Analysis of Metastatic-Related Gene Expression in Gastric Cancer by Low-Density cDNA Microarrays

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OBJECTIVE To screen metastatic-related genes in human gastric cancer by a low-density cDNA microarray technique.

METHODS A total of 18 paired gastric cancer and adjacent normal mucosa were examined by a low-density cDNA microarray containing 23 genes. RT-PCR was used for further verification.

RESULTS The mRNA expression of MMP-7, heparanase, S100A4, hTERT, hRad17 in gastric cancers was higher than that in coupled normal mucosa (P=0.002, 0.00011, 0.000072, 0.002, 0.00016 respectively), whereas nm23H1, and CDH1 were lower (P=0.003, 0.012 respectively). The concordance was verified further by RT-PCR with a correlation coefficient of 0.774. In gastric primary lesions the mRNA expression of MMP-7, heparanase and S100A4 was higher in the serosa involved compared to non-involved (P=0.003, 0.009, 0.012 respectively), whereas nm23H1, CDH1, KAI1 were lower (P=0.001, 0.001, 0.006 respectively). With respect to the area of serosa involvement, MMP-7 and heparanase expressions were higher in an area of more than 20 cm^2 compared to an area of less than 20 cm^2 (P=0.001, 0.02 respectively), whereas nm23H1, CDH1 and KAI1 were lower (P=0.030, 0.041, 0.031 respectively). MMP-7 and hTERT expressions were higher in the heavier lymph node metastatic cases (no less than 7) than in the lighter lymph node metastatic cases (no more than 6, P=0.001, 0.005 respectively).

CONCLUSION Expression of MMP-7, S100A4, heparanase, hTERT, KAI1, CDH1 and nm23H1 correlated closely with invasion and metastasis in gastric carcinomas. The low-density cDNA microarrays can be used to examine the expression of many genes simultaneously, parallely and quickly.

KEYWORDS: gastric carcinoma, low-density cDNA microarray, metastasis.

Gastric cancer is very frequent in China and is often in an advanced stage at first presentation. Only 30%~40% of patients undergoing surgery have a curative resection. Metastasis and relapse are the common reasons for its poor prognosis, which involve many processes including adhesion, degradation, motility, angiogenesis, lymphogenesis and escape from immune surveillance controlled by many genes. Further studies and elucidation of the interaction among the genes would contribute to an understanding of the mechanisms of tumor metastasis. In this study we established a low-density cDNA microarray in which the target genes are closely related to metastasis based on past literature.[1-9] In order to evaluate the whole status and role in metastasis, the mRNA expression of these genes was examined simultaneously in paired gastric carcinoma primary lesions and normal mucosa. Some genes were studied further for confirmation by RT-PCR.

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MATERIALS AND METHODS

Patients and tissue samples

A total of 18 primary gastric cancers and corresponding normal mucosas were obtained randomly with informed consent from the patients who underwent gastrectomy in the Surgical Oncology Department, the First Hospital of China Medical University. The patients received no chemotherapy and/or radiotherapy prior to surgery. Tissue samples were snap-frozen in liquid nitrogen, then stored at -70℃. Each sample was examined histologically by using H&E-stained cryostat sections. Clinical and pathological features were recorded in detail.

Construction of the low-density cDNA microarray

Such genes as beta actin, glyceraldehyde phosphate dehydrogenase (GAPDH), heparanase, matrix metalloproteinase 2 (MMP-2) and matrilysin (MMP-7), vascular endothelial growth factor C (VEGF-C), S100A4, hRad17, human telomerase reverse transcriptase (hTERT), transforming growth factor beta (TGF-beta), CD44s, integrin beta, E-cadherin (CDH1), KAI1 (CD82), tissue inhibitors of metalloproteases 1 and 2 (TIMP1,2), pTEN, nm23H1 and hepatitis B were selected as targets. The primers were designed with Primer Premier 5.0, and the product length was between 189 and 1078 bp. Beta actin and GAPDH were selected as internal controls, hepatitis B as a negative control and spotting solution as a blank control. The products were obtained by RT-PCR, and purified using ethanol and then dissolved in 50% dimethyl sulfoxide (DMSO). The concentration of the targets were adjusted above 0.5 μg/μl. The low-density cDNA microarray was prepared by printing targets onto the aminoslides (CEL Co.) using Micro Grind II gene chip spotting equipment (England). The array was 14×14.[10,11]

Probe preparation

Total RNA from 18 fresh gastric carcinomas and coupled normal mucosas were extracted with Trizol (Invitrogen Co.) reagents according to the protocol supplied by the manufacturer. A UV300 spectrophotometer was used to determine the A_{260}/A_{280} ratio and 1% formaldehyde denaturing agarose gel electrophoresis was used to assess the purity, quality and integrity of total RNA. For each labeling reaction, 100 μg high-quality total RNA was used. First-strand cDNA synthesis was primed with oligo $dT_{(18)}$ primer, and simultaneously incorporated fluorescently labeled deoxyribonucleotides (Cy5-dUTP, Pharmacia Co.). Reactions were quenched by the addition of 0.5 mol/L EDTA and the RNA template was hydrolyzed by the addition of 1 mol/L NaOH followed by heating at 68℃ for 30 min. Reactions were neutralized with 1 mol/L Tris-HCl, and the labelled cDNA precipitated by ethanol after which it was dissolved in hybridization buffer.

Hibridization, washing, detection and analysis

The probe mixtures were denatured by heating at 95℃ for 3 min and placed on ice. Then the fluorescently-labeled cDNA probe (gastric carcinoma or normal mucosa) was added onto the prepared chip and covered with silicon alkylating glass. The chips were incubated at 60℃ for 18 h. After hybridization the chips were washed in a solution of 1×SSC/0.1%SDS and 0.1×SSC for 5 min at room temperature respectively, then dried and scanned by a Gene TAC™ LS (USA). The mean signal intensity of each spot was analyzed with analysis soft ware in a scanner.

Verification by RT-PCR

In order to know the reliability of the low-density cDNA microarray, 5 genes were selected randomly, such as GAPDH, heparanase, MMP-7, KAI1 and TIMP-1, and the mRNA expressions in the above samples were examined by the reverse transcription polymerase chain reaction (RT-PCR). The two results were compared to determine the concordance.

Statistical analysis

The comparison of different gene expression between gastric carcinoma and coupled normal mucosa was conducted by a paired-samples *t* test. The comparison of different groups in gastric carcinoma was conducted by one-way ANOVA. The correlation of the gene expression between the microarray and RT-PCR was conducted by Pearson Correlation Analysis. All the analysis was finished by SPSS 11.5 soft ware.

RESULTS

The target genes on microarry

Figs. 1 and 2 show the purified target genes on ployacrylamide gel electrophoresis and silver staining. The purity of target genes was very good and there was no apparent additional bands. Each band in DL2000 and 100 bp markers was about 50 ng as the control. The A_{260} of the purified product was assayed and the concentrations of the target genes were adjusted above 0.5 μg/μl.

Results of the low-density cDNA microarray

The hybridization had a good specificity as there were no signals in both blank and negative control. Beta actin and GAPDH as the internal controls had good and accordant signals. The house keeping genes were selected to normalize in the same microarray, then the normalized signals of all target genes were compared.

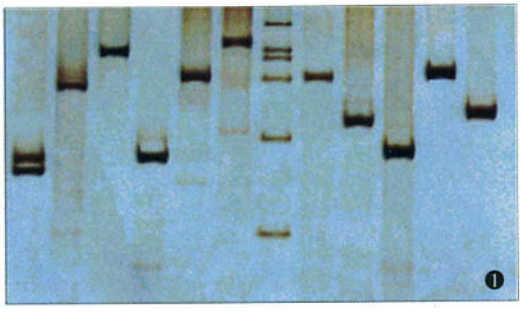
Comparison of all the gene expression in gastric cancer and coupled normal mucosa

The expressions of MMP-7, heparanase, S100A4, hTERT and hRad17 in gastric carcinoma primary lesions were higher than the coupled normal mucosa (P=0.002, 0.00011, 0.000072, 0.002, 0.00016 respectively); whereas the expressions of nm23H1 and CDH1were lower (P=0.003, 0.012 respectively). There was no significant difference among other genes. Figs. 3 and 4 show the typical low-density cDNA microarray hybridizations in a gastric carcinoma primary lesion and normal mucosa respectively.

The correlation between gene expression in primary lesions and biobehavior of gastric carcinomas

According to the invasion depth of the gastric cancers, the samples were divided into 2 groups: one was serosa-involved including se (serosa) and si (serosa infiltration); the other was non-serosa involved including m (mucosa), sm (submucosa), mp (muscularis propria) and ss (subserosa). Among the serosa-involved group the expression of MMP-7, heparanase and S100A4 increased more markedly than the non-serosa involved (P=0.003, 0.009, 0.012 respectively), but the expression of nm23H1, CDH1 and KAI1 decreased more (P=0.001, 0.001, 0.006 respectively). There was no significant difference among other genes.

On the basis of area of serosa involvement the samples were also divided into 2 groups: less than 20 cm^2 was regarded as the lighter; more than 20 cm^2 as the heavier. Among the heavier group the expression of MMP-7 and heparanase markedly increased (P=0.001, 0.02 respectively), but nm23H1, CDH1 and KAI1 ex-



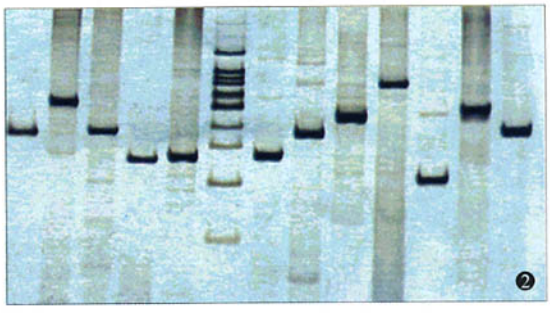


Fig.1. The purified target genes on ployacrylamide gel electrophoresis and silver staining (from left to right was actin1,2,3, GAPDH1,2,3, DL2000 marker, integrin-beta, VEGF-C, TGF-beta, heparanase, hTERT).

Fig.2. The purified target genes on ployacrylamide gel electrophoresis and silver staining (from left to right was HBV, TIMP-1, MMP-7, MMP-2, TIMP-2, 100 bp marker, S100A4, hRad17, CD44s, CDH1, pTEN, KAI1, nm23H1).

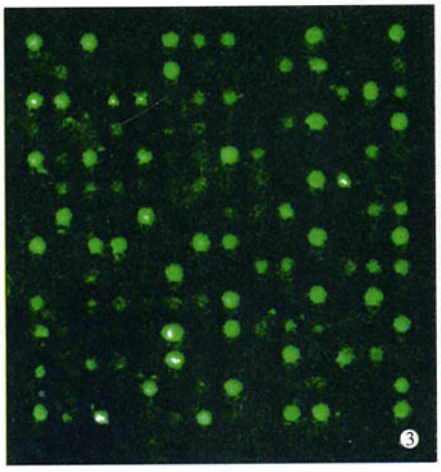


Fig.3. Low-density cDNA microarray hybridization from a gastric carcinoma primary lesion.

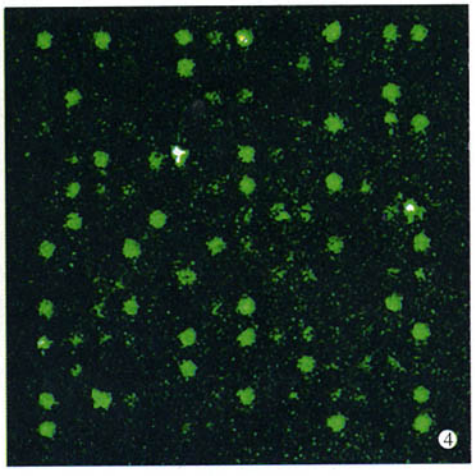


Fig.4. Low-density cDNA microarray hybridization from coupled gastric normal mucosa.

pression decreased (P=0.030, 0.041, 0.031 respectively). There was no significant difference among other genes. With respect to the number of lymph node metastasis, 2 groups were formed: no more than 6 was regarded as the lighter; no less than 7 as the heavier. The expressions of MMP-7 and hTERT in the heavier group were higher than that in the lighter group (P= 0.001, 0.005 respectively), whereas the expression of CDH1 was lower (P=0.063), perhaps due to the lower number of patients. There was no significant difference among other genes. There was no significant correlation between gene expression and other clininco-pathological factors, such as surgery type, growth pattern, differentiation degree, serosa type, histology type, lymphatic cancer embolus or vein cancer embolus, etc.

Reliability of hybridization

The expression of GAPDH, heparanase, MMP-7, KAI1, TIMP-1 in 18 samples were examined by RT-PCR. The intensity of each gene amplification band was measured by Chemi Imager 5500, and then the expression index ($I=A_{gene}/A_{GAPDH}$) was calculated. A concordance result was seen between the cDNA microarray (normalized) and RT-PCR, The Pearson Correlation Index was 0.774 (P<0.0001).

DISCUSSION

Gene chips have received widespread attention over the last few years, and have been used for studies in many fields.[12-16] Because of the simultaneous, parallel and rapid advantage, expression gene chips have been used widely and made an important contribution to expression profiling analysis. During the course of the onset and development of some diseases only a fraction of genes (20~30 or hundreds) varied in their expression profile from recent literature reports. [17-19] However, with respect to high density cDNA microarrays, due to the high expense, fixed and many target genes and lack of individuation there is a waste of resources for research. So we built a stable low-density cDNA microarry technique in which all target genes had a close relationship to metastasis of gastric cancer based on past research. This low-density cDNA microarray was designed with ideal characteristics, so it could be used for early diagnosis of metastasis and relapse in gastric cancer.[11]

Nowadays, most conventional cDNA microarray techniques use double fluorescence labeling (Cy3 and Cy5). However, Cy3 and Cy5 fluorescein differ in their incorporating rate and stimulating wave-length during reverse transcription and scanning. Cy3-dUTP has a higher incorporating rate than Cy5-dUTP, so the ratio of Cy3 and Cy5 is used for normalization. In this study, in order to avoid the influence of different fluorescein incorporating rates, we adopted single fluorescein labeling, and normalized the results with house keeping genes as an internal control. The total RNA quantity of different specimens was equal (100 μg). So the results were more comparable. Moreover, 5 genes chosen randomly were examined in corresponding samples by RT-PCR. The normalized results were in accord with the low-density cDNA microarray resulting in a correlation coefficient of 0.774. The conclusion therefore was quite credible.

Invasion and metastasis of carcinoma is a multi-gene process, multi-factor and multi-step complex course, including adhesion, degradation of the extracellular matrix and vessel basement membrane, angiogenesis and lymphogenesis, tumor cell movement and so on. In this study we showed the following results: when heparanase, MMP-7 and S100A4 were overexpressed and KAI1, CDH1 and nm23H1 were underexpressed in gastric primary carcinomas, the cancer cells had more invasiveness and always penetrated the serosa. With MMP-7 and heparanase overexpression and nm23H1, CDH1 and KAI1 underexpression, the area of serosa involvement by the cancer cell was bigger, and more than 20 cm^2. When MMP-7 and hTERT were overexpressed and CDH1 underexpressed, the number of metastatic lymph nodes was more and the extent was heavier. In a word, the gastric carcinoma had a stronger invasiveness and metastatic potential if the expression of heparanase, MMP-7 and S100A4 were higher and KAI1, CDH1 and nm23H1 were lower. The degree of lymph node metastasis was more serious in patients with MMP-7 and hTERT mRNA overexpression and CDH1 underexpression. So we concluded that above genes have a close relationship with gastric cancer invasion and metastasis. These genes may have a fundamental role in tumor development and may be regarded as better molecular targets for early diagnosis and treatment in gastric carcinoma.

Heparanase, an endoglycosidase , can degrade heparan sulfate (HS) and heparan sulfate proglycans (HSPGs), which constitute the main components of the extracellular matrix (ECM) and vessel basement membrane. MMP-7, also called matrilysin, can degrade the structural proteins of the ECM, which is an essential step for cancerous cell invasion and metastasis. [2,3] S100A4 (h-mts1) is a member of the S100 gene fami-

ly, coding for a calcium-binding protein. Filamentous actin, non-muscle myosin, and non-muscle tropomyosin had been suggested as target molecules for S100A4 so as to alter cytoskeletal dynamics and cellular motility. It has been called a metastatic-associated gene whose expression showed a strong correlation with the proliferative potential, invasion and metastatic ability of cancers.[1,20] A decrease in adhesiveness of cancer cells can lead to cell scatter, which is believed to be a first step of tumor metastasis. E-cadherin (CDH1), calcium-dependent transmembrane glycoprotein, is the strongest molecule for homophilic adhesion of epithelial cells. Its reduction in expression results in the decrease of cancer cell adhesive ability and cell dispersion occured. This has been shown in many carcinomas. S100A4 has a close relationship with E-cadherin, the invasion potential of S100A4 overexpression maybe partly due to E-cadherin underexpression,[20] which was also observed in this study.

Human telomerase reverse transcriptase (hTERT) had been identified as a putative catalytic subunit of human telomerase and is the major determinant of human telomerase activity. hTERT mRNA expression has a coincidence with telomerase activity, which is associated with clinicopathological and lymph node metastasis in many tumors.[7,21] Nm23H1 is a generally accepted metastasis-suppressor gene, which can alter cytoskeletal dynamics so as to suppress tumor cell motility. KAI1 specifies a protein of 267 amino acids, with four hydrophobic and presumably transmembrane domains and one large extracellular hydrophilic domain with three potential N-glycosylation sites, which correlates with the tumor metastatic phenotype and may take a role in the interaction of cells or between cells and the extracellular matrix.[22] Down-regulation of KAI1 was observed in a variety of malignancies, such as colon, prostate, esophagus, gastric, hepatic, bladder and ovary cancers and so on, which was concordant with low differentiation, lymph node metastasis, blood dispersion or peritoneum implantation of carcinoma.[23,24]

Presently there have been some reports that high density cDNA microarrays have been used to detect gene expression profiling of different tumors for molecular stage, molecular type and prognosis assessment.[16,25,26] Although the selected genes in this study were less in comparison with all the genes involved in gastric cancer invasion and metastasis, we had established a stable low-density cDNA microarray technique. Newly discovered genes related to gastric cancer can be added continuously, then the specific metastatic related microarray for gastric cancer will have been accomplished. This can help us to know the molecular type and stage, biobehavior and prognosis, to make an early diagnosis of metastasis and relapse, to select the molecular targets for treatment and to elevate diagnosis and therapy levels for gastric cancer patients.

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