Gene Expression Profile Differences in Gastric Cancer and Normal Gastric Mucosa by Oligonucleotide Microarrays

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OBJECTIVE To study the difference of gene expression in gastric cancer (T) and normal tissue of gastric mucosa (C), and to screen for associated novel genes in gastric cancers by oligonucleotide microarrays.

METHODS U133A (Affymetrix, Santa Clara, CA) gene chip was used to detect the gene expression profile difference in T and C. Bioinformatics was used to analyze the detected results.

RESULTS When gastric cancers were compared with normal gastric mucosa, a total of 270 genes were found with a difference of more than 9 times in expression levels. Of the 270 genes, 157 were up-regulated (Signal Log Ratio [SLR] $\geqslant 3$), and 113 were down-regulated (SLR $\leqslant -3$). Using a classification of function, the highest number of gene expression differences related to enzymes and their regulatory genes (67, 24.8%), followed by signal-transduction genes (43,15.9%). The third were nucleic acid binding genes (17, 6.3%), fourth were transporter genes (15, 5.5%) and fifth were protein binding genes (12, 4.4%). In addition there were 50 genes of unknown function, accounting for 18.5%. The five above mentioned groups made up 56.9% of the total gene number.

CONCLUSION The 5 gene groups (enzymes and their regulatory proteins, signal transduction proteins, nucleic acid binding proteins, transporter and protein binding) were abnormally expressed and are important genes for further study in gastric cancers.

KEYWORDS: gastric cancer, normal gastric mucosa, gene-expression profile.

Differentially expressed genes in diverse tissue specimens may be detected with parallel analysis using gene chips, which have greatly improved the traditional experiments in which only a single, or several gene expressions can be observed for each test. More and more cDNA microarray methods are now being applied in the study of gene expression. In the present paper, the gene chip technique was used to analyze different gene expression patterns between gastric carcinomas and normal tissue of the gastric mucosa. In addition we have explored the tumor-associated gene-cluster and their functions involved in the process of formation and development of the gastric carcinomas. These studies will be helpful to comprehensively understand the mechanism of carcinogenesis at the molecular level with the hope that this research will provide molecular markers and target genes for clinical diagnosis, prevention, prognosis and treatment of gastric cancer.

MATERIALS AND METHODS

Materials

All the tissue specimens including gastric carcinomas (T) and gastric mucosa, which were from the distant cutting margin (C), were taken from 5 patients being operated in our hospital. For each specimen one part was frozen immediately in liquid nitrogen after surgical resection, and another part was used for histopathological examination to ensure that all normal gastric mucosa was devoid of cancer cells and that it had maintained the corresponding histological appearance. The clinical and pathological data from these patients are shown in Table 1.

Methods

Oligonucleotide microarray gene chips

Human full- length genome U133A chips (Affymetrix, Santa Clara, CA) were used. This array contains about 18,000 full-length genes from the Unigene GenBank.

Sample preparation

RNA was extracted from the tissues by a single-step method.[1] Briefly, after removing the T and C tissues from the liquid nitrogen, the specimens were ground completely into a tiny powder in a ceramic mortar while adding liquid nitrogen. TRIZOL was used to extract total RNA followed by use of a QIAGEN'S reagent kit for its purification. Spectrophotometric analysis was employed (one optical density unit at A 260 nm equals 40 μg/ml of RNA) to calculate the total RNA concentration. An equivalent of a total RNA, from the T and C samples, then was mixed. Using the T7- (dT) 24 (oligonucleotide) for a primer, the first strand of cDNA was synthesized through retro-transcription, then the first strand was used as a template to synthesize the second strand. After the double stranded DNA was purified, a BioAssay High Yield RNA Transcript Labeling kit was employed to transcribe the synthesis of cRNA directly, and at the same time to biotin-label the cRNA. Then a certain quantity of cRNA product was taken to make a 35~200 bp fragment of cRNA which was produced under high temperature and high salt conditions.

Hybridization and washing

The fragmentated cRNA was mixed with its control solution to prepare a hybridization solution. The hybridization solution was placed on the chips, and the 2 chips (T, C) placed into the hybridization 640 oven for 16 h to finish the hybridization procedure. The chips were removed from the hybridization oven, washed and stained, and eluted automatically in the Fluidics Station 400 .

Fluorescence scanning and analysis of results

The chip was scanned with a GeneChip Scanner and the intensity value of the fluorescent signals obtained from the expressed genes. Using an internal reference gene (housekeeping gene) the primary signal data were normalized and corrected. The images produced were analyzed by Microarray Suite Software using digital computation, and the intensity of the fluorescence signals and their ratios calculated.

RESULTS

Quantity judgment of the test chip

For each cRNA sample a scanning profile was produced after hybridization with the test chip (Fig.1). A clearly printed character "GeneChip TEST3" was on the upper portion of the profile. Many spots and well-distributed lines were around the profile. Some spots

Table 1. Clinical features and pathological diagnosis of 5 cases with gastric carcinoma

No. of In-P	Sex	Age	Pathological diagnosis	Lymph metastasis	Clinical stage
113702	F	45	Lesser curvature of stomach invasion ulcer type middle differentiation adenocarcinoma invasion of serosa	1/35	III
123730	M	48	Anterior wall in body of stomach middle differentiation adenocarcinoma invasion of muscular layers and nerve	1/17	III
123673	F	57	Lesser curvature in cardia of stomach invasion ulcer type middle differentiation adenocarcinoma invasion of serosa	1/14	III
123808	F	49	Anterior wall in cardia of stomach low- middle differentiation adenocarcinoma, a part of mucinous adenocarcinoma and esophagus of extremities inferior	0/35	II
123733	M	60	Cardia of stomach node type middle differentiation adenocarcinoma invasion of esophagus muscular layer	5/22	III

distributed on the 4 corners of the profile and the character "+" was clear and obvious. It indicated that the profile had a good quantity between the gene chip and the samples of RNA showing that the results of the detecting gene chip were reliable. Then, the samples from the 2 groups were hybridized with a U133A gene chip and scanned.

Hybridization results of the sample chip

A scanning profile was produced from the samples of the 2 groups after hybridization with the U133A gene chip (Figs.2,3). Fig.4 is a compared scatter plot of the gastric cancer with the control. The results were collected and analyzed by bioinformatics, and the number of differentially expressed genes in the gastric cancer (T) compared to the normal gastric mucosa (C) , as shown in Table 2.

Because of the large number of genes, the T was compared to the C only if the expression difference was (SLR ≥ 3 or SLR≤ -3). The genes were classified based on their molecular function. The group with the highest number of genes contained enzymes and their regulator genes (67, 24.8%) followed by these groups in decreasing order: signal transduction genes (43, 15.9%), nucleic acid binding genes (17, 6.3%), transporter genes (15, 5.5%) and protein binding genes (12, 4.4%). In addition there were 50 genes of unknown function, accounting for 18.5% of the total, as shown in Table 3.

Table 2. Comparison of the number of differentially expressed genes in T *vs.* C

	Up-regulated		Down- regulated	
	SLR ≥ 2	SLR ≥ 3	SLR ≤ -2	SLR ≤ -3
T *vs.* C	530	157	236	113

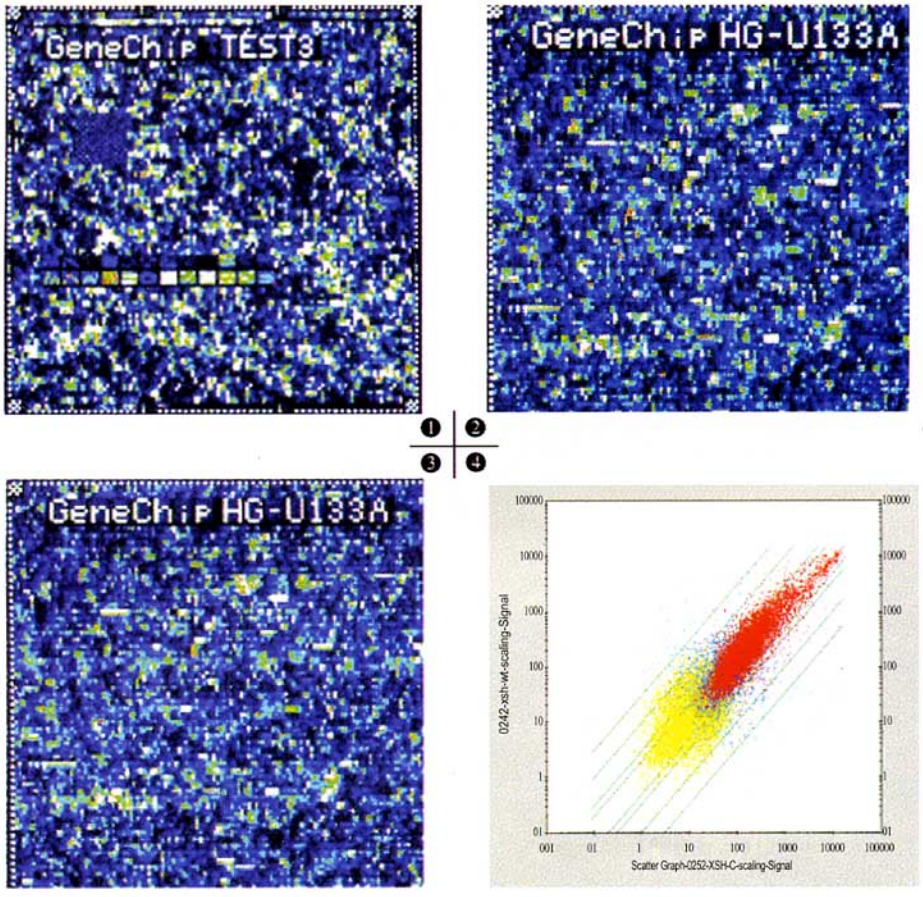

Fig.1. Scanning results of control cRNA *vs.* test chip post-hybridization. **Fig.2.** Scanning results of post-hybridization in control cRNA *vs.* U133A chip. **Fig.3.** Scanning results of post-hybridization in gastric cancer group cRNA *vs.* U133A chip. **Fig.4.** Scatter plots in gastric cancer tissue *vs.* control.

Table 3. Comparison of the molecular function of the genes in T vs. C (SLR≥3 or SLR≤−3)

No. Molecular function	Up-regulated	Down-regulated
1 Enzyme and enzyme regulatory	41	26
2 Defense/immune protein	1	5
3 Structural molecule	7	2
4 Transcription factor	3	7
5 Nucleic acid binding (RNA, DNA)	9	8
6 Protein binding (Ca, GF, cell skeleton, cell cycle)	7	5
7 Carbohydrate and lipid binding	2	1
8 Metal ion binding	8	2
9 Tumor suppressor	2	0
10 Cell sport	3	4
11 Transporter (electron, ion, protein)	8	7
12 Signal trasduction (receptor, GF)	24	19
13 Cell adhesion molecule	9	3
14 Apoptosis suppressor	1	0
15 Tumor antigen	5	1
16 Unknown	27	23
Total	157	113

Comparison of the differential gene expressions which were up-regulated between T and C (SLR ≥ 5) are shown in Table 4.

Comparison of the differential gene expressions which were down-regulated between T and C (SLR ≤ -5) are shown in Table 5.

DISCUSSION

Gastric carcinoma mortality accounts for 23% of all malignant tumor deaths in China and is a caused of world-wide morbidity. Up to the present time, treatment for gastric carcinoma and its prognosis have been greatly improved, but it still remains as a major health problem. Studies at the molecular level provide a new approach to explore its etiology, to seek an ideal early stage molecular marker, to open the possibility for gene therapy and to promote treatment and prevention of this disease.

Carcinogenesis results from a series of molecular changes caused by abnormal expression of tumor-associated genes or inactivation of tumor suppression genes. The gene chip has been widely used to detect gene expression differences in various specimens by parallel analysis. The greatest advantage of this technique is that it changes the traditional experiment where only a single or several gene expression differences can be observed in one procedure. Therefore, more and more cDNA microarrays have been applied to the study of gene expression. For example, the gene chip has been used to study multidrug-resistance and chemotherapy sensitivity,[2,3] to establish carcinogenic models of gastric carcinoma,[4] to find genes associated with gastric carcinoma and its metastases [5-8] and to study the relationship between *Helicobacter pylori* and gastric malignancy.[9] The gene chip also has been used to compare gastric carcinoma with normal gastric mucosa,[10,11] and in another study a difference in expression in different types of gastric carcinoma analyzed and developed prognoses based on their gene expression profile.[12]

We used the gene chip technique to analyze the gene expression profile difference in gastric cancers versus normal mucosa. The results were as follows: when gastric carcinomas were compared with normal gastric mucosa, a total of 270 genes were found with a difference of more than 8 times in expression levels. There were 157 up-regulated (SLR ≥ 3), and 113 down-regulated (SLR ≤ -3) genes. The functions of these genes were classified into the following groups. Most belonged to enzymes and regulatory genes (67, 24.8%) followed by signal transduction genes (43, 15.9%), nucleic acid binding genes (17, 6.3%), transporter genes (15, 5.5%) and protein-binding genes (12, 4.4%). In addition there were 50 genes (18.5%) of unknown function. The 5 groups mentioned above made up of 56.9% the total gene number. Following is a discussion of the genes in the 5 groups showing a large expression difference.

First of all, the enzyme and enzyme regulatory genes had the largest number of changes (67, 24.8%), including genes for heparan sulfate proteoglycan, serine protein kinase and its inhibitor, alcohol dehydrogenase and Na^+/K^+-ATPase. Heparan sulfate proteoglycan (HSPG) is an important compositional component of the extra cellular matrix and basement membrane. Heparinase, an endoglucosidase, can hydrolyze heparan sulfate which linked up with a nuclear form of HSPG, can destroy the structure of the extra cellular matrix and basement membrane. It can play an important action in promoting cancer invasion and metastasis. Some investigators have shown a positive heparinase ratio of about 80% in gastric carcinomas. An increase in haparinase mRNA expression in gastric carcinomas promots growth, metastasis and invasion.[13] Our results showed the HSPG gene to be markedly up-regulated (SLR was 3.1).

Serine (or cysteine) proteinase takes part in constitutive cell skeleton formation, which is connected with the extracellular signal proteins and the intracellular

Table 4. Comparison of up-regulated differential gene expression between T and C (SLR ≥5)

Probe Set ID	Gene name	T vs. C SLR
204673_at	mucin 2, intestinal/tracheal	5
204705_x_at	aldolase B, fructose-bisphosphate	5
5207714_s_at	serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	5
209875_s_at	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	5
214183_s_at	transketolase-like 1	5
217564_s_at	carbamoyl-phosphate synthetase 1, mitochondrial	5
220639_at	hypothetical protein FLJ22800	5
206156_at	gap junction protein, beta 5 (connexin 31.1)	5.1
211885_x_at	fucosyltransferase 6 [alpha (1,3) fucosyltransferase]	5.1
218717_s_at	myxoid liposarcoma associated protein 4	5.4
206067_s_at	Wilms tumor 1	5.5
203757_s_at	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	5.6
206000_at	meprin A, alpha (PABA peptide hydrolase)	5.6
206023_at	neuromedin U	5.6
206430_at	caudal type homeo box transcription factor 1	5.8
210398_x_at	fucosyltransferase 6 [alpha (1,3) fucosyltransferase]	5.8
202888_s_at	alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal aminopeptidase, CD13, p150)	5.9
211657_at	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	5.9
205774_at	coagulation factor XII (Hageman factor)	6.1
202504_at	tripartite motif-containing 29	6.2
202790_at	claudin 7	6.3
208250_s_at	deleted in malignant brain tumors 1	6.3
209844_at	homeo box B13	6.5
214612_x_at	melanoma antigen, family A, 6	6.5
203953_s_at	claudin 3	6.6
209942_x_at	melanoma antigen, family A, 3	6.7
213432_at	mucin 5, subtypes A and C, tracheobronchial/gastric	6.9
213201_s_at	troponin T1, skeletal, slow	7
205892_s_at	fatty acid binding protein 1, liver	7.2
209847_at	cadherin 17, LI cadherin (liver-intestine)	7.2
209792_s_at	kallikrein 10	7.3
204855_at	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5	7.7
206664_at	sucrase-isomaltase	7.7
200072_s_at	heterogeneous nuclear ribonucleoprotein M	7.7

skeleton. It functions as a protein for translocation and localization, as well as intercellular signal transfer. Oien et al.[9] reported that serine proteinase expression levels were markedly up-regulated in human gastric carcinoma tissues. Wang et al.[14] also reported that if cysteine proteinase was over expressed in patients with gastric carcinoma, they would have a poor prognosis and lower survival rate. In the present study, we found that the serine proteinase inhibitor clade B (SLR was 7.7), clade H (SLR was 5) and clade E (SLR was 4) all were up-regulated. Kallikrein-10 also a serine proteinase was found to be up-regulated (SLR was 7.3).

Alcohol dehydrogenase and aldehyde dehydrogenase are 2 key enzymes in ethanol metabolism. Their genetic polymorphism is related to formation of liver cancer, gastric carcinoma and esophageal cancer. Abe et al.[4] reported that the aldehyde dehydrogenase expression level was dramatically down-regulated in gastric carcinoma. Our study also found that alcohol dehydrogenase IB expression was remarkably down-reg-

Table 5. Comparison of down-regulated differential gene expression between T and C (SLR ≤-5)

Probe Set ID	Gene name	T *vs.* C SLR
218087_s_at	sorbin and SH3 domain containing 1	-5
207356_at	defensin, beta 4	-5.1
214465_at	ecotropic viral integration site 81B	-5.1
207909_x_at	deleted in azoospermia 4	-5.2
213071_at	ecotropic viral integration site 39B	-5.3
201497_x_at	myosin, heavy polypeptide 11, smooth muscle	-5.4
208791_at	clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	-5.5
209966_x_at	estrogen-related receptor gamma	-5.7
207912_s_at	deleted in azoospermia 4	-5.9
220630_s_at	eosinophil chemotactic cytokine	-6.3
207139_at	ATPase, H+/K+ exchanging, alpha polypeptide	-6.4
207546_at	ATPase, H+/K+ exchanging, beta polypeptide	-6.4
207981_s_at	estrogen-related receptor gamma	-6.4
209612_s_at	alcohol dehydrogenase IB (class I), beta polypeptide	-6.9
205261_at	progastricsin (pepsinogen C)	-7.4
221095_s_at	potassium voltage-gated channel, Isk-related family, member 2	-7.7
213265_at	ecotropic viral integration site 41B	-8.1
206334_at	lipase, gastric	-8.5
202018_s_at	lactotransferrin	-8.6
207033_at	gastric intrinsic factor (vitamin B synthesis)	-8.9
220191_at	18 kDa antrum mucosa protein	-8.9

ulated (SLR was -6.9).

Na^+/K^+-ATPase, a widely distributed enzyme on the cell membrane, is a key enzyme for maintaining Na^+/K^+ ion gradients of cells and aids in protein translocation. Membrane translocation of H^+, Na^+, K^+, and Cl^- is an energy regulating process, that depends on adequate ATP and Na^+/K^+-ATPase activity, which is of importance in the gastric mucosa for production of HCl needed for digestion. Lee et al.[10] reported that Na^+/K^+-ATPase expression levels were up-regulated in an intestinal type of gastric cancer. In our study we found that ATPase, Na^+/K^+ transporting, alpha 1 polypeptide (SLR was 2.4), ATPase, V group 10B type also was up-regulated (SLR was 3.5). ATPase, H^+/K^+ exchanging, α and β polypeptides were down-regulated (their SLR all were -6.4).

The second class of genes that showed expression changes involved signal transduction (43, 15.9%). Signal transduction is crucial for many life processes such as cellular propagation and differentiation, neuron activity and immune function. Our discussion will touch on the gene expression differences in the low-density lipoprotein receptor, G-proteins and G-protein receptor.

The low-density lipoprotein receptor (LDL) is a multiple function protein found in many mammalian cells with considerable variation in activity. The LDL receptor functions in transport of cholesterol into the cell from the serum for use in cell proliferation and for synthesis of steroid hormones and bile salts. Homocysteine and low-density lipoprotein are involved in co-inducing an apoptotic effect [15] in atheromas which are found in the vascular endothelium. Our study showed that 2 LDL receptor gene expressions were obviously up-regulated (SLR was 4.5 and 3.4, respectively).

G-proteins are part of a large of family of proteins all of which combine with GTP, and are involved in signal transduction. Alterations in the G-protein structure can result in diseases, such as genetic endocrine disease or oncogenesis. [16] In our research we found that a number of the G-protein family genes were down-regulated as follows: G-protein-connexin 18 and 30 (SLR was -3.2 and -3.6, respectively); G-protein signal-transduction regulator 5 (SLR was -3.1); the membrane-spanning 4-domains, subfamily A, member 1 (SLRs were -3.4); the other V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (SLR was -

4.2); ArgBP2 (Arg/Abl binding protein 2) (SLR was -4.5) and G-protein signal transduction regulator 13 (SLR was -4.5). Expression of these genes was up-regulated: amiloride binding protein 1 (SLR was 3.6); striatin, calmodulin binding protein 3 (SLR was 3.8); Rho GTPase activation protein (SLR was 3.9) and liver fatty acid binding protein 1 (SLR was 7.2).

Changes in gene expression of the third group involved nucleic acid binding genes (17, 6.3%). The genes of interest for discussion which showed a larger expression difference were: a DEAD/H box protein gene, a RNA binding motif protein 10 gene and a deleted azoospennnia (DAZ) gene.

The DEAD/H box proteins are part of a helicase protein family[17] which are involved in embryogenesis, spermatogenesis and cell division. Their action encompasses unfolding of double stranded and folding function, and to introduce a secondary structure into single strand RNA (unwindase) such as: initiation transformation, nuclei and mitochondria adhering to each other, and ribosome assembly in the spliceosome and so on. We found that the DEAD/H (Asp-Glu-Ala-His) box protein polypeptide 8 gene expression was markedly up-regulated (SLR was 3.1), whereas the DEAD/H (Asp-Glu-Ala-Asp) box protein polypeptide 3 gene expression was significantly down-regulated (SLR was -4.9).

The RNA binding motif protein 10 gene, situated at chromosome Yq11.23, functions in binding Zn^{+}, RNA and DNA, and its expression was remarkably up-regulated (SLR was 3.5).

Zhu reported[18] that a Yq 11 septum in a micro deletion frequently appeared which was an azoospermia factor (AZFc) in a patient with infertility. The DAZ (deleted in azoospennia) gene was a likely candidate for the AZFc. It contained a lot of functional gene clusters, so it is called a DAZ family. The DAZ gene possessed high homology with the Drosophila's male infertility gene (boula gene). If the latter had a mutation, it resulted in prevention of spermatogenesis. In our study, we found the expression of two DAZ2 genes (SLR was -3.3 and -4.3) and 2 other DAZ4 gene expressions (SLR was -5.2 and -5.9) were all significantly down-regulated.

The fourth, category of genes showing marked differences were transporter genes (15, 5.5%). Our discussion will cover genes for the intrinsic factor (IF) and gap junction (GJ).

Intrinsic factor is a 17,000 molecular weight mucin produced by the gastric mucsa; its essential function is and to promote vitamine B12 resorption by the gastrointestinal tract. About one unit of IF can increase 1 ng of B12 absorption. When the IF is deficient, it may result in pernicious anemia. Yassion et al.[19] reported that an 11 year old girl who had a profound anemia had a gastric IF gene deletion. In the present study we found that gastric IF gene expression was markedly down-regulated (SLR was -8.9).

Recent findings suggest that the cell GJ gene is a kind of non-mutated type suppressor. At present, it is well known that there are more than 10 family members. The cell gap junction pathway is composed of connexin (Cx), which can mediate the use of energy and the movement of messages in intercellular transport. It plays a role in cell growth and control of differentiation. After a cell becames malignant, abnormal expression of Cx will generally occur to a degree related to its malignancy. Ma et al.[20] reported that Cx32 and Cx 43 were connective genes, involved in a close relationship with the liver cells. Both produced a high expression product in the normal liver QZS cells line (99.0% and 99.1%, respectively), maintaining normal liver cell function. The Cx32 and Cx43 protein expression was significantly decreased in a liver carcinoma cell line. We found two GJ genes were significantly up-regulated (SLRs were 3.6 and 5.1).

Protein binding genes (12, 4.4%) made up the fifth group of which we will discuss cell cycle protein genes. Cyclin is involved in the control of cell division so its expression in tumors, which are chacterizaed by uncontrolled growth, decreased apoptosis, increased cell proliferation and dedifferentiation is of great interest. Many cellular oncogene and suppressor genes are linked to cell cycle control. Yasuda et al.[21] reported that gastric carcinoma Cyclin B1 gene over-expression was negatively correlated with its biological behaviour. We found that the cell cycle associated genes such as Ki 67(SLR was 3.2), Cyclin D2 (SLR was 4.1), Cyclin E1 (SLR was 4.2) and Cyclin M4 (SLR was 4.5), all were significantly up-regulated.

In addition, in our study we found that the carcino-embryonic antigen (CEA) associated cellular adhesion molecule 5 (SLR was 4.6) and adhesion molecule 6 (SLR was 5.6) expressions were significantly up-regulated. CEA is a soluble glycoprotein with a complex structure and molecular weight of about 200,000. CEA is present in the fetal gastrointestinal tract, pancreas and liver, but after birth its level is very low. With the appearance of a gastrointestinal malignant tumor the patient's serum CEA will increase, as is the case with breast or lung cancer and other malignant tumors. Therefore, even though CEA is a broad-spectrum tu-

mor marker, it still has important clinical value in differential diagnosis, to monitor the degree of illness, and to estimate therapeutic efficacy and so on. Both Terashima et al.[6] and Sakakura et al.[22] reported that the CEA gene was up-regulated in metastatic gastric cancer. Oue et al.[7] also reported that the CEA CAM-6 gene expression was up-regulated in 50% of patients with gastric cancer.

Our study indicated that using the gene chip technique for detecting the difference of gene expression may provide a new direction for diagnosis, therapy and prevention for human gastric carcinomas.

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