A microarray-based gastric carcinoma prewarning system

Da-Xiang Cui, Li Zhang, Xiao-Jun Yan, Ling-Xia Zhang, Jun-Rong Xu, Yan-Hai Guo, Gui-Qu Jin, Giovani Gomez, Ding Li, Jin-Rong Zhao, Fen-Chan Han, Ju Zhang, Jia-Le Hu, Dai-Ming Fan, Hua-Jian Gao

Abstract
AIM: To develop a microarray-based prewarning system consisting of gastric cancer chip, prewarning data and analysis software for early detection of gastric cancer and pre-cancerous lesions.

METHODS: Two high-density chips with 8 464 human cDNA sites were used to primarily identify potential genes specific for normal gastric mucosa, pre-cancerous lesion and gastric cancer. The low-density chips, composed of selected genes associated with normal gastric mucosa, precancerous lesion and gastric cancer, were fabricated and used to screen 150 specimens including 60 specimens of precancerous lesion, 60 of gastric cancer, pre-cancerous lesion from normal gastric tissues. All data were compiled into a prewarning database by CGO software. Northern blot and immunohistochemistry analysis confirmed that gene and protein of \textit{brca1} and \textit{ndr1} identified may be used to distinguish gastric cancer status and non-cancer status.

RESULTS: A total of 412 genes associated with three stages of gastric cancer development were identified. There were 216 genes displaying higher expression in gastric cancer, 85 genes displaying higher expression in pre-cancerous lesion and 88 genes displaying higher expression in normal gastric mucosa. Also 15 genes associated with metastasis of gastric cancer and 8 genes associated with risk factors were screened out for target genes of diagnosis chip of early gastric cancer. The threshold values of 412 selected genes to distinguish gastric cancer, pre-cancerous lesion from normal gastric mucosa were defined as 6.01±2.40, 4.86±1.94 and 5.42±2.17, respectively. These selected 412 genes and critical threshold values were compiled into an analysis software, which can automatically provide reports by analyzing the results of 412 genes obtained by examining gastric tissues. All data were compiled into a prewarning database for gastric cancer by CGO software. Northern blot and immunohistochemistry analysis confirmed that gene and protein of \textit{brca1} displayed lower expression in normal gastric mucosa and higher expression in gastric cancer tissues, conversely, \textit{ndr1} displayed lower expression in gastric cancer and higher expression in normal gastric mucosa.

CONCLUSION: The microarray-based prewarning system for gastric cancer was developed. This system consisted of gastric cancer-associated gene chip, prewarning data and analysis software, which has a high potential for applications in the early detection of gastric cancer. The two potential markers \textit{brca1} and \textit{ndr1} identified may be used to distinguish cancer status and non-cancer status.

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Key words: Microarray; Prewarning; Gastric cancer

INTRODUCTION
Gastric cancer has high incidence in China and in the whole world. Understanding the biological processes of cancer initiation at the gene expression level is very important for early cancer detection. Study of gene expression levels at different stages of growth, disease, cell cycle, and response to stimulation may help to answer why different stages of cancerous development occur[1]. We have been trying to establish a prewarning system of gastric cancer as a part of a larger effort to develop effective and economical diagnostic tools capable of distinguishing different stages of cancer.
development. This system consists of three important parts: a gastric cancer microarray, a prewarning data library and a data analysis software.

Screening characteristic differentially expressed genes associated with different stages of cancer development is of central significance to this study. In our previous studies\(^\text{(5,6)}\), some differentially expressed genes between gastric cancer tissues and precancerous lesions have been obtained. Genes that have been shown to correlate with gastric cancer were used as a part of the target genes in the microarray. Commercially available microarrays with 8 464 human cDNA sites have also been used for identifying specific genes associated with normal mucosa, precancerous lesions and gastric cancer.

The gene microarray technique has the advantage of simultaneously monitoring the expression of thousands of genes in one hybridization experiment. This technique has greatly facilitated the detection of differentially expressed genes and the construction of gene expression profiles. Since 1995, the DNA microarray technique has been widely employed to investigate the functions of genes, especially those genes involved in tumor generation and growth\(^\text{(7)}\). This technique has a great potential as a practical clinical tool for medical diagnosis\(^\text{(8)}\). Although many genes are known to be related to the pathological process of gastric carcinoma, so far very few prognostic biomarkers of gastric cancer have actually been used in clinical medicine. In our present study, we tried to identify specific genes involved in gastric carcinogenesis, with the objective of establishing a prewarning system for early diagnosis, therapy and prevention of gastric cancer.

**MATERIALS AND METHODS**

**Resource of tissue specimens**

Specimens used in this study were classified into three different categories: those of gastric cancer (including all types of pathologic gastric cancers such as diffuse type and intestinal type), those of paracancerous lesions (according to international classified standard including atrophic gastritis, intestinal gland metaplasia, atypical hyperplasia) and those of normal gastric mucosa (including slight superficial gastritis). A total of 150 specimens including 60 gastric cancers, 60 pre-cancerous lesions and 30 normal gastric mucosa were obtained. Genes from normal gastric mucosa tissues with Cy3-dUTP, those from gastric cancer tissues and precancerous tissues were labeled with Cy5-dUTP, those from normal gastric mucosa tissues with Cy3-dUTP. The labeled probes were mixed, fragmented and precipitated by ethanol and dissolved in 20 μL hybridization solution (5×SSC+2 g/L SDS).

**Fabrication of microarrays**

Microarrays consisting of 2 435 fragment sites including 412 genes were fabricated. These synthesized oligonucleotide DNAs were first dissolved in 3× SSC solution. Spot report oligo array validation system (Cat # 252170-7) was used as quality control. Spots with pure 3× SSC solution were selected as background control. The target genes were spotted on silylated slides by MicroGridII spotting robotics (BioRotics Inc.). After spotting, the slides were hydrated (2 h), dried (0.5 h, RT), UV crosslinked (65 mJ/cm), and then treated with 2 g/L SDS (10 min), H₂O₂ (10 min), and 2 g/L NaBH₄ (10 min). The slides were dried before being made ready for usage.

**Extraction of total RNAs and probe preparation**

Total RNA extraction was performed by using total RNA extract kit from Promega Inc. Final total RNA templates were dissolved with non-RNase and non-DNase Milli-Q H₂O. Fluorescent cRNA probes were prepared through reverse transcription and then purified, referring to the protocol of Schena et al.

**Hybridization and washing**

After denatured at 95 °C for 5 min, the probes were added onto slides, covered with a cover glass and incubated at 42 °C for 17 h. The slides were subsequently washed in solutions of 2× SSC+2 g/L SDS, 0.1× SSC+2 g/L SDS and 0.1× SSC, 10 min each time, and then dried at room temperature.

**Detection and analysis**

Microarrays were scanned by using Affymetrix® 428™ array scanner. ImageGene 3.0 software (BioDiscovery Inc.) was used to quantify, correct for background noise and normalize the signals from post-hybridization chip.

**Construction of prewarning data library**

The data files were incorporated into a computer database by CGO software, including patient disease history and all screened results, such as, name, file number, sex, age, address, telephone, e-mail address, marital status, blood type, body mass, disease history, imaging examination, pathological examination, serum examination, blood examination, cytogenetic report, and gene array report.

**Threshold values of expression profiles**

Expression gene profiles were established according to the acquired data. CAD software was used in the selection of discriminating candidate genes by their correlation with three kinds of gastric tissues, determination of the optimal set of reporter genes by using a leave-one-out validation procedure, determination of the threshold values of selected gene expression levels to distinguish normal gastric mucosa from pre-cancerous lesions and gastric cancer, and metastatic cancer and no-metastatic cancer.
Analysis software for gastric cancer prewarning data
A total of 412 genes and critical threshold values to distinguish normal gastric mucosa from pre-cancerous lesion and gastric cancer were compiled into an analysis software, which could provide analysis reports by analyzing the microarray test results.

Northern blot analysis
Five micrograms of mRNA was resolved by denaturing formaldehyde agarose gel and transferred onto hybrid membranes (Amersham). The membranes were hybridized with \(^\text{32}^\text{p}\)-labeled fragments of cDNA overnight, washed twice in 1 g/L standard saline citrate and 1 g/L SDS for 20 min and then exposed to Kodak BioMax film at -80 °C with an intensifying screen for 24 h.

Immunohistochemistry analysis
Standard avidin-biotin complex (ABC) technique was used for immunohistochemical staining of formalin-fixed, paraffin-embedded gastric cancer tissues. Specific antibody (10 mg/L) and PBS were added onto tissue slides previously blocked with rabbit serum and incubated overnight. After washing with PBS, the slides were incubated with a rabbit anti-human IgG conjugated to biotin at room temperature for 1 h, alkaline phosphatase substrate was then added for color development. The slides were counterstained with hematoxylin-eosin.

Statistical analysis
A two-way clustering analysis was performed by using Cluster software and Tree view software from http://www.microarray.org (PNAS 1998; 95:14863). Statistical analysis was performed by using the \(t\) test. All \(P\) values were based on two-sided testing, and a significant difference was defined as \(P\) less than 0.05.

RESULTS
Screened genes associated with normal gastric mucous, pre-cancerous lesion and gastric cancer
Two high-density chips were used to primarily screen differential genes associated with normal gastric mucosa, pre-cancerous lesion and gastric cancer. According to the obtained partial biochip hybridization results, 393 genes closely associated with three stages of gastric cancer development were primarily screened out (Figure 1). Fifteen genes associated with gastric cancer metastasis and 8 genes associated with risk factor genes of gastric cancer, such as cagA, vacA, Ure, EB, were selected according to the literature\(^9\). These genes were used as main target genes on the prewarning chip. The oligonucleotides associated with 412 genes were designed, synthesized and fabricated into low-density chip.

One hundred and fifty specimens screened by low-density chip
All the 150 specimens with clear pathological results were screened with the fabricated low-density microarrays. Among these, 60 were known to be cancerous, 60 precancerous and 30 normal (Figure 2). In the 60 cancer specimens, 216 genes were found to exhibit higher expression levels than those in normal gastric mucosa. Among the 216 genes, 156 also exhibited higher expression levels than those in the precancerous lesions (Table 1). In the 60 specimens of Precancerous lesions, 126 genes exhibited higher expression levels than those in the normal tissues. Among those, 85 genes also showed higher expression levels than those in the gastric cancer tissues (Table 1). Contrary to our initial expectations, selected risk factor genes such as cagA, vacA, Ure, EB did not show overexpression levels in gastric cancer tissues in comparison with the normal tissues and precancerous lesions. In fact, these genes showed lower expression levels in gastric cancer tissues than in normal tissues and precancerous lesions. This result demonstrated that the risk factor genes due to \(H\) pylori infection might be more closely associated with the progression of precancerous lesion. Eighty-eight genes in normal tissues exhibited higher expression levels than those found in gastric cancer tissues and pre-cancerous tissues (Table 1). These genes are helpful for distinguishing normal gastric mucosa from precancerous lesions. This is very important in diagnosing the precancerous lesion among common gastric diseases, such as superficial gastritis, because the treatment of precancerous lesion requires special focused methods. If left untreated, precancerous lesion might result in gastric cancer in a limited time.

Construction of prewarning database library of gastric cancer
The gene expression profiles of each specimen obtained by biochip were stored together with patient clinical data.
including follow-up treatments until death. The data files were incorporated into a computer database by CGO software, including patients’ disease history and all screened results such as name, file number, sex, age, address, telephone, e-mail address, marital status, blood type, body mass, disease history, imaging examination, pathological examination, serum examination, blood examination, cytogenetic report, gene array report. The prewarning data were added with new content. These data would be available on Gastric Cancer Information Web presided by Dr. Cui at http://www.37c.com.cn.

Critical threshold values to distinguish normal gastric mucosa from pre-cancerous lesion and gastric cancer

A total of 412 genes were selected as the main diagnostic genes, including 216 genes that displayed higher expression levels in cancer tissues than in non-cancer tissues, 85 genes with higher expression levels in precancerous lesions than in cancer tissues and 88 genes that exhibited higher expression levels in normal tissues than in gastric cancer tissues and pre-cancerous tissues. We selected 15 genes associated with metastasis of gastric cancer as metastasis biomarkers, 8 risk factor genes as reference biomarkers to predict the development of pre-cancerous lesions (Table 1). The critical threshold values to distinguish normal gastric mucosa from pre-cancerous lesion and gastric cancer were decided and were summarized in Table 2.

**Table 1 Differentially expressed genes in prewarning microarray of gastric cancer**

| GenBank | Number | Description of gene |
|---------|--------|---------------------|
| Highly expressed genes in gastric cancer |
| 1       | NM_001962 | Homo sapiens ephrin-A5 (EFNA5) |
| 2       | XM_017384 | Homo sapiens matrix metalloproteinase 7 (MMP7) |
| 3       | NM_008610 | Mus musculus matrix metalloproteinase 2 (Mmp2) |
| 4       | XM_004995 | Homo sapiens matrix metalloproteinase 14 (MMP14) |
| 5       | AF003573 | Bos taurus angiopoietin-1 (ang-1) |
| 6       | AF004327 | Homo sapiens angiopoietin-2 |
| 7       | M11730  | Human tyrosine kinase-type receptor (HER2) |
| 8       | U13948  | Human zinc finger/leucine zipper protein (AF10) |
| 9       | XM_049646 | Homo sapiens similar to octamer-binding transcription factor 3B (OCT-3B) |
| 10      | XM_055784 | Homo sapiens fibroblast growth factor 2 (basic) (FGF2) |
| 11      | XM_056035 | Homo sapiens proliferating cell nuclear antigen (PCNA) |
| 12      | L24203  | Homo sapiens ataxia-telangectasia group D-associated protein |
| 13      | XM_087201 | Homo sapiens similar to RED protein, IK cytokine |
| 14      | X00663  | Human mRNA fragment for epidermal growth factor (EGF) receptor |
| 15      | NM_002607 | Homo sapiens platelet-derived growth factor alpha polypeptide (PDGFA) |
| 16      | XM_165656 | Homo sapiens matrix metalloproteinase 2 (MMP2) |
| 17      | NM_005918 | Homo sapiens malate dehydrogenase 2, NAD (mitochondrial) (MDH2) |
| 18      | AF503165 | Homo sapiens HUS1 checkpoint homolog (HUS1) gene |
| 19      | XM_045667 | Homo sapiens antigen identified by monoclonal antibody Ki-67 (MK67) |
| 20      | XM_05913 | Homo sapiens frequently rearranged in advanced T-cell lymphomas (FRAT1) |
| 21      | XM_032866 | Homo sapiens signal transducer and activator of transcription 5A (STAT5A) |
| 22      | NM_004103 | Homo sapiens protein tyrosine kinase 2beta (PTK2B) |
| 23      | XM_008355 | Homo sapiens membrane protein, palmitoylated 2 (MPP2) |
| 24      | L18920  | Human MAGE-2 gene exon 2, 3, 4 |
| 25      | M12174  | Homo ras-related rho |
| 26      | NM_01233 | Homo sapiens c-myc binding protein (MYCBP) |
| 27      | BC016514 | Homo sapiens, similar to translocated promoter region (to activated MET oncogene) |
| 28      | NM_004324 | Homo sapiens BCL-2 associated X protein (BAX) |
| 29      | Z26500  | Cyclin A |
| 30      | D45906  | LIMK-2 |
| 31      | D21255  | OB-cadherin |
| 32      | X54925  | Type I interstitial collagenase |
| 33      | X05232  | Stromelysin, matrix metalloproteinase 3 |
| 34      | M26126  | Human pancreatic trypsin 1 (TRP1) |
| 35      | XM_055254 | Homo sapiens fibronectin 1 (FN1) |
| 36      | AF081127 | Danio rerio fibronectin (fn2) |
| 37      | M15796  | Human cyclin protein gene |
| 38      | HSFBEDA | Human fibronectin gene ED-A region |
| 39      | HSU6406 | Human putative EPH-related PTK receptor ligand LERK-8 (Epig8) |
| 40      | AF06846 | Homo sapiens scaffold attachment factor A (SAF-A) |
| 41      | HS8TRCP | Homo sapiens mRNA for beta-transducin repeat containing protein |
| 42      | AI10763 | Homo sapiens skeletal muscle LIM- protein 1 (FHL1) gene |
| 43      | HUMHO2SC01 | Human mRNA for home oxygenase-2 |
| 44      | HNMISH16 | Human mutator hMSH2 gene |
| 45      | HSHEKH1 | Homo sapiens mRNA for EHK-1 receptor tyrosine kinase |
| 46      | HSKLCN30 | Homo sapiens mRNA for unknown antigen |
| 47      | AF009304 | Homo sapiens mRNA for SH3 binding protein |
| 48      | AF070561 | Homo sapiens clone 24703 beta-tubulin |
| 49      | HUMCAMIV | Homo vascular cell adhesion molecule 1 |
| 50      | HSRNASMG | Homo sapiens mRNA for Smn protein G |
| 51      | X83228  | Homo sapiens mRNA for L1-cadherin |
| 52      | AF12100 | Homo sapiens HSPC039 protein |
| 53      | HSU97018 | Homo sapiens echinoderm microtubule-associated protein homolog HuEMAP |
| 54      | HUS41388 | Human Ets transcription factor (NERF-2) |
| 55      | HSY17392 | Homo sapiens mRNA for prefoldin subunit 1 |
| 56      | HSU08316 | Human insulin-stimulated protein kinase 1 (SPK-1) |
| 57      | HZNF232G2 | Homo sapiens zinc finger protein ZNF232, exons 2 and 3 |
| 58      | HUMP3ST | Homo sapiens mRNA for L1-cadherin |
| 59      | J03040  | Human mRNA |
| 60      | XM_035809 | Homo sapiens similar to chondroitin sulfate proteoglycan 2 (versican) |
| 61      | L40379  | Homo sapiens thyroid receptor interactor (TRIP10) |
| 62      | HSU72069 | Human karyopherin beta2 |
| 63      | HUMPCK2 | Human phosphoglycinate kinase (pgk) mRNA, exons 2 to last |
| 64      | HSU07139 | Human insulin-stimulated protein kinase 1 (SPK-1) |
| 65      | XM_01472 | Homo sapiens v-jun sarcoma virus 17 oncogene homolog (avian) (JUN) |
| 66      | AU100088 | Human phosphoglucomutase (PGDM) gene |
| 67      | HUMLRCPZ | Human Kruppel related zinc finger protein (KLF10) |
| 68      | AF07050 | Homo sapiens neuroendocrine-specific protein C homolog |
| 69      | HUMSC35A | Human splicing factor SC35 |
| 70      | HUMPTPB | Homo sapiens protein tyrosine phosphatase (CIP2) |
| 71      | AF049608 | Homo sapiens monocarboxylate transporter 2 (MC2T2) |
| 72      | HUMHEK | Human receptor tyrosine kinase (HER) |
| 73      | J03210  | Human collagenase type IV |
| 74      | HSRA8P90 | Homo sapiens mRNA for Rab8a effector p0 |
| 75      | AF184924 | Homo sapiens zinc finger transcription factor BTER2 gene |
| Accession       | Description                                                                 | Reference                                                                 |
|-----------------|------------------------------------------------------------------------------|---------------------------------------------------------------------------|
| HUMC5A2A        | Human fibrillar collagen (pro2A (V) gene)                                    |                                                                            |
| HUMC5APA        | Human GTPase-activating protein ras p21 (RASAJ)                               |                                                                            |
| HUMCGLBLS       | Human glutamate receptor subunit (GluH1)                                     |                                                                            |
| AF047715        | Homo sapiens A-kinease anchoring protein (AKAP18)                            |                                                                            |
| HSU40282        | Homo sapiens integrin-linked kinase (LIK)                                    |                                                                            |
| HSATPFLM        | Human mRNA for mitochondrial ATP synthase (F1-ATPase) alpha subunit           |                                                                            |
| AF152485        | Homo sapiens protochelin alpha 7 short form protein (PCDH-alpha7)            |                                                                            |
| HSRP19          | Human mRNA for 19 ku protein of signal recognition particle (SRP)            |                                                                            |
| U17195          | Homo sapiens A-kinease anchor protein (AKAP100)                              |                                                                            |
| HSU72999        | Human neuronal o lactomedin-related ER localized protein                      |                                                                            |
| XM_037859       | Human focal adhesion kinase (FAK)                                            |                                                                            |
| HSU04209        | Human-associated microfilibril protein                                       |                                                                            |
| D82878          | Hemicentinotus pulherrimus mRNA for p34cdc2                                  |                                                                            |
| AF060515        | Homo sapiens cyclin K (CPR4)                                                 |                                                                            |
| D21262          | Human mRNA for KIAA0035 gene                                                 |                                                                            |
| NM_005641       | Homo sapiens TATA box binding protein-associated factor, RNA polymerase II, 85 ku |                                                                            |
| HSU07550        | Human chaperonin 10                                                         |                                                                            |
| X82153          | Homo sapiens mRNA for calretinin 0                                          |                                                                            |
| HSU41766        | Human metalloprotease/disintegrin/cysteine-rich protein precursor (MDC9)     |                                                                            |
| AB017019        | Homo sapiens mRNA for JKTBP2                                                |                                                                            |
| HUMNC           | Human cellular fibroenectin                                                  |                                                                            |
| U93303          | Homo sapiens thyroglobulin (TG)                                              |                                                                            |
| AF0504354       | Homo sapiens protocollagen-3 (PRC3) gene                                     |                                                                            |
| HSUMC0LIX       | Homo sapiens collagen alpha 3 type IX (COL9A3)                               |                                                                            |
| NM_002427       | Homo sapiens metalloproteinase 13 (MMP13)                                    |                                                                            |
| AF039747        | Homo sapiens cadherin-10 (CDH10)                                            |                                                                            |
| AF072242        | Homo sapiens methyl-CpG binding protein MDB2 (MDB2)                          |                                                                            |
| HSMYCC          | Human c-myc oncogene                                                        |                                                                            |
| HSTSPM          | Homo sapiens tissue specific mRNA                                            |                                                                            |
| HSU64517        | Human Crk-associated substrate related protein Cas-L                          |                                                                            |
| HSVACM1         | Homo sapiens mRNA for vasopresin-activated calcium mobilizing receptor-like protein |                                                                            |
| HUMPA1H         | Homo pro-alpha-1 (V) collagen                                                |                                                                            |
| AF059611        | Homo sapiens nucleotidase protein NRP/B (NRPB)                               |                                                                            |
| HSU304845       | Homo alfa (I) collagen (COL4A6)                                             |                                                                            |
| M87860          | Human S-lac lectin L-14-II (LGALS2) gene                                     |                                                                            |
| AF492837        | Human mRNA for osteopontin                                                  |                                                                            |
| HSFCOXB         | Homo sapiens coxsubIII mRNA for cytochrome c oxidase subunit IIib            |                                                                            |
| U01244          | Human fibulin-1D                                                            |                                                                            |
| U52153          | Human invasory rectifying potassium channel Kir3,2                           |                                                                            |
| S66427          | RBP1=retinoblastoma binding protein 1 [human, Nalm-6 pre-B cell leukemia, mRNA, 4854 nt] |                                                                            |
| AF117108        | Homo sapiens IGF-II mRNA-binding protein                                     |                                                                            |
| HSU40983        | Human cell surface heparin binding protein HIP                               |                                                                            |
| HSU59289        | Human I3-cadherin                                                           |                                                                            |
| HSU95032        | Human growth-arrest-specific protein                                         |                                                                            |
| HSU18018        | Homo E1 enhancer binding protein (E1A-F)                                    |                                                                            |
| HUMCGRPB        | Homo sapiens (clone HSNM29) CGRP type 1 receptor                             |                                                                            |
| X59543          | Human mRNA for M1 subunit of ribonucleotid reductase                         |                                                                            |
| AF072810        | Homo sapiens transcription factor WSTF                                       |                                                                            |
| AF005068        | Homo sapiens breast and ovarian cancer susceptibility protein splice variant (BRCA1) |                                                                            |
| HSU66197        | Human fibroblast growth factor homologous factor 1 (FHF-1)                   |                                                                            |
| HUMVTRN         | Human cell adhesion protein (vitronectin) receptor                            |                                                                            |
| Gene Accession | Gene Description |
|---------------|------------------|
| HSU08316      | Human insulin-stimulated protein kinase 1 (ISPK-1) |
| HUMAAE        | Homo sapiens dbpB-like protein |
| HSU44839      | Human putative ubiquitin C-terminal hydrolase |
| HUMACTIIIA    | Human activin type II receptor |
| HSU46857      | Human RNA polymerase II boloenzyme component |
| HSU72621      | Human LOT1 |
| HUMHSPCA      | Human proliferating cell nuclear antigen (PCNA) gene |
| HUMPMOR       | Human NAD(P)H: menadione oxidoreductase |
| HSU41765      | Human metalloprotease/disintegrin/cysteine-rich protein |
| HUMHGLUT1     | Human mRNA for glutamate transporter |
| HSU66243      | Human CD36 glycoprotein |
| HUMHSPCA      | Human mRNA for platelet activating factor |
| HUMIL8RB      | Human metalloproteinase 3 |
| HUMCALBETB    | Human voltage-dependent calcium channel beta-1 subunit |
| HUMATPSAS     | Human gene for ATP synthase alpha subunit (exon 1-12) |
| HUMBAFAA      | Human mRNA for platelet activating factor |
| HSU62010      | Human keratinocyte growth factor |
| HUMTPARN      | Human mRNA for tissue plasminogen activator |
| HUMKGF        | Human keratinocyte growth factor |
| HUMHSPCA      | Human mRNA for phosphatidylinositol 4-kinase (PI4K) |
| HUMHGLUT1     | Human mRNA for glutamate transporter |
| HUMKGF        | Human keratinocyte growth factor |
| HUMCALBETB    | Human voltage-dependent calcium channel beta-1 subunit |
| HUMPBC       | Human ADP/ATP carrier protein (ANT-2) gene |
| HUMUPTP       | Human plasminogen activator |
| HUMCALBETB    | Human voltage-dependent calcium channel beta-1 subunit |
| HUMHSPCA      | Human mRNA for platelet activating factor |
| HUMHSPCA      | Human mRNA for platelet activating factor |
| HUMHGLUT1     | Human mRNA for glutamate transporter |
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| HUMHSPCA      | Human mRNA for platelet activating factor |
| HUMHGLUT1     | Human mRNA for glutamate transporter |
| HUMHSPCA      | Human mRNA for platelet activating factor |
15 U05259 Human MB-1 gene
13 X59770 Homo sapiens IL-1R2 mRNA for type II interleukin-1
12 M57732 Human hepatic nuclear factor 1 (TCF1)
11 NM_005522 Homo sapiens homeo box A1 (HOXA1)
10 U52191 Human SMCY (H-Y)
9 Z49107 Homo sapiens galectin
8 L15533 Homo sapiens pancreatitis-associated protein (PAP)
7 M10942 Human metallothionein-Ie gene (hMT-Ie)
6 M61853 Human cytochrome p4502C18 (CYP2C18)
5 L07518 Homo sapiens mucin
4 AF043909 Homo sapiens gastric mucin (MUC5AC)
3 M63154 Human intrinsic factor
2 U75272 Human gastricsin
1 X05997 Human mRNA for gastric Lipase

Highly expressed genes in normal gastric mucous

1 X05997 Human mRNA for gastric Lipase
2 U75272 Human gastricsin
3 M63154 Human intrinsic factor
4 AF043909 Homo sapiens gastric mucin (MUC5AC)
5 L07518 Homo sapiens mucin
6 M61853 Human cytochrome p4502C18 (CYP2C18)
7 M10942 Human metallothionein-Ie gene (hMT-Ie)
8 L13533 Homo sapiens pancretatitis-associated protein (PAP) gene
9 Z49107 Homo sapiens galectin
10 U52191 Human SMY1 (H-Y)
11 NM_005522 Homo sapiens homoe box A1 (HOXA1)
12 M57732 Human hepatic nuclear factor 1 (TCF1)
13 X59770 Homo sapiens IL-1R2 mRNA for type II interleukin-1 receptor
14 X76223 Homo sapiens MAL gene exon 4
15 U05259 Human MB-1 gene

Highly expressed genes in GC7901 and GES-1

16 XM_052013 Homo sapiens polymeric immunoglobulin receptor (PIQR)
17 U90065 Homo sapiens ATP sulfurylase/APS kinase 2
18 M55422 Human Krueppel-related zinc finger protein (H-plk)
19 S78825 Id1, transcription regulator helix-loop-helix protein
20 U19948 Homo sapiens protein disulfide isomerase (PDIP)
21 U43522 Human cell adhesion kinase beta (CAKbeta)
22 U12139 Human alphal (XI) collagen (COL11A1) gene, 5' region and exon 1
23 M14539 Human factor XIII subunit
24 X65614 Homo sapiens mRNA for calcium-binding protein S100P
25 AF000560 Homo sapiens TFF-1 interacting peptide 20
26 AF002224 Homo sapiens Angiogen Syndrome Gene, 6b-AP ubiquitin protein ligase 3A
27 U57096 Human janus kinase 3 (Jak3)
28 U42600 Human calcium-activated potassium channel beta subunit
29 NM_017406 cAMP responsive element binding protein-like 1
30 U4806 Homo sapiens FLT3/FLK2 ligand
31 D8436 Homo sapiens p52 and p64 isoforms of N-Shc
32 Z30425 Homo sapiens orphan nuclear hormone receptor
33 M16346 Human creatine kinase-B
34 X96294 Homo sapiens encoding mitochondrial citrate transport protein
35 HSN223H1 Homo sapiens nm23H1 gene
36 NM_014792 Homo sapiens KIAA0125 gene product (KIAA0125)
37 M34041 Human alpha-2-adrenergic receptor (alpha-2 c2) gene
38 XM_002444 Homo sapiens serine threonine kinase 39 (Skt39)
39 NM_001690 Homo sapiens ATPase, H+ transporting, lysosomal
40 L12398 Human sapiens dopamine receptor D4 (DRD4)
41 L76465 Homo sapiens NAD+ dependent 15 hydroxyprostaglandin dehydrogenase (PGDH)
42 U57094 Homo sapiens small GTP-binding protein
43 Z14978 Homo sapiens mRNA for actin-related protein
44 X53961 Human lactotransferrin
45 M2628 Homo sapiens alpha-1 Ig germline C-region membrane-coding region
46 MB8426 Homo sapiens adipsin/ complement factor D
47 X04391 Human lymphocyte glycoprotein T1/Leu-1
48 X04553 Homo sapiens sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (sema3p) 48 (SEMA4B)
49 AF071054 GCys-1, mRNA differentially expressed in cell lines GC7901 and GES-1
50 AF063015 Homo sapiens cell division protein
51 AF071056 GCys-17, mRNA differentially expressed in cell lines GC7901 and GES-1
52 AF002130 Homo sapiens mRNA for cell division protein
53 NM_001730 Homo sapiens Kruppel-like factor 5 (intestinal)
54 AB047278 Arabidopsis thaliana AtNdr1 mRNA for Ndr kinase
55 HMIGFBP1 Human insulin-like growth factor binding protein-1 (IGFBP1) gene
56 HUM20D9 Human gene for 2-oxoglutarate dehydrogenase
57 HSCDC2 Human CDC2 gene involved in cell cycle control
58 XM_061005 Homo sapiens similar to Mucin 2 precursor
59 XM_052013 Homo sapiens polymeric immunoglobulin receptor (PIQR)
60 D80419 Homo sapiens OTH18
61 HSU89870 Homo sapiens homoe box A1 (HOXA1)
62 NM_013942 Homo sapiens cell division cycle associated 7 (CDC7)
63 HSU09716 Human mannose-specific lectin (MBP)
64 HSU09716 Human mannose-specific lectin (MBP)
All 412 genes and critical threshold values to distinguish normal gastric mucosa from precancerous lesion and gastric cancer were compiled into an analysis software, which can automatically provide analysis reports by analyzing the provided microarray test results. The analysis software for examination results of prewarning system of gastric cancer locates on the website http://shasta.mpi-stuttgart.mpg.de/array/form.html. The software cannot be downloaded until it is confirmed to be very effective and complete.

**Northern blot analysis of brca1 and ndr1**

Two new biomarkers brca1 and ndr1 (NM_007271) were identified. Brca1 (AF208045) showed no or low-expression levels in normal gastric mucosa and high-expression level in gastric cancer. There was a statistically significant difference in expression levels between normal gastric mucous tissues and gastric cancer tissues (P<0.01, Figure 3), indicating that higher expression of brca1 was closely associated with gastric cancer stage. Further analysis indicated that higher expression of brca1 appeared to have no correlation with pathological types of gastric cancer (P>0.05, data not shown). Conversely, ndr1 (NM_007271) displayed higher expression levels in normal gastric tissues and no or lower expression in gastric cancer, and there was a statistically significant difference in expression levels between normal gastric mucous tissues and gastric cancer tissues (P<0.01), indicating that higher expression level of ndr1 was closely associated with normal stage of gastric mucosa tissues.

**Immunohistochemistry analysis of brca1 and ndr1**

Brca1 protein exhibited higher expression in 60 gastric cancer tissues, lower or no expression in 30 normal gastric cancer tissues, and there was no expression or lower expression in 30 normal tissues. Brca1 and ndr1 were analyzed by western blotting and immunohistochemistry, respectively. brca1 and ndr1 were over-expressed in 60 gastric cancer tissues, while the expression level was lower or no expression in 30 normal gastric tissues. (Figure 3)

**Table 2 Gene expression threshold for distinguishing three kinds of gastric mucosa**

| Gene classification | Gastric cancer tissue (GC/N) | Precancerous lesion (PC/N) | Normal gastric mucosa (N*/GC or N*/PC) |
|---------------------|-----------------------------|---------------------------|---------------------------------------|
| 246 genes associated with gastric cancer | 6.01±2.40                   | 1.18±0.47                 | <0.75                                 |
| 85 genes associated with precancerous lesions | 1.32±0.53                   | 4.86±1.94                 | 2.54±0.41                             |
| 88 genes associated with normal mucosa | 1.31±0.54                   | 2.50±0.75                 | 5.42±2.17                             |
| 15 genes associated with metastasis of gastric cancer | 5.81±2.32 (M)               | 1.13±0.58                 | 0.65±0.35                             |
| 8 genes associated with risk factors | 2.32±1.19 (N*)              |                           | >2.0                                  |

Specification: The above data indicate the relative expression levels between GC/N, PC/N, N/PC and N/GC mean ratio and minimum values. M: Metastasis; N*: No metastasis. GC: Gastric cancer; PC: Precancerous lesion; N: Normal mucosa. N*: Selected gene expression levels in normal gastric mucosa.
mucosa tissues. There was a statistically significant difference in expression levels between gastric cancer tissues and normal gastric mucosa tissues \((P<0.01, \text{Figure 4A})\). The result indicated that higher expression of \(brca1\) was associated with gastric cancer stage. \(Ndr1\) protein exhibited higher expression in 30 normal gastric mucosa tissues, lower or no expression in 60 gastric cancer tissues. There was a statistically significant difference in expression levels between normal gastric mucosa tissues and gastric cancer tissues \((P<0.01, \text{Figure 4B})\). The result indicated that higher expression of \(ndr1\) was closely associated with normal stage of gastric mucous tissues.

**DISCUSSION**

The development of normal gastric mucosa into gastric cancer is a complex process. Previous research in the pathology of gastric cancer demonstrated that normal gastric mucosa could gradually develop into pre-cancerous lesions under special conditions, eventually evolving toward gastric carcinoma. During the periods from normal gastric mucosa to gastric cancer, it has not been shown how many genes are involved at different stages of cancer development. The cDNA microarray technology could provide an efficient tool to address the difficulties in screening and quantifying expression levels of a large number of genes\(^{[7-10]}\). So far there are some reports associated with gene expression profiles of gastric cancer based on biochip\(^{[11,12]}\). However, the problem of early gastric cancer detection is still not solved satisfactorily. In the present study, we tried to establish a prewarning system of gastric cancer based on biochip and CAD technique to solve the problem of early gastric cancer detection.

Firstly, two high-density microarrays with 8 464 human cDNA sites were used to screen two pairs of gastric cancer tissues and 389 genes associated with three stages of gastric cancer development such as normal gastric mucosa, precancerous lesion and gastric cancer were obtained. The selected 389 genes were used as main diagnostic genes on the prewarning chip, 15 genes associated with metastasis of gastric cancer as diagnostic genes of metastasis stages, 8 risk factor genes as reference biomarkers to predict the development of precancerous lesions.

A total of 412 genes were selected to fabricate the low-density chip, which was used to screen 150 clinical specimens. It was found that the gene expression levels in normal, precancerous lesion and cancer tissues were significantly different as expected. CAD software and statistical methods were used to identify key genes and their critical threshold values characterizing different tissue status. Two hundred and sixteen genes displayed higher expression levels in cancer tissues than in non-cancer tissues, 85 genes exhibited higher expression levels in precancerous lesions than in cancer tissues, and 88 genes exhibited higher expression levels in normal tissues than in gastric cancer and precancerous tissues (Table 1). The critical threshold values to distinguish normal gastric mucosa from precancerous lesion and gastric cancer were identified (Table 2). With the above-mentioned standards, the 150 specimens could be clearly grouped according to their tissue status determined in pathology diagnosis. Therefore, we considered that the established standard had a great potential in the detection of early gastric cancer. Based on these selected genes and critical threshold values characterizing three stages of gastric cancer development, an analysis software was developed which could analyze the examination results of 412 genes achieved by biochip and provide automatically an analysis report. The software remained to be optimized. These expression profiles obtained from all these specimens and available clinical data had been compiled into a prewarning data library of gastric cancer by CGO software, and these detailed data would be very useful for the further research and therapy of gastric cancer.

From Table 2, it appeared reasonable to define integrate markers of GC, PC, NU consisting of many genes, instead of individual genes, to distinguish three kinds of gastric tissues status. Once gastric cancer was diagnosed, the expression levels of 15 metastasis genes could be subjected to focal studies to identify whether the cancer metastasized, and to speculate the prognosis of the cancer patients. These results could also be complemented with supporting evidence from patient’s disease history, for example, discomfort or pain in the gastric area, body mass loss in a short time, etc. If a precancerous lesion was diagnosed, the expression levels of risk factor genes might be analyzed as indicators on how fast such lesion would lead to cancer\(^{[13]}\). One may also establish and search the prewarning database library to compare similar patients to make a best treatment plan. The diagnosis and treatment information associated with...
gastric cancer can also be obtained from gastric cancer information web presided over by Dr. Cui http://www.37c.com.cn. The prewarning database of gastric cancer is available on gastric cancer information web. The analysis software of examination results of the prewarning system of gastric cancer locates on the website http://shasta.mpi-stuttgart.mpg.de/array/form.html.

Two new biomarkers have been identified of diagnostic value, braa1 (AF208045)[14] and ndr1 (NM_007271). Braa1 showed no or low-expression levels in normal gastric mucosa and high-expression level in gastric cancer, and appeared to have no correlation with pathological types of gastric cancer. Conversely, ndr1 displayed high-expression levels in normal gastric tissues and no or lower expression in gastric cancer. These results were also confirmed by Northern blot and immunohistochemistry analysis. These two biomarkers may be very useful for distinguishing benign from malignant gastric mucosa lesions.

Gastric cancer specimens from different patients were found to display some variability in gene expression profiles. The reasons could be attributed to variations in specimens, lesion types and the number of cells collected. Moreover, variations among individuals may pose a serious challenge to diagnosis accuracy. In cases of doubt, it would be advisable to analyze microarray results together with clinical symptoms of patients and pathological results. It is very difficult to devise gene expression profiles to further classify the specimens consistent with pathology types such as atrophic gastritis, intestinal gland metaplasia, atypical hyperplasia, etc.

Of course, new methods of disease classification can be defined according to gene expression profiles and DNA levels (mutation, deletion and amplification). Such methods may not be fully consistent with pathology classification, but nevertheless may be appropriate for future clinical applications. In the near future, pathological diagnosis will remain a useful and complementary diagnostic tool.

To test the generality of this standard, we collected randomly some autopsy specimens and screened them with fabricated gastric microarrays. Simultaneously, pathology diagnosis was performed on the same specimens. We found that the results achieved by the microarray were highly identical with traditional pathological results. In another paper, we have reported these results in detail[15,16].

In summary, further studies will lead to a more complete prewarning database library. The prewarning database, together with miniaturized microarray techniques, will be used to further improve the accuracy and reliability of the prewarning system for gastric cancer[10].

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