Different molecular pathways determining extrahepatic and intrahepatic recurrences of hepatocellular carcinoma

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Abstract. Recent genome-wide screens have identified genes associated with the metastatic potential of hepatocellular carcinoma (HCC); however, there is little overlap between the identified genes, and interpretations of the results remain controversial. These inconsistencies may be related to differences in the sample populations, use of distinct microarray platforms and algorithms, and the complicated modes of HCC recurrence. We investigated the gene expression profiles of extrahepatic recurrence (EHR) and early intrahepatic recurrence (IHR), which are two representative modes of recurrence of HCC attributable to metastasis. We used DNA microarray analysis and identified 46 signature genes for EHR in 35 HCCs in a supervised learning manner. The obtained gene expression profile was compared with that for early IHR that was determined previously in the same manner. The 46 signature genes for EHR included many cell adhesion-related genes (*ITGA6*, *SPP1*, *DNMBP*, *CD44* and *POSTN*), which all showed higher expression in HCC with EHR than in HCC without EHR. The 46 signature genes for early IHR included 10 immune response-related genes, which all showed lower expression in HCC with early IHR than in HCC without early IHR. The signature genes for EHR included only two immune response-related genes (P=0.013). These results suggest that alteration of the cell adhesion system plays a central role in EHR and that reduction of the immune response is a specific step in early IHR. These results indicate that the metastatic processes in EHR and early IHR involve different molecular pathways.

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Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; LC, liver cirrhosis

Key words: intrahepatic recurrence, extrahepatic recurrence, hepatocellular carcinoma, microarray

Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of cancer with an estimated 564,000 new cases worldwide in year 2000 and it represents a major health problem because its incidence is increasing in many countries (1-3). Despite many advances in the treatment of HCC, the recurrence rate at 5 years after curative treatment exceeds 70% (4). Given the high frequency of recurrence, it is critical to better understand the mechanisms underlying HCC recurrence in order to improve the outcome.

The mode of recurrence of HCC is complicated (5). There are two representative modes of recurrence of HCC after surgery, intrahepatic recurrence (IHR) and extrahepatic recurrence (EHR). IHR can be further classified into two subtypes, early IHR and late IHR (6,7). Early IHR, most of which can be attributed to the intrahepatic metastasis of cancer cells and is detected in 30-50% of patients within 1 or 2 years of surgery, limits the potential for a surgical cure of HCC (6,7). Late IHR is a *de novo* primary tumor, which accounts for the majority of HCC recurrence after 3 years of surgery (8). In contrast, it was reported that HCC recurred in distant organs such as the lung and bone in only 3 (7%) of 42 patients who underwent a liver transplantation, one of the radical curative treatment strategies (9). Thus, the frequency of EHR is markedly lower than that of early IHR; however, once HCC progresses to EHR, it is difficult to control the lesions in most cases. Indeed, the frequency of death due to respiratory failure resulting from lung metastasis of HCC has increased for the last 30 years in the Japanese population (10).

Recently, genome-wide approaches have attracted a great deal of attention in the field of cancer research. Several investigators, including ourselves, have identified gene signatures linked to HCC recurrence (6,11-14). Unfortunately, there was little overlap between the genes identified in the various studies, and interpretations of the data remain controversial (15). These inconsistencies may be related to differences in the sample populations, use of distinct microarray platforms and algorithms, and the complicated modes of HCC recurrence (5). There have been no studies

Table I. Clinicopathologic background of 35 HCCs.

Factors	Extrahepatic recurrence		P-value
	Positive (n=10)	Negative (n=25)	
Sex (male/female)	5/5	18/7	P=0.257[a]
Age (years) (mean±SD)	57.6±17.9	61.8±8.6	P=0.760[b]
Viral infection (HBV/HCV/non-B non-C)	3/6/1	7/16/2	P=1.00[a]
Cirrhosis (presence/absence)	6/4	10/15	P=0.454[a]
Histological grading (well/moderate/poor)	0/6/4	1/21/3	P=0.211[a]
Venous invasion (presence/absence)	4/6	17/8	P=0.150[a]
Stage (I/II/III-IV)	2/2/6	9/11/5	P=0.112[a]

[a]Fisher's exact test, [b]Mann-Whitney U test.

evaluating signature genes on the basis of the complicated recurrence modes of HCC. In the present study, to clarify the molecular features associated with EHR and early IHR of HCC, we investigated the genes linked to EHR of HCC in a supervised learning manner and then compared these genes with those linked to early IHR that was identified previously in the same manner (6,11).

Materials and methods

Samples. We analyzed 12,600 genes in 76 HCC samples using huU95A DNA Chips® (Affymetrix, Santa Clara, CA, USA) (16,17). Written informed consent was obtained from all the patients prior to surgery. The study protocol was approved by the Institutional Review Board for Human Use at the Yamaguchi University School of Medicine.

Given that differences in the hepatitis virus infection pattern, coexisting liver disease, and sex can largely affect the identification of signature genes for HCC (18-21), sample backgrounds for HCC with EHR and HCC without EHR must be adjusted to decrease any bias or noise. Moreover, the sample size and event rate between the EHR cohort and the previous cohort (n=33) for early IHR (11) have to be adjusted. We repeated random sampling out of the above 76 samples and selected a sample set (n=35) showing no differences in the patient backgrounds between HCC with EHR and HCC without EHR (Table I) on the basis of pTNM classification of the International Union Against Cancer (22).

The 35 HCC patients were followed for more than 5 years after surgery. Among them, 5 had extrahepatic metastases at surgery, and 5 had EHR after curative surgery. These 10 patients were classified as the EHR group. The remaining 25 patients, who had no EHR during the follow-up period, were classified as the non-EHR group. In the EHR group, lung metastases were found in 8 patients and bone metastases were found in 3. One patient had both lung and bone metastases. Among the 5 patients who had EHR after curative surgery, 2 had early IHR. In the non-EHR group, 9 patients had early IHR.

Gene selection procedure. The preparation of specimens, synthesis of cDNA and cRNA, and oligonucleotide microarray procedure (huU95A DNA Chips®, Affymetrix) were described previously (6,16,23). For the samples, the quality of the extracted RNA was confirmed by the appearance of characteristic 28S and 18S rRNA fragments on agarose gels (data not shown).

Given our previous finding (23) that the expression of genes with average differences (ADs) of <40 on the huU95A DNA Chips® was not reproducible by RT-PCR, we selected genes with ADs of >40 in at least half of the 35 HCC samples. With filtering, we identified 8,407 genes. We used the Fisher ratio (6,18) to evaluate the potential of each selected gene in discriminating HCC with EHR from HCC without EHR, and the genes were then ranked in order of the decreasing Fisher ratio. Of the 8,407 genes, the 46 genes with the highest Fisher ratios were selected. The mean of the AD of all the 46 genes was 2-fold higher or 0.5-fold lower in HCC with EHR than in HCC without EHR (Table I). This gene signature for EHR was compared with that for early IHR determined previously in the same manner (6,11). To examine the changes in expression during hepatocarcinogenesis, the expression data of the cell adhesion-related genes (*ITGA6*, *SPP1*, *DNMBP*, *CD44*, and *POSTN*) in HCCs were compared with those for 16 non-cancerous liver tissues (16).

Statistical analysis. The Chi-square test, Fisher's exact test, Student's t-test, and Mann-Whitney U test were used to evaluate the differences between groups. Data were analyzed with SPSS 11.0J software (SPSS, Inc., Chicago, IL). P-values <0.05 were considered significant.

Results

As we expected, the 46 signature genes for EHR were quite different from those for early IHR (Tables II and III). We believe that this result is reliable because we performed an adjustment for the sample backgrounds between the two cohorts. The expression levels of several cell adhesion-related genes (*ITGA6*, *SPP1*, *DNMBP*, *CD44* and *POSTN*) were significantly higher in HCC with EHR than in HCC without EHR. When the expression levels were compared with those in non-cancerous liver tissues, the 5 cell adhesion-related genes could be classified into two groups (Fig. 1). The levels of *ITGA6* and *SPP1* were higher in HCC without EHR than in non-cancerous liver tissue, and expression

Table II. Forty-six feature genes responsible for EHR of HCC.

Fisher ratio	Probe	GB	Symbol	Function	EHR(-)	EHR(+)
2.06	37181_at	X76538	MPV17	metabolism	<40	248.6
1.47	39549_at	AI743090	EST	unknown	<40	117.4
1.44	37137_at	M17016	GZMB	immune system	132.7	63.3
1.37	33411_g_at	S66213	ITGA6	cell adhesion	68.5	217.3
1.31	38199_at	AI289489	EST	unknown	116.8	46.9
1.30	31894_at	M95724	CENPC1	DNA binding	137	67.3
1.29	40574_at	AA868268	EST	unknown	91.7	<40
1.22	34342_s_at	AF052124	SPP1	cell adhesion	3914.6	11311.6
1.19	38436_at	D87440	RTF1	transcritpion	59.5	134.9
1.17	34339_at	AB009282	CYB5-M	metabolism	71.5	184.6
1.16	34390_at	U90441	P4HA2	metal ion binding	110.8	222.2
1.16	34712_at	AB023227	DNMBP	cell adhesion	49.7	153.9
1.11	32389_at	W25892	EST	unknown	87.1	<40
1.10	33533_at	U40992	DNAJB4	unknown	96.8	43.5
1.10	31634_at	M13057	PRH1	extracellular matrix	127.5	45.6
1.08	37133_at	AF027406	STK23	signal transduction	201.3	78
1.08	33621_at	X71348	TCF2	transcription	164.2	74.4
1.07	1451_s_at	D13666	POSTN	cell adhesion	233.6	989.6
1.04	2092_s_at	J04765	SPP1	cell adhesion	2389.4	6532
1.03	33718_at	AC006128	WIZ	metal ion binding	155	76.8
1.02	1648_at	U60805	OSMR	cell proliferation	56.9	156.1
1.02	39815_at	AA883101	EST	unknown	40.9	96.6
1.02	1076_at	M28983	ILIA	immune system	82.3	<40
1.01	2036_s_at	M59040	CD44	cell adhesion	55.9	203.9
1.01	39447_f_at	W27095	EST	unknown	93.2	<40
1.00	301_at	Mucin6,Gastr	MUC6	cell protection	58	144.1
0.98	40074_at	X16396	MTHFD2	molecule transport	90.8	202.6
0.97	757_at	D28364	ANXA2	cell proliferation	204.2	557.8
0.97	41020_at	M95971	PCSK2	proteolysis and peptidolysis	<40	98.2
0.96	38634_at	Ml1433	RBP1	molecule transport	320.2	747.2
0.93	35275_at	AL050025	EST	unknown	132.4	316.9
0.90	33410_at	S66213	ITGA6	cell adhesion	125.6	328.5
0.86	34310_at	Y00486	APRT	metabolism	103.7	277.8
0.86	39690_at	AF002282	PDUM3	protein binding	65.4	175
0.85	32314_g_at	M12125	TPM2	cytoskelton	110.3	250.7
0.85	33979_at	X55990	RNASE3	defense to bacteria	108.7	<40
0.84	39277_at	U60805	OSMR	cell proliferation	49.8	168
0.84	31404_at	AF019765	GRK1	signal transduction	167.7	70.5
0.84	31587_at	X96969	SLC14A	molecule transport	<40	97
0.83	33101_g_at	AB017551	FETUB	unknown	1194.4	453.6
0.83	31684_at	M62896	ANXA2P1	pseudogene	196	519.8
0.83	33068_f_at	U08854	UGT2B15	detoxification	4901.9	2002.1
0.82	39525_at	AL120687	EST	unknown	45.2	90.5
0.82	40298_at	AB014603	KIAA0703	unknown	182.2	90.1
0.82	36632_at	U00957	AKAP10	signal transduction	47.4	95.9
0.81	667_at	L22206	AVPR2	signal transduction	104.4	<40

GB, GenBank; EHR, extrahepatic recurrence; expression levels represent the mean of the average difference by Affymetrix.

Table III. Representative function and EHR- and early IHR-related genes.

Functional category	EHR-related genes	Early IHR-related genes
Cell adhesion	7[a]	1
Signal transduction	4	4
Cell proliferation	3[a]	1
Metabolism	3	4
Molecule transport	3	0
Transcription	2	5
Immune system	2	10
Others	22	21
Total	46	46

EHR, extrahepatic recurrence; IHR, intrahepatic recurrence; [a]number including overlapping genes.

levels were further increased in HCC with EHR. In contrast, the expression levels of *DNMBP*, *CD44*, and *POSTN* in HCC without EHR were similar to those in non-cancerous liver tissue but increased in HCC with EHR.

The 46 EHR-related genes included only 2 immune system-related genes, *GZMB* and *IL1A*, both of which were expressed at lower levels in HCC with EHR than in HCC without EHR (Table II). The number of immune system-related genes was significantly lower in the EHR-related profile than in the early IHR-related profile (2/46 vs 10/46, P=0.013 by the Chi-square test) (Table III).

Discussion

Despite recent progress in available treatments, the recurrence of HCC, including the appearance of new HCC in the liver, occurs in ~70% of cases within 5 years after curative treatment (3). Because recurrence (i.e. EHR and early IHR) attributable to the metastatic spread of HCC cells limits the efficacy of various therapeutic options, a precise understanding of EHR and early IHR may improve the prognosis of HCC. On the basis of this concept, we previously identified 46 genes associated with early IHR of HCC within 1 year after curative surgery (11) and developed 12-gene predictors with high accuracy for early IHR (6). Kurokawa *et al* (12) focused on early IHR within 2 years after surgery and identified 92 genes related to early IHR with the use of PCR array technology. An elegant microarray study by Lee *et al* (13) identified 406 genes associated with the survival of HCC patients; however, it was unclear whether the identified

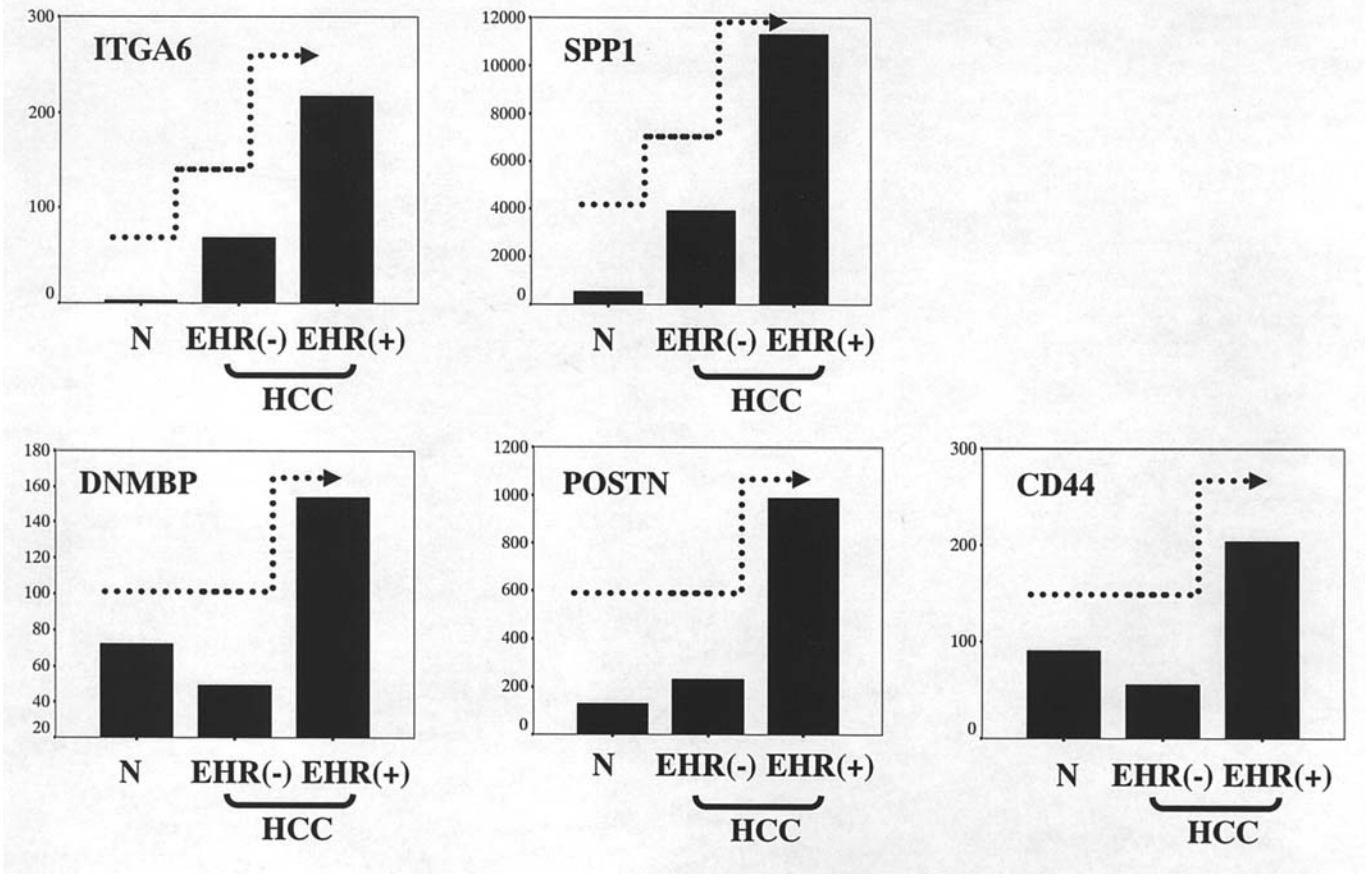

Figure 1. Expression levels of 5 cell adhesion-related genes in HCC and non-cancerous liver tissues. Mean expression levels of *ITGA6*, *SPP1*, *DNMBP*, *CD44* and *POSTN* in HCCs with EHR and HCCs without EHR was compared with those of 16 non-cancerous liver tissues (15). Note that these genes can be classified into two subgroups, i.e., the first (*ITGA6* and *SPP1*) and the second (*DNMBP*, *POSTN* and *CD44*), by their expression patterns. N, non-cancerous livers (n=16); EHR(-), HCC without extrahepatic recurrence (n=25); EHR(+), HCC with extrahepatic recurrence (n=10).

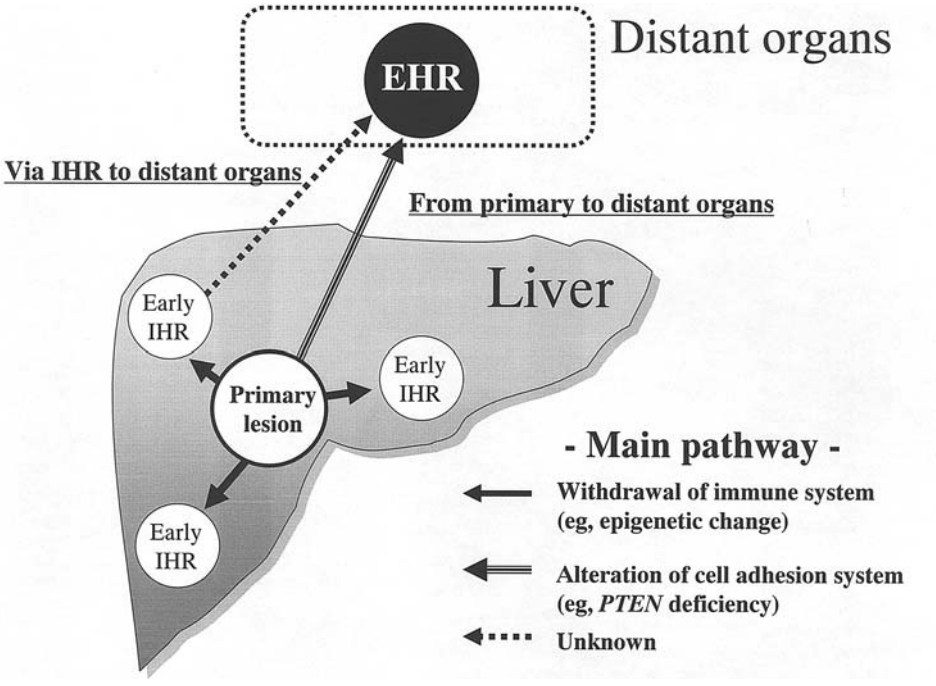

Figure 2. Schematic representation of the proposed mechanisms underlying HCC recurrence due to metastasis. EHR, extrahepatic recurrence; IHR, intrahepatic recurrence.

genes were related to true recurrence attributable to metastasis. Ye *et al* (14) identified 153 genes linked to the intrahepatic spread of HCC at the time of surgery and highlighted the role of the *SPP1* gene in metastasis of HCC. Unfortunately, few genes were identified in these studies (11-14), resulting in a lack of information regarding features common to the metastatic process of HCC (15). To address this problem, it is necessary first to investigate the differences in the molecular patterns between early IHR and EHR in the same DNA microarray platform and cohort. Thus, our present study examined the gene specific to early IHR and EHR of HCC. The most striking finding of the present study was that signature genes for EHR included many cell adhesion-related genes (*ITGA6*, *SPP1*, *DNMBP*, *CD44* and *POSTN*), all of which increased significantly in HCC with EHR compared with HCC without EHR.

ITGA6 encodes the integrin alpha chain alpha 6, which is an integral cell-surface protein that participates in cell adhesion and cell-surface mediated signaling in combination with other integrins (24). The increased expression of *ITGA6* may allow HCC cells to metastasize easily to organs other than the liver. To our knowledge, *ITGA6* has not been reported to correlate with distant metastasis of HCC.

A transciptome study by Ye *et al* (14) identified *SPP1* (osteopontin) as a signature gene with elevated levels in HCC with intrahepatic spread at surgery; however, they also showed that the increased production of *SPP1* by HCC cells after transfection increased lung metastasis but not intrahepatic metastasis in mice. This finding is consistent with the expression pattern of *SPP1* in EHR in our present study. In the present study, the levels of *ITGA6* and *SPP1* were higher in HCC without EHR than in non-cancerous liver, and these levels were further increased in HCC with EHR. This result suggests that these molecules are related to oncogenesis and the progression of HCC. It was reported that the expression of *ITGA6* and *SPP1* was induced in liver of PTEN (phosphatase and tensin homolog) deficient mice (25), which suffered from fatty liver, steatohepatitis, liver fibrosis, and HCC (26). These results suggest that PTEN deficiency may play an important role in EHR but not early IHR of HCC (Fig. 2).

CD44 encodes a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion, and migration. *CD44* can also interact with *SPP1* (27). The altered expression of *CD44* in HCC has been reported (28-30). It was reported that the expression of a variant of *CD44* was upregulated in distant metastasis of HCC (27), which supports our present findings for *CD44*. *SPP1* also binds to the alpha5 beta3-integrin to increase plasma membrane levels of *CD44*v6 on cancer cells (29). Thus, *CD44*, *ITGA*, and *SPP1* may be the markers of metastatic potential of HCC. Unfortunately, the roles of *DNMBP* and *POSTN* in EHR of HCC remain unclear, and therefore, further studies are needed.

Another important finding of the present study was that only 2 immune system-related genes, *GZMB* and *IL1A*, were identified as signature genes for EHR of HCC. In contrast with the gene profile for early IHR (11), the host immune response plays a less significant role in EHR. Although it was reported that an immune therapy decreased the frequency of IHR and was effective for HCC (31,32), there have been no reports regarding the efficacy of immune therapy for the distant metastasis of HCC. Thus, withdrawal of immune response at the primary site of HCC may be a pathway specific for early IHR. This concept is also supported by the result of the transcriptome study of Kurokawa *et al* that showed the downregulation of several MHC class I genes in HCC with early IHR (12).

In our previous study, we found that 4 MHC class II genes (*HLA-DRA*, *HLA-DRB1*, *HLA-DG*, and *HLA-DQA*) were coordinately downregulated in HCC with early IHR

(11). We found that the levels of *CIITA*, a transactivator of MHC class II genes, were markedly lower in HCC than in non-cancerous liver (33). This downregulation may be due to the epigenetic inactivation of *CIITA*. More recently, it was reported that expression of each of the above-mentioned MHC class II genes was induced by the histone deacetylase inhibitor trichostatin A and that the histone acetylation status of the gene promoters was associated with expression in a *CIITA*-independent manner (34). On the basis of these findings, we propose that the epigenetic pathways play a central role in early IHR of HCC via downregulation of immune-system related genes (Fig. 2).

In the present study, we investigated the genetic signature only at the primary site of HCC, and therefore, we did not identify the signature genes of the IHR site. Although it is currently controversial whether metastases themselves have the capacity to metastasize, it is important to understand how the gene expression changes when HCC cells metastasize indirectly to distant organs. Further studies are needed to clarify the molecular basis of each individual step in the process of the recurrence of HCC. Such information may promote the development of a robust system to predict recurrence of HCC and provide HCC patients with more personalized therapies (35).

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