Identify lymphatic metastasis-associated genes in mouse hepatocarcinoma cell lines using gene chip

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AIM: In order to obtain lymphogenous metastasis-associated genes, we compared the transcriptional profiles of mouse hepatocarcinoma cell lines Hca-F with highly lymphatic metastasis potential and Hca-P with low lymphatic metastasis potential.

METHODS: Total RNA was isolated from Hca-F and Hca-P cells and synthesized into double-stranded cDNA. In vitro transcription double-stranded cDNA was labeled with biotin (i.e., biotin-labeled cRNA, used as the probe). The cRNA probes hybridized with Affymetrix GeneChip® MOE430A (containing 22 690 transcripts, including 14 500 known mouse genes and 4 371 ESTs) respectively and the signals were scanned by the GeneArray Scanner. The results were then analyzed by bioinformatics.

RESULTS: Out of the 14 500 known genes investigated, 110 (0.8%) were up regulated at least 2 fold. Among the total 4 371 ESTs, 17 ESTs (0.4%) (data were not presented) were up regulated at least 2 fold. According to the Gene Ontology and TreeView analysis, the 110 genes were further classified into two groups: differential biological process profile and molecular function profile.

CONCLUSION: Using high-throughput gene chip method, a large number of genes and their cellular functions about angiogenesis, cell adhesion, signal transduction, cell motility, transport, microtubule-based process, cytoskeleton organization and biogenesis, cell cycle, transcription, chaperone activity, motor activity, protein kinase activity, receptor binding and protein binding might be involved in the process of lymphatic metastasis and deserve to be used as potential candidates for further investigation. Cyclin D1, Fosl1, Hsp47, EGFR and AR, and Cav-1 are selected as the possible candidate genes of the metastatic phenotype, which need to be validated in later experiments. ESTs (data were not presented) might indicate novel genes associated with lymphatic metastasis. Validating the function of these genes is helpful to identify the key or candidate gene/pathway responsible for lymphatic metastasis, which might be used as the diagnostic markers and the therapeutic targets for lymphatic metastasis.

INTRODUCTION
Metastasis is the major cause of cancer morbidity and mortality[1]. Metastasis formation is a complex process, involving invasion, transport, arrest, adherence, extravasation and tumor cell proliferation[2]. High-throughput methods are needed to display the molecular changes involved in this complicated series of steps. Recent development of cDNA microarray technology has opened a new era in this field[3]. It can provide massive datasets simultaneously. Except this, suitable models for cancer metastasis are necessary for analysis of mechanisms[4]. Because majority of malignant tumors are carcinomas and lymph node metastases often represent the first step in the metastatic process, whereas the molecular mechanism of lymphatic metastasis remains poorly understood, the clones of lymphatic metastasis are prone to be established. A mouse hepatocarcinoma cell line named Hca-F with highly lymphogenous metastatic potential and its syngeneic cell line named Hca-P[5] with low lymphogenous metastatic potential have been isolated from hepatocarcinomas in mice. Using gene chip combination with lymphatic metastasis models, we investigated the transcriptional profiles of the mouse hepatocarcinoma cell lines Hca-F with a metastasis rate over 70% and its syngeneic cell line Hca-P with a metastasis rate less than 30% in order to identify lymphatic metastasis-associated genes. Although several metastasis-associated genes have already been
screened with these two cell lines using suppression subtractive hybridization method, we decided to detect the expression profiles of cell lines Hca-F and Hca-P using Affymetrix Genechip® array technology in purpose of extending the panel of candidate genes.

MATERIALS AND METHODS

Animals and cell lines

Hepatocarcinoma cell lines, Hca-F and Hca-P were established and stored by our department. Inbred 615-mice were bred and provided by our department. Forty 615-mice were equally divided into two groups. Hca-F and Hca-P cells were inoculated in each group respectively (2×10⁶ cells per mouse). On the 28th d after inoculation, mice were killed and their lymph nodes were collected and stained using HE and examined by light microscope. Then the lymph node metastasis rates of Hca-F and Hca-P cell lines were calculated and tested.

RNA collection and probe preparation for oligonucleotide array hybridization

Total RNA was isolated from Hca-F and Hca-P cells respectively using TRIzol reagent (Invitrogen Life Technologies, P/N 15596-018) and cleaned with Rneasy Mini Kit (Qiagen, P/N 74104). cDNA was synthesized using the T7-Oligo(dT)₄ primer (5'-GGCCAGTTAATTGT AATACGACTCACTATAGGGAGGCGG-(dT)₂₀₃'). Double-stranded cDNA was purified with Phase Lock Gel (Eppendorf, P/N 0032 007.953)-phenol/chloroform extraction (Ambion, P/N 9732). Then in vitro transcription labeling was performed using the Enzo RNA Transcription Labeling Kit (Affymetrix, P/N 900182). The biotin-labeled cRNA was purified with the Qiagen Rneasy Mini Kit and fragmented randomly to an average size of approximately 50-200 bases by mild alkaline treatment at 94 °C for 35 min in fragmentation buffer. The hybridization solution was composed of 0.05 μg/μL fragmented cRNA, 1 μL herring sperm DNA, 1 μL acetylated BSA and 50 μL 2× hybridization buffer. In addition, the hybridization solution contained a mixture of four control cRNAs for bacterial and phage genes (bioB, bioC, bioD and cre at 5, 5, 25 and 100 pmol/L, respectively) to serve as positive controls of spiked bacterial bioB, bioC, bioD and cre (Figure 1). Double-stranded cDNA was purified with Phase Lock Gel (Eppendorf, P/N 0032 007.953)-phenol/chloroform extraction (Ambion, P/N 9732). Then in vitro transcription labeling was performed using the Enzo RNA Transcription Labeling Kit (Affymetrix, P/N 900182). The biotin-labeled cRNA was purified with the Qiagen Rneasy Mini Kit and fragmented randomly to an average size of approximately 50-200 bases by mild alkaline treatment at 94 °C for 35 min in fragmentation buffer. The hybridization solution was composed of 0.05 μg/μL fragmented cRNA, 1 μL herring sperm DNA, 1 μL acetylated BSA and 50 μL 2× hybridization buffer. In addition, the hybridization solution contained a mixture of four control cRNAs for bacterial and phage genes (bioB, bioC, bioD and cre at 5, 5, 25 and 100 pmol/L, respectively) to serve as positive controls of spiked bacterial bioB, bioC, bioD and cre (Figure 1). Figure 2A, B indicate the scanning result of real chip (Hca-F and Hca-P, respectively).

RESULTS

The lymph node metastasis rates of Hca-F and Hca-P were 75% (15/20) and 25% (5/20), respectively. The quality of GeneChip® was tested and verified by the positive controls of murine housekeepers β-actin and GAPDH and externally positive controls of spiked bacterial bioB, bioC, bioD and cre (Figure 1). Figure 2A, B indicate the scanning result of real chip (Hca-F and Hca-P, respectively). Figure 3 indicates the comparison of gene expression signal in cell line Hca-F with Hca-P.

To identify genes associated with the lymphatic metastasis, we analyzed the transcriptional profiles of 14 500 mouse genes and 4 371 ESTs from highly lymphatic metastasis potential cell line Hca-F and low lymphatic metastasis potential cell line Hca-P using the Affymetrix GeneChip® array method. On the basis of the selection criteria for up-regulated described above, 110 genes (132 transcripts) and 17 ESTs (21 transcripts) (data were not presented) were obtained. The results about differentially expressed genes are presented in Table 1.

Statistical analysis

The data obtained through GeneChip® scanning was analyzed using Affymetrix® Microarray Suit Software 5.0⁶⁷. Before the two arrays were compared, the GeneChip® software conducted normalization and scaling of the data for each array. The mRNA expression level of a transcript is directly related to the signal which is a quantitative metric calculated for each probe set and measures the mean difference of fluorescence intensity between perfect match and central mismatch oligonucleotides of a probe set. Signal log ratio, which estimates the magnitude and direction of change of a transcript when two arrays are compared, of at least three (that indicates an increase of the transcript level by 2³-fold change), and changing P-value, which measures the probability that the expression levels of a probe set in two different arrays are the same or not, ≤0.05 (that means the expression level in the experiment array is higher than that of the baseline array) were used to select differentially expressed genes. In the following, only up-regulated genes were presented and the assignment “up-regulated” refers to Hca-F in comparison with Hca-P.
Figure 2 (A) Scanning result of real chip after hybridization with cRNA from Hca-F cell line; (B) Scanning result of real chip after hybridization with cRNA from Hca-P cell line.

Figure 3 Comparison of gene expression signal in Hca-F cell line with that in Hca-P cell line.

Table 1 Differential gene expression profile in cell lines Hca-F vs Hca-P

| Gene          | Symbol descriptions | F vs P_Signal log ratio |
|---------------|---------------------|-------------------------|
| Slc38a4       | Solute carrier family 38, member 4 | 9                       |
| Krt2-8        | Keratin complex 2, basic, gene 8    | 3.4                     |
| Krt1-19       | Keratin complex 1, acidic, gene 19  | 7.4                     |
| Cldn9         | Claudin 9            | 7.4                     |
| Gja1          | Gap junction membrane channel protein alpha 1 | 7.2                     |
| Fbp2          | Fructose bisphosphatase 2          | 6.4                     |
| R75183        | Expressed sequence R75183       | 5.5                     |
| Egfr          | Epidermal growth factor receptor | 6.9                     |
| Lepr          | Leptin receptor        | 6.7                     |
| Tm4sf3        | Transmembrane 4 superfamily member 3 | 6.5                    |
| Pla2g1b       | Phospholipase A2, group IB, pancreas | 6.4                    |
| Ripk3         | Receptor-interacting serine-threonine kinase 3 | 6.4                    |
| Igfbp4        | Insulin-like growth factor binding protein 4 | 6.3                     |
| Piwil2        | Piwi-like homolog 2 (Drosophila) | 6.3                     |
| IL24          | Interleukin 24         | 5.9                     |
| Daf1          | Decay accelerating factor 1      | 5.9                     |
| Cav           | Caveolin, caveolae protein      | 5.8                     |
| Arhgef3       | Rho guanine nucleotide exchange factor (GEF) 3 | 5.7                     |
| Efna1         | Ephrin A1               | 5.7                     |
| Ptp48         | Protein tyrosin phosphatase, non-receptor type 8 | 4.2                     |
| Rab3b         | RAB3B, member RAS oncogene family | 5.6                     |
| 1190003K14Rik | RIKEN cDNA 1190003K14 gene   | 5.6                     |
| Sh2bp5        | SH2-domain binding protein 5    | 5.5                     |
| Fscn1         | Fascin homolog 1, actin bundling protein (strongly lomolvent purpuratus) | 5.1                     |
| Krt2-7        | Keratin complex 2, basic, gene 7 | 5.1                     |
| Cnm2          | Calponin 2              | 5.2                     |
| Sema3b        | Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B | 5.1                     |
| Cd109         | CD109 antigen           | 5.3                     |
| Gb20476       | cDNA sequence BC02006    | 5.3                     |
| Ldb4yh        | Leukotriene B4 12-hydroxydehydrogenase | 5.2                     |
| Krt1-18       | Keratin complex 1, acidic, gene 18 | 5.1                     |
| Fosl1         | Fos-like antigen 1       | 5.1                     |
| Snog          | Synuclein, gamma         | 5.1                     |
| Col8a1        | Procollagen, type VIII, alpha 1 | 4.9                     |
| Pcd2b       | Proctocardin beta 7     | 4.9                     |
| Msln          | Mesothelin              | 4.9                     |
| IL23a         | Interleukin 23, alpha subunit p19 | 4.9                     |
| …             | Mus musculus adult male tongue cDNA | 4.8                     |
| Ppp1r14a      | Protein phosphatase 1, regulatory (inhibitor) subunit 1A4 | 4.7                     |
| Cdx3          | Colony stimulating factor 3 (granulocyte) | 4.7                     |
| Nlfi          | Nuclear factor, interleukin 3, regulated | 4.6                     |
| Procr         | Protein C receptor, endothelial | 4.6                     |
| Nrl1d1        | Nuclear receptor subfamily 1, group D, member 1 | 4.6                     |
| 2810003C17Rik | RIKEN cDNA 2810003C17 gene | 4.6                     |
| Mgc27770      | Hypothetical protein MGC27770 | 4.5                     |
| Eng           | Endoglin                | 4.5                     |
| F2r           | Coagulation factor II (thrombin) receptor | 4.5                     |
| Cdc42ep5      | CDC42 effector protein   | 4.4                     |
| Pla2g7        | Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) | 4.4                     |
| D18Ertd653e   | DNA segment, Chr 18, ERATO Doi 653, expressed | 4.4                     |
| Il2rg         | Interleukin 2 receptor, gamma chain | 4.4                     |
According to the Gene Ontology (GO) classification and TreeView analysis, the genes are further divided into two groups: differential biological process profile and molecular function profile, as shown respectively in Tables 2, 3. Biological process refers to a biological objective to which the gene or gene product contributes. Molecular function is defined as the biochemical activity (including specific binding to ligands or structures) of a gene product.[8]

Table 2  Differential biological process profile in cell lines Hca-F vs Hca-P

| Development                          | Hca-F | Hca-P |
|--------------------------------------|-------|-------|
| Itgb5 gb: NM_010580 14117533_a_at    | 3.3   | 3.5   |
| Mlf1 gb: NM_010580 14117534_a_at     | 3.3   | 3.5   |
| Mta1 gb: NM_010580 14117535_a_at     | 3.3   | 3.5   |
| Tnfaip2 gb: NM_009153 4.7            |       |       |
| Igfbp6 gb: BC019836 1423756_s_at     | 3.3   | 4.1   |
| Efna1 gb: BC019836 1423757_s_at      | 3.7   | 4.1   |
| Cnd1 gb: M64403 3.2                  |       |       |
| Rrad gb: NM_007631 3.3               |       |       |
| Zdhhc2 gb: BC019836 1423758_x_at     | 3.3   | 4.1   |
| A530090O15Rik gb: BC002046 4.2       | 3.3   | 4.1   |
| Chs3l3 gb: M64403 3.2                |       |       |
| Chs1 gb: Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1 | 3.2   |       |
| Timp1 gb: Tissue inhibitor of metalloproteinase 1 | 3.2   |       |
| Sgk2 gb: Serum/glucocorticoid regulated kinase 2 | 3.1   |       |
| Panx1 gb: Panx1 3.6                  |       |       |
| Dok1 gb: Downstream of tyrosine kinase 1 | 3.6   |       |
| Abhd3 gb: Abhd3 3.6                  |       |       |
| Wsc1 gb: Wsc1 3.6                    |       |       |
| Ihrg gb: Igf2 5.4                    |       |       |
| Myo1b gb: Myo1b 3.4                  |       |       |
| Myo1g gb: Myo1g 3.3                  |       |       |
| Siat10 gb: Siat10 3.3                 |       |       |
| Nod2 gb: Nod2 3.3                    |       |       |
| Tptih gb: Tptih 3.3                  |       |       |
| Igfbp6 gb: Igfbp6 3.3                |       |       |
| Cnd1 gb: Cnd1 3.3                    |       |       |
| BC003236 gb: BC003236 3.2            |       |       |

Table 3  Molecular function profile in cell lines Hca-F vs Hca-P

| Development                          | Hca-F | Hca-P |
|--------------------------------------|-------|-------|
| Itgb5 gb: NM_010580 14117533_a_at    | 3.3   | 3.5   |
| Mlf1 gb: NM_010580 14117534_a_at     | 3.3   | 3.5   |
| Mta1 gb: NM_010580 14117535_a_at     | 3.3   | 3.5   |
| Tnfaip2 gb: NM_009153 4.7            |       |       |
| Igfbp6 gb: BC019836 1423756_s_at     | 3.3   | 4.1   |
| Efna1 gb: BC019836 1423757_s_at      | 3.7   | 4.1   |
| Cnd1 gb: M64403 3.2                  |       |       |
| Rrad gb: NM_007631 3.3               |       |       |
| Zdhhc2 gb: BC019836 1423758_x_at     | 3.3   | 4.1   |
| A530090O15Rik gb: BC002046 4.2       | 3.3   | 4.1   |
| Chs3l3 gb: M64403 3.2                |       |       |
| Chs1 gb: Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1 | 3.2   |       |
| Timp1 gb: Tissue inhibitor of metalloproteinase 1 | 3.2   |       |
| Sgk2 gb: Serum/glucocorticoid regulated kinase 2 | 3.1   |       |
| Panx1 gb: Panx1 3.6                  |       |       |
| Dok1 gb: Downstream of tyrosine kinase 1 | 3.6   |       |
| Abhd3 gb: Abhd3 3.6                  |       |       |
| Wsc1 gb: Wsc1 3.6                    |       |       |
| Ihrg gb: Igf2 5.4                    |       |       |
| Myo1b gb: Myo1b 3.4                  |       |       |
| Myo1g gb: Myo1g 3.3                  |       |       |
| Siat10 gb: Siat10 3.3                 |       |       |
| Nod2 gb: Nod2 3.3                    |       |       |
| Tptih gb: Tptih 3.3                  |       |       |
| Igfbp6 gb: Igfbp6 3.3                |       |       |
| Cnd1 gb: Cnd1 3.3                    |       |       |
| BC003236 gb: BC003236 3.2            |       |       |
Table 3 Differential molecular function profile in cell lines Hca-F vs Hca-P

| Function                        | Hca-F       | Hca-P       |
|---------------------------------|-------------|-------------|
| Transporter activity            | Gja1 (6.4)  | Slc38a4 (6.4) |
|                                 | Ramp3 (3.6) | Rab3b (5.6)  |
|                                 | Scn8a (4)   |             |
|                                 | Tptih (3.3) |             |
| Structural molecule activity     | Eppl1 (3.6) | Cldn9 (7.4) |
|                                 | Col8a1 (4.9) | Krt1-19 (7.4) |
|                                 | Tub4 (3.3)  | Krt2-7 (5.2) |
|                                 | Krt2-8 (4.8) | M21836 (8.6) |
| Chaperone activity              | Serpinh1 (4) | Snog (5) |
|                                 |             | Motor activity |
|                                 |             | Myo1g (3.3) |
|                                 |             | AI25526 (3.4) |
| Catalytic activity              |             |             |
|                                 |             |             |
| Hydrolase activity              |             |             |
|                                 |             |             |
| Kinase activity                 |             |             |
|                                 |             |             |
**DISCUSSION**

We used an Affymetrix GeneChip® MOE430A to identify lymphatic metastasis-associated genes in two hepatocarcinoma cell lines with different lymphatic metastasis potential. Based on the selection criteria for up-regulated expression discussed in “MATERIALS AND METHODS”, 110 differential genes were observed in the highly lymphogenous metastatic cell line. The over expressed genes were then classified according to the GO classification and TreeView analysis.

In the category development, we found three genes associated with angiogenesis: endoglin (EDG; CD105), ephrin A1 and Tnfaip2. Tumor angiogenesis plays an important role in tumor growth and metastasis[9] and certain angiogenesis markers may be useful as metastasis markers and/or the targets for antiangiogenic therapy[10]. EDG was thought to be a proliferation-associated antigen of endothelial cells and essential for angiogenesis. Elevated serum EDG was associated with metastasis in patients with colorectal, breast, and other solid tumors and chemotherapy exerts a suppression effect on the serum EDG[11-13]. In endometrial carcinoma, EDG counts correlated significantly with the presence of angiolympathic invasion, lymph nodes metastasis and tumor stage[14]. Ephrin-A1, formerly called B61, was noted up-regulation of MCAM (CD146; Mel-CAM; Muc18) in Hca-F cell line. Mcam, a member of the immunoglobulin superfamily and homologous to several cell adhesion molecules, was identified as a tumor necrosis factor alpha-inducible gene (TNFα) and the targets for antiangiogenic therapy. Mcam is highly expressed in marrow from patients with acute myelogenous leukemia French-American-British subtypes M2-M4[15], but its correlation with metastasis requires to be elucidated.

Adaptation of cell adhesion functions of the tumor cells to successfully overcome the different hurdles in the metastatic cascade is a prerequisite for metastasis[16]. We noted up-regulation of MCAM (CD146; Mel-CAM; Muc18) in Hca-F cell line. Mcam, a member of the immunoglobulin superfamily and homologous to several cell adhesion molecules, was associated with tumor progression and the development of metastasis in human malignant melanoma and also was an important determinant in increasing metastasis of human prostate cancer LNCaP cells to distant organs in a nude mouse model[17-19]. We also noted over expression of integrin β3, Col8A1 (procollagen, type VIII, alpha 1) and Pdcdh7 (protocadherin beta) in the Hca-F cell line.

In the category signal transduction, we observed up-
adaptation to increased motility and invasion of the Hca-F cell line. Changes in the expression of genes for pancreatic carcinoma cell line BSp73-ASML were up-regulated in highly lymphogenous metastatic be involved in tumor metastasis. Hsp27, which encodes nucleotide sugar transporters, has been shown to metastatic pancreatic carcinoma cell line BSp73-ASML, the ras-related Rab proteins and protein tyrosine phosphatases were all over expressed. Lepr positive correlated significantly with distant metastasis and lower survival in breast cancer. F2r, protease-activated receptor 1, a G protein-coupled receptor for thrombin, was shown to be preferentially expressed in highly lymphogenous metastatic pancreatic carcinoma cell line BSp73-ASML and correlated with breast carcinoma cell invasion and metastasis. Booden et al. also reported that altered trafficking of proteolytically activated PAR1 (F2r) caused sustained activation of phosphoinositide hydrolysis and extracellular signal-regulated kinase signaling, even after thrombin withdrawal, and enhanced breast carcinoma cellular invasion.

The ability to locomote and migrate is fundamental to the acquisition of invasive and metastatic properties by tumor cells. D7Ertd458e (necl-5), one of the five nectin-like molecules (necls), which have domain structures similar to those of nectins, has recently been identified and appears to play different roles from those of nectins. Experiments showed that enhanced motility and metastasis of V12Ras-NIH3T3 cells (NIH3T3 cells transformed by an oncogenic Ki-Ras) were at least partly the result of up-regulated Necl-5, which does not homophilically trans-interact, but heterophilically trans-interacts with nectin-3, regulates cell migration and adhesion.

In the category transport, Slc38a4 was detected to overexpress in the highly metastatic cell line. Recent work has considered SLC38 transporters as therapeutic targets in neoplasia. Although to date Slc38a4 has not been reported to be correlated with tumor metastasis straightly, the member of the solute carrier family SLC35, which encodes nucleotide sugar transporters, has been shown to be involved in tumor metastasis and SLC16 and SLC2 were up-regulated in highly lymphogenous metastatic pancreatic carcinoma cell line BSp73-ASML. Meanwhile, the reason Sk38a4 deserves further attention is that it differs most in our study.

The state of tubulin polymerization associates with tumor metastasis and increased depolymerized form of tubulin could promote metastasis. We noted Tuba4 over expression in Hca-F cell line. Changes in the expression of genes for the cytoskeleton organization and biogenesis mediate adaptation to increased motility and invasion of the metastatic tumor cell. Krt1-19 (keratin 19), Krt1-18 (keratin 18), Krt2-7 (keratin 7) and Krt2-8 (keratin 8) were up regulated in the highly metastatic cell line Hca-F. Expressive changes of these genes have been reported to be correlated with the invasive and metastatic phenotype.

A remarkable feature in our study is the increased steady state level of the mRNA for cyclin D1 in the category cell cycle. Cyclin D1 is a nuclear protein that plays an important role in regulating the cell cycle by promoting entry of cells from the G1 to S phase due to interaction with its catalytic partner cdk4 or with the extradiol receptor. Over expression of cyclinD1 was associated with the liability of lymph node metastasis and the poor prognosis for patient with laryngeal squamous cell carcinoma, esophageal carcinoma, mammary infiltrating duct carcinoma, oral squamous cell carcinoma and papillary thyroid carcinoma. mRNA for cyclin D1 was also found to be over expressed in lymph node metastases of breast carcinoma by comparison of gene expression profiles with their primary counterparts.

In the category transcription, we observed another feature of our system, i.e., the increased expression of Fosl1 (Fra1; fra-1). Fosl1 encodes a transcription factor, which was found over expressed in highly aggressive breast carcinoma cell lines and lymphogenous metastatic pancreatic carcinoma. It was reported that Fosl1 induces transformation and invasiveness of human epithelial adenocarcinoma cells. In addition, we identified up-regulation of NR1D1, a member of the orphan receptor superfamily. It was coexpressed with ERBB2 in 34 breast cancer biopsies and also mapped within the same chromosomal location as the ERBB2 gene.

In the present study, we found over expression of heat-shock protein Serpin h1 (HSP47) and SNCG (persyn; breast cancer-specific protein 1) in the category chaperone activity. HSP47 is a stress-inducible glycoprotein of Mr 47000 molecular weight and is assumed to be a collagen-specific molecular chaperone. Tumor cell lines, which were derived from metastatic carcinomas and were still metastatic in animals, synthesized higher levels of HSP47. SNCG, the third member of a neuronal protein family synuclein, is a new chaperone protein in the Hsp-based multiprotein chaperone complex for the stimulation of ligand-dependent ER-alpha signaling and thus stimulates hormone-responsive mammary tumorigenesis, and is also highly associated with breast or ovarian cancer progression. In addition, aberrant SNCG gene expression can occur via CpG island demethylation, and tends to occur during the more progressive stages of gastric carcinogenesis.

The motor activity of tumor cell plays an important role in invasiveness and metastasis. Our results revealed the up-regulation of Myosin IB and Myosin IG which are two members of the myosin I family of motor proteins. Myosins are a large family of structurally diverse motor proteins. Each myosin utilizes energy from ATP hydrolysis to generate force for indirectional movement along actin filaments. It has been reported that myosin VI, a motor protein that regulates border cell migration, was abundantly expressed in high-grade ovarian carcinomas but not in normal ovary and ovarian cancers that behave indolently. Inhibiting myosin VI expression in high-grade ovarian carcinoma cells impeded cell spreading and migration in vitro.

Another hallmark of our system is the overexpression of mRNAs coding for kinase activity, such as Sgk2, AXL, Mak and EGFR. EGFR belongs to the family of type I receptor tyrosine kinase. Over expression of EGFR often correlates with an aggressive tumor phenotype and poor prognosis. AXI, another member of a family of
receptor tyrosine kinases, has been described to act as a mitogenic factor along with its ligand Gas-6 and has also shown to have a role in apoptosis, cell adhesion, and chemotaxis. There was a significant increase in the steady-state levels of Axl or its mRNA in a variety of cancers. Meanwhile, in colon cancer Axl receptor tyrosine kinase was expressed highly in a peritoneal metastatic nodule than in primary malignant tissues and in papillary thyroid carcinomas solid component and invasive front tended to over express Axl[50-54]. These indicated that Axl might be related to the tumorigenesis and tumor progression. Sgk, a serine/threonine protein kinase, was found up-regulation in the tumorigenic HeLa cells compared to nontumorigenic HeLa cells which came from fusion of tumorigenic HeLa cells with human skin fibroblasts[55]. Male germ cell-associated kinase (Mak) was shown to be up-regulated in prostate cancer cell lines than those of normal prostate epithelial cells[56].

In the category binding, Loxl2 gene expression was up regulated. Loxl2, a copper-containing amine oxidase, belongs to the LOX family which functions as extracellular matrix modulating enzyme. LOX and LOX family members Loxl2, Loxl3, and Loxl4 were observed only in breast cancer cells with a highly invasive/metastatic phenotype but not in poorly invasive/nonmetastatic breast cancer cells[57]. We also found Areg (AR) over expressed in the highly metastatic hepatocarcinoma cell line. Areg is one of the ligands of EGFR. Concomitant presence of the EGF receptor and its ligands EGF, TGF-alpha, and/or amphiregulin Areg is associated with enhanced tumor aggressiveness and shorter postoperative survival[58,59]. EGF and AR might modulate invasion by increasing the expression of MMPs[60] or stimulating directional (chemotactic) and/or random (chemokinetic) motility in malignant cells[61]. In addition, the mRNA for caveolin (Cav; Cav-1) was up regulated in the highly metastatic cell line. Cav-1 is a major structural component of caveolae of plasma membranes. It was identified as a metastasis-related gene and/or a worse prognostic predictor in prostate carcinoma, renal cell carcinoma, esophageal squamous cell carcinoma, lung adenocarcinoma and colorectal cancer[62-64]. Cav-1 was reported to be necessary for mediating filopodia formation in lung adenocarcinoma, which may enhance the invasive ability of cancer cells[65]. In an other study, caveolin-1 was shown to affect angiogenesis during the progression of clear cell renal cell carcinoma[66].

Taken together, we found that the metastatic phenotype of the highly metastatic mouse hepatocarcinoma cell line Hca-F is accompanied by marked differences in its transcriptional profile in comparison with the low metastatic cell line Hca-P. A large number of genes and their cellular functions, such as angiogenesis, cell adhesion, signal transduction, cell motility, transport, microtubule-based process, cytoskeleton organization and biogenesis, cell cycle, transcription, chaperone activity, motor activity, protein kinase activity, receptor binding and protein binding, might be involved in the process of lymphatic metastasis and deserve to be used as potential candidates for further investigation. We selected cyclin D1, Fosl1, Hsp47, EGFR and AR, and Cav-1 as the possible candidate/key genes of the metastatic phenotype, which needed to be validated in later experiments. Besides these genes, several other genes which were not validated to contribute to enhanced tumor metastatic properties should be further investigated for example, Sk38a4 and Cldn9. ESTs (data were not presented) might indicate novel genes associated with lymphatic metastasis and also need attention. Our next work is to identify the candidate genes/pathway responsible for lymphogenous metastasis, because although a large number of genes are associated with the metastasis, some of the changes are believed to be the secondary events; the expression changes as a result of metastasis rather than as an initiator of the metastasis event[67]. The elucidation of the candidate genes/pathway might not only provide useful diagnostic markers for tumor lymphogenous metastasis, but also more importantly, provide novel therapeutic targets.

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