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Identification of novel hub genes associated with liver metastasis of gastric cancer

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Understanding hub genes involved in gastric cancer (GC) metastasis could lead to effective approaches to diagnose and treat cancer metastasis. In this study, 272 differentially expressed genes between synchronous liver metastasis and the paired GC were selected from microarray assays. KEGG pathway analysis indicated that of 13 enriched pathways, 8 were involved in cancer metastasis. Literature-based annotations showed that the differentially expressed genes significantly enriched known metastasis-related genes. With the use of protein–protein interaction network, we found a subnetwork significantly enriching the metastasis-related genes and hubs. Unannotated hubs in this subnetwork were predicted to be novel metastasis-associated genes. Nine hubs in this subnetwork were validated by using quantitative RT-PCR, and 4 hubs were further validated by immunohistochemistry. *NR4A2* was significantly down-regulated in synchronous liver metastasis compared with the paired GC at both transcriptional and translational levels. NR4A2 immunostaining was apparent in the mesenchymal cells of pathologically normal gastric mucosa and in the epithelial cells of primary GC. *HSP90AA1* was not only up-regulated in the metastatic GC compared with primary GC at both transcriptional and translational levels, but also up-regulated in primary GC compared with the normal mucosa at the translational level. *NR4A2, NR3C1, ARF3, XAB2*, and alternatively spliced variants of *NR4A2*, SP8 and SP-novel, were significantly down-regulated, whereas *CCNE1* significantly up-regulated, in primary GC compared with the normal gastric mucosa. Conclusively, *NR4A2* and *HSP90AA1* stand out as promising diagnostic markers and therapeutic targets for liver metastasis of GC. *CCNE1* and *NR3C1* indicate primary GC, rather than distant metastasis.

Key words: gastric cancer; metastasis; hub; gene expression

Gastric cancer (GC) is the second leading cause of cancer death worldwide.[1] Surgical resection is the most effective treatment for GC without distant metastasis. However, relapse after surgical treatment and distant metastasis contribute to the high GC-associated fatality. A fully understanding of the molecular genetics and signaling pathways involved in GC metastasis is a prerequisite for the development of early diagnostic and adjuvant therapeutic options. Recently, up-regulation of a group of molecules such as human epidermal growth factor receptor 2, urokinase-type plasminogen activator receptor, maspin, mucin, tensin 4, osteopontin, secretory leukocyte protease inhibitor, phosphatase of regeneration liver-3 and Mac-2 binding protein in tumor tissues or sera has been demonstrated to be correlated to the metastasis and poor outcome of GC.[2–10] Down-regulation and/or promoter methylation of some tumor suppressors such as 30-kDa Tat-interacting protein, T-lymphocyte maturation associated protein, and ZIP kinase in tumor tissues have been associated with poor outcome of GC.[11–13]

It is not single molecule that can predict biological behavior of GC. The combination of diverse genetic alterations may be reliable for the prediction of GC prognosis. Gene expression analysis with microarrays may reveal important molecules associated with GC progression and dissemination.[14–17] However, different experimental protocols and diverse genetic background of tumor samples or cells complicate the profiling of differentially expressed genes associated with GC metastasis, resulting in few genes in common. This lack of agreement doubts the reliability of reported predictive gene lists. A recent study reported a mathematical method—probably approximately correct sorting for evaluating the robustness of such gene lists. With the use of this method, it was estimated that thousands of samples were needed to generate a robust gene list for predicting outcome in cancer.[18] Obviously, it is impossible to meet this demand in a single study. With the development of bioinformatics, it is becoming possible to identify important metastasis-associated genes from several microarray assays.

Proteins do not function in isolation, but interact with one another to form molecular networks. The protein–protein interaction (PPI) network has a small number of highly connected protein nodes (known as hubs), which have high probabilities of engaging in essential biological function.[19,20] Human proteins translated from cancer-associated genes show an increase in the number of proteins they interact with, and also appear to participate in central hubs rather than peripheral ones.[21] The PPI subnetwork markers are more reproducible than individual marker genes selected without network information, and achieve higher accuracy in the classification of metastatic versus (*vs.*) non-metastatic tumors.[22] Through PPI analysis signal transduction in functional pathways can be achieved. Signaling pathways and processes are becoming important in cancer research, such as the relevance of pathway-driven approaches in whole genome testing,[23] the usefulness of pathway-driven research to characterize cancer and identify biomarkers with high sensitivity to targeted drugs.[24,25] In this report, we presented an integrated analysis of gene lists from expression profile data of the patients with liver metastatic and orthotopic GC, and found a PPI subnetwork that significantly enriched hub genes involved in metastasis-associated signaling pathways, and 2 important hub genes were proved to be associated with liver metastasis of GC in clinical samples.

Material and methods

Tissue specimen

Tumor tissues were obtained from GC patients who had undergone curative surgery at Department of General Surgery of the 1st affiliated hospital, Second Military Medical University, from January 2006 to February 2009. Our study was approved by the Institutional Review Board, and only the patients with the written

Additional Supporting Information may be found in the online version of this article.

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TABLE I – CLINICAL AND PATHOLOGICAL FEATURES OF THE INCLUDED 67 PATIENTS WITH GASTRIC ADENOCARCINOMA

	Metastasis sites				Total
	None	Lymph node	Liver	Spleen	
Case number	13	40	13	1	67
Age (y)					
Median	61	61	60	60	61
Range	53–81	30–85	30–74	60	30–85
Sex					
Male	5	30	11	0	46
Female	8	10	2	1	21
Location					
Upper	2	15	5	0	22
Middle	7	16	5	0	28
Lower	4	9	3	1	17
Stages[1]					
I	11	1	0	0	12
II	2	4	0	0	6
III	0	23	0	0	23
IV	0	12	13	1	26
Histopathological subtypes					
Moderately differentiated	7	14	3	0	24
Poorly differentiated	6	26	10	1	43
CEA (ng/mL)					
Median	1.78	2.55	4.75	–	2.57
Range	0.20–12.71	0.59–125.70	0.79–107.80	–	0.20–125.70
CA199 (U/mL)					
Median	9.28	21.40	60.95	–	21.13
Range	2.19–35.00	1.82–1,000	5.32–1,000	–	0.76–1,000
CA724 (U/mL)					
Median	3.47	5.15	9.29	–	4.64
Range	0.71–110.80	0.23–300	0.23–64.04	–	0.23–300

–, no data.
[1]According to the sixth edition of UICC staging system.

informed consents were included. GC was obtained from non-necrotic tumor area. The gastric mucosa not continuous with the tumor was selected as pathologically normal mucosa. All specimens were frozen in liquid nitrogen immediately after resection and stored at −80°C.

A total of 67 Chinese patients with GC were included in this study. The H&E slides of all cases were reviewed by 1 investigator (Y.Y). All cancer samples were pathologically identified as adenocarcinoma. The detailed characteristics of the patients are provided in Table I. Synchronous liver metastasis and the matched GC specimens were harvested from 11 of the 67 patients. Primary GC and paired pathologically normal gastric mucosa were harvested from 57 patients. Two GC patients with liver metastasis and 1 GC patient with spleen metastasis, neither with paired GC nor with paired normal mucosa, were also included. None of the included patients had received chemotherapy or radiotherapy before surgical treatment.

Microarray assays

The synchronous liver metastasis and their matched GC were used for double-colored cDNA microarray assay. RNA extraction, integrity analysis, reverse transcription and hybridization to the 16K cDNA Chip were performed as previously described.[26] Data were deposited in GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/) in series GSE11072. The differentially expressed genes were extracted from 3 qualified assays (GEO Accession: GSM253488, GSM253489 and GSM253490) in accordance with MIAME standards. We selected differentially expressed genes with fold-change 1.6 or higher (logarithm base $2 \geq 0.678$-fold) in each microarray data for further analysis.

Pathway analysis

Pathway information was downloaded from Kyoto Encyclopedia of Genes and Genomes (KEGG) database (www.genome.jp/kegg/pathway.html). A total of 14,784 genes contained in this chip were compared with those of different pathways in KEGG database to analyze pathway enrichment of the differentially expressed genes.

Literature-based annotation

Annotations of the differentially expressed genes and 382 cancer census genes (www.sanger.ac.uk/genetics/CGP/Census, up to December 6, 2008)[27] were online retrieved using defined terms "metastasis" or "metastatic" or "metastases" from the Microarray Literature-based Annotation (MILANO) (http://milano.md.huji.ac.il) and Chilibot (http://www.chilibot.net) on Medline (up to January 20, 2009). The number of co-occurrences of each gene on the list with the defined terms was quickly counted in abstracts via MILANO. Genes with co-occurrence once or more in MILANO were then used to retrieve sentences with the defined terms in abstract via Chilibot. After carefully reading the retrieved sentences, we identified genes associated or not associated with metastasis. Cancer-associated genes from the differentially expressed genes were also identified via Chilibot with the defined terms "cancer" or "tumor" or "carcinoma." PMID numbers of related articles were recorded.

Prediction of metastasis-associated genes by using PPI network

The differentially expressed genes were online introduced to UniHI database (http://www.mdc-berlin.de/unihi) (the fourth version) to search for PPI data. We deleted repeated interactions, and calculated the gene degree. The interactions among the differentially expressed genes were visualized by using Cytoscape 2.5.1 software.[28] Network-neighbors of the known metastasis-related genes were predicted to be candidate metastasis-related genes. Genes with 30 or more gene degree in the PPI network were considered as hubs.

Prioritization of candidate metastasis-associated genes by using Endeavour

We used online Endeavour software (http://homes.esat.kuleuven.be/~bioiuser/endeavor/endeavorweb.php) to prioritize the

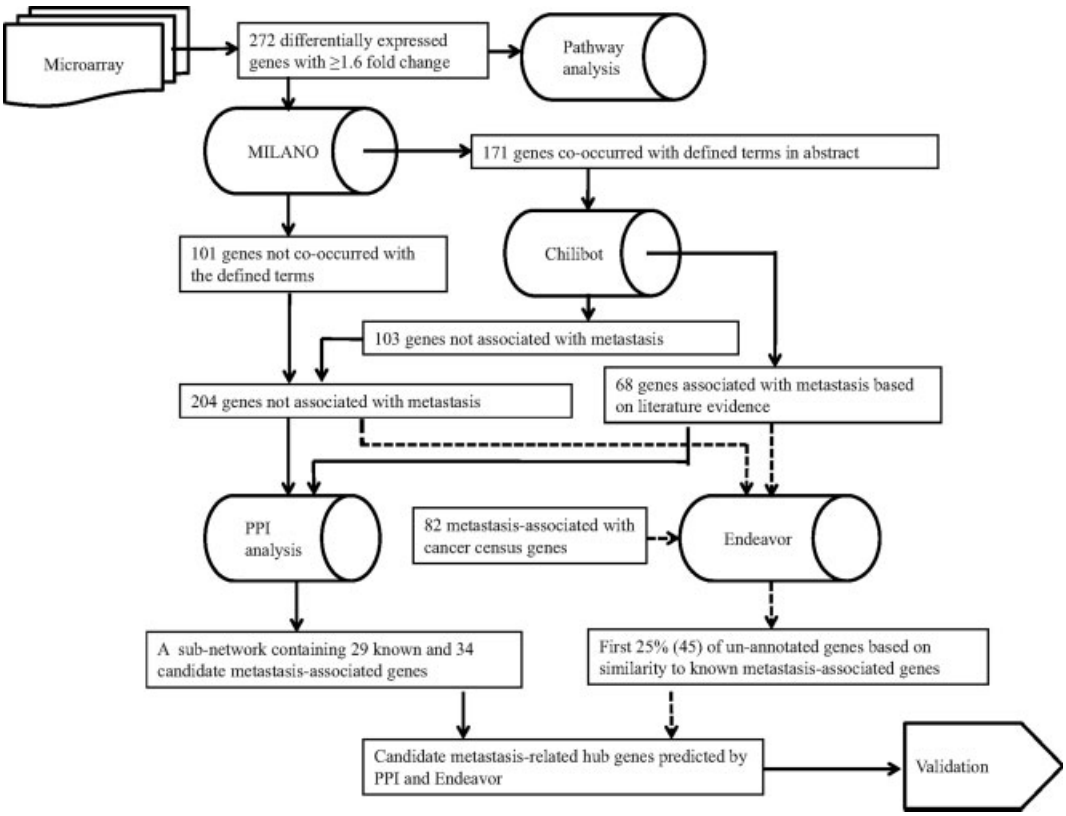

FIGURE 1 – Schematic diagram of the study.

candidate genes based on their similarity to the known metastasis-related genes. On the basis of Chilibot searches, the known metastasis-related genes from the cancer census genes and from our differentially expressed genes were grouped as Group A and Group B, respectively. The differentially expressed genes not co-occurred with "metastasis" or "metastatic" or "metastases" in Chilibot were grouped as Group C. We used Groups A and B as training sets, Group C as a testing set. The training sets and the testing set were online introduced to Endeavour software for prioritization. The data sources used for this assay were EnsemblEst, GeneOntology, Interpro, Kegg, Swissprot, Blast, CisRegModule, SonEtAl, SuEtAl, Bind, BioGrid, Hprd, InNetDB, Intact, Mint, String, Motif, Ouzounis, Prospectr and Text. As compared with the training sets, the first 25% of the testing set in the prioritization were selected as candidate metastasis-associated genes. Diagram of the integrated method of isolating GC metastasis-related hubs was shown in Figure 1.

Real-time quantitative reverse transcription PCR

Quantitative reverse transcription PCR (qRT-PCR) was used to validate expression patterns of the selected genes between synchronous liver metastasis and their primary counterparts from the remaining 8 patients or between GC and their matched pathologically normal gastric mucosa from 57 of the 67 patients. The PCR primers were designed by using Primer Premier 5.0 software (PREMIER Biosoft International, Canada) and synthesized by Shanghai Invitrogen (Shanghai, China). Primer pairs used in this study were listed in Supporting Information Table I. *GAPDH* was used as endogenous controls. Total RNA (1 μg) was used for the assay. Reverse transcription of mRNA was carried out by using the reverse transcriptase XL (AMV) and oligo(dT)18 primer (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instruction. A 20-μL reaction mixture contained 2-μL single-stranded cDNA, 10-μL SYBR Premix Ex Taq (Takara Biotechnology) and 1-μL 5-μM PCR primers. The LightCycler™ 480 real-time PCR Thermoblock (Roche, Basel, Switzerland) was programmed to initially denature the samples for 30 sec at 95°C, followed by 40–45 cycles consisting of 95°C for 5–15 sec, 56–63°C for 10–15 sec, 72°C for 15–30 sec (Supporting Information Table I). The specificity of the amplification was checked by melt-curve analysis from 70°C to 95°C by 0.2°C interval. All reactions were run in triplicates. Relative levels of mRNA expression were calculated according to the E-methods.[29]

Analysis of NR4A2 alternatively spliced variants

Alternatively spliced variants of *NR4A2* were assessed by using Alternative Splicing Database (ASD) at http://www.ebi.ac.uk/asd. Nine possible splicing patterns from SP1 to SP9 of *NR4A2* were downloaded, and used as references for the design of Primer sets A-E (Supporting Information Table I). SP1 with GenBank accession number NM_006186.3 was considered as prototype *NR4A2*. Message RNA from 18 samples (6 metastatic GC, 6 primary GC and 6 matched gastric mucosa samples) was mixed, reversely transcribed and amplified by using Primers from A to E, respectively. After electrophrosis with 2% agarose gel, sizes of PCR products were used to evaluate expression pattern of these variants. PCR products were cloned into a cloning vector pMD18T (TaKaRa Biotechnology) and then sequenced. The sequences were used for the design of primers for qRT-PCR to assess expression patterns of each spliced variant in the 8 pairs of primary and liver metastatic GC specimens, and the 57 pairs of primary GC and the matched mucosa specimens.

Immunohistochemistry

Rabbit polyclonal antibody for NR4A2 (Abcam, Cambridge, UK), rabbit antibody for HSP90 (Entrez-Gene ID: 3320) (Cell Signaling Technology, Danvers, MA), rabbit polyclonal antibody

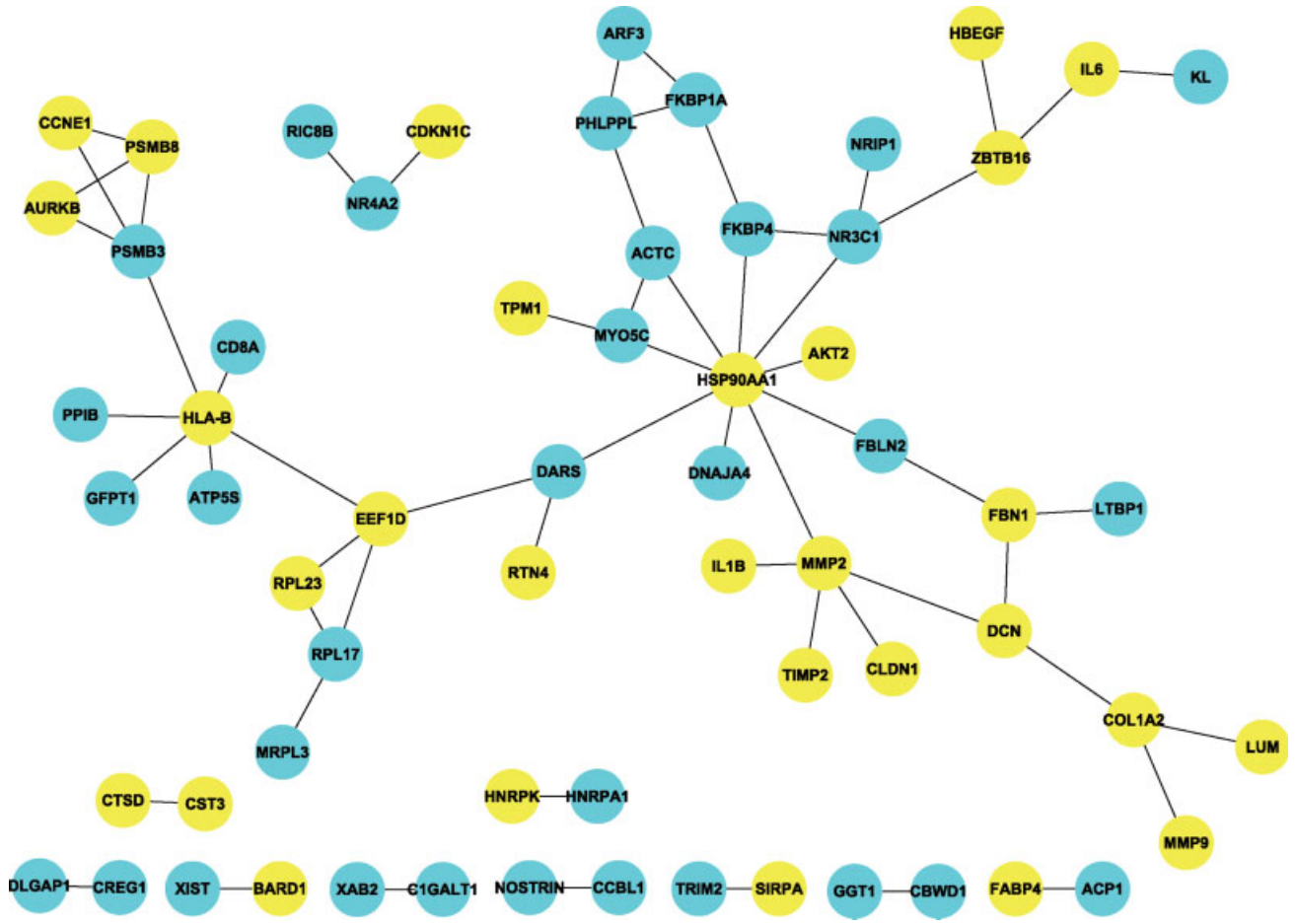

FIGURE 2 – A subnetwork containing 60 interactions among 63 genes from the differentially expressed genes. Nodes and links represent human proteins–protein interactions (PPI). Yellow nodes indicate known genes associated with metastasis; blue nodes indicate unannotated genes.

for CCNE1 (cyclin E, M-20) and mouse monoclonal antibody for NR3C1 (3D5) (Santa Cruz Biotechnology, Santa Cruz, CA) were used for immunohistochemical analyses. Briefly, the paraffin blocks were cut at 4-6 μm, dried overnight at 60°C and deparaffinised in xylene. Sections were rehydrated through graded alcohol in water, and heated in an autoclave oven at 121°C for 10 min. The sections were incubated with 20% goat serum for 30 min to block nonspecific binding. The diluted primary antibodies were incubated for 30 min in room temperature. The immunostaining was performed using the EnVision method (DAKO, Glostrup, Denmark) according to the manufacturer's instructions. The images were obtained with a BX50 microscope (Olympus, Tokyo, Japan) equipped with a digital color video camera. All of the samples were analyzed by 3 independent observers (Y.Y., W.C. and G.C.) who were blinded to the clinical information. Scoring of intensity of the immunostaining was done semiquantitatively (1+ = weak staining, 2+ = moderate staining and 3+ = strong staining). The cases were interpreted as negative if <10% of the cells were positive. There was close agreement (>90%) among the 3 investigators. Disagreements were resolved by consensus.

Statistic analysis

Chi-square test was used for KEGG pathway enrichment of the differentially expressed genes and the analyses of metastasis-related genes and hub genes in the subnetwork. Independent-samples *t* test was used to compare rank positions of gene sets by using Endeavor software. Relative expression fold values from qRT-PCR were transformed into logarithm base 2, and the paired *t* test was used to compare expression patterns of genes of interest in synchronous liver metastasis *vs*. their paired GC, as well as in primary GC *vs*. the matched mucosa tissues. Kruskal-Wallis H test was used to evaluate positive degrees of the immunostaining for HSP90, NR4A2, CCNE1 and NR3C1 in gastric mucosa, primary and metastatic GC, respectively. All statistical tests were 2-sided, and performed by using Statistical Program for Social Sciences (SPSS 15.0 for Windows, SPSS, Chicago, IL). $P < 0.05$ was considered as statistically significant.

Results

Differentially expressed genes involved in KEGG pathways

A total of 272 differentially expressed genes, containing 177 genes down-regulated and 95 genes up-regulated in the metastatic GC, were selected for further analysis. The differentially expressed genes significantly clustered in 13 pathways (Supporting Information Table II) in KEGG database. Of the 13 pathways, "cell communication," "ECM-receptor interaction," "focal adhesion," "toll-like receptor signaling," "GnRH signaling," "MAPK signaling," "VEGF signaling" and "leukocyte transendothelial migration" have been involved in cancer metastasis and aggression.[30–35] This result indicates that the genes from our microarray data are reliable and consistent with current knowledge on cancer metastasis.

TABLE II – POTENTIAL METASTASIS-RELATED HUB GENES PREDICTED BY ENDEAVOUR AND PROTEIN–PROTEIN INTERACTION

Gene name	Gene description	Rank position in Endeavour	Degree in the subnetwork	Degree in UniHI	Metastasis-associated partner
NR3C1	Glucocorticoid receptor	89	4	110	*HSP90AA1*/*ZBTB16*
NR4A2	Orphan nuclear receptor NR4A2	135	2	30	*CDKN1C*
HNRPA1	Heterogeneous nuclear ribonucleoprotein A1	147	1	141	*HNRPK*
PSMB3	Proteasome subunit beta Type 3	148	4	194	*HLA-B*/*PSMB8*/*CCNE1*/*AURKB*
FBLN2	Fibulin-2 precursor.	155	2	53	*HSP90AA1*/*FBN1*
DARS	Aspartyl-tRNA synthetase, cytoplasmic	156	3	136	*HSP90AA1*/*RTN4*/*EEF1D*
XAB2	XPA-binding protein 2	157	1	94	no
CD8A	T-cell surface glycoprotein CD8 alpha chain precursor	158	1	32	*HLA-B*

Cancer- or metastasis-associated genes hunted from the differentially expressed genes

The differentially expressed genes were online introduced into MILANO. A total of 171 genes had co-occurrences with "metastasis" or "metastatic" or "metastases." The 171 were then online introduced into Chilibot to hunt the metastasis-associated genes. Sixty-eight (Supporting Information Table III) of the 171 genes were associated with metastatic phenotype, including 19 genes associated with GC metastasis and 49 genes associated with metastasis of other cancer types. Of the 272 differentially expressed genes, 118 (Supporting Information Table IV) including the 68 metastasis-associated genes were determined to be cancer-associated genes in Chilibot. Of the 382 cancer census genes, 337 had co-occurrences with "metastasis" or "metastatic" or "metastases" in MILANO. Of the 337 genes, 82 (Supporting Information Table V) were determined to be associated with metastasis in Chilibot. The proportion of the known metastasis-related genes in our cancer-related differentially expressed genes (68/118, 57.63%) was 2.68-fold higher ($P < 0.001$) than that in the cancer census genes (82/382, 21.47%). A total of 204 differentially expressed genes which have not been reported to be related to metastasis (unannotated) served as the sources for novel genes associated with GC metastasis.

Candidate hub genes associated with liver metastasis of GC

Of the 272 differentially expressed genes, 248 with Entrez-Gene ID were introduced into UniHI database for PPI analysis. Of the 248 genes, 209 had PPI information, and 203 of the 209 genes had at least 1 interaction partner. Of the 203 genes, 49 with gene degree 30 or more were hub genes. Of the 49 hub genes, 21 were known metastasis-associated genes (Supporting Information Table VI). The proportion of known metastasis-associated genes in the 49 hub genes (42.9%) was significantly higher than that in the 272 differentially expressed genes (25.0%) ($p = 0.015$), indicating that hub genes in our differentially expressed genes enriched metastasis-related genes. After deleting the interacted genes not listed in our differentially expressed genes, we obtained a subnetwork containing 63 genes with 60 interactions. This subnetwork included 29 known metastasis-related genes, as shown in Figure 2. The proportion of the known metastasis-related genes in this subnetwork (29/63) was significantly higher than that in total genes with PPI information (61/209) ($p = 0.014$), indicating that this subnetwork enriched the known metastasis-related genes, and 34 unannotated genes in this subnetwork were predicted to be metastasis-related. The proportion of hub genes in this subnetwork (37/63) was significantly higher than that in total genes with PPI information (49/209) ($p < 0.001$), indicating that the subnetwork also enriched hub genes. *HSP90AA1*, *HLA-B* and *MMP2* interacted with 9, 6 and 5 genes in this subnetwork (Fig. 2), respectively, indicating that these genes play a central role in this subnetwork.

Prioritization of candidate genes associated GC metastasis

The 82 metastasis-related genes (Group A) from the census cancer genes and the 68 metastasis-related genes (Group B) from our differentially expressed genes shared 4 genes: *HSP90AA1, AKT2, PDGFRB* and *ZBTB16*. Of the 204 unannotated genes in our differentially expressed genes, 179 (Group C) could be identified in Endeavour. When the 2 training sets (Groups A and B) and 1 testing set (Group C) were online introduced into Endeavour, significant differences were determined between any 2 groups ($p < 0.001$ for each), indicating that genes of different sources could be separated easily using Endeavour. The first quartile of the distribution, 45 genes in the testing set, was taken as candidate metastasis-associated genes because these genes overlapped with some genes in the 2 training sets in the prioritization (Supporting Information Fig. 1). Of the 45 genes, 8 were also included in the subnetwork as candidate metastasis-related hubs (Table II). Of the 8 hubs, *NR4A2*, *NR3C1*, *HNRPA1* and *XAB2* were selected for validation. Another 5 hub genes included in this subnetwork, but not included in the 45 genes, were also selected for validation because of high gene degree (Supporting Information Table VI). Four up-regulated hubs (*HSP90AA1*, *HNRPA1*, *RPL17* and *XAB2*) and 5 down-regulated hubs (*NR4A2*, *ARF3*, *FKBP1A*, *NR3C1* and *CCNE1*) from the microarray data were finally selected for validation.

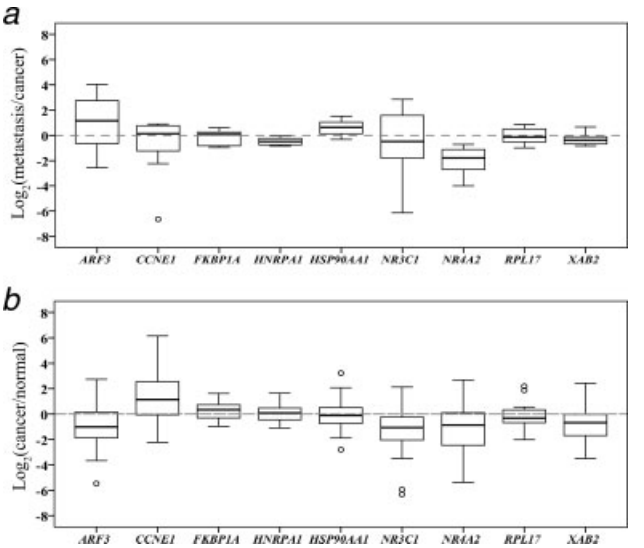

FIGURE 3 – Box plot of real-time quantitative RT-PCR results. (*a*) fold changes among the 8 paired samples (liver metastasis *vs*. orthotopic gastric cancer) are plotted in logarithmic scale. (*b*) fold changes among the 57 paired samples (gastric cancer *vs*. matched mucosa) are plotted in logarithmic scale. Score of zero in this scale indicates no change, whereas score of 1 indicates a 2-fold change. The box area contains 50% of the data samples, and 99.3% of the samples are within the upper and lower boundary markers.

Validation of gene expression by using qRT-PCR

Expression of above 9 genes in synchronous liver metastasis *vs*. the matched GC from the 8 patients indicated that *NR4A2* ($p = 0.001$) was significantly down-regulated, whereas *HSP90AA1*

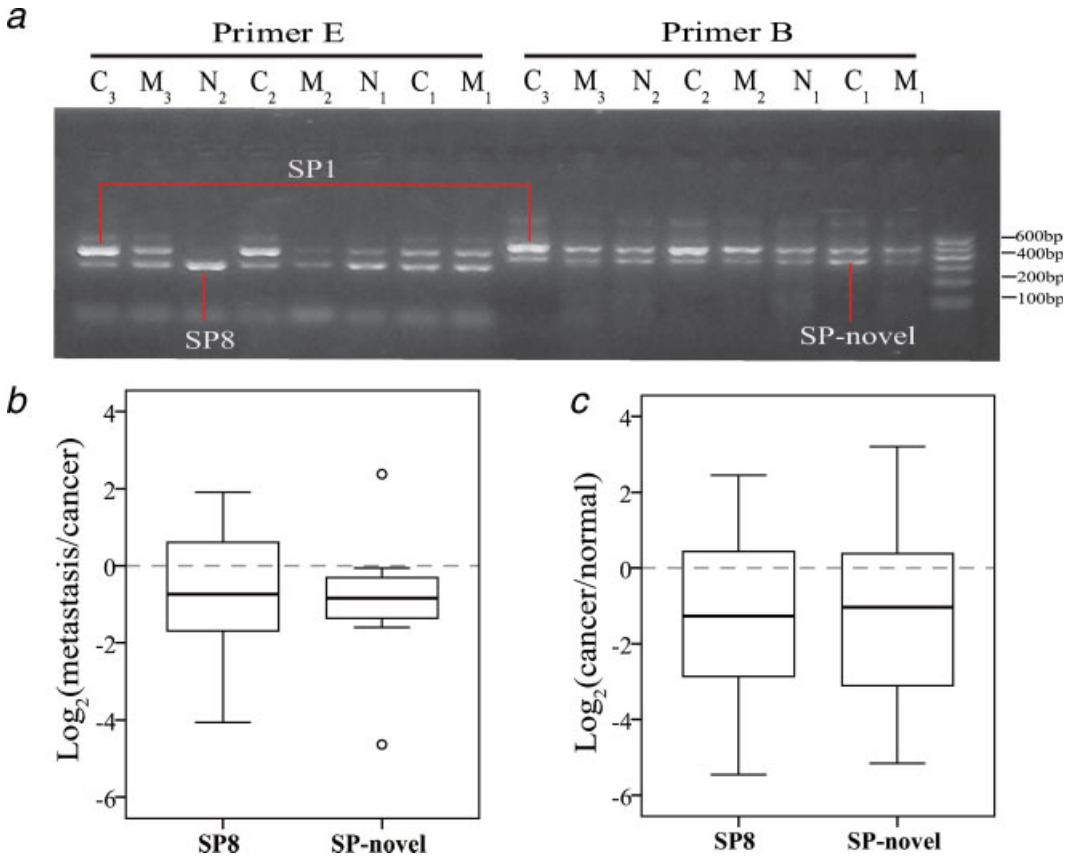

Figure 4 – Identification and expression patterns of NR4A2 alternative spliced variants in clinical gastric samples. (*a*) RT-PCR results of SP1 (prototype *NR4A2*), SP8, and SP-novel in the independent gastric samples (M, liver metastasis; C, primary gastric cancer; N, pathologically normal gastric mucosa) by using Primer B and Primer E. (*b*) Box plot of real-time quantitative RT-PCR results of SP8, and SP-novel between liver metastatic gastric cancer and orthotopic gastric cancer in the 8 paired samples. (*c*) Box plot of real-time quantitative RT-PCR results of SP8, and SP-novel between primary gastric cancer and the pathologically normal mucosa in the 57 paired samples. Score of zero in this scale indicates no change, whereas score of 1 indicates a 2-fold change. The box area contains 50% of the data samples, and 99.3% of the samples are within the upper and lower boundary markers. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

($p = 0.029$) significantly up-regulated, in synchronous liver metastasis, which was consistent with the microarray data. Expression of *HNRPA1* ($p = 0.002$) was down-regulated with low fold-change in synchronous liver metastasis, in contrast to the microarray data. No significant differences were found in the expression ratios of other 6 genes between synchronous liver metastasis and the matched GC. *NR4A2*, *NR3C1, ARF3* and *XAB2* were down-regulated ($P < 0.001$ for each), whereas *CCNE1* ($p = 0.001$) up-regulated, in primary GC compared with the paired gastric mucosa from the 57 patients. Figure 3 shows the box plot analysis of the real-time qRT-PCR results of above 9 genes in synchronous liver metastasis *vs*. the paired GC (Fig. 3*a*), and in primary GC *vs*. the paired gastric mucosa (Fig. 3*b*). From these results, *NR4A2* stands out as the most probable prognostic and even diagnostic marker inversely associated with GC. *HSP90AA1* may be a viable biomarker for GC metastasis. *CCNE1* seems to be a viable marker for primary GC. *NR3C1, ARF3* and *XAB2* are inversely associated with primary GC.

Expression patterns of *NR4A2, NR3C1, XAB2, ARF3* and *CCNE1* in primary GC *vs*. the paired gastric mucosa at the early stage (Stages I–II, $n = 18$) were consistent with those at the advanced stage (Stages III–IV, $n = 39$) (data not shown). However, significantly differential expression of *ARF3* and *CCNE1* was found at the Stages I–III ($n = 23$; $p = 0.009$, $p = 0.001$, respectively), not at the Stage IV ($n = 16$; $p = 0.217$, $p = 0.648$, respectively), indicating that *ARF3* and *CCNE1* are apt to be markers of GC without distant metastasis.

Identification of alternatively spliced variants of NR4A2 and their expression patterns in GC

From the mixed mRNA, 3 alternatively spliced variants of *NR4A2* were found, including SP8 and SP-novel. SP1 was identified as prototype *NR4A2*. SP-novel was partially identical to a gene with GenBank accession number NM_173172, but not included in ASD database. The 3 variants were amplified by using Primers B and E (Fig. 4*a*), rather than by using other primer sets. SP-novel could be specially amplified with Primer B. DNA sequence of SP-novel was deposited in GenBank database with accession number FJ643485. Expression patterns of SP8 and SP-novel were examined by using qRT-PCR. Although the expression of SP8 and SP-novel was not statistically different between synchronous liver metastasis and the matched GC, SP8 and SP-novel were significantly down-regulated in primary GC compared with the paired mucosa ($p < 0.001$ for each), as shown in Figure 4*b*. Down-regulation of SP8 and SP-novel was found in GC at the Stages I–II ($p = 0.004$, $p = 0.009$, respectively) and at the Stages III–IV ($p = 0.003$, $p = 0.005$, respectively).

Expression patterns of NR4A2, HSP90AA1, CCNE1 and NR3C1 encoding proteins in GC

Expression patterns of NR4A2, HSP90, CCNE1 and NR3C1 encoding proteins in 26 pathologically normal mucosa, 26 primary GC and 11 GC metastasized to liver were examined by immunostaining. Immunoreactivities of HSP90, NR4A2 and NR3C1 were

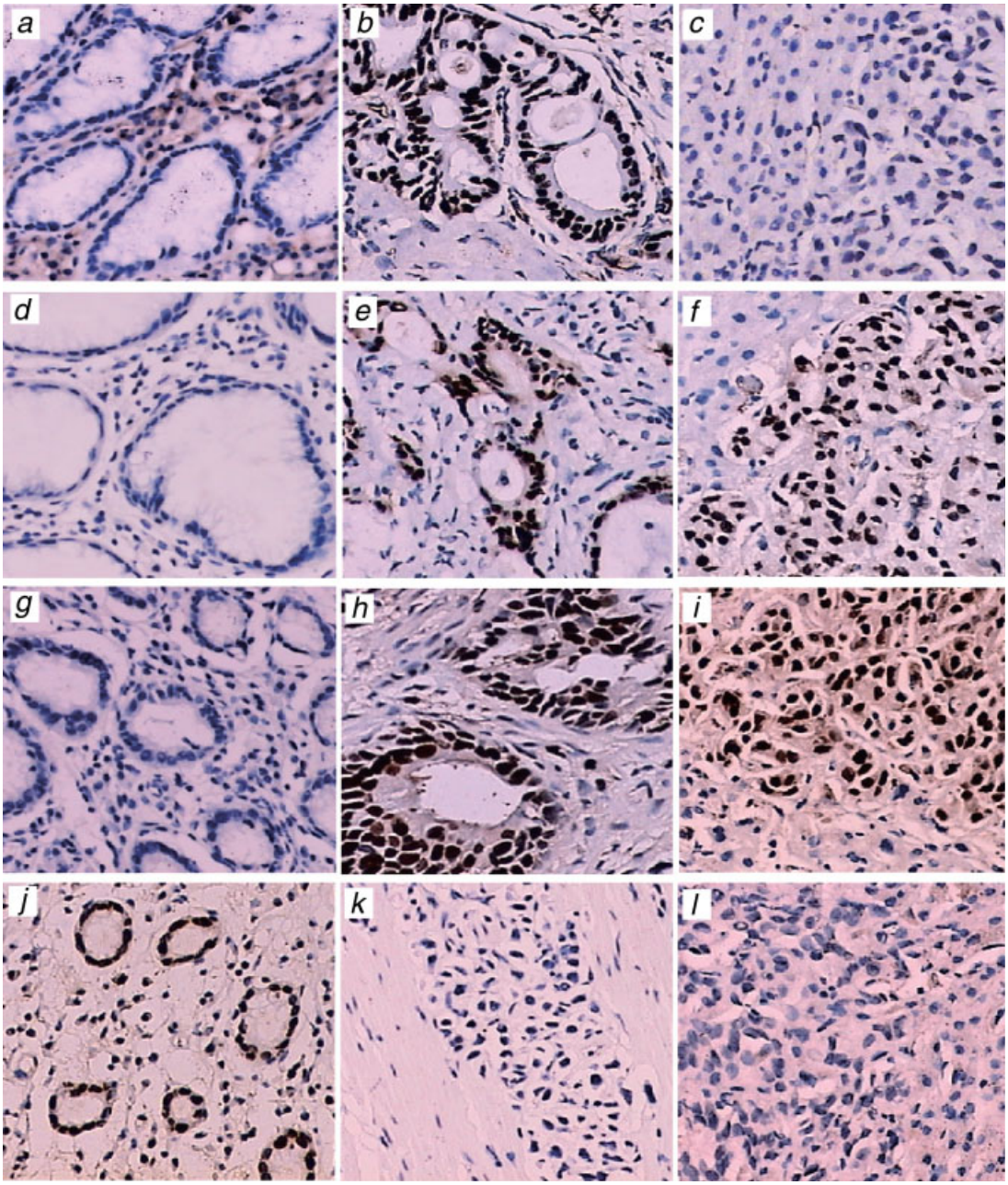

Figure 5 – Immunostaining for NR4A2, HSP90, CCNE1 and NR3C1 in the pathologically normal gastric mucosa, primary gastric cancer and gastric cancer metastasized to liver. Expression of NR4A2 (*a–c*), HSP90 (*d–f*), CCNE1 (*g–i*), and NR3C1 (*j–l*) in the pathologically normal gastric mucosa (*a–j*), primary gastric cancer (*b–k*), and gastric cancer metastasized to liver (*c–l*). For all images, ×400.

mainly nuclear and slightly cytoplasmic, whereas CCNE1 immunoreactivity was only nuclear, in GC or gastric epithelial cells (Fig. 5). However, NR4A2 immunostaining was apparent in mesenchymal cells of pathological normal gastric mucosa (Fig. 5*a*). Kruskal-Wallis H test demonstrated that NR4A2 protein in gastric epithelial cells was significantly down-regulated, whereas HSP90 protein significantly up-regulated, in the liver metastasis than in primary GC (Table III). These results are quite consistent with those of qRT-PCR. HSP90 protein was also significantly up-regulated in the primary GC compared with the normal gastric mucosa. NR4A2 protein was significantly up-regulated in the epithelial cells of primary GC than in those of the normal mucosa, and significantly up-regulated in mesenchymal cells of the normal gastric mucosa than in those of primary GC. Expression patterns of CCNE1 and NR3C1 proteins were quite consistent with those of qRT-PCR.

Discussion

In this study, we took a loose criterion to include the differentially expressed genes between synchronous liver metastasis and their matched GC, and then developed an innovative, integrated method to enrich candidate hub genes associated with liver metastasis of GC (Fig. 1). KEGG pathway analysis demonstrated that of the 13 enriched pathways, 8 were involved in cancer metastasis

TABLE III – IMMUMOHISTOCHEMISTRICAL RESULTS OF HSP90AA1, CCNE1, NR4A2, AND NR3C1 IN LIVER METASTATIC GASTRIC CANCER, PRIMARY GASTRIC CANCER AND PATHOLOGICAL NORMAL GASTRIC MUCOSA

Specimen types	Positive degree				p-value[1]	p-value[2]
	−	+	++	+++		
NR4A2 (gastric cancer or epithelial cell)						
Liver metastasis	9	1	1	0	0.003	–
Primary gastric cancer	6	12	6	2	–	0.000
Pathological normal gastric mucosa	24	1	0	1	–	–
NR4A2 (gastric mesenchymal cell)						
Primary gastric cancer	19	5	2	0	–	0.005
Pathological normal gastric mucosa	9	10	5	2	–	–
HSP90AA1						
Liver metastasis	1	4	2	4	0.043	–
Primary gastric cancer	13	3	7	3	–	0.003
Pathological normal gastric mucosa	22	4	0	0	–	–
CCNE1						
Liver metastasis	4	2	3	2	0.945	–
Primary gastric cancer	8	6	10	2	–	0.000
Pathological normal gastric mucosa	21	3	2	0	–	–
NR3C1						
Liver metastasis	7	3	1	0	0.802	–
Primary gastric cancer	15	6	4	1	–	0.005
Pathological normal gastric mucosa	4	12	7	3	—	—

[1]Liver metastasis *vs*. primary gastric cancer.–[2]Primary gastric cancer *vs*. pathological normal gastric mucosa.

and aggression. Literature-based annotation analyses showed that the proportion of known metastasis-related genes in our cancer-related differentially expressed genes was 2.68-fold higher than that in the cancer census genes, the most common genes that are causally implicated in cancer development.[24] These results indicate that our differentially expressed genes enrich cancer metastasis-related genes. With the use of PPI network, we found that hub genes in our differentially expressed genes significantly enriched the metastasis-related genes. This PPI subnetwork significantly enriched a number of hub genes that supported functional pathways of cancer metastasis, such as *MMP2* in leukocyte transendothelial migration and GnRH signaling pathways and *AKT2* in focal adhesion, VEGF signaling and MAPK signaling pathways (Supporting Information Table II). Unannotated genes in this subnetwork were predicted to be metastasis-related. By integrating PPI network and Endeavour prioritization, 8 unannotated genes (Table II) were predicted to be novel metastasis-related at high priority. The enrichment of the function-related genes from large amount of differentially expressed genes dramatically minimizes the false positivity of microarray data and increases the reliability of hub gene prediction. This integrated technology represents an increasingly important method in hunting for disease genes.

Nine hub genes were selected for validation by using qRT-PCR. Four of them were further validated by using immunohistochemistry. Some metastasis-associated genes are also up-regulated in the primary cancers compared with the adjacent tissues.[26] Metastatic potential of human tumors is encoded in the bulk of a primary tumor.[36] Thus, we examined the expression patterns of the metastasis-associated genes in primary GC *vs*. the normal gastric mucosa. It was found that *HSP90AA1* was not only up-regulated in synchronous liver metastasis compared with its matched GC at both transcriptional and translational levels, but also up-regulated in primary GC compared with its matched gastric mucosa at the translational level. These results indicate that *HSP90AA1* expression is closely associated with malignant phenotype of GC. HSP90 is an ATP-dependent molecular chaperone that maintains the active conformation of client oncoproteins in cancer cells. Inhibition of HSP90 leads to inhibit tumor growth and metastasis.[37] Thus, HSP90AA1 might serve as a potential prognostic marker or even a candidate therapeutic target of GC. *CCNE1* was up-regulated in primary GC at the Stages I–III compared with the matched mucosa, indicating that *CCNE1* might be a viable marker for GC without distant metastasis. *CCNE1* encoding cyclin E1 has been considered to be the key oncogene within amplification at 19q12 in multiple human tumor types including GC.[38] *NR3C1, ARF3* and *XAB2* were down-regulated in primary GC compared with the matched mucosa. *NR3C1* immunostaining was only found in the epithelial cells of the normal mucosa, not in GC. *NR3C1* encodes glucocorticoid receptor which functions as a transcriptional factor and participates in the regulation of several molecular processes including inflammation and differentiation. Cancer-specific CpG island hypermethylation resulting in transcriptional silencing of *NR3C1* might be of clinical significance for human GC.[39] *XAB2* interacted with *HLA-B* (Table II). Expression of *HLA-B*/*C* heavy chain is associated with GC progression and recurrence.[40] *XAB2* encodes xeroderma pigmentosum group A-binding protein 2 which is involved in pre-mRNA splicing, transcription-coupled DNA repair, and cellular differentiation. *ARF3* encodes a guanine nucleotide-binding protein functioning in vesicular trafficking, phospholipase D activation and intracellular transport. *HNRPA1* encodes the heterogeneous nuclear ribonucleoprotein core protein A1 which functions as a carrier for RNA during export of RNA to the cytoplasm. The clinical significance of *XAB2*, *ARF3* and *HNRPA1* remain to be elucidated.

In this study, we found that *NR4A2* was an important hub gene inversely associated with liver metastasis of GC. *NR4A2* was not only down-regulated in liver metastatic GC compared with primary GC, but also down-regulated in primary GC compared with the normal gastric mucosa. NR4A2 protein was also proved to be significantly down-regulated in liver metastatic GC compared with primary GC. However, NR4A2 immunostaining was apparent in mesenchymal cells of the normal gastric mucosa, and in the epithelial cancer cells of primary GC. Epithelial cells can convert into mesenchymal cells by a process known as the epithelial-mesenchymal transition (EMT), and invasion of adenocarcinoma is accompanied by the release of signal cells through a EMT process.[41] NR4A2 may be regulated by Wnt signaling which are involved in EMT process.[41,42] Thus, we hypothesized that role of NR4A2 in liver metastasis of GC might be partially related to EMT process. This study also revealed that 2 alternatively spliced variants of NR4A2, SP8 and SP-novel, were significantly down-regulated in primary GC compared with their matched mucosa. These results indicate that SP8 and SP-novel might be involved in carcinogenesis of GC. Expression of the NR4A2 variants was not significantly different between primary tumor and metastasis, possibly because of small size of liver metastatic samples or alternatively, the spliced variants of NR4A2 play a different role in carcinogenesis and metastasis of GC. NR4A2 belongs to an orphan nuclear receptor family including NR4A1 and NR4A3. NR4A transactivates target genes, and plays a key role in maintaining midbrain dopaminergic neurons, regulating cytokine and growth

action in chronic inflammatory diseases, stabilizing DNA integrity, regulating matrix metalloproteinase expression.[41–44] Abrogation of *NR4A1* and *NR4A3* in mice leads to development of acute myeloid leukemia.[45] Although NR4A2 expresses in bladder cancer cells, activation of NR4A2 leads to inhibition of bladder cancer.[46] NR4A2 has been found to be significantly down-regulated in prostate cancer compared with the normal samples.[47] Thus, we suggest that *NR4A2* is a putative tumor suppressor. Down-regulation of NR4A2 and its spliced variants in gastric epithelial cells might be indicative for malignant phenotype and poor outcome of GC. Introduction of NR4A2 might be useful for the treatment of GC metastasis.

Our study has several limitations. First, only the patients with solitary, resectable metastatic foci were able to receive surgical treatment, resulting in small number of the participants with synchronous liver metastasis and their paired GC were included in microarray analysis and subsequent validation study. Second, possible bias in data mining might exist because current studies tend to report positive findings. Third, incompleteness of current PPI databases might limit the reliability of hub genes prediction. Fourth, the 16 K cDNA chip used in the study only contained 14,784 unigenes. Some metastasis-associated genes were not included as probes, resulting in loss of data.

Conclusively, we developed an innovative, integrated method to hunt novel hubs associated with liver metastasis of GC at low cost. Up-regulated hub genes *HSP90AA1* and *CCNE1*, down-regulated hub genes *NR4A2*, *NR3C1*, and alternatively spliced variants SP8 and SP-novel of *NR4A2* might play roles in liver metastasis and/or carcinogenesis of GC. These hubs might be new prognostic or diagnostic biomarkers, and/or candidate therapeutic targets for GC. Further clinical investigation is necessary to confirm these preliminary findings.

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