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

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Abstract

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 2^3 fold. Among the total 4 371 ESTs, 17 ESTs (0.4%) (data were not presented) were up regulated at least 2^3 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.

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Key words: Hepatocarcinoma; Lymphatic metastasis; Cell lines Hca-F and Hca-P; Gene chip

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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 into 20 mice in each group respectively (2×10^6 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$(dT)_{24}$ primer (5'-GGCCAGTGAATTGT AATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3'). 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 Transcript 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 ℃ 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 comparison tools for hybridization efficiency between arrays. A biotinylated oligonucleotide B2, which specifically hybridized to features at the center and corners of the chip, was also added to the solution to allow correct orientation and recognition of the probe sets.

Array hybridization and scanning

The hybridization cocktail was heated to 99 ℃ for 5 min in a heat block, followed by a 45 ℃ heat block for 5 min and centrifugation for 5 min to remove any insoluble material. Meanwhile, the arrays were wet with appropriate volume 1× hybridization and incubated by 1× hybridization buffer at 45 ℃ for 10 min with rotation. The buffer solution was then removed from the probe array and the clarified-hybridization cocktail was added. Fragmented cRNA (5 μg) was hybridized to Affymetrix MOE430A array (containing 22 690 transcripts, almost 14 500 known genes and 4 371 ESTs) for 16 h in Affymetrix®Fluidics Station 400. The arrays were then scanned using the GeneArray Scanner (G2500AgeneArray Scanner, Affymetrix). The cRNA probe was first hybridized to a "test chip" before to the MOE430A array and the quality was confirmed.

Statistical analysis

The data obtained through GeneChip® scanning was analyzed using Affymetrix® Microarray Suit Software 5.0[6,7]. 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 2^3-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, $\leqslant$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.

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.

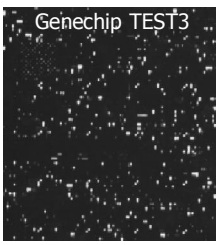

Figure 1 Scanning result of test chip.

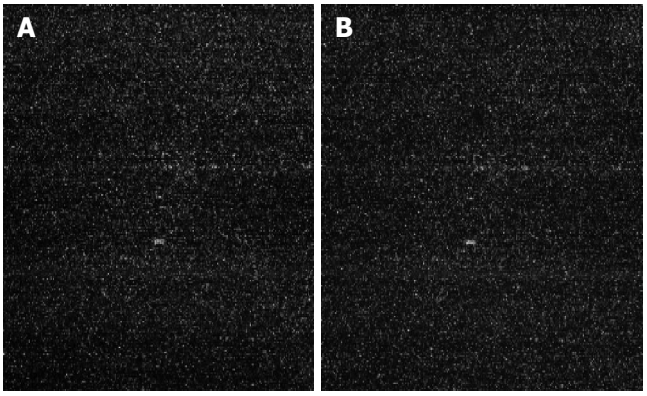

Figure 2 (A) Scanning result of real chip after hybridization with cRNA from Hca-F cell line; (B) Scanning result of real chip after hybridization with cRNA from Hca-P cell line.

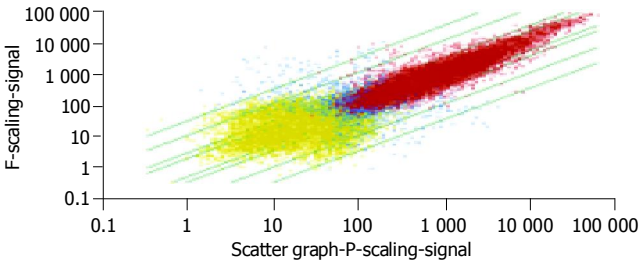

Figure 3 Comparison of gene expression signal in Hca-F cell line with that in Hca-P cell line.

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	
	[1]gb: NM_027052	9
	gb: AK003626	3.4
Krt2-8	Keratin complex 2, basic, gene 8	
	gb: M21836	8.6
	gb: NM_031170	4.8
Krt1-19	Keratin complex 1, acidic, gene 19	7.4
Cldn9	Claudin 9	7.4
Gja1	Gap junction membrane channel protein alpha 1	
	gb: M63801	7.2
	gb: BC006894	6.4
Fbp2	Fructose bisphosphatase 2	7.1
R75183	Expressed sequence R75183	
	gb: BC004774	7
	gb: BB324973	5.5
Egfr	Epidermal growth factor receptor	
	gb: AF275367	6.9
	gb: U03425	3.6
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	
	gb: NM_010517	6.3
	gb: BC019836 [2]1423756_s_at	5.8
	gb: BC019836 1423757_x_at	4.1
	gb: AA119124	4
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	
	gb: D38146	5.7
	gb: BC002046	4.2
Ptpn8	Protein tyrosine phosphatase, non-receptor type 8	5.6
Rab3b	RAB3B, member RAS oncogene family	5.6
1190003K14Rik	RIKEN cDNA 1190003K14 gene	5.6
Sh3bp5	SH3-domain binding protein 5 (BTK-associated)	5.5
Fscn1	Fascin homolog 1, actin bundling protein (Strongylocentrotus purpuratus)	
	gb: NM_007984 1416514_a_at	5.4
	gb: BE952057	4.2
	gb: NM_007984 1448378_at	3.7
Fgf15	Fibroblast growth factor 15	5.4
Ehox	ES cell derived homeobox containing gene	5.3
Ankrd1	Ankyrin repeat domain 1 (cardiac muscle)	
	gb: NM_013468	5.3
	gb: AK009959	3.6
1600023A02Rik	RIKEN cDNA 1600023A02 gene	5.3
Cd109	CD109 antigen	5.3
BC037006	cDNA sequence BC037006	5.3
Ltb4dh	Leukotriene B4 12-hydroxydehydrogenase	5.2
Krt2-7	Keratin complex 2, basic, gene 7	5.2
Cnn2	Calponin 2	5.2
Sema3b	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	
	gb: BB116052	5.1
	gb: NM_009153	4.7
Krt1-18	Keratin complex 1, acidic, gene 18	5.1
…	Mouse gene for 18S rRNA	5.1
Fosl1	Fos-like antigen 1	
	gb: NM_010235	5
	gb: U34245	4.4
Sncg	Synuclein, gamma	5
Col8a1	Procollagen, type VIII, alpha 1	4.9
Pcdhb7	Protocadherin 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 14A	4.7
Csf3	Colony stimulating factor 3 (granulocyte)	4.7
Nfil3	Nuclear factor, interleukin 3, regulated	4.6
Procr	Protein C receptor, endothelial	4.6
Nr1d1	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 (Rho GTPase binding) 5	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	

	gb: L20048 1416296_at	4.3
	gb: L20048 1416295_a_at	3.3
Smpd2	Sphingomyelin phosphodiesterase 2, neutral	4.3
Areg	Amphiregulin	4.3
Mcam	Melanoma cell adhesion molecule	4.2
Rtn2	Reticulon 2 (Z-band associated protein)	4.2
Gcnt2	Glucosaminyltransferase, I-branching enzyme	4.2
D7Ertd458e	DNA segment, Chr 7, ERATO Doi 458, expressed	
	gb: NM_009310	4.2
	gb: BB049138 1423904_a_at	4
	gb: BC013673 1451160_s_at	4
	gb: BB049138 1423905_at	3.9
	gb: BC013673 1423903_at	3.8
Chi3l3	Chitinase 3-like 3	4.1
D4Ertd765e	DNA segment, Chr 4, ERATO Doi 765, expressed	4.1
Ptprr	Protein tyrosine phosphatase, receptor type, R	4.1
Scn8a	Sodium channel, voltage-gated, type VIII, alpha polypeptide	4
AXL	AXL receptor tyrosine kinase	4
Serpinh1	Serine (or cysteine) proteinase inhibitor, clade H, member 1	4
Panx1	Pannexin 1	3.9
Tm4sf9	Transmembrane 4 superfamily member 9	3.9
Mak	Male germ cell-associated kinase	3.9
…	Mus musculus transcribed sequences	3.8
9130017N09Rik	RIKEN cDNA 9130017N09 gene	3.8
Loxl2	Lysyl oxidase-like 2	3.7
Dok1	Downstream of tyrosine kinase 1	3.6
Abhd3	Abhydrolase domain containing 3	3.6
Ramp3	Receptor (calcitonin) activity modifying protein 3	3.6
Fibp	Fibroblast growth factor (acidic) intracellular binding protein	3.6
Wscr5	Williams-Beuren syndrome chromosome region 5 homolog (human)	3.6
Eppk1	Epiplakin 1	3.6
2010004A03Rik	RIKEN cDNA 2010004A03 gene	3.6
Itgb5	Integrin beta 5	
	gb: NM_010580 1417534_at	3.5
	gb: NM_010580 1417533_a_at	3.3
Mlf1	Myeloid leukemia factor 1	3.5
Tnfrsf22	Tumor necrosis factor receptor superfamily, member 22	3.5
Zdhhc2	Zinc finger, DHHC domain containing 2	3.5
Tnfaip2	Tumor necrosis factor, alpha-induced protein 2	3.4
Rrad	Ras-related associated with diabetes	3.4
A530090O15Rik	RIKEN cDNA A530090O15 gene	3.4
Chi3l4	Chitinase 3-like 4	3.4
Tnfrsf19	Tumor necrosis factor receptor superfamily, member 19	3.4
Myo1b	Myosin IB	
	gb: AI255256	3.4
	gb: BI080370	3.2
Tuba4	Tubulin, alpha 4	3.3
Myo1g	Myosin IG	3.3
Siat10	Sialyltransferase 10 (alpha-2,3-sialyltransferase VI)	3.3
Nol3	Nucleolar protein 3 (apoptosis repressor with CARD domain)	3.3
Tpt1h	tRNA splicing 2′ phosphotransferase 1 homolog (S. cerevisiae)	3.3
Igfbp6	Insulin-like growth factor binding protein 6	3.3
Ccnd1	Cyclin D1	
	gb: M64403	3.2
	gb: NM_007631	3.1
BC003236	cDNA sequence BC003236	3.2
2010001C09Rik	RIKEN cDNA 2010001C09 gene	3.2
Chst1	Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	3.2
Timp1	Tissue inhibitor of metalloproteinase 1	3.2
4921530G04Rik	RIKEN cDNA 4921530G04 gene	3.2
Lxn	Latexin	3.1
Sgk2	Serum/glucocorticoid regulated kinase 2	3.1
2310047E01Rik	RIKEN cDNA 2310047E01 gene	3.1
2200002N01Rik	RIKEN cDNA 2200002N01 gene	3
Ptpre	Protein tyrosine phosphatase, receptor type, E	3
Psmb8	Proteosome (prosome, macropain) subunit, beta type 8 (large multifunctional protease 7)	3
Tm7sf1	Transmembrane 7 superfamily member 1	3

[1]Accession number of each gene obtained from PubMed; [2]Affymetrix probe identification number.

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					
Itgb5	gb: NM_010580 1417533_a_at 3.3		Ccnd1	gb: M64403	3.2
Mlf1	gb: NM_010580 1417534_at 3.5			gb: NM_007631	3.1
Morphogenesis					
Gja1	gb: BC006894	6.4	Sema3b	gb: BB116052	5.1
	gb:M63801	7.2		gb:NM_009153	4.7
Tnfaip2		3.4	Tm4sf9		3.9
Igfbp6		3.3	Igfbp4	gb: NM_010517	6.3
Egfr	gb: AF275367	6.9		gb: AA119124	4
	gb: U03425	3.6		gb: BC019836 1423756_s_at	5.8
Efna1	gb: D38146	5.7		gb: BC019836 1423757_x_at	4.1
	gb: BC002046	4.2	Eng		4.5
Cellular process					
Cell communication					
Cell adhesion					
Mcam		4.2	Itgb5	gb: NM_010580 1417533_a_at	3.3
Tm4sf9	3.9			gb: NM_010580 1417534_at	3.5
D7Ertd					
458e	gb: BC013673 1423903_at 3.8		Col8a1		4.9
	gb: BB049138 1423904_a_at 4		Eng		4.5
	gb: BB049138 1423905_at 3.9		Pcdhb7		4.9
	gb: NM_009310 4.2				
	gb: BC013673 1451160_s_at 4				
Cell–cell signaling					
Gja1	gb: BC006894	6.4			
	gb: M63801	7.2			
Signal transduction					
IL2rg	gb: L20048 1416295_a_at 3.3		Efna1	gb: BC002046	4.2
	gb: L20048 1416296_at 4.3			gb: D38146	5.7
Itgb5	gb: NM_010580 1417533_a_at 3.3		Egfr	gb: AF275367	6.9
	gb: NM_010580 1417534_at 3.5			gb: U03425	3.6
Dok1		3.6	Fgf15		5.4
Ptpre		3	Cdc42ep5		4.4
Ramp3		3.6	Sh3bp5		5.5
Lepr		6.7	Ptprr		4.1
Eng		4.5	Ripk3		6.4

F2r		4.5		R75183	gb: BC004774	7
Rrad		3.3			gb: BB324973	5.5
Rab3b		5.6				
Cellular physiological process						
Cell death						
Ripk3		6.4		Nol3		3.3
Cell motility						
Tm4sf9		3.9	D7Ertd458e	gb: BC013673 1423903_at		3.8
Cnn2		5.2		gb: BB049138 1423904_a_at		4
				gb: BB049138 1423905_at		3.9
				gb: NM_009310 4.2		
				gb: BC013673 1451160_s_at		4
Cell growth and/or maintenance						
Transport						
Tuba4		3.3	Slc38a4	gb: NM_027052		9
Ramp3		3.6		gb: AK003626		3.4
Rab3b		5.6	Cav			5.8
Scn8a		4				
Cell organization and biogenesis						
Igfbp6		3.3	Igfbp4	gb: NM_010517		6.3
Egfr	gb: AF275367	6.9		gb: AA119124		4
	gb: U03425	3.6		gb: BC019836 1423756_s_at		5.8
…	AFFX-18SRNAMur/X00686_M_at	5.1		gb: BC019836 1423757_x_at		4.1
Microtubule-based process						
Tua4		3.3				
Cytoskeleton organization and biogenesis						
Krt1-19		7.4	Krt2-8	gb: M21836		8.6
Tuba4		3.3		gb: NM_031170		4.8
Krt2-7		5.2	Myo1b	gb: AI255256		3.4
Krt1-18		5.1		gb: BI080370		3.2
Cell proliferation						
Tm4sf9		3.9	Pla2g1b			6.4
Cell cycle						
Ccnd1	gb: M64403	3.2	Egfr	gb: AF275367		6.9
	gb: NM_007631	3.1		gb: U03425		3.6
Axl		4				
Regulation of cellular process						
Igfbp6		3.3	Igfbp4	gb: NM_010517		6.3
Ramp3		3.6		gb: AA119124		4
				gb: BC019836 1423756_s_at		5.8
				gb: BC019836 1423757_x_at		4.1
Cell differentiation						
Ccnd1	gb: M64403	3.2	Mlf1			3.5
	gb: NM_007631	3.1				
Regulation of biological process						
Regulation of cellular process						
Igfbp6		3.3	Igfbp4	gb: NM_010517		6.3
Ramp3		3.6		gb: BC019836 1423756_s_at		5.8
Sh3bp5		5.5		gb: BC019836 1423757_x_at		4.1
				gb: AA119124		4
Regulation of physiological process						
Gja1	gb: BC006894	6.4	Procr			4.6
	gb: M63801	7.2				
Behavior						
Scn8a		4				
Nr1d1		4.6				
Physiological process						
Metabolism						
Pla2g1b		6.4	Pla2g7			4.4
Psmb8		3	2310047E01Rik			3.1
Fbp2		7.1				3.3
Protein metabolism/phosphorus metabolism						
A530090O15Rik		3.4	Axl			4
Ccnd1	gb: M64403	3.2	Egfr	gb: AF275367		6.9
	gb: NM_007631	3.1		gb: U03425		3.6
Krt2-8	gb: M21836	8.6	Mak			3.9
	gb: NM_031170	4.8	Ptpn8			5.6
Ptpre		3	Ptprr			4.1
Ripk3		6.4	Sgk2			3.1
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism						
Nol3		3.3	Lepr			6.7
Transcription						
Fosl1	gb: NM_010235	5	Ankrd1	gb: NM_013468		5.3
	gb: U34245	4.4		gb: AK009959		3.6
Nfil3		4.6	Nr1d1			4.6
Regulation of physiological process						
Gja1	gb: BC006894	6.4	Procr			4.6
	gb: M63801	7.2				
Coagulation						
Procr		4.6	F2r			4.5
Organismal physiological process						
Chi3l3		4.1	Chi3l4			3.4
Csf3		4.7	Daf1			5.9
F2r		4.5	Gja1	gb: BC006894		6.4
IL24		5.9		gb: M63801		7.2
Pla2g7		4.4	Procr			4.6
… Mus musculus transcribed sequences		3.8	Psmb8			3
Response to stimulus						
Chi3l3		4.1	Chi3l4			3.4
Csf3		4.7	Daf1			5.9
F2r		4.5	IL24			5.9
Gja1	gb: BC006894	6.4	Krt2-8	gb: NM_031170		4.8
	gb: M63801	7.2		gb: M21836		8.6
Pla2g1b		6.4	Pla2g7			4.4
Psmb8		3	Serpinh1			4
… Mus musculus transcribed sequences		3.8				

Table 3 Differential molecular function profile in cell lines Hca-F *vs* Hca-P

Transporter activity					
Gja1	gb: BC006894	6.4	Slc38a4	gb: AK003626	3.4
	gb: M63801	7.2		gb: NM_027052	9
Ramp3		3.6	Rab3b		5.6
Scn8a		4			
Tptih		3.3			
Structural molecule activity					
Eppk1		3.6	Cldn9		7.4
Col8a1		4.9	Krt1-19		7.4
Tuba4		3.3	Krt2-7		5.2
Krt2-8	gb: NM_031170	4.8	Krt1-18		5.1
	gb: M21836	8.6			
Chaperone activity					
Serpinh1		4	Sncg		5
Motor activity					
Myo1b	gb: BI080370	3.2	Myo1g		3.3
	gb: AI255256	3.4			
Catalytic activity					
Gcnt2		4.2	Siat10		3.3
Chst1		3.2	Loxl2		3.7
Ltb4dh		5.2	2310047E01Rik		3.1
Hydrolase activity					
Pla2g1b		6.4	Smpd2		4.3
Abhd3		3.6	Ptpn8		5.6
Ptpre		3	Chi3l3		4.1
Rrad		3.4	Rab3b		5.6
Chi3l4		3.4	Ptprr		4.1
Pla2g7		4.4	Fbp2		7.1
Psmb8		3			
Kinase activity					
Ccnd1	gb: M64403	3.2	Krt2-8	gb: NM_031170	4.8
	gb: NM_007631	3.1		gb: M21836	8.6
A530090O15Rik		3.4			
Protein-tyrosine kinase activity					
Sgk2		3.1	Egfr	gb: AF275367	6.9

Axl		4		gb: U03425	3.6
Ripk3		6.4			
Transmembrane receptor protein kinase activity					
Egfr	gb: AF275367	6.9			
	gb: U03425	3.6			
Protein serine/threonine kinase activity					
Sgk2		3.1	Mak		3.9
Axl		4	Egfr	gb: AF275367	6.9
Ripk3		6.4		gb: U03425	3.6
Transferase activity					
A530090O15Rik		3.4	Axl		4
Ccnd1	gb: M64403	3.2	Egfr	gb: AF275367	6.9
	gb: NM_007631	3.1		gb: U03425	3.6
Chst1		3.2	Gcnt2		4.2
Krt2-8	gb: NM_031170	4.8	Mak		3.9
	gb: M21836	8.6	Ripk3		6.4
Sgk2		3.1	Siat10		3.3
Enzyme regulator activity					
Ccnd1	gb: M64403	3.2	Lxn		3.1
	gb: NM_007631	3.1	Timp1		3.2
Sh3bp5		5.5	Cd109		5.9
1600023A02Rik		5.3	Serpinh1		4
Binding					
Nucleic acid binding					
Fosl1	gb: NM_010235	5	Ankrd1	gb: NM_013468	5.3
	gb: U34245	4.3		gb: AK009959	3.6
Nr1d1		4.6			
Metal ion binding					
Pla2g1b		6.4	Smpd2		4.3
Ltb4dh		5.2	Scn8a		4
2310047E01Rik		3.1	Pcdhb7		4.9
2810003C17Rik		4.6	Loxl2		3.7
Zdhhc2		3.5			
Nucleotide binding					
A530090O15Rik		3.4	Axl		4
Egfr	gb: AF275367	6.9	Krt2-8	gb: NM_031170	4.8
	gb: U03425	3.6		gb: M21836	8.6
Mak		3.9	Rab3b		5.6
Myo1b	gb: BI080370	3.2	Ripk3		6.4
	gb: AI255256	3.4	Rrad		3.4
Scn8a		4	Sgk2		3.1
Tuba4		3.3			
Receptor binding					
Areg		4.3	Csf3		4.7
Dok1		3.6	Fgf15		5.4
Il24		5.9	Pla2g1b		6.4
Protein binding					
Ankrd1	gb: NM_013468	5.3	Ccnd1	gb: M64403	3.2
	gb: AK009959	3.6		gb: NM_007631	3.1
Cav		5.8	Cdc42ep5		4.4
Col8a1		4.9	Eng		4.5
D7Ertd458e					
	gb: BC013673 1423903_at	3.8	Egfr	gb: AF275367	6.9
	gb: BB049138 1423904_a_at	4		gb: U03425	3.6
	gb: BB049138 1423905_at	3.9	Fibp		3.6
	gb: NM_009310	4.2	IL2rg	gb: L20048 1416295_a_at	3.3
	gb: BC013673 1451160_s_at	4		gb: L20048 1416296_at	4.3
Itgb5	gb: NM_010580	3.5	Igfbp6		3.3
	gb: NM_010580	3.3	Lepr		6.7
Nol3		3.3	Pcdhb7		4.9
Procr		4.6	Ptpre		3
Rab3b		5.6	Rrad		3.4
Sh3bp5		5.5	Tnfrsf22		3.5
Fscn1	gb: NM_007984 1416514_a_at	5.4	Myo1b	gb: BI080370	3.2
	gb: BE952057	4.2		gb: AI255256	3.4
	gb: NM_007984 1448378_at	3.7	Cnn2		5.2
Carbohydrate binding					
Chi3l3		4.1	Chi3l4		3.4
Signal transducer activity					
Dok1		3.6	Ripk3		6.4
Receptor activity					
Axl		4	Itgb5	gb: NM_010580.1	3.5
D7Ertd458e	gb: BC013673 1423903_at	3.8		gb: NM_010580.1	3.3
	gb: BB049138 1423904_a_at	4	Nr1d1		4.6
	gb: BB049138 1423905_at	3.9	Procr		4.6
	gb: NM_009310	4.2	Ptpn8		5.6
	gb: BC013673 1451160_s_at	4	Ptprr		4.1
Ramp3		3.6	Tnfrsf19		3.4
Tnfrsf22		3.5	Lepr		6.7
IL2rg	gb: L20048.1 1416295_a_at	3.3	Egfr	gb: AF275367.1	6.9
	gb: L20048.1 1416296_at	4.3		gb: U03425.1	3.6
F2r		4.5			

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[11,12]. In endometrial carcinoma, EDG counts correlated significantly with the presence of angiolymphatic invasion, lymph nodes metastasis and tumor stage[9]. Ephrin-A1, formerly called B61, was found to be up-regulated during melanoma progression and implicated in angiogenesis[13,14]. Tnfaip2 (B94), originally identified as a tumor necrosis factor alpha-inducible gene in endothelial cells, was highly expressed in marrow from patients with acute myelogenous leukemia French-American-British subtypes M_0-M_2[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 β5, Col8A1 (procollagen, type VIII, alpha 1) and Pcdhb7 (protocadherin beta) in the Hca-F cell line.

In the category signal transduction, we observed up-

regulation of Cdc42ep5 (CEP5; Borg3), Rab3b, Lepr (leptin receptor), Ptprr (protein tyrosine phosphatase, receptor type, R) and F2r (coagulation factor II (thrombin) receptor; Par1; ThrR). Cdc42ep5, one member of CEPs which acts downstream of Cdc42 to induce actin filament assembly leading to cell shape changes, induced pseudopodia formation in NIH-3T3 fibroblasts[20]. In tumor, it might promote the ability of invasiveness and metastasis. In the highly lymphogenous metastatic pancreatic carcinoma cell line BSp73-ASML, the ras-related rab proteins and protein tyrosine phosphatases were all over expressed[16]. Lepr positive correlated significantly with distant metastasis and lower survival in breast cancer[21]. 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[16] and correlated with breast carcinoma cell invasion and metastasis[22,23]. Booden *et al*[24] 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[25]. 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[26,27].

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[28]. 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[29] and SLC16 and SLC2 were up-regulated in highly lymphogenous metastatic pancreatic carcinoma cell line BSp73-ASML[16]. Meanwhile, the reason Slc38a4 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[16]. 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[16,30].

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[31-35]. 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[36].

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[16,37]. It was reported that Fosl1 induces transformation and invasiveness of human epithelial adenocarcinoma cells[38]. 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[39].

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 M_r 47 000 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[40]. 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[41]. In addition, aberrant SNCG gene expression can occur via CpG island demethylation, and tends to occur during the more progressive stages of gastric carcinogenesis[42].

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[43]. 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*[44].

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[16,45-49]. AXL, 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[16,58-60]. EGF and AR might modulate invasion by increasing the expression of MMPs[61] or stimulating directional (chemotactic) and/or random (chemokinetic) motility in malignant cells[62]. 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[63-68]. Cav-1 was reported to be necessary for mediating filopodia formation in lung adenocarcinoma, which may enhance the invasive ability of cancer cells[67]. In an other study, caveolin-1 was shown to affect angiogenesis during the progression of clear cell renal cell carcinoma[69].

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 have not been validated to contribute to enhanced tumor metastatic properties straightly deserve further attention, for example, Slc38a4 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[1]. 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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