2).[14,15] In Table 1, among the p53 mutations, a missense mutation, R249S in exon7 of *p53* gene has been reported that its protein has a gain of function via c-Myc activation on CDK4 phosphorylation at serine 249 and consequent PIN1 binding to promote HCC progression.[16] However, most of these mutations were served as dominant-negative function.

Interactions of metabolic and immune response pathways are considered as a critical homeostatic mechanism that when perturbed, will lead to metabolic disorders and diseases.[17] Results from this study revealed that p53 mutant HCC has initiated HK2 and ENO1 activation in metabolic pathway, and HK2 and ENO1 expression levels can serve as predictive markers for poor prognosis in HCC. HK2 is the enzyme that catalyzes the conversion of glucose to glucose-6-phosphate (G6P) in the first step of glycolysis. HK2 is regulated by p53 transcriptional target gene[18] and is required for initiation and maintenance of tumor. Its systemic deletion inhibited glucose-derived ribonucleotides and damaged glutamine-derived carbon use in anaplerosis of mouse models of cancer.[18] ENO1, also known as pyruvate dehydrogenase, catalyzes the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate.[19] ENO1 silencing by shRNA has been shown to effectively suppress the proliferation and increase chemosensitivity of gastric cells.[20]

We proposed big data sets collected from TCGA to obtain complete gene expression profiles in p53 status. The GSEA method is responsible for 11 799 genes expression of an entire database evaluation that can execute the calculation of an ES, estimate the significance level of ES, and adjust for multiple hypothesis testing.[21] Enrichment score distribution

(A)

Core enrichment in Poor prognosis
PITRM1
NDC80
GNAZ
CCNE2
CENPA
MTDH
UCHL5
NUSAP1
MELK
DTL
PRC1
MCM6
RUNDC1
DIAPH3
MMP9
ORC6
CDC42BPA
DCK

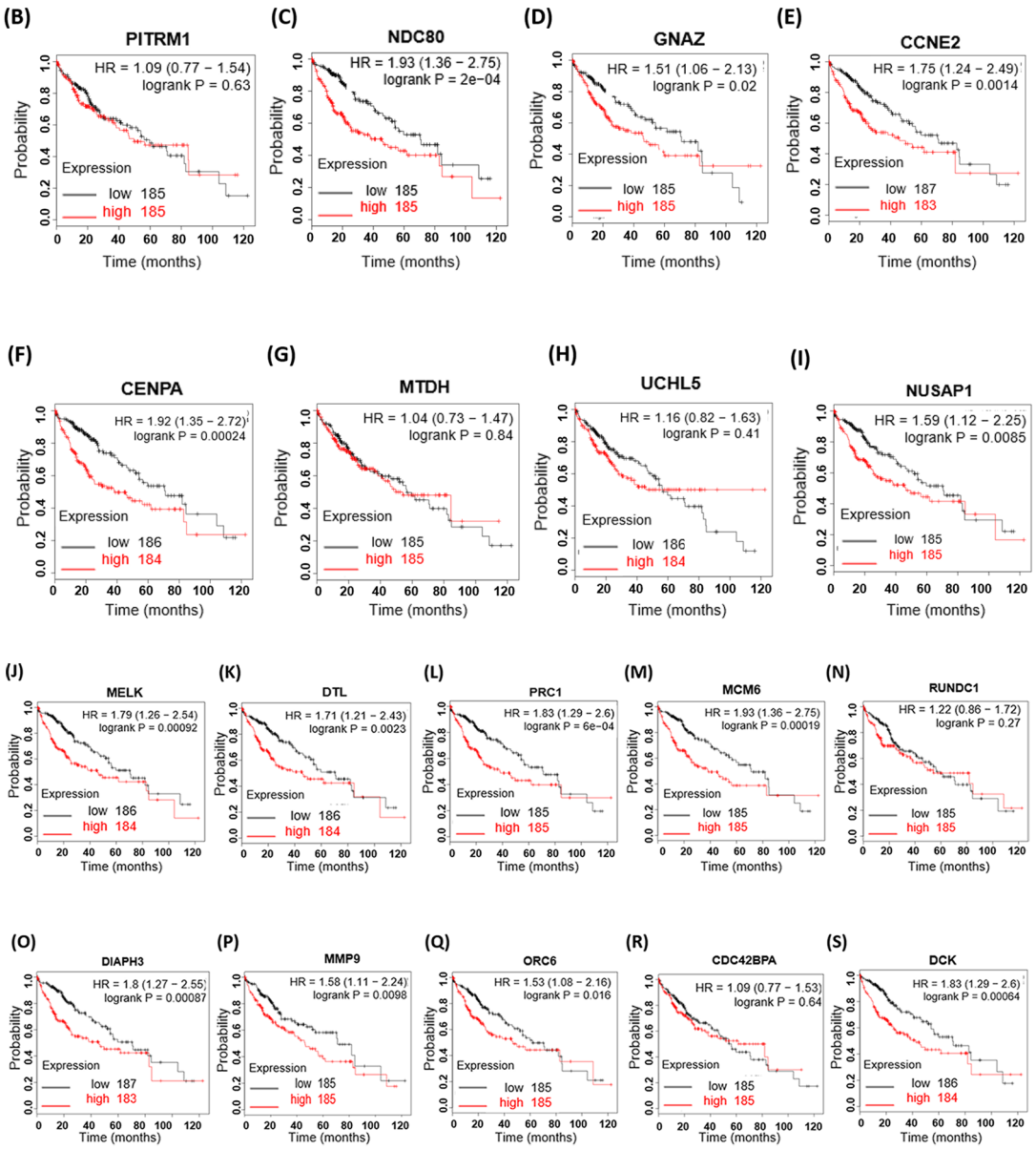


Figure 4. The 18 genes were significantly enriched in VANTVEER_BREAST_CANCER_POOR_PROGNOSIS. (A) The significantly enriched genes list. (B) to (S) Kaplan-Meier curves of patients with HCC with low- versus high expression of 18 genes. HCC indicates hepatocellular carcinoma.

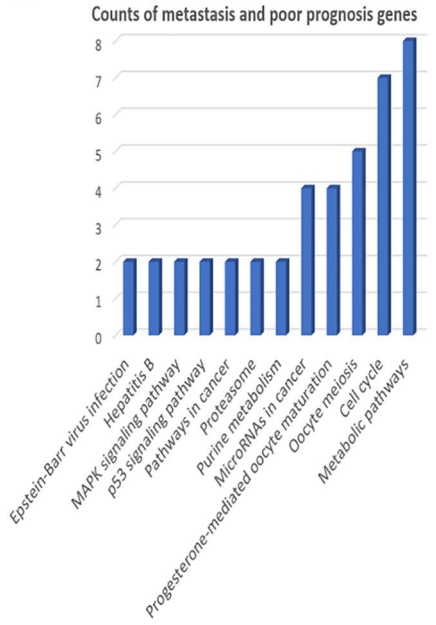
of VANTVEER_BREAST_CANCER_METASTASIS_DN and VANTVEER_BREAST_CANCER_POOR_PROGNOSIS sets from the C2 functional collection in the list of genes in the HCC is ranked by their correlation with p53 status.

Despite the modest sample size of HCC samples analyzed, this study successfully used genome-wide transcriptome profiling of p53 wild-type and p53 mutants and provided prognosis marker and therapeutic targets for HCC. In addition, the raw data generated by TCGA consortium was mapped to identify gene difference in metabolic pathway. The expressions of the 2 genes, *HK2* and *ENO*, have been proved to be of significance in HCC by other researchers.

Conclusions

In conclusion, in this proof-of-concept study, we provide a convenient and feasible approach for identifying novel potential prognosis and therapeutic target pathways/genes for human diseases using the abundant RNA-Seq data worldwide. The transcriptome profiling of specific disease target may be beneficial for seeking adjuvant therapy in the precision medicine era.

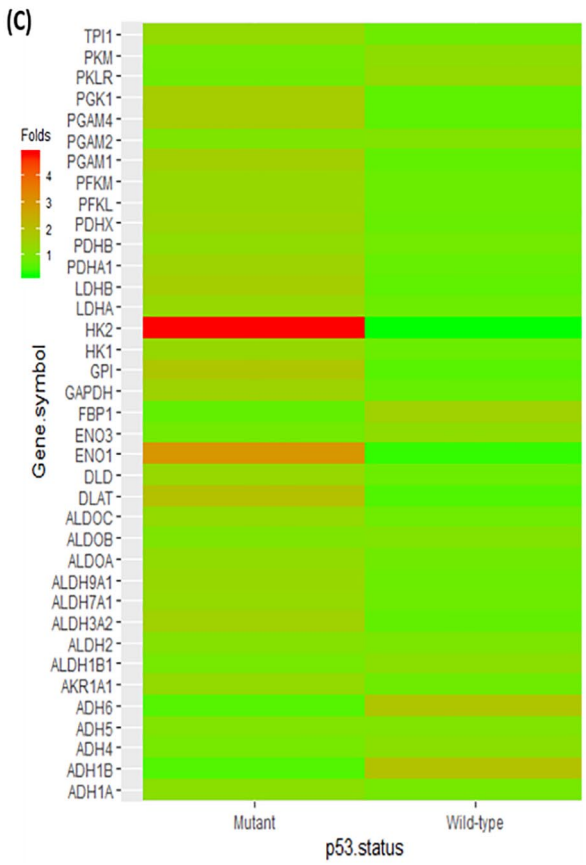
(A)



(B)

List of 8 genes of metabolic pathways that involved breast cancer metastasis and poor prognosis of GSEA database.	
ASNS	asparagine synthetase (glutamine-hydrolyzing)(ASNS)
DEGS1	delta 4-desaturase, sphingolipid 1 (DEGS1)
DCK	deoxycytidine kinase (DCK)
EXT1	exostosin glycosyltransferase 1 (EXT1)
GMPS	guanine monophosphate synthase (GMPS)
MCCC1	methylcrotonoyl-CoA carboxylase1 (MCCC1)
PTDSS1	phosphatidylserine synthase 1 (PTDSS1)
PGK1	phosphoglycerate kinase 1 (PGK1)

(C)



(D)

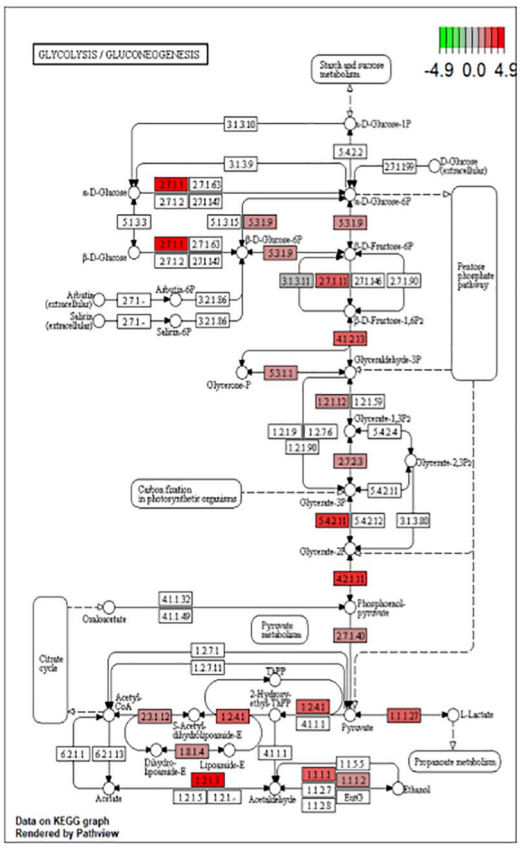


Figure 5. Enriched pathway terms of significantly enriched metastasis and poor prognosis genes in HCC. (A) Counts of metastasis and poor prognosis genes distributed in different pathways from KEGG datasets. (B) List of 8 genes of metabolic pathways that involved breast cancer metastasis and poor prognosis of GSEA database. (C) All enzyme mRNA expression of glycolysis/gluconeogenesis in p53 wild-type and mutant HCC. (D) A colored KEGG pathway of glycolysis/gluconeogenesis will be drawn and color means genes that p53 mutant versus p53 wild-type group in TCGA HCC database. HCC indicates hepatocellular carcinoma; GSEA, Gene Set Enrichment Analysis.

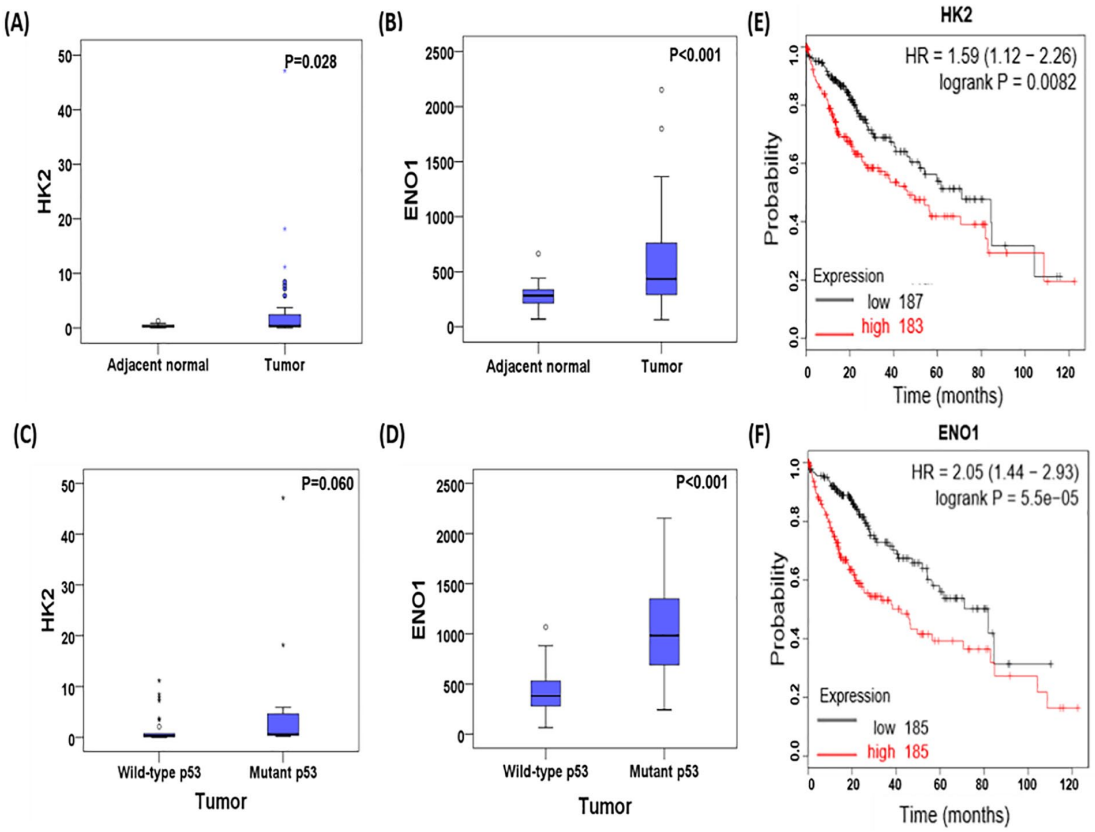


Figure 6. Expressions of HK2 and ENO1 correlate with HCC poor prognosis. (A) and (B) HK2 and ENO1 expression in HCC tissues and adjacent normal tissues. HK2 and ENO1 expression levels were positively associated with HCC tissues ($P = .028$ and $P < .001$). (C) and (D) HK2 and ENO1 expression in mutant p53 HCC and wild-type p53 HCC. HK2 and ENO1 expression levels were positively associated with mutant p53 HCC tissues ($P = .060$ and $P < .001$). (E) and (F) Kaplan-Meier curves of patients with HCC with low- versus high expression of HK2 and ENO1 (n = 370; $P < .001$, log-rank test). HCC indicates hepatocellular carcinoma.

Author Contributions

E-PIC, F-YT, and C-CL conceived the study; P-MC and J-RL performed the data collection and analysis; E-PIC and P-MC redesigned the experiment and data analysis; and E-PIC and P-MC wrote the paper. All authors contributed to the revision and approval of the final manuscript.

Availability of Data and Material

The data set and materials presented in this investigation are available on request from the corresponding author.

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