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Identification of the differential expressive tumor associated genes in rectal cancers by cDNA microarray

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Abstract

AIM: To identify tumor associated genes of rectal cancer and to probe the application possibility of gene expression profiles for the classification of tumors.

METHODS: Rectal cancer tissues and their paired normal mucosa were obtained from patients undergoing surgical resection of rectal cancer. Total RNA was extracted using Trizol reagents. First strand cDNA synthesis was indirectly labeled with aminoallyl-dUTP and coupled with Cy3 or Cy5 dye NHS mono-functional ester. After normalization to total spots, the genes which background subtracted intensity did not exceed 2 SD above the mean blank were excluded. The data were then sorted to obtain genes differentially expressed by $\geqslant$ 2 fold up or down in at least 5 of the 21 patients.

RESULTS: In the 21 rectal cancer patients, 23 genes were up-regulated in at least 5 samples and 15 genes were down-regulated in at least 5 patients. Hierachical cluster analysis classified the patients into two groups according to the clinicopathological stage, with one group being all above stage Ⅱ and one group all below stage Ⅱ.

CONCLUSION: The up-regulated genes and down-regulated genes may be molecular markers of rectal cancer. The expression profiles can be used for classification of rectal cancer.

Key words: Rectal cancer; Tumor associated genes; cDNA microarray; Differential expression genes

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common causes of cancer related deaths, the majority of which are secondary to liver metastasis. Whereas significant improvements have been made in patient survival, the mortality is still quite high in patients with metastatic cancer[1]. Clinical trials of new therapeutic agents are constantly ongoing and drug effectiveness is improving. However, a complete cure for patients with advanced colorectal cancer awaits new targets and strategies. To achieve this goal, a comprehensive understanding of the mechanisms of colorectal carcinogenesis is essential. In the last two decades these mechanisms have been intensively studied. At the molecular level, activation of oncogenes[2] and inactivation of tumor suppressor genes are processes known to be involved in colorectal cancer[3,4]. CRC typically develops over decades and involves multiple genetic events. This has led to the development of a multi-step model of colorectal tumorigenesis[5]. It is generally accepted that one of the initiating steps in colorectal carcinogenesis is mutation in APC tumor suppressor gene[6].

APC is known to bind to a cell signaling/transcription factor β catenin. Activation of the Wnt pathway normally signals the association of β-catenin with members of the T-cek factor/lymphocyte-enhancer factor family and translocation to nucleous[7,8]. This complex can activate the transcription of a variety of target genes including c-Myc[9] and cyclin D1[10,11]. Loss of APC function leads to an abnormal accumulation of β-catenin and dys-regulates Wnt signaling[12]. Traditional methods of identifying novel targets that are involved in colon cancer progression are based on the studies of individual

genes. The developments of oligonucleotide or cDNA microarrays have made it possible to permit the expression levels of tens of thousands of genes to be monitored simultaneously and rapidly. These new methods have been used to identify the genes involved in colorectal cancer development. By using cDNA microarray with different number of elements, many genes have been identified. Kitahara[13] identified 235 genes of differential expression in 9216 human genes by using laser-capture micro-dissection. These differentially expressed genes include those associated with signal transduction, metabolic enzymes, production of reactive oxygen species, cell cycle, transcription, mitosis and apoptosis[13]. In another study, Williams identified 2632 genes (574 up-regulated and 2058 down-regulated) of totally 9592 genes microarray were consistently expressed in one-third patients[14]. In an effort to use gene expression profiles to find the molecular marker of rectal cancer, we constructed cDNA microarray with 373 known genes and 73 expressed sequence tags (ESTs) that are related to the pathogenesis of tumor.

MATERIALS AND METHODS

Fabrication of tumor associated genes cDNA microarray. Totally 447 cDNA clones were obtained from the Research Genetics (Invitrogen, life Technologies, USA). *E. coli* with cDNA clones were cultured with LB supplemented with ampicillin (50 mg/L) or chloramphenicol (170 mg/L). Clone plasmids were extracted with Edge BioSystems plasmid extraction kit. Clone inserts were PCR amplified from the plasmids with M13 vector specific universal primer (M13F: 5'-GGT GTA AAA CGA CGG CCA GTG-3'; M13R: 5'-CAC ACA GGA AAC AGC TAT G-3') in 96-well PCR microtiters. The PCR products were purified according to protocols published previously, and resuspended in Arrayit Spot solution [DNA precipitations. http://cmgm.stanford.edu/pbrown/protocols/2_DNA.html[15]. The re-suspended PCR products were printed in duplicate on silianated glass slides (Cel associated) with Cartisan Spot machines and UV-cross-linked at 3500 mJ using Stratagene cross-linker. Printed slides were post-processed using an online method[16].

Tissue samples

Rectal cancer tissues and their corresponding normal rectal tissues were obtained with informed consent from 21 patients who underwent surgical resection of their tumor in Shandong Tumor Hospital. The tissues were stored at -80℃. Tissues were homogenized with Trizol (Life Technologies) in homogenizer of IKA. Total RNA was extracted according to the manufacturer's protocols. The $A_{(260/280)}$ of total RNA was between 1.8-2.0.

Probe preparation and hybridization and data analysis

Twenty microgram of total RNA from matched tumor/normal sample was reverse transcribed into cDNA using Superscript Ⅱ (Invitrogen, Carlsbad CA) with random hexamers (Invitrogen, Carlsbad CA). Probe preparation was done based on online protocols and as described by Yang[17] and slightly modified by our laboratory[18]. First strand cDNA synthesis was primed with 6 μg random hexamers (Life Technologies) by heating at 70℃ for 10 min, snap-cooling on ice for 30 s and incubated at room temperature for an additional 5-10 min. Reverse transcription was performed in the presence of 500 μmol/L each of dATP, dCTP and dGTP, 200 μmol/L aminoalyl-dUTP (Sigma), 300 μmol/L dTTP, 1 × first strand buffer, 10 μmol/L dithiolhreitol, and 400 U superscript Ⅱ (Life Technologies, Carlsbad, CA) in 30 μL reaction at 42℃ overnight. Reactions were quenched with 0.5 mol/L EDTA and RNA template was hydrolyzed by addition of 10 μL of 1 mol/L NaOH followed by heating at 70℃ for 10 min. Reactions were neutralized with 10 μL 1 mol/L HCl and cDNA was purified with Amicon Microcon YM100 according to the manufacturer's protocol. cDNA was lyophilized in speed vaccum Centrifuger (Eppendorf 5301) and resuspended in 4.5 μL of 0.1 mol/L sodium carbonate(pH 9.0) buffer. NHS ester (4.5 μL) Cy3 or Cy5 dye (Amersham Pharmacia, GE Healthcare) in DMSO [dye from one tube (1 mg) was dissolved in 73 μL of DMSO] was added and reactions were incubated at room temperature in the dark for 1 h. Coupling reactions were quenched by addition of 41 μL of 0.1 mol/L sodium acetate, pH 5.2, and unincorporated dye was removed using QiaQuick PCR purification kits according to the manufacturer's instruction.

Hybridization and image processing

Each slide was printed with duplicate microarrays. Slides were prehybridized in 1% BSA, 5 × SSC, 0.1% SDS for 45 min, washed by dipping in double distilled deionized H_2O and 2-propanol twice and air dried and used in 1 h. Fluorescent cDNA probes were lyophylized to dryness and resuspended in 10 μL hybridization buffer (formamide 5 μL, 20 × SSC 2.5 μL, RGDDH 201 μL, 2% SDS 0.5 μL and 1 μL human cot-1 DNA). Combined Cy dye labeled probes were denatured at 100℃ water bath for 2 min and cooled at room temperature for 5 min. Room temperature probes were applied to the pre-hybridized microarray and covered with hybridized coverslip (Sigma) and placed in the hybridization chamber (Corning). Hybridizations were carried out at 42℃ water bath for 20-22 h followed by washing in 2 × SSC and 0.1% SDS for 3 min, 1 × SSC for 2 min and 0.2 × SSC for 1 min and 0.05 × SSC for 10 s, and dried by spinning in plate centrifuge at 800 rotation/min for 4 min. Arrays were scanned using a Scan-array 4000 dual color confocal laser scanner (Packard Bioscience). Data were saved as paired TIFF images. Paired images were analyzed with Quantarray 3.0 provided by the Scanner. After normalized to the total, genes of which the background subtracted intensity did not exceed 2 SD above the mean blank were excluded. The data were then sorted to obtain genes differentially expressed by $\geqslant$ 2 fold or $\leqslant$ 0.5 in at least 5 of the patients.

Semi-quantitative RT-PCR

Some of the differentially expressed genes were selected for validation of the microarray results with semi-quantitative RT-PCR. The sequences of the primers were designed with online primer design software (Primer 3 old

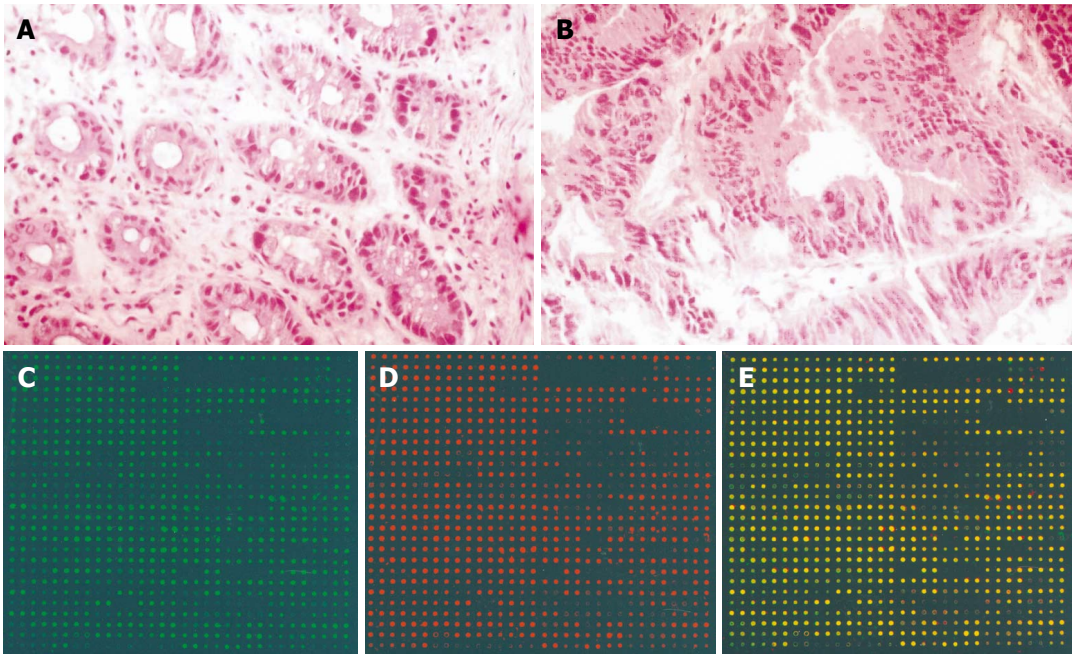

Figure 1 Histopathological examination of the tumor tissues of rectal cancer. **A**: Normal rectal mucosa tissue; **B**: Adenocarcinoma of rectal tissues; **C**, **D** and **E**: images of normal tissue, tumor tissues and overlay image of the tumor to normal of patient No.19 respectively. The density of the spot reflects the fluorescent intensity of the genes. In the overlay images, the yellow spots represent the expression of the genes in tumor tissues and normal tissues were not significant. The red spots represent the up-regulated genes in rectal cancer tissues. The green spots represent the down-regulated genes in rectal cancer tissues.

Version http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi#PRIMER_self_any[19]. The sequences of primers used for RT-PCR are as follows: MTA1 forward, 5'-AGCTACGAGCAGCACAACGGGGT-3', reverse, 5'-CACGCTTGGTTTCCGAGGAT-3'; β-actin forward, 5'-GTGGGGCGCCCCAGGCACCA-3', reverse, 5'-CTCCTTAATGTCACGCACGATTTC-3'[12]. RT-PCRs were performed with TAKARA version 2.1 RT-PCR kit using random primers in 25 μL volume. The PCR reaction was performed at 94℃ for 2 min, followed by 30 cycles at 94℃ for 30 s, 56℃ for 30 s and 72℃ for 30 s and a final extension at 72℃ for 5 min. β-actin was used as internal control. The products were electrophoresed in 1% agarose (Spanish).

Immunohistochemistry analysis of the BCL-2 protein expression in all patients

All the samples of rectal cancer and paired normal tissues were subject to immunohistochemistry examination for the expression of BCL-2 to validate the results of microarray. The primary antibody of BCL-2 was a product of Santa Cruz (1:100 dilution). The expressions of BCL-2 in tumor and normal tissues were read by two pathologists blindly. The image was captured with NIKON H600L microscope with digital camera.

Cluster analysis and principal component analysis

The normalized ratios of all pairs of samples of rectal cancer were put into an Excel-sheet. And every ratio was log2 transformed and saved as a tab-delimited Txt. file that was ready to be analyzed with Version 5.0 kindly provided online free of charge by Professor Leif E Peterson (http://mbcr.bcm.tmc.edu/genepi)[20].

RESULTS

Totally 21 pairs of rectal samples were analyzed. Their pathological and clinical characteristics are shown in Table 1. The tissue samples were all confirmed to be rectal cancer and normal paired tissues (Figure 1A and B). The differential expression genes were identified by a tumor associated genes microarray fabricated in our laboratory. The representative scan images are shown in Figure 1C, D and E which represented the image of normal tissue, rectal cancer and the overlay of the image respectively. In the overlay images of microarray, red color spots represented genes that were up-regulated in rectal cancer tissues, green color spots represented down-regulated genes and yellow color spots represented the expression of genes in cancer

Table 1 Clinical data of the rectal cancer patients

Patient No.	Age (yr)	Sex	TNM	Clinical stage
1	31	Male	T4N2M0	Ⅲ
2	51	Female	T3N1M0	Ⅲ
3	47	Male	T3N1M0	Ⅲ
4	67	Female	T3N0M0	Ⅱ
6	49	Male	T2N2M0	Ⅲ
7	38	Male	T3N0M0	Ⅱ
8	59	Female	T3N0M0	Ⅱ
9	53	Male	T3N0M0	Ⅱ
10	62	Male	T2N0M0	Ⅰ
11	62	Male	T3N1M0	Ⅲ
12	41	Male	T3N1M0	Ⅲ
13	33	Male	T4N0M0	Ⅱ
14	76	Male	T3N1M0	Ⅲ
15	75	Female	T2N0M0	Ⅰ
16	32	Female	T3N1Mo	Ⅲ
17	65	Female	T3N1Mo	Ⅱ
18	48	Female	T3N0Mo	Ⅱ
19	32	Male	T3N2M0	Ⅲ
20	68	Male	T2N0M0	Ⅰ
21	76	Male	T3N0M0	Ⅱ
22	76	Male	T3N0M0	Ⅱ

T1-4 represent the size of the tumor; N0-4 represent the level of node metastasis; M1-4 represent the level of metastasis.

Table 2 Commonly up-regulated genes in rectal cancer patients showing ≥ 2 fold over expression

Accession No.	Gene description	Patients (*n*)
NM_000184	Hs.23763, hemoglobin, gamma G (186624)	8
NM_000633	B-cell CLL/lymphoma 2 (BCL2), nuclear gene encoding mitochondrial protein, transcript variant alpha (3950016)	6
Not found	Hs.77579, ESTs (2811005)	6
Not found	Hs.87497, ESTs (1216395)	5
BB366468	Hs.126701, ESTs, Weakly similar to homolog of the Aspergillus nidulans sudD gene product [H.sapiens] (1082187)	5
NM_000600	Interleukin 6 (interferon, beta 2) (IL6) (3876176)	5
NM_004469	c-fos induced growth factor (vascular endothelial growth factor D) (FIGF) (160946)	5
BM440387	KIAA0712 gene product (539641)	5
BB217505	Hs.106513, Homo sapiens mRNA; cDNA DKFZp586I1518 (from clone DKFZp586I1518) (585388)	5
NM_002210	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51) (ITGAV) (3878920)	5
L12168	Adenylyl cyclase-associated protein (2217802)	5
NM_007047	Hs.19301, butyrophilin, subfamily 3, member A2 (1070574)	5
NM_018844	b-cell receptor associated protein (2746778)	5
U41371	Spliceosome associated protein 145 (1964680)	5
NM_001022	Hs.5621, ribosomal protein S19 (728984)	5
NM_002506	Nerve growth factor, beta polypeptide (NGFB) (72869)	5
NM_000121	Erythropoietin receptor (EPOR) (114048)	5
NM_003371	Vav 2 oncogene (VAV2) (3861692)	5
NM_000267	Neurofibromin 1 (neurofibromatosis, von Recklinghausen disease, Watson disease) (NF1) (3868563)	5
AF071400	Plasminogen activator inhibitor, type Ⅱ (arginine-serpin) (PAI2) (323255)	5
NM_006059	Laminin, gamma 3 (LAMC3) (2497685)	5
NM_002865	Hs.234726, RAB2, member RAS oncogene family (728947)	5
NM_013230	Hs.180414, CD24 antigen (small cell lung carcinoma cluster 4 antigen) (115306)	5

The number in the brackets represents IMAGE clone number.

Table 3 Commonly down-regulated genes in rectal cancers showing < 0.5 under expression

Accession No.	Gene description	Patients (*n*)
Not found	Hs.170311, EST (126182)	5
NM_004689	Metastasis associated 1 (MTA1) (4054392)	5
BC001766	S100 calcium-binding protein, beta (neural) (S100B) (759948)	5
NM_005246	Fer (fps/fes related) tyrosine kinase (phosphoprotein NCP94) (FER) (3886018)	5
NM_001920	Decorin (DCN) (274397)	5
NM_001951	E2F transcription factor 5, p130-binding (E2F5) (701492)	5
NM_002211	Homo sapiens integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12) (ITGB1) (531839)	5
Not found	Hs.23954, ESTs (132543)	5
Not found	Hs.5621, ESTs (2947053)	5
NM_007191	Wnt inhibitory factor-1 (WIF-1) (40908)	5
NM_021785	Retinoic acid induced 2 (RAI2) (501868)	5
Not found	Tachykinin, precursor 1 (substance K, substance P, neurokinin 1, neurokinin 2, neuromedin L, neurokinin alpha, neuropeptide K, neuropeptide gamma) (TAC1), transcript variant beta (784179)	5
NM_004345	Cathelicidin antimicrobial peptide (CAMP) (3057931)	5
AF385430	Rho GTPase activating protein 1 (ARHGAP1) (3896574)	5
NM_005252	v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS) (755279)	5

The numbers in the brackets represent IMAGE clone number.

tissue, which were between 0.5-2.0. If the ratio of the duplicate spots was the same, we defined it the significantly differentially expressed gene. The expression ratios of tumor associated genes in tumor and paired normal rectal tissues were analyzed with QuantaArray 3.0 and normalized to the total. The 2-fold up- or down-regulated genes were defined as differential expression genes.

The results showed that 23 genes were up-regulated in at least 5 samples (Table 2) and 15 genes were down regulated at least in 5 samples (Table 3). The up-regulated genes included growth factors, growth factor receptor, nuclear transcription factor, oncogenes, adhesion molecules, and some metabolic enzymes.

To validate the results of microarray examination, the expression of MTA1 mRNA was measured with semi-quantitative RT-PCR using β-actin as internal control. The results showed that the MTA1 expression was down-regulated (Figure 2). The up-regulation of BCL-2 was also validated by immunohistochemistry and significant expression of BCL-2 was shown in tumor tissues and less expression in normal rectal mucosa (Figure 3A-D).

For the cluster analysis, the Claufavor version 6.0 supplied kindly by Professor Leif Peterson was used to classify the patients or genes by gene expression profiles. As a result, the patients were classified into two groups, with patients No 22, 13, 12, 1, 11, 21, 19, 2, 6, 16, 15, 3 into one group which were all above grade Ⅱ except patient No 15 and the other patients into another group. The top 100 genes that were related with rectal cancer are shown in Figure 4A and B. Principal component analysis showed that all the genes were belonged to 7 factors, in which 17 genes were positively correlated with factor 1 ($r > 0.45$). They are as follows: apoptosis associated protein, xeroderma pigmentosum, complementation group A (XPA), early endosome antigen 1162 kDa (EEA), lactate dehydrogenase A, spectrin, alpha non-erythrocytic 1 (alpha-fodrin), small nuclear ribonucleoprotein associated protein n (human), phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1) , RAS p21 protein activator (GTPase activating protein) 1 (RASA1), cathepsin D (lysosomal aspartyl protease) (CTSD), v-ski avian sarcoma viral oncogene homolog (SKI), Hs.271616, ESTs, small nuclear ribonucleoprotein associated proteins b and b' (human); contains mer22.

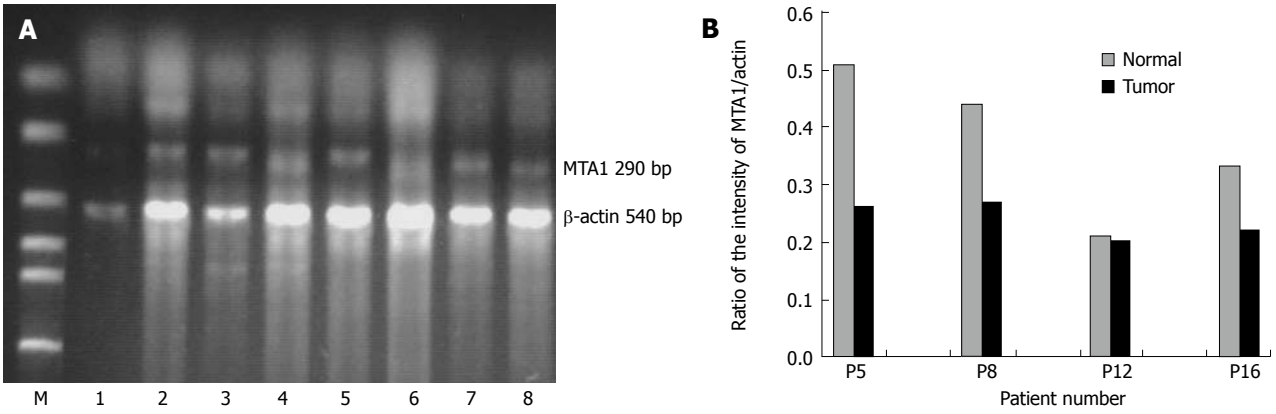

Figure 2 Semi-quantitative RT-PCR of MTA1 mRNA. **A**: Electrophoresis image of MTA1 expression, lanes 1-8 represent the mRNA expression of MTA1 in 4 pairs of rectal cancer patients, respectively. Lanes 1, 3, 5, 7 were normal tissues and 2, 3, 6, 8 were tumor tissues. **B**: The semi-quantitative analysis results with spot intensity software. The ratios of MTA1 in tumor tissues were lower than that of paired normal tissues.

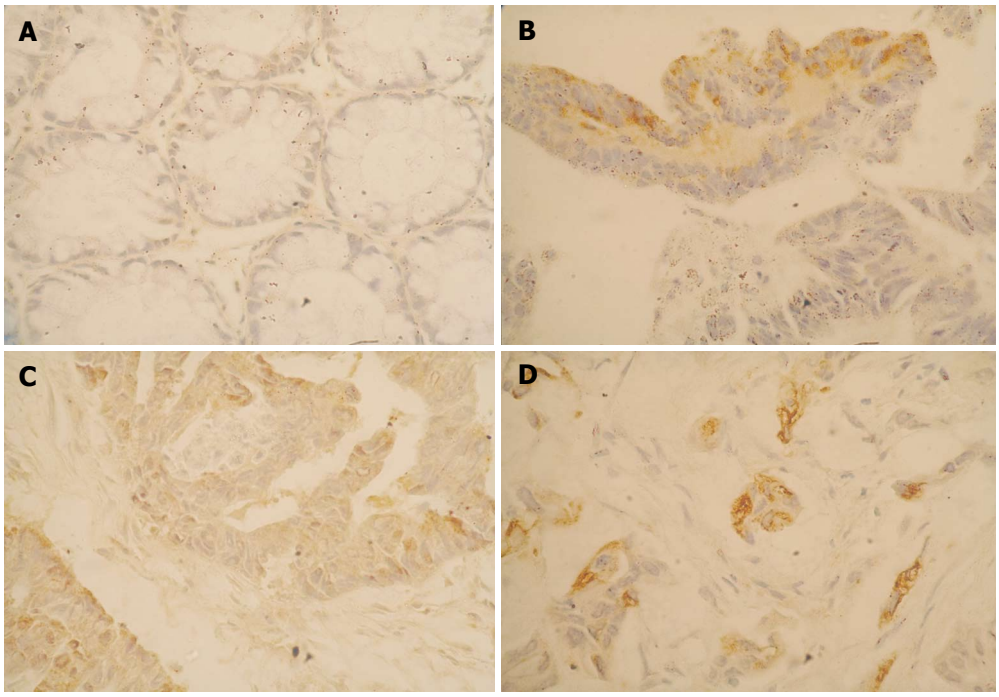

Figure 3 Validation of the expression of BCL-2 with immunohistochemistry method. **A**: The normal rectal mucosa. There was no expression of BCL-2 in all the cells; **B**: The expression of BCL-2 in well differentiated rectal cancer. The tumor cells were stained yellow-brown by DAB; **C**: The expression of BCL-2 in another intermediately differentiated rectal cancer. The expression of BCL-2 was also significant in tumor cells; **D**: The expression of BCL-2 in poorly differentiated rectal tissues.

t1 mer22 repetitive element, CD83 antigen (activated B lymphocytes, immunoglobulin superfamily) (CD83), bone marrow stromal cell antigen 1 (BST1), Wnt inhibitory factor-1 (WIF-1), histone deacetylase 1 (HDAC1), microvascular endothelial differentiation gene 1 (MDG1).

Nine genes were negatively correlated with factor 3 ($r < -0.45$) (Figure 4B); they are as follows: apoptosis associated protein, phospholipase C, delta 1 (PLCD1), small nuclear ribonucleoprotein associated protein n (human), RAS p21 protein activator (GTPase activating protein) 1 (RASA1), cathepsin D (lysosomal aspartyl protease) (CTSD), met proto-oncogene (hepatocyte growth factor receptor) (MET), 65 KDa yes-associated protein; contains Alu repetitive element; contains element MER31 repetitive element, KIAA0712 gene product, ESTs (Weakly similar to homolog of the Aspergillus nidulans sudD gene product). These results showed that microarray expression profiles may be used for the classification of rectal cancer.

DISCUSSION

Differential gene expression can be detected in several ways, including differential screening of cDNA libraries[21], subtraction hybridization[22] differential display of RNA[23], serial analysis of gene expression (SAGE)[24] and cDNA microarray[25]. Among these cDNA microarray is the most commonly used technique now for surveying many samples with thousands of genes[26]. By using cDNA microarray methods and laser capture microdissection, 235 differential expression genes were identified from colorectal cancers[14]. A colonchip has been constructed for the identification of genes that are related to colorectal cancer tumorigenesis, 59 genes showing two-fold increase of altered expression with 23 genes commonly up-regulated and 36 genes commonly down-regulated in colorectal cancer[27]. All these studies were about colorectal carcinoma, while few were concerned with rectal cancer. Moreover, rectal cancer has the tendency to occur in young

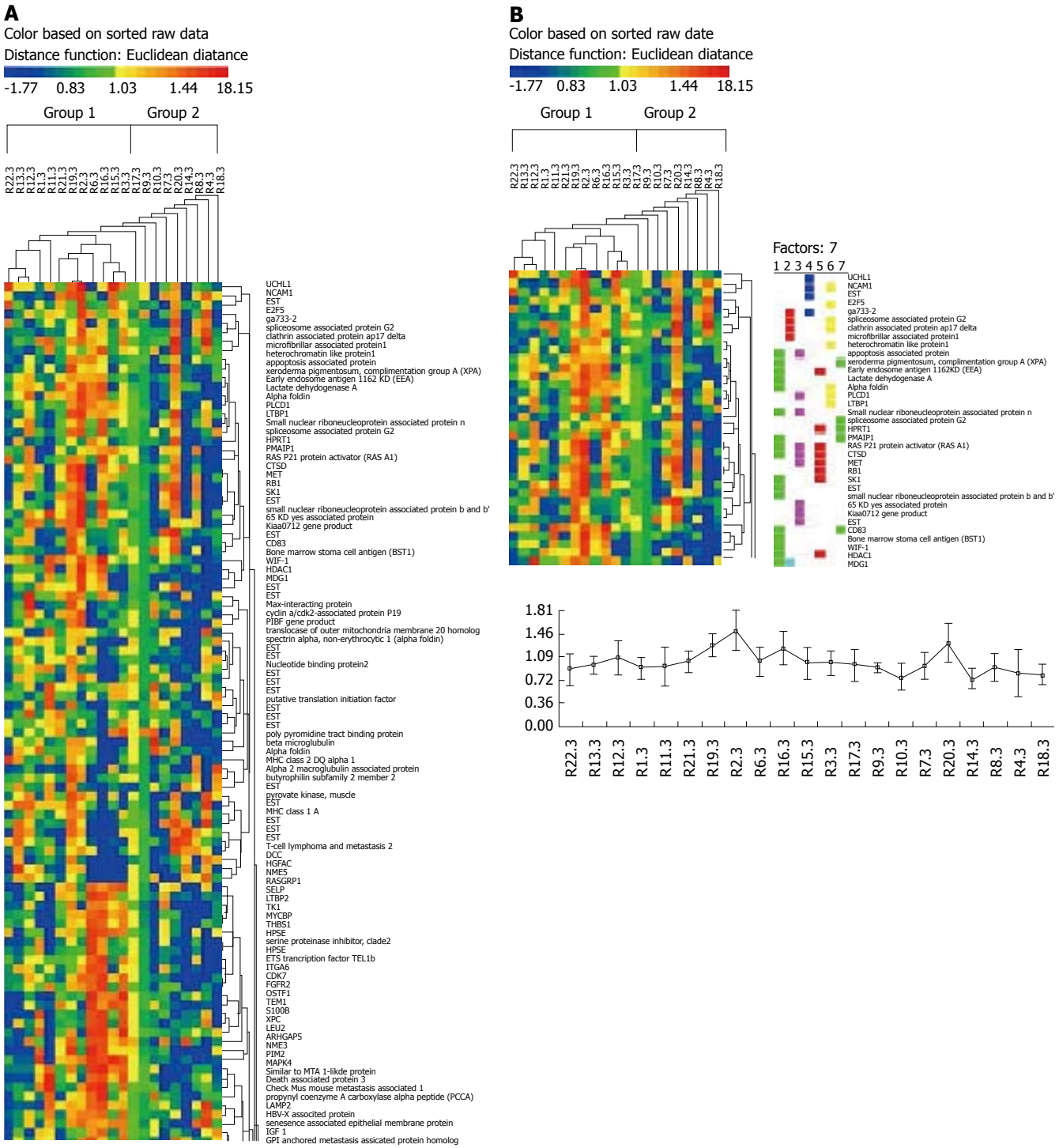

Figure 4 Hierarchial cluster analysis and principal component analysis results. **A**: Hierachial cluster analysis of patients and genes. The patients could be classified into two groups: one group of clinicopathological stage above grade Ⅱ and one group below grade Ⅱ. **B**: Principal component analysis of the genes related to rectal cancer.

age. To identify the molecular markers of rectal cancer we constructed a tumor associated genes cDNA microarray for the evaluation of the predisposition to metastasis with gene expression profiles. All the tumor associated genes were selected from literatures that are related with the growth, invasion and metastasis process of tumor and some ESTs. We used this cDNA microarray to detect the differential expression genes that play important roles in the pathogenesis of rectal cancer. We found that 23 genes were up-regulated in rectal cancers compared with their paired normal tissues. The most commonly expressed gene in rectal cancers was BCL-2. It was validated by immunohistochemistry. We also found that oncogene vav-2 was up-regulated in 5 of the 21 patients. Vav-2 acts as a guanosine nucleotide exchange factor (GEF) for RhoG and RhoA-like GTPases in a phosphotyrosine-dependent manner. Vav-2 oncogenic activation correlates with the acquisition of phosphorylation-independent exchange activity. *In vivo*, wild-type Vav-2 is activated oncogenically by tyrosine kinases, an effect enhanced further by co-expression of RhoA. Likewise, the Vav-2 oncoprotein synergizes with RhoA and RhoB proteins in cellular

transformation. Transient transfection assays in NIH-3T3 cells show that phosphorylated wild-type Vav-2 and the Vav-2 oncoprotein induce cytoskeletal changes resembling those observed by the activation of the RhoG pathway. In contrast, the constitutive expression of the Vav-2 oncoprotein in rodent fibroblasts leads to major alterations in cell morphology and to highly enlarged cells in which karyokinesis and cytokinesis frequently are uncoupled[28].

There were 15 down-regulated genes including PIGF gene products, MTA1, WIF-1 ras-guanine releasing protein and all pre-mRNA cleavage factor Im. There was a controversial report that MTA1 are down-regulated in rectal cancer and it has been validated by RT-PCR. Toh *et al*[29] reported a relative over-expression of MTA1 mRNA (tumor/normal ratio $\geqslant$ 2) was observed in 14 of 36 (38.9%) colorectal carcinomas. Tumors over-expressing MTA1 mRNA exhibited a significantly deeper wall invasion and a higher rate of metastasis to lymph nodes, and tended to be at an advanced Dukes' stage with frequent lymphatic involvement[29].

In addition, Nicolson has reported the over-expression of MTA1 in several cell lines (the breast, ovarian, lung, gastric and colorectal cancer but not melanoma or sarcoma) and cancerous tissues (breast, esophageal, colorectal, gastric and pancreatic cancer)[30]. Giannini *et al*[31] have reported that MTA1 increased in tumor tissues than in normal tissues by real-time PCR. This difference may be attributed to the different function MTA1 exerts in different tumor stage. Further studies are needed at protein levels.

To investigate the possibility of classification of tumor with gene expression profiles, hierarchical cluster analysis was done and the results showed that the patients could be divided into two groups: clinicopathological grade higher than grade Ⅱ and the other below grade Ⅱ.

Many studies have done to uncover mechanisms underlying progression of colorectal carcinogenesis and to identify genes associated with the liver metastasis. Li *et al*[32] analyzed expression profiles of 14 primary colorectal cancers with liver metastases, and compared them with profiles of 11 non-metastatic carcinomas and those of 9 adenomas of the colon. A hierarchical cluster analysis using data from a cDNA microarray containing 23040 genes indicated that the cancers with metastasis had different expression profiles from those without metastasis although a number of genes were commonly up-regulated in primary cancers of both categories. They found that 54 genes were frequently up-regulated and 375 genes were frequently down-regulated in primary tumors with metastases to liver, but not in tumors without metastasis. Subsequent quantitative PCR experiments confirmed that PRDX4, CKS2, MAGED2, and an EST (GenBank accession number BF696304) were expressed at significantly higher levels in tumors with metastasis[32]. Koehler has studied the expression profiles of 25 colorectal carcinomas (CRCs, pT1-4), corresponding normal colonic mucosa, and 14 liver metastases using cDNA arrays containing 1176 cancer-related genes (Clontech). They found no specific expression signature in matching metastases, but a set of 23 classifier genes with statistically significant expression patterns in high- and low-stage tumors was identified[33].

Komori *et al*[34] constructed the gene expression profiles of 50 colorectal cancers (CRCs) and 12 normal colorectal epithelia using a cDNA microarray specially constructed for CRC. Hierarchical clustering analysis and principal component analysis could clearly distinguish the gene profiles of cancer tissues from those of normal tissues. They identified 22 up-regulated genes and 32 down-regulated genes in CRC. Many of these genes have been previously identified in association with human carcinogenesis, being 68% and 78%, respectively[34].

Moreover, D'Arrigo *et al*[35] compared the transcriptional profiles of 10 radically resected primary CRCs from patients who did not develop distant metastases within a 5-year follow-up period with those of 10 primary/metastatic tumor pairs from patients with synchronous liver metastases. Arrays of 7864 human cDNAs were utilized using laser-microdissected bioptic tissues. Of 37 gene expression differences found between the 2 groups of primary tumors, 29 were also different between non-metastasizing tumors from metastases. The gene encoding mannosyl (alpha-1, 3-)-glycoprotein beta-1, 4-N-acetyl-glucosaminyl-transferase (GnT-Ⅳ) became significantly up-regulated in primary/metastatic tumor pairs ($P < 0.001$)[35]. All these results were obtained from colorectal cancer. However, colon cancer and rectal cancer may have different molecular markers. Our methods have similar ability in classifying tumor with gene expression profiles.

In conclusion, we have screened 23 up-regulated genes and 15 down-regulated genes from 21 rectal cancers. Hierarchical cluster analysis divided the patients into one group with clinicopathological stage above Ⅱ, and one group below stage Ⅱ. These genes may be the molecular markers of rectal cancer. In addition, we have also found gene expression profiles in different stage of rectal cancers and to further screen the molecular markers in each stage of the cancer larger samples are needed.

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