***RRM2* Computational Phosphoprotein Network Construction and Analysis between No-Tumor Hepatitis/Cirrhotic Liver Tissues and Human Hepatocellular Carcinoma (HCC)**

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Key Words

RRM2 • Human hepatocellular carcinoma • Phosphoprotein network construction and analysis

Abstract

RRM2 computational phosphoprotein network construction and analysis of human hepatocellular carcinoma (HCC) is very useful to identify novel markers and potential targets for prognosis and therapy. By integration of gene regulatory network infer (GRNInfer) and the database for annotation, visualization and integrated discovery (DAVID) we identified and constructed significant molecule *RRM2* phosphoprotein network from 25 no-tumor hepatitis/cirrhotic liver tissues and 25 HCC patients in the same GEO Dataset GSE10140-10141. We gained the negative result of *RRM2* phosphoprotein module through the net numbers of activation minus inhibition compared with no-tumor hepatitis/cirrhotic liver tissues and predicted possibly the decrease of *RRM2* phosphoprotein module in HCC. Our integrative result showed that *RRM2* phosphoprotein cluster of HCC contained both in human no-tumor hepatitis/cirrhotic liver tissues and HCC terms of phosphoprotein (with *RRM2*) and cell cycle (without *RRM2*), only in HCC

terms of cell-cell signaling, cell projection part, glycoprotein, cell projection, cell adhesion, biological adhesion, integral to plasma membrane, plasma membrane, kinase and phosphorus metabolic process (without *RRM2*), and none in HCC terms of cell death (without *RRM2*) and ion binding (with *RRM2*) compared with human no-tumor hepatitis/cirrhotic liver tissues, all the condition is vital to invasion of HCC. Therefore, we deduced the weaker *RRM2* phosphoprotein function in HCC consistent with our above computation. It would be necessary of *RRM2* phosphoprotein function decrease to invasion of HCC. *RRM2* phosphoprotein interaction module construction in HCC can be a new route for studying the pathogenesis of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related death. So to develop novel drugs in HCC has become a challenge for biologists.

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RRM2 computational phosphoprotein network construction and analysis of human hepatocellular carcinoma (HCC) is very useful to identify novel markers and potential targets for prognosis and therapy.

RRM2 is one out of 50 genes identified as high expression in human hepatocellular carcinoma (HCC) vs no-tumor hepatitis/cirrhotic liver tissues. *RRM2* has been proved to be concerned with molecular function of translation initiation factor, ribosomal protein, guanyl-nucleotide exchange factor, reductase, major histocompatibility complex antigen, non-receptor serine/threonine protein kinase, membrane traffic regulatory protein, and biological process of amino acid biosynthesis, protein biosynthesis, protein modification, protein phosphorylation, protein targeting, T-cell and MHC-I-mediated immunity, developmental processes (DAVID). *RRM2*'s relational study also can be seen in these papers [1-4]. However, the molecular mechanism concerning *RRM2* phosphoprotein network construction in HCC has little been addressed.

In this paper, by integration of gene regulatory network infer (GRNInfer) and the database for annotation, visualization and integrated discovery (DAVID) we identified and constructed significant molecule *RRM2* phosphoprotein network from 25 no-tumor hepatitis/cirrhotic liver tissues and 25 HCC patients in the same GEO Dataset GSE10140-10141. We gained the negative result of *RRM2* phosphoprotein module through the net numbers of activation minus inhibition compared with no-tumor hepatitis/cirrhotic liver tissues and predicted possibly the decrease of *RRM2* phosphoprotein module in HCC. Our integrative result showed that *RRM2* phosphoprotein cluster of HCC contained both in human no-tumor hepatitis/cirrhotic liver tissues and HCC terms of phosphoprotein (with *RRM2*) and cell cycle (without *RRM2*), only in HCC terms of cell-cell signaling, cell projection part, glycoprotein, cell projection, cell adhesion, biological adhesion, integral to plasma membrane, plasma membrane, kinase and phosphorus metabolic process (without *RRM2*), and none in HCC terms of cell death (without *RRM2*) and ion binding (with *RRM2*) compared with human no-tumor hepatitis/cirrhotic liver tissues, all the condition is vital to invasion of HCC. Therefore, we deduced the weaker *RRM2* phosphoprotein function in HCC consistent with our above computation. It would be necessary of *RRM2* phosphoprotein function decrease to invasion of HCC. *RRM2* phosphoprotein interaction module construction in HCC can be a new route for studying the pathogenesis of HCC. *RRM2* phosphoprotein interaction module

construction in HCC can be a new route for studying the pathogenesis of HCC. Our construction of *RRM2* phosphoprotein network may be useful to identify novel markers and potential targets for prognosis and therapy of HCC.

Materials and Methods

Microarray Data

We used microarrays containing 6144 genes from 25 no-tumor hepatitis/cirrhotic liver tissues and 25 HCC patients in the same GEO Dataset GSE10140-10141 [5].

Gene Selection Algorithms

50 potential HCC molecular markers were identified using significant analysis of microarrays (SAM). SAM is a statistical technique for finding significant genes in a set of microarray experiments. The input to SAM is gene expression measurements from a set of microarray experiments, as well as a response variable from each experiment. The response variable may be a grouping like untreated, treated, and so on. SAM computes a statistic d_i for each gene i , measuring the strength of the relationship between gene expression and the response variable. It uses repeated permutations of the data to determine if the expression of any genes is significantly related to the response. The cutoff for significance is determined by a tuning parameter delta, chosen by the user based on the false positive rate. We normalized data by log2, and selected two class unpaired and minimum fold change=2.78. Here we chose the 50 top-fold significant (high expression genes of HCC compared with no-tumor hepatitis/cirrhotic liver tissues) genes under the false-discovery rate and q-value are 0%. The q-value (invented by John Storey [6]) for each gene is the lowest false discovery rate at which that gene is called significant. It is like the well-known p-value, but adapted to multiple-testing situations.

Network Establishment of Candidate Genes

The entire network was constructed using GRNInfer [7] and GVedit tools. GRNInfer is a novel mathematic method called GNR (Gene Network Reconstruction tool) based on linear programming and a decomposition procedure for inferring gene networks. The method theoretically ensures the derivation of the most consistent network structure with respect to all of the datasets, thereby not only significantly alleviating the problem of data scarcity but also remarkably improving the reconstruction reliability. The following Equation (1) represents all of the possible networks for the same dataset.

$$J = (X^T - A)U\Lambda^{-1}V^T + YV^T = \hat{J} + YV^T \quad (1)$$

We established network based on the 50 top-fold distinguished genes and selected parameters as lambda 0.0 because we used one dataset, threshold 0.000001. Lambda is a positive parameter, which balances the matching and sparsity terms in the objective function. Using different thresholds, we can predict various networks with different edge density.

Fig. 1. *RRM2* up- and down-stream network construction in human no-tumor hepatitis/cirrhotic liver tissues by infer (A, C). *RRM2* up- and down-stream network construction in HCC by infer (B, D). Arrowhead represents activation, cycle represents inhibition.

Functional Annotation Clustering

The DAVID Gene Functional Clustering Tool provides typical batch annotation and gene-GO term enrichment analysis for highly throughput genes by classifying them into gene groups based on their annotation term co-occurrence [8, 9]. DAVID uses a novel algorithm to measure relationships among the annotation terms based on the degrees of their co-association genes to group similar annotation contents from the same or different resources into annotation groups. The grouping algorithm is based on the hypothesis that

similar annotations should have similar gene members. The functional annotation clustering integrates the same techniques of Kappa statistics to measure the degree of the common genes between two annotations, and fuzzy heuristic clustering to classify the groups of similar annotations according to kappa values. The tool also allows observation of the internal relationships of the clustered terms by comparing to the typical linear, redundant term report, over which similar annotation terms may be distributed among many other terms.

Fig. 2. *RRM2* up- and down-stream phosphoprotein cluster in human no-tumor hepatitis/cirrhotic liver tissues by DAVID (A, C). *RRM2* up- and down-stream phosphoprotein cluster by DAVID in HCC (B, D). Gray color represents gene-term association positively reported, black color represents gene-term association not reported yet.

Results

Identification of HCC Molecular Markers

RRM2 is one out of 50 genes identified as high expression in human hepatocellular carcinoma (HCC) vs no-tumor hepatitis/cirrhotic liver tissues. The 50 significant genes included *SPINK1* (serine peptidase inhibitor Kazal type 1); *AFP* (alpha-fetoprotein); *PAGE4* (P antigen family member 4); *MYBL2* (v-myb myeloblastosis viral

oncogene homolog-like 2); *CDKN3* (cyclin-dependent kinase inhibitor 3); *TSHB* (thyroid stimulating hormone beta); *DKK1* (dickkopf homolog 1); *RRM2* (ribonucleotide reductase M2 polypeptide); *S100P* (S100 calcium binding protein P); *ACTN2* (actinin alpha 2); *TROAP* (trophinin associated protein); *ELAVL3* (ELAV-like 3); *DLG7* (discs large homolog 7); *NEK2* (NIMA-related kinase 2); *LCN2* (lipocalin 2); *HIST1H3H* (histone cluster 1 H3); *NINJ2* (ninjurin 2); *PRSS1* (protease serine 1);

Term	RRM2 upstream				RRM2 downstream			
	con(act)	con(inh)	exp(act)	exp(inh)	con(act)	con(inh)	exp(act)	exp(inh)
Ion Binding					2	1		
Mutagenesis Site	2	4			4	3	0	2
Cytoplasm	5	3	2	4	2	3	0	3
Phosphoprotein	6	7	3	5	7	4	0	3

Table 1. Up- and down-stream gene numbers of activation and inhibition of each module with *RRM2* gene in *RRM2* phosphoprotein cluster between human no-tumor hepatitis/cirrhotic liver tissues and HCC. con represents control (human no-tumor hepatitis/cirrhotic liver tissues), exp: experiment (HCC), act: activation, inh: inhibition.

NUSAP1 (nucleolar and spindle associated protein 1); *IGF2BP3* (insulin-like growth factor 2 mRNA binding protein 3); *SEMA3B* (sema domain immunoglobulin domain short basic domain secreted 3B); *FOXM1* (forkhead box M1); *REG3A* (regenerating islet-derived 3 alpha); *FLJ33790*; *SFRP4* (secreted frizzled-related protein 4); *BIRC5* (baculoviral IAP repeat-containing 5); *SLC17A7* (solute carrier family 17 member 7); *BRCA1* (breast cancer 1); *TOP2A* (topoisomerase (DNA); II alpha); *NR5A1* (nuclear receptor subfamily 5 group A member 1); *XRCC2* (X-ray repair complementing defective repair in Chinese hamster cells 2); *E2F1* (E2F transcription factor 1); *DMN* (desmuslin); *ROBO1* (roundabout axon guidance receptor homolog 1); *ALDH3A1* (aldehyde dehydrogenase 3 family member A1); *NCAPH* (non-SMC condensin I complex subunit H); *BUB1B* (budding uninhibited by benzimidazoles 1 homolog beta); *HOXA5* (homeobox A5); *PTTG1* (pituitary tumor-transforming 1); *GDPD5* (glycerophosphodiester phosphodiesterase domain containing 5); *TK1* (thymidine kinase 1); *AMELY* (amelogenin Y-linked); *PRKG2* (protein kinase cGMP-dependent type II); *UBE2C* (ubiquitin-conjugating enzyme E2C); *SULT1C2* (sulfotransferase family cytosolic 1C member 2); *GRM1* (glutamate receptor metabotropic 1); *SCML2* (sex comb on midleg-like 2); *TTK* (TTK protein kinase); *CCNE2* (cyclin E2); *CDC20* (cell division cycle 20 homolog).

RRM2 Up- and Down-stream Network Construction in Human No-tumor hepatitis/cirrhotic liver tissues and HCC

In the no-tumor hepatitis/cirrhotic liver tissues, *RRM2* upstream network appeared that *ACTN2*, *AMELY*, *DMN*, *FOXM1*, *GRM1*, etc. activate *RRM2*, and

ALDH3A1, *BUB1B*, *DKK1*, *GDPD5*, *HOXA5*, etc. inhibit *RRM2*, as shown in Fig. 1 (A), whereas in HCC, *RRM2* upstream network showed that *ACTN2*, *DLG7*, *ELAVL3*, *LCN2*, *PRKG2*, etc. activate *RRM2*, and *ALDH3A1*, *AMELY*, *DKK1*, *DMN*, *FLJ33790*, etc. inhibit *RRM2*, as shown in Fig. 1 (B).

In the no-tumor hepatitis/cirrhotic liver tissues, *RRM2* downstream network reflected that *RRM2* activates *BIRC5*, *BRCA1*, *BUB1B*, *CCNE2*, *DLG7*, etc. and inhibits *ACTN2*, *ALDH3A1*, *AMELY*, *CDKN3*, *DKK1*, etc., as shown in Fig. 1 (C), whereas in HCC, *RRM2* downstream network appeared that *RRM2* activates *ALDH3A1*, *AMELY*, *BIRC5*, *BRCA1*, *CCNE2*, etc. and inhibits *ACTN2*, *BUB1B*, *DKK1*, *ELAVL3*, *FOXM1*, etc., as shown in Fig. 1 (D).

Identification of RRM2 Up- and Down-stream Phosphoprotein Cluster in Human No-tumor hepatitis/cirrhotic liver tissues and HCC by DAVID

RRM2 phosphoprotein cluster terms only in human no-tumor hepatitis/cirrhotic liver tissues cover cell death (without *RRM2*) and ion binding (with *RRM2*), as shown in Fig. 2 (A, C). However, *RRM2* phosphoprotein cluster terms only in HCC contain cell-cell signaling, cell projection part, glycoprotein, cell projection, cell adhesion, biological adhesion, integral to plasma membrane, plasma membrane, kinase and phosphorus metabolic process (without *RRM2* gene), as shown in Fig. 2 (B, D). *RRM2* phosphoprotein cluster terms both in human no-tumor hepatitis/cirrhotic liver tissues and HCC include phosphoprotein (with *RRM2*) and cell cycle (without *RRM2*), as shown in Fig. 2 (A, B, C, D).

In the no-tumor hepatitis/cirrhotic liver tissues, *RRM2* upstream modules mainly include phosphoprotein

Term	RRM2 upstream			
	con(act)	con(inh)	exp(act)	exp(inh)
Phosphoprotein	<i>ACTN2, AMELY, NEK2, NR5A1, TROAP, XRCC2</i>	<i>BUB1B, CDC20, GRM1, HIST1H3H, IGF2BP3, PTTG1, ROBO1</i>	<i>IGF2BP3, MYBL2, NR5A1</i>	<i>ACTN2, E2F1, GRM1, NINJ2, ROBO1</i>

Term	RRM2 downstream			
	con(act)	con(inh)	exp(act)	exp(inh)
Phosphoprotein	<i>ACTN2, BRCA1, CCNE2, FOXM1, HIST1H3H, TTK, XRCC2</i>	<i>BIRC5, CDC20, TOP2A, TROAP</i>		<i>BRCA1, BUB1B, CDKN3</i>

Table 2. Activation and inhibition gene names of *RRM2* up- and down-stream phosphoprotein cluster in human no-tumor hepatitis/cirrhotic liver tissues and HCC. con represents control (human no-tumor hepatitis/cirrhotic liver tissues), exp: experiment (HCC), act: activation, inh: inhibition.

(*ACTN2, AMELY, NEK2, NR5A1, TROAP, XRCC2, BUB1B, CDC20, GRM1, HIST1H3H, IGF2BP3, PTTG1, ROBO1, RRM2*), etc., as shown in Fig. 2 (A). In HCC, *RRM2* upstream modules mainly cover phosphoprotein (*IGF2BP3, MYBL2, NR5A1, ACTN2, E2F1, GRM1, NINJ2, ROBO1, RRM2*), etc., as shown in Fig. 2 (B). In the no-tumor hepatitis/cirrhotic liver tissues, *RRM2* downstream modules mainly consist of phosphoprotein (*ACTN2, BRCA1, CCNE2, FOXM1, HIST1H3H, TTK, XRCC2, BIRC5, CDC20, TOP2A, TROAP, RRM2*), etc., as shown in Fig. 2 (C). In HCC, *RRM2* downstream modules mainly contain phosphoprotein (*BRCA1, BUB1B, CDKN3, RRM2*), etc., as shown in Fig. 2 (D).

Discussion

We have already done some works in this relative field about gene network construction and analysis presented in our published papers [10-13]. By integration of gene regulatory network infer (GRNInfer) and the database for annotation, visualization and integrated discovery (DAVID) we constructed significant molecule *RRM2* phosphoprotein network and compared *RRM2* up- and down-stream gene numbers of activation and inhibition between no-tumor hepatitis/cirrhotic liver tissues and HCC (Table 1).

In *RRM2* phosphoprotein module of upstream network of human no-tumor hepatitis/cirrhotic liver tissues, our integrative result verified that *ACTN2, AMELY, NEK2, NR5A1, TROAP, XRCC2* activate *RRM2*, and *BUB1B, CDC20, GRM1, HIST1H3H, IGF2BP3, PTTG1, ROBO1* inhibit *RRM2*, whereas in that of HCC, *IGF2BP3, MYBL2, NR5A1* activate *RRM2*, and *ACTN2, E2F1, GRM1, NINJ2, ROBO1* inhibit

RRM2. In *RRM2* phosphoprotein module of downstream network of human no-tumor hepatitis/cirrhotic liver tissues, our integrative result illustrated that *RRM2* activates *ACTN2, BRCA1, CCNE2, FOXM1, HIST1H3H, TTK, XRCC2* and inhibits *BIRC5, CDC20, TOP2A, TROAP*, whereas in that of HCC, *RRM2* inhibits *BRCA1, BUB1B, CDKN3* (Fig. 1, 2; Table 2). *BUB1B* has been proved to be concerned with molecular function of kinase, protein kinase and non-receptor serine/threonine protein kinase, and biological process of protein metabolism and modification, protein modification, protein phosphorylation, developmental processes, cell cycle, chromosome segregation, cell proliferation and differentiation, embryogenesis, oncogenesis and mitosis (DAVID). *BUB1B*'s relational study also can be presented in these papers [14-18]. *CDKN3*'s molecular function consists of phosphatase and protein phosphatase, and it is involved in biological process of protein metabolism and modification, protein modification, protein phosphorylation and cell cycle (DAVID). *CDKN3*'s relational study also can be presented in these papers [19-21]. *MYBL2* is relevant to molecular function of transcription factor and nucleic acid binding, and biological process of nucleoside, nucleotide and nucleic acid metabolism, mRNA transcription, mRNA transcription regulation, apoptosis, cell cycle, cell cycle control, cell proliferation and differentiation, inhibition of apoptosis (DAVID). *MYBL2*'s relational study also can be presented in these papers [22, 23]. *NINJ2* has been reported to be relevant to molecular function of cell adhesion molecule, and it is involved in biological process of cell adhesion, neuronal activities, neurogenesis, ectoderm development (DAVID). *NINJ2*'s relational study also can be presented in these papers [24-27]. *E2F1* is identified by molecular function of transcription factor and nucleic acid binding, and it is involved in biological process of nucleoside,

nucleotide and nucleic acid metabolism, mRNA transcription, mRNA transcription regulation, apoptosis, cell cycle, cell cycle control, cell proliferation and differentiation (DAVID). *E2F1*'s relational study also can be presented in these papers [28-31]. We gained the negative result of *RRM2* phosphoprotein module through the net numbers of activation minus inhibition compared with no-tumor hepatitis/cirrhotic liver tissues and predicted possibly the decrease of *RRM2* phosphoprotein module in HCC.

Otherwise, our integrative result showed that *RRM2* phosphoprotein cluster of HCC contained both in human no-tumor hepatitis/cirrhotic liver tissues and HCC terms of phosphoprotein (with *RRM2*) and cell cycle (without *RRM2*), only in HCC terms of cell-cell signaling, cell projection part, glycoprotein, cell projection, cell adhesion, biological adhesion, integral to plasma membrane, plasma membrane, kinase and phosphorus metabolic process (without *RRM2*), and none in HCC terms of cell death (without *RRM2*) and ion binding (with *RRM2*) compared with human no-tumor hepatitis/cirrhotic liver tissues, all the condition is vital to invasion of HCC (Fig. 2). As we all know, relationships of annotation terms in one cluster have similar annotation contents and correlative functions. Some researchers indicated that overexpression of *RRM2*

significantly enhances the invasive and metastatic potential of tumor [1, 32]. Therefore, we deduced the weaker *RRM2* phosphoprotein function in HCC consistent with our above computation. It would be necessary of *RRM2* phosphoprotein function decrease to invasion of HCC.

In conclusion, we deduced the weaker *RRM2* phosphoprotein module in HCC. It would be necessary of *RRM2* phosphoprotein function decrease to invasion of HCC. *RRM2* phosphoprotein interaction module construction in HCC can be a new route for studying the pathogenesis of HCC.

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