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Changes in Global Gene Expression Induced by NOR1 Over-expression in HepG2 Cells*

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Abstract Previous work from this laboratory has cloned a novel gene NOR1 and showed its extensive expression in normal tissues and down-regulation in carcinomas. To further investigate its downstream target genes and better understand its function, NOR1 was over-expressed in HepG2 hepatoma cells and global changes in gene expressions from a stable line were identified by cDNA microarrays. The results discovered 59 genes up-regulated in these cells compared with the original cells, including Grb2, HBP17, TNFRSF11B genes that have been implicated in tumorigenesis and cancer development. In addition, 103 down-regulated genes were also identified, including genes encoding Bik, MAP2K6 and ZFP95 proteins. The expression patterns of certain genes identified by microarrays were validated by quantitative real-time PCR and the results showed that expression difference were statistically significant ($P < 0.05$). These data suggest that NOR1 may influence the biology and cancerous behaviors of HepG2 cells by regulating expression of a set of genes involved in signal transduction, cell cycle regulation, transcription and translation controls.

Key words NOR1, hepatoma, cDNA microarray, quantitative real-time PCR.

Previously, a novel gene named NOR1 which located in chromosome 1p36-35 has been cloned in our laboratory. Earlier studies showed that it was significantly down-regulated in several cancer cell lines and carcinoma tissues[1], and reduced the growth rate of cancer cells when over-expressed by gene transfection. Flow cytometry analysis revealed that the growth rate reduction was due to its effects on cell cycle progression[2, 3]. Based on these previous findings, we hypothesized that NOR1 might be a tumor suppressor gene associated with liver cancer and nasopharyngeal carcinoma[4].

In order to identify downstream genes regulated by NOR1 and to provide molecular mechanisms underlining NOR1 effects on cancer cells, we investigated the global changes in gene expression induced by over-expression of NOR1 in HepG2 cells by gene profiling with microarray technology.

1 Materials and methods

1.1 Cell culture and gene transfection

The human hepatocellular carcinoma cells, HepG2, were maintained in RPMI1640 supplemented with 10% calf serum in a humidified culture incubator with 5% CO_2 and 95% air at 37℃. The cDNA sequence of NOR1 coding region was constructed in the eukaryotic expression vector pcDNA3.1(+) plasmid (Invitrogen) between two restriction sites *Bam*HⅠ and *Xho* Ⅰ. The NOR1- pcDNA3.1 expression plasmid was then transfected into HepG2 cells by the liposome-medicated method (Lipofectamine purchased from Invitrogen). Transfected cells were selected by media supplemented with 400 mg/L G418 for three weeks. Clones resistant to G418 were then picked. One stable clone was selected and expanded for the stable cell line for all subsequent experiments. In parallel, HepG2 cells were also transfected with the empty plasmid vector pcDNA3.1(+), and a stable line without NOR1 overexpression was also established to use as the control cell line. RT-PCR was then used to confirm the expressions of NOR1 mRNA in the two cell lines.

1.2 RT-PCR detection for NOR1 mRNA

Total RNAs were isolated from cells with TRIzol reagent (Invitrogen) and then reverse-transcribed into

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cDNA with the M-MLV reverse transcriptase (Promega) according to manufacturer's protocols. The condition for PCR was 94℃ for 5 min, followed by 30 cycles of 94℃ for 30 s, 55℃ for 45 s and 72℃ for 45 s, then a final extension at 72℃ for 5 min. The primers for PCR amplification of NOR1 cDNA were: 5′ CAGAATAATAACGGTCGCTTTG 3′ and 5′ CGAGGTTTGGGAGAACGATT 3′ for a 305 bp product. At the same time, β-actin gene was also amplified from each cDNA sample as an internal control for PCR efficiency. The β-actin primers were: 5′ CAGGCTGTGCTATCCCTGTA 3′ and 5′ CATACCCCTCGTAGATGGGC 3′ for a 280 bp product. All PCR reactions were carried out in the 25 μl volume containing 1×PCR Buffer, 100 μmol/L $MgCl_2$, 2 μl cDNA templates, 200 nmol/L of each dNTPs, 1 μl of each primer, 0.5 μl (2.5 U/μl) Taq polymerase (Roche). The final products were electrophoresized on 1% agarose gel, stained with ethidium bromide, and detected under UV light.

1.3 cDNA microarray

The cDNA microarray was made by Shanghai Biochip Limited Company. We chose the model RBC-R-HC-100-23 microarrays that represent total 17 101 genes including 16 420 unigenes, 12 581 known genes and 3 869 ESTs. Each microarray was also spotted 10 positive controls and 6 negative controls. We used NOR1 over-expressed HepG2 cells as the experimental group, and empty plasmid vector transfected cells as the control group. Total RNA isolation and cDNA reverse-transcription were carried out as described above for RT-PCR. After purification, the cDNAs form the experimental group and the control group were paired and labeled with respective fluorescent dyes Cy5 and Cy3, followed by hybridization to the same microarrays. Hybridized array slides were scanned and fluorescence signal intensities were quantified with Agilent scanning software. The ratios of signal intensities of two dyes were then generated and represented mRNA levels of the experimental group relative to the control groups. A ratio > 2 indicates at least two fold increases or a ratio < 0.5 indicates at least two fold decreases in the mRNA level of the experimental group.

1.4 Validation of differentially expressed genes by quantitative real-time PCR

Three genes identified from microarray analysis as up-regulated genes in NOR1 over-expressed cells, Grb2, HBP17 and TNFRSF11B, were selected to evaluate by quantitative real-time PCR. Their relative mRNA levels were compared among the following three cell lines: pcDNA3.1 (+) NOR1 / HepG2, pcDNA3.1(+)/HepG2, and HepG2 and denoted as C1, K1 and H1 respectively in the results figures. Total RNAs were isolated from these cells and cDNA were generated as described earlier. A 20 μl PCR reaction contained 1 μl cDNA, 0.4 μl of each primer pair specific to each gene, and SYBR Premix Ex Tq 10 μl. The reaction was carried out in the Rotor-Gene RG-3000 Real time Thermal Cycler. The condition for PCR was 95℃ for 2 min, then 60 cycles of 95℃ for 20 s, 62℃ for 30 s, followed by a final extension at 72℃ for 30 s. The 18 S RNA was used as internal control and the distilled water in replacement of the primers and the templates was used as the negative control. The relative abundance of mRNAs was then compared by the values of the threshold cycles, defined as the number of cycles at which amplified DNA products reached the preset threshold amount amplified from a specific cDNA.

Table 1 List of genes selected for validation by real-time PCR and their specific primer sequences used for reverse transcription PCR

Gene name	Primer sequence	Length of product/bp
Grb2	F5′ ATTGTGTGTCCCAGAGTG 3′	167
	R5′ AGTAGCTTTGAAGTCATATTTG 3′	
HBP17	F5′ TGCTCTCCTTCCTCCTAC 3′	180
	R5′ AGTTGGCTTGGTCTTTGG 3′	
TNFRSF11B	F5′ TGACCACTACTACACAGACAG 3′	160
	R5′ CAAGCAGAACTCTATCTCAAGG 3′	

*The primer sequences were shown 5′ to 3′. ** F and R denote forward and reverse primers respectively.

1.5 Statistic analyses

The statistic analyses for all the results were performed by SPSS12.0 software. Analysis of variance (ANOV) was adopted for group comparison and S-N-K was for the further comparison (C1-K1, C1-H1, H1-K1). We used the standard $P < 0.05$ to denote a significant difference for changes between samples.

2 Results

2.1 The establishment of NOR1 over-expressed HepG2 cell line

We transfected HepG2 cells by the liposome-medicated method, selected the stable clones that were resistant to G418, and established a stable cell line that over-express NOR1 gene. Using RT-PCR, we detected the increased expression of NOR1 mRNA in the stable HepG2 clone transfected with pcDNA3.1 (+) NOR1 but only the basal level of NOR1 mRNA in the control cells transfected with the empty vector pcDNA3.1 alone. As seen in Figure 1, RT-PCR products of the internal control β-actin were equally amplified from both cell samples, validating the changes in NOR1 mRNA levels in the same experiment.

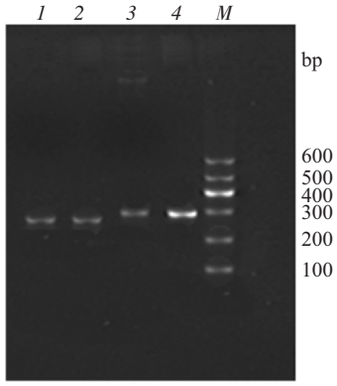

Fig. 1 NOR1 over-expression in HepG2 cells transfected with pcDNA3.1(+)NOR1

RT-PCR amplified cDNA products from total RNAs isolated from HepG2 cell lines stably transfected with either pcDNA3.1(+)NOR1 (lane *2*, *4*) or pcDNA3.1(+) empty vector (lane *1*, *3*). Lane *3*, *4* are products for NOR1; Lane *1*, *2* are products for β-actin internal controls. *M* is the DNA molecular weight marker with DNA sizes labeled for each band by the numbers in base-pairs (bp) next to them.

2.2 Screening for the differentially expressed genes in NOR1 over-expressed HepG2 cells by cDNA microarrays

Microarray analyses of the gene expression profiles were performed by dual-color hybridization for the two cell lines: the experimental cells pcDNA3.1 (+)NOR1 / HepG2 and the control cells pcDNA3.1 (+)/HepG2. Total RNAs were isolated and cDNAs were synthesized and labeled by two fluorescent dyes. Equal amounts of labeled cDNAs from the experimental and control groups were then paired to hybridize to the same set of microarrays. The resulting microarray images after hybridization were shown in Figure 2. Using the two fluorescence channels (Cy3 and Cy5) obtained from two cDNA samples, the ratios of fluorescence intensities at each

Fig. 2 Scanned dual-fluorescence images of the microarray hybridized with Cy3 labeled cDNA from NOR1 over-expressed cells and Cy5 labeled cDNA from the control cells

Red spots represent up-regulated genes in the NOR1 over-expressed cells and green spots represent down-regulated genes. Yellow spots represent genes with similar expression levels in both cell lines.

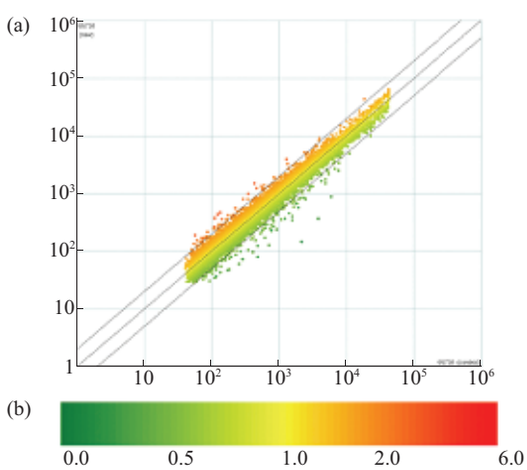

Fig. 3 Scatter diagram representing all the hybridization signals from a microarray after normalization (a) and a chromatic aberration graph showing the color scheme (b)

The *x*-axis represents signal intensities from the green fluorescence channel (Cy5 labeled control cDNA) and the *y*-axis from the red fluorescence signal intensities (Cy3 labeled cDNA from the experimental group). The diagonal line cross the center indicates where equal amounts of fluorescent signals from the two channels were obtained. The two lines parallel to the center line specifies a two fold differences in fluorescence signals between the two samples. Only spots outside these two lines were considered to represent genes with at least two fold changes in mRNA expression levels. (b) A chromatic aberration graph showing the color scheme used in (a). Numbers below each changed color denote the corresponding fold changes in fluorescence intensities, also reflex the changes in gene expression levels.

spot indicate the different levels of mRNA for each gene from these two samples. Ratios bigger than 2 or smaller than 0.5 thus indicate more than 2 fold changes in mRNA expression levels between the two samples (Figure 3). The gene lists in Tables 2 and 3 showed that there were 59 genes up-regulated and 103 genes down-regulated in the NOR1 overexpressed cells (Tables 2, 3) compared with the control.

Table 2 List of up-regulated genes in the NOR1 over-expressed HepG2 cells

Apoptosis-associated gene			Metabolism-related gene			Cell cycle control gene		
GenBank ID	Name	Fold change	GenBank ID	Name	Fold change	GenBank ID	Name	Fold change
NM-004342	CALD1	2.039	NM-003129	SQLE	3.552	AJ238555	C21orf107	2.218
NM-015715	PLA2G3	2.028	NM-015715	PLA2G3	2.028	NM-006142	SFN	2.151
NM-002546	TNFRSF11B	6.022	NM-000859	HMGCR	2.361	NM-133377	RAD1	2.146
AJ238555	C21orf107	2.218	NM-001159	AOX1	2.071	BC014469	CDKN2B	2.899
NM-006142	SFN	2.151	NM-002340	LSS	2.012	NM-005130	HBP17	3.411
NM-000450	SELE	2.152	NM-002837	PTPRB	2.218			
NM-002086	GRB2	4.772	BC000349	GBA	2.612			
BC014469	CDKN2B	2.899	NM-006745	SC4MOL	2.101			
NM-005362	MAGEA6	2.119	NM-058004	PIK4CA	2.321			
NM-002640	SERPINB8	2.280	NM-012199	EIF2C1	2.788			
NM-018970	GPR85	3.454	NM-005130	HBP17	3.411			
NM-058004	PIK4CA	2.321						

Table 3 List of the down-regulated genes in the NOR1 over-expressed HepG2 cells

Cell cycle control gene			Signal transduction gene			Binding protein gene		
GenBank ID	Name	Fold change	GenBank ID	Name	Fold change	GenBank ID	Name	Fold change
NM-005980	S100P	0.423	D86962	GRB10	0.498	NM-000596	IGFBP1	0.485
U39675	MAP2K6	0.227	D86962	GRB10	0.498	NM-018337	ZNF444	0.409
NM-012222	MUTYH	0.496	AB007867	PLXNB1	0.342	AB033092	MTA3	0.363
NM-004095	EIF4EBP1	0.365	NM-006681	NMU	0.474	NM-007327	GRIN1	0.432
NM-031459	SESN2	0.332	NM-004864	GDF15	0.169	NM-012395	PFTK1	0.394
NM-012385	P8	0.265	NM-001393	ECM2	0.241	NM-3714	STC2	0.298
BF224349	ASNS	0.149	NM-007327	GRIN1	0.432	NM-014476	PDLIM3	0.263
NM-000596	IGFBP1	0.485	NM-0019056	P17.3	0.441	BC013128	CEBPG	0.428
AB033092	MTA3	0.363	BC007339	DHRS2	0.406			
NM-014569	ZNF95	0.324	NM-004915	ABCG1	0.414			
NM-005628	SLC1A5	0.201	NM-001179	BIK	0.457			

2.3 Validation of the differentially expressed genes by quantitative real-time PCR

Three up-regulated genes: Grb2, HBP17 and TNFRSF11B were chosen for validation by real-time PCR. Figures 4 ～6 show the sets of amplification curves for three genes. Each gene was measured in triplicate. Higher copy numbers of the target mRNA result in lower values of the threshold cycles (*C*t). Table 4 listed the values of threshold cycles (*C*t) for amplification of above three genes and the inner control 18S RNA from three cell lines pcDNA3.1(+) NOR1/HepG2 (C1), pcDNA3.1(+)/HepG2 (K1) and the original HepG2 cells (H1). Table 5 showed the relative expression levels of the three genes in the three cell lines. The statistic analysis showed that the expression of Grb2, HBP17 and TNFRSF11B were 4.87, 5.23 and 6.33 fold up-regulated respectively in pcDNA3.1(+)NOR1/HepG2 cells compared with the HepG2 cells, and all these changes were significant with the *P* values smaller than 0.05.

The statistic analysis also showed the differences in the mRNA levels of all three genes between pcDNA3.1 (+) NOR1 / HepG2 cells and either pcDNA3.1(+)/HepG2(K1) or HepG2 (H1) cells were significant ($P < 0.05$); however, there were no significant differences for these same genes between pcDNA3.1(+)/HepG2 (K1) and HepG2 (H1) cells ($P_{all} > 0.05$). Therefore, the results of the real-time PCR for all three genes were in consistence with the results obtained from cDNA microarray.

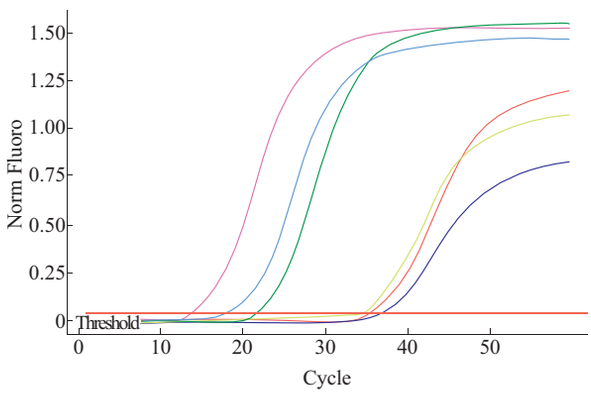

Fig. 4 Amplification curves of Grb2 in three samples from a quantitative real-time PCR reaction

■: H1; ■: K1; ■: C1 ; ■: H1 (18S RNA); ■: K1 (18S RNA); ■: C1(18S RNA).

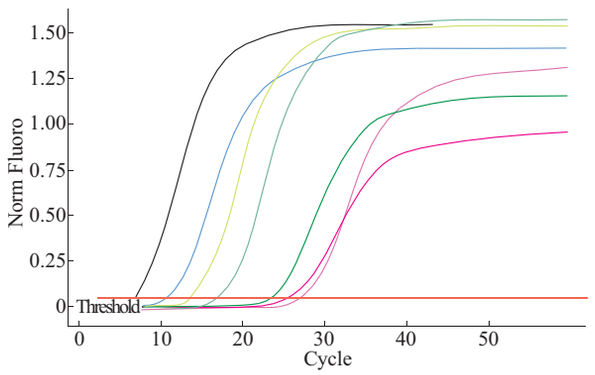

Fig. 5 Amplification curves of HBP17 in three samples from a quantitative real-time PCR reaction

■: C1; ■: K1; ■: H1 ; ■: C1 (18S RNA); ■: K1 (18S RNA); ■: H1(18S RNA).

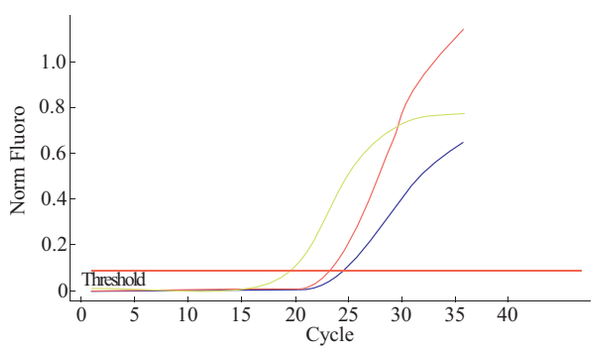

Fig. 6 Amplification curves of TNFRSF11B in three samples from a quantitative real-time PCR reaction

■: C1; ■: K1; ■: H1.

Table 4 Values of the threshold cycles (*Ct*) measured by real-time PCR for the three selected genes

Cells	Grb2	HBP17	18S RNA	TNFRSF11B	18S RNA[1)]
K1	35.87 ± 1.22	27.71 ± 2.34	16.33 ± 1.04	23.72 ± 1.98	10.11 ± 1.11
C1	32.93 ± 1.38	25.39 ± 1.05	17.79 ± 1.34	21.98 ± 2.03	12.66 ± 1.63
H1	35.78 ± 1.56	26.78 ± 1.77	15.98 ± 1.02	22.04 ± 1.54	9.88 ± 1.33

The C_t of 18SRNA marked with [1)] was just for TNFRSF11B. Data were obtained from three repeated measurements for each gene and expressed as $\bar{x} \pm s$ deviation.

Table 5 The relative mRNA levels of the three genes in the three cell lines

Gene name	H1 Cells	K1 Cells	C1 Cells
GRB2	1.000 ± 0.224	1.147 ± 0.067	4.873 ± 0.119[1,2)]
HBP17	1.000 ± 0.056	1.163 ± 0.129	5.230 ± 0.052[1,2)]
TNFRSF11B	1.000 ± 0.224	1.137 ± 0.070	6.330 ± 0.060[1,2)]

Data were obtained from three repeated measurements for each gene and expressed as $\bar{x} \pm s$ deviation. The mean values for mRNA levels in the HepG2 cells (H1) were set to one, then all other mRNAs were normalized based on these values. Statistic analysis was applied to all data and revealed that there was no difference in mRNA levels of the three genes between HepG2 (H1) and pcDNA3.1(+)/HepG2 (K1) ($P_{all} > 0.05$), but there were significant differences ($P < 0.05$) between C1 and H1 (1) and between C1 and K1 (2) for all three genes.

3 Discussion

To search for downstream targets of NOR1 gene, we utilized hepatocarcinoma cell HepG2 to establish a cell line stably integrated with the NOR1 expression plasmid pcDNA3.1(+)NOR1. We identified the set of genes regulated by over-expression of NOR1 through gene expression profiling of the NOR1 over-expressing cells in comparison with its original cells or the control cells that transfected the empty plasmid vector alone.

There were total 162 genes regulated by NOR1 and they could be classified into several functional categories, such as apoptosis genes, tumor-associated genes, genes for the regulation of certain enzyme activities, genes playing roles in mRNA transcription or protein translation, genes regulating cell cycles, genes involved in metabolic processes, genes encoding signal transducers or their associative proteins. Examples for the highly up-regulated genes by NOR1 are genes encoding for three proteins Grb2, HBP17, TNFRSF11B, which have all been shown to play various direct or indirect roles in tumor biology.

Grb2, abbreviated from growth factor receptor-bound 2, is a ubiquitously expressed adapter protein linking the growth factor receptors at the cell surface to the Ras signaling pathway inside cells. In addition to its role as a receptor-proximal adaptor protein, Grb2 participates directly in the regulation of actin filament formation and actin-based cell mobility[5,6]. It has long been implicated in the oncogenesis of several human malignancies. Studies over the last decade also found its other fundamental roles in cell mobility and angiogenesis, which can also contribute to tumor growth, invasiveness and metastasis[7]. In endothelial cells, many signaling pathways mediated by Grb2, particularly through its translation from the cytoplasm to the cell surface are critical for vasculogenesis, angiogenesis and lymphoangiogenesis. For examples, binding of the angiogenic factor VEGF-A to its receptor VEGFR-2 induces phosphorylation of VEGFR2, then phosphorylated VEGFR-2 recruits and forms complex with a series of signaling molecules including Grb2 at the cell surface. The FLT4L kinase, an alternatively spliced form of the VEGFR3/FLT4 receptor, also binds Grb2 directly through its cytoplasmic region and recruits a second pool of Grb2 upon phosphorylation of Shc[8]. In non-endothelial cell types, Grb2 translocation is also believed to link receptor activation to cell cycle progression and cell mobility *via* the Sos/Ras and Rac1/Rho pathways, respectively[9~11]. Due to the primary effects of Grb2 on cell motility and the related processes, and the role of HGF in angiogenesis[12,13], Grb2 SH2 domain-binding antagonists have been investigated for their ability of to block HGF-stimulated Grb2 signaling.

Since NOR1 gene contains two casein phosphorylation sites, which may bind to the SH2 domain of Grb2, we speculate that NOR1 may enhance the Grb2 signaling pathways by the induction of Grb2 gene expression as seen here in our study, and also by interaction to the over-expressed proteins *via* its SH2-domains. Therefore, NOR1 gene may influence the occurrence, invasion, metastasis and angiogenesis of various tumors through the Grb2-mediated pathways.

Another highly induced gene by NOR1 is HBP17 encoding fibroblast growth factor binding protein (FGF-BP). Many studies have reported its over-expression in many cancers and its vital role in the initial stage of tumor angiogenesis. In head and neck cancers, FGF-BP acts as an angiogenesis factor to accelerate tumor angiogenesis and its high expression correlates with the microvessel density in the tumor[14, 15]. In other cancers, such as pancreatic cancer and colon cancer, FGF-BP is also found to be over-expressed[16]. FGF-BP first binds to b-FGF and releases it from the extracellular matrix, then b-FGF can participate the process of angiogenesis. Our study showed up-regulation of HBP17 gene by NOR1, it is likely that FGF-BP protein may also be up-regulated. Based on the functions of FGF-BP, we speculate that NOR1 may also influence the neovascularization of tumors *via* the action of FGF-BP.

Another highly induced gene TNFRSF11B encodes the 11B member of tumor necrosis factor receptor superfamily, also known as osteoprotegerin. The protein has two known ligands in the TNF family: the receptor activator of nuclear factor-κB ligand (RANKL), and the TNF-related apoptosis inducing ligand (TRAIL). Many studies[17~19] showed that TNFRSF11B has potential biological effects on tumorigenesis and tumor deterioration. Because it lacks the cytoplasm death domain of other TNF receptors, TNFRSF11B may act as a soluble decoy receptor of TRAKL. In this scenario, TRSAL can compete with TRAKL for their bindings to TNFRSF11B and DR4/DR5. The process may play an important role in protecting tumor cells from the cytotoxic effects induced by lymphocytes and their apoptosis. Since we found that TNFRSF11B is over-expressed in the tumor cells with NOR1 over-expression, thus it is possible that NOR1 may be involved in the signal transduction pathway leading to apoptosis in the tumors, in turn, influencing the entire process of tumorigenesis and tumor development.

It is very interesting to note that all these genes are inter-related in one way or another. For instances, PDGF and b-FGF are known to induce the expression

of TNFRSF11B; on the other hand, Grb2 combined with PDGF can amplify and strengthen the functions of PDGF[20]. FGF-BP encoded by HBP17 can also interact with b-FGF, then in turn regulates the quantity and activity of b-FGF. So we hypothesize that during the entire procedure of tumorigenesis and tumor development, there may exist a signal network among Grb2, FGF-BP and TNFRSF11B. Based on this, NOR1 gene may play a regulatory role in this signal network upstream of the signal transduction pathway.

Compared with the traditional methods of genetic analysis, cDNA microarray is a superior technology for analyzing gene expression in the genome-wide scale, thus providing a broader view to address more complex biological problems at the molecular levels. However, this technology examines gene expression at the level of transcription, which may not reflect the level of protein expression and protein function that may involve further post-translational modification. For this reason, further work is needed to investigate NOR1 protein levels and its biological functions.

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NOR1 基因转染对肝癌细胞 HepG2 基因表达谱的影响 *

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摘要 NOR1 基因是一在正常组织中广泛表达且在肿瘤组织中表达下调的新基因．为进一步研究 NOR1 基因的功能和寻找其下游基因，利用脂质体技术将 NOR1 基因转染进 HepG2 细胞，采用 cDNA 微阵列技术分析其基因表达谱的改变．试验表明 NOR1 基因的转染能使 Grb2，HBP17，TNFRSF11B 等 59 个基因上调，同时也下调 Bik，MAP2K6，ZFP95 等 103 个基因．随后用实时荧光定量 PCR 对 cDNA 微阵列结果中上述 3 个上调表达基因进行验证，结果表明，基因表达差异具有统计学意义($P < 0.05$)，荧光定量 PCR 结果与微阵列结果相符．这些结果提示，NOR1 基因对肝癌 HepG2 细胞的生物学行为的影响可能与它对细胞信号转导，细胞周期调控，转录、翻译调控相关基因的表达影响有关．

关键词 NOR1 基因，肝癌，cDNA 微阵列，实时荧光定量 PCR

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