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Genes differentially expressed in responsive and refractory acute leukemia

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1. ABSTRACT

DNA microarray in 22 patients with acute leukemia revealed genes which were differentially expressed. Ribosomal protein SA (RPSA), minichromosome maintenance deficient 2 (MCM2) and heterogeneous nuclear ribonucleoprotein A1 (HNRPA1) were significantly upregulated ($p<0.05$, t test) in refractory patients, suggesting that they may play a role in refractoriness in acute leukemia and could be biomarkers of prognosis.

2. INTRODUCTION

Understanding complex biological processes, such as disease initiation and progression at the molecular level, has become an exciting area in biomedicine. Alterations in gene expression patterns accompanying different stages of growth, disease onset, cell cycle progression, responses to environmental stimuli, provide important clues about these complex processes. Identifying stereotypical expression patterns may allow elucidation of the mechanisms that underlie cancer and provide important molecular markers for diagnostic purposes.

Further progress in the management of acute leukemia (AL) is currently limited by our incomplete understanding of the molecular pathways involved in leukemia pathogenesis and by the lack of useful prognostic markers for most patients. cDNA microarray technology (1,2), which allows the simultaneous analysis at gene expression levels for thousands of transcripts, has significantly accelerated the rate of progress in our understanding of the molecular basis of AL. Currently, considerable findings have been made by applying cDNA microarray to leukemia molecular classification, diagnosis and prognosis. For instance, leukemia classification can be based solely on microarray gene expression monitoring (3); lipoprotein lipase (LPL) and a disintegrin and metalloproteinase domain 29 (ADAM29) gene expression is a strong prognostic indicator in chronic lymphocytic leukemia (4); some of the biological pathways affected by farnesyl protein transferase inhibitors (FTI) treatment may provide a proof of principle for identifying candidate genes

that might be used as surrogate biomarkers of drug activity (5).

However, most of these experiments did not describe the holistic effects and inter-regulation of those differentially expressed genes in the disease progression, but only focused on certain genes or pathways related to leukemogenesis, classification, prognosis and drug-induced changes according to clinical materials and statistical methods. Through this investigation, we aimed to find the key genes related to leukemogenesis and to refractoriness which could potentially serve as biomarkers.

3. MATERIALS AND METHODS

3.1. RNA isolation

The acute leukemia patient and normal specimens used in this study were provided by Changhai Hospital at the Second Military Medical University. Institutional approval was obtained for all specimens. Bone marrows from 22 patients diagnosed with *de novo* AL were obtained as well as normal bone marrow from 20 voluntary donors with no history of AL that were used as reference samples. Tissue samples were ground into a fine powder in a 10 cm ceramic mortar (RNase-free) and then were homogenized in TRIzol (Biostar, Shanghai, China). After centrifugation, the supernatant was separated from the organic phase and was extracted in an equal volume of chloroform. The aqueous phase was then precipitated by an equal volume of isopropanol at 4°C, centrifuged to pellet the RNA and dissolved in Milli-Q H_2O.

3.2. Construction of microarrays and probe preparations

The construction of the microarrays used in this study (12800 chip) was carried out following Brown's method (6). The 12800 microarrays consisted of 12,800 sequences including full-length and partial cDNAs representing known, novel, and control genes provided by United Gene Holdings. All the sequences were verified. The known genes were selected from the NCBI Unigene set and cloned into a plasmid vector. The novel genes were obtained through systematic full-length cloning efforts carried out at United Gene Holding. The control spots of non-human origin in the 12800 chip included the rice U2 RNA gene (8 spots), the hepatitis c virus (HCV) coat protein gene (8 spots), and spotting solution alone without DNA (32 spots). The cDNA inserts were amplified by use of the polymerase chain reaction (PCR) using universal primers to plasmid vector sequences and were then purified (7). All PCR products were examined by agarose gel electrophoresis to ensure the quality and the identity of the amplified clones as expected. Then the amplified PCR products were dissolved in a buffer containing 3· SSC solution. The solution with amplified PCR products were spotted onto silylated slides (CEL Associates, Houston, TX., USA) using a Cartesian PixSys 7500 motion control robot (Cartesian Technologies, Irvine, CA., USA) fitted with ChipMaker Micro-Spotting Technology (TeleChem International, Sunnyvale, CA., USA). The glass slides were then hydrated for 2 hrs in 70% humidity, dried for 0.5 hrs at room temperature, and UV crosslinked (65 mj/cm). They were further processed at room temperature by soaking in 0.2% sodium dodecyl sulfate (SDS) for 10 min, distilled H_2O for 10 min, and 0.2% sodium borohydride ($NaBH_4$) for 10 min. The slides were dried again and ready for use. The fluorescent cDNA probes were prepared through reverse transcription of the isolated mRNAs and then purified according to the methods of Schena et al (1,2). The RNA samples from normal bone marrows were labeled with Cy3-dUTP and those from leukemia patients with Cy5-dUTP.

3.3. Hybridization

The probe was dissolved in 20 µl of Hybridization Solution [5X SSC (0.75M NaCl and 0.075M sodium citrate), 0.4% SDS, 50% formamide]. Microarrays were pre-hybridized with a hybridization solution containing 0.5 mg/ml denatured salmon sperm DNA at 42ºC for 6 hrs. Fluorescent probe mixtures were denatured at 95ºC for 5 minutes, and then applied onto the pre-hybridized chip under a cover glass. Chips were hybridized at 42ºC for 15-17 hours. Next, the hybridized chips were each washed at 60°C for 10 min in solutions of 2X SSC and 0.2% SDS, 0.1X SSC and 0.2% SDS, and 0.1X SSC, then dried at room temperature.

3.4. Detection and Analysis

The chips were scanned with a ScanArray 4000 (GSI Lumonics, Bellerica, MA) at two wavelengths, 635nm and 532 nm, to detect emission from both Cy5 and Cy3 respectively. The acquired images were analyzed using GenePix Pro 3.0 software. The intensities of each spot at the two wavelengths represent the quantity of Cy3-dUTP and Cy5-dUTP. Ratios of Cy5 to Cy3 were computed using the GenePix Pro 3.0 median of ratio method. Overall intensities were normalized using the corresponding GenePix default normalization factor. All spots flagged "Bad" or "Not Found" by GenePix software were removed from the final data. Only genes with raw intensity values for both Cy3 and Cy5 of >200 counts were chosen for differential analysis. Genes were identified as differentially expressed if the ratio was >2 or <0.5, or the absolute value of base 2 logarithm of the ratio was >1.

4. RESULTS AND DISCUSSION

Samples from sixteen males and 6 females, from ages 15 to 86, were used in this study (AML M1 1 case, AML M2 4 cases, AML M3 5 cases, AML M4 1 case, AML M5 3 cases, AML M6 3 cases, ALL L2 5 cases). The information regarding these specimens is provided in table 1. The tumor sample gene expression was compared to the normal references (i.e. no history of acute leukemia) and the ratio of these samples was converted into a log scale with base 2. Supplement raw data can be downloaded at http://www.chinagenenet.com/weiqing/.

4.1. Genes differentially expressed in acute leukemia

A patient was defined to be refractory according to the standard proposed in the 2nd National Refractory Leukemia Conference (Fuzhou, China, Oct, 1999). In summary, a patient was regarded as refractory

Table 1. Clinical subtype and response

Subtype	Treatment [1]	Number of treatment(s)
Responsive		
AML-M1	DA	1
AML-M2	MA	1
AML-M2	DA, MA	2
AML-M2	DA	1
AML-M2	DA	1
AML-M3	ATRA	1
AML-M3	ATRA	1
AML-M3	ATRA	1
AML-M3	ATRA	1
AML-M3	ATRA	1
AML-M5	DA	1
AML-M5	DA	1
AML-M5	MA, HA	2
AML-M6	DA	1
AML-M6	DA	1
ALL-L2	VDCP	1
ALL-L2	VDCP	1
ALL-L2	VDCP	1
Refractory		
ALL-L2	VDCP	1
ALL-L2	CMVP,CODP,VDCP	3
AML-M4	DA, IDA	2
AML-M6	MA, HA	2

[1] Letters represent a combination of chemotherapy treatments where the drugs used were: D (Daunorubicin, DNR), M (Mitoxantrone), H (Homoharringtonine), A (Arabinoside), IDA (Idarubicn), V (Vincristine Sulfate, VCR), C (Cyclophosphamide, CTX), P (Prednisone), O (VCR) and ATRA (All-trans retinoic acid).

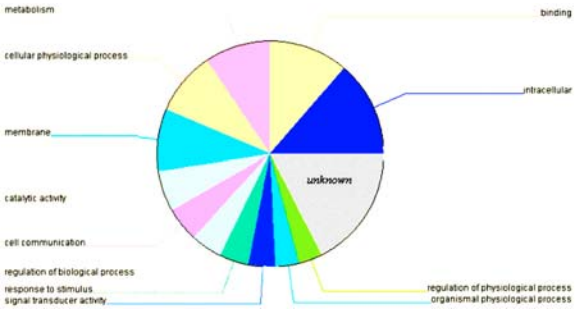

Figure 1. Gene Ontology distribution of the 114 differentially expressed genes compared to normal samples.

if he or she (a) was an AML subtype and did not show complete remission (CR) after 2 courses of classical inducement, or (b) was an ALL subtype and did not show CR 4 or 5 weeks after classical inducement, or (c) had a relapse within 6 months after the first CR, or (d) had a relapse 6 months after the first CR and failed in classical inducement, or (e) had more than one relapse.

We found 114 genes which were differentially expressed in 9 samples with an accordance rate of over 80%, including 51 downregulated genes and 63 upregulated genes. Explored by Onto-express software (8), the distribution of these 114 genes in gene ontology (GO) terms is shown in figure 1, of which the three largest known components are the intracellular, the binding and the metabolism groups. The expression of 32 key genes, was consistently changed in more than 15 samples, or were differentially regulated between responsive and refractory patients ($p<0.05$) (Table 2, Figure 2). All these genes were first reported in acute leukemia except MCM2, HNRPA1, RTN4, MAPK14, ANXA2 and NUP214.

S100 calcium binding protein A11 (S100A11), which has not been reported in leukemia yet, was downregulated in all 22 patients. The protein encoded by S100A11 gene is a member of the S100 family and contains 2 EF-hand calcium-binding motifs. S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. Downregulation of S100C expression is associated with increasing tumor aggressiveness and poor prognosis in bladder cancer patients (9).

4.2. Genes differentially expressed in refractory versus responsive acute leukemia

RPSA has not been previously reported in acute leukemia. RPSA regulates laminins, a family of extracellular matrix glycoproteins and the major noncollagenous constituent of basement membranes. Laminins have been implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signaling, neurite outgrowth, metastasis and multi-drug resistance. It has been observed that the level of the laminin receptor transcript is higher in colon carcinoma tissue and lung cancer cell line than that of their normal counterparts. Also, there is a correlation between the upregulation of this polypeptide in cancer cells and their invasive and metastatic phenotype (10-13). In our experiments, RPSA was upregulated, especially in refractory patients, with a significant difference ($p<0.01$, t test). CDKN2D, CLU, MAPK14, B2M, TXNIP and SAT induce differentiation or apoptosis and are involved in proliferation (14-19).. MCM2 is a biomarker of proliferation in human malignant fibrous histiocytoma (MFH), and its expression is ubiquitous in proliferating cells, regardless of the expression of p53 (20). This gene was differentially expressed in refractory patients and others with a significant level ($p=0.0225$). FBLN1 can inhibit proliferation by inhibiting secreted form of amyloid precursor protein (sAPP) (21), but it was downregulated in 90% of the samples, especially in refractory patients ($p=0.0396$). HNRPA1, which was reported to be upregulated in many tumors and have the function of inhibiting apoptosis (22), was markedly upregulated in refractory patients ($p=0.0065$). TNFSF1B, CTSD and ANXA2, which are involved in cell proliferation (23-25), were downregulated. Protein kinase C delta (PRKCD) which inhibits the expression of glutamine synthetase (26) and inhibits EI24 (27) and promote apoptosis (28-29)

Taken together, high density cDNA microarray, showed changes in expression profile of genes implicated in proliferation and differentiation and

Table 2. Genes differentially expressed in acute leukemia compared to normal references.

Gene	Definition	Differentially Expressed Genes in Refractory Patients	Differentially Expressed Genes in Responsive Patients	p-value [2]	Other tumors with altered gene expression
CLU	clusterin	↓ (-3.2856) [1]	# (-0.3508)	0.0003	esophageal squamous cell carcinoma, prostate cancer
MCM2	minichromosome maintenance deficient 2	↑ (1.8120)	# (0.6168)	0.0225	fibrous histiocytoma, lung cancer, **leukemia**
FBLN1	fibulin 1	↓ (-3.6710)	↓ (-2.3932)	0.0396	ovarian cancer, breast cancer
EI24	etoposide induced 2.4 mRNA	↑ (1.9820)	# (0.6699)	0.0047	breast cancer
RPSA	ribosomal protein SA	↑ (2.1115)	# (0.2410)	0.0051	colon carcinoma, lung cancer
HNRPA1	heterogeneous nuclear ribonucleoprotein A1	↑ (2.1512)	# (0.0830)	0.0065	**leukemia**,, colorectal cancer, breast cancer, ovarian cancer
ICMT	isoprenylcysteine carboxyl methyltransferase	↓ (-2.0879)	# (-0.7039)	0.0072	neuroblastoma
MDM4	transformed 3T3 cell double minute 4, p53 binding protein	# (-0.3684)	# (0.9170)	0.0248	breast cancer
RTN4	reticulon 4	↓ (-2.0737)	↓ (-1.2786)	0.0254	**leukemia**, various cancers
PGEA1	PKD2 interactor, golgi and endoplasmic reticulum associated 1	# (-0.6283)	# (0.5373)	0.0349	colon carcinoma
EIF3S3	eukaryotic translation initiation factor 3, subunit 3 gamma	# (0.6788)	↓ (-1.0178)	0.0364	breast cancer, prostate cancer
FEZ1	fasciculation and elongation protein zeta 1	↓ (-1.1001)	# (0.0748)	0.0398	oral squamous cell carcinoma
F2R	coagulation factor II (thrombin) receptor	# (-0.8225)	# (0.4709)	0.0467	ovarian cancer, prostate cancer, pancreatic cancer
DUSP1	dual specificity phosphatase 1	↓ (-4.5299)	↓ (-3.0530)	0.0487	lung cancer, renal cell cancer, HCC
LAPTM5	lysosomal associated multispanning membrane protein 5	↓ (-1.9525)	↓ (-2.5199)		multiple myeloma
CTSD	cathepsin D (lysosomal aspartyl protease)	↓ (-2.5468)	↓ (-2.0994)		breast cancer, ovarian cancer, colon carcinoma
CDKN2D	cyclin-dependent kinase inhibitor 2D	↓ (-2.3551)	↓ (-2.3010)		glioma
RXRB	Retinoid X receptor, beta	↓ (-1.9595)	↓ (-1.9434)		prostate cancer, lung cancer
ACTR3	ARP3 actin-related protein 3 homolog	↓ (-1.0718)	↓ (-2.0890)		gastric cancer
CTNNA1	Catenin (cadherin-associated protein), alpha 1, 102kDa	↓ (-1.0587)	↓ (-1.5569)		pancreatic cancer
B2M	beta-2-microglobulin	↓ (-2.0144)	↓ (-2.6824)		melanoma, multiple myeloma
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	↓ (-2.8468)	↓ (-2.3734)		thyroid cancer
VIM	Vimentin	↓ (-3.1986)	↓ (-2.6383)		prostate cancer, breast cancer
HLA-A	Major histocompatibility complex, class I, A	↓ (-1.9226)	↓ (-2.3328)		bladder cancer, prostate cancer, meningioma
ARPC1B	Actin related protein 2/3 complex, subunit 1B, 41kDa	↓ (-2.4730)	↓ (-2.8175)		gastric cancer
MAPK14	mitogen-activated protein kinase 14	↓ (-2.5919)	↓ (-2.2791)		Ewing's sarcoma, breast cancer, HCC, **leukemia**
TXNIP	thioredoxin interacting protein	↓ (-2.8192)	↓ (-2.5735)		prostate cancer
ANXA2	annexin A2	↓ (-2.4306)	↓ (-2.8155)		osteosarcoma, prostate cancer, **leukemia**
SAT	spermidine/spermine N1-acetyltransferase	↓ (-2.9216)	↓ (-3.2173)		colon carcinoma
PRKCD	Protein kinase C, delta	↓ (-1.7967)	↓ (-1.9125)		colon carcinoma, cervical carcinoma
S100A11	S100 calcium binding protein A11	↓ (-3.9894)	↓ (-3.8912)		squamous-cell carcinoma, bladder cancer, gastric cancer
NUP214	nucleoporin 214kDa	↓ (-1.6465)	↓ (-1.5961)		**leukemia**

[1] "↑" signifies upregulation, "↓" signifies downregulation and "#" signifies normal level of expression. The number in the parentheses indicates the average log2 ratio of the refractory or responsive groups compared with normal samples. [2] The p-value was calculated using all of the data for each particular gene and comparing the refractory and responsive groups.

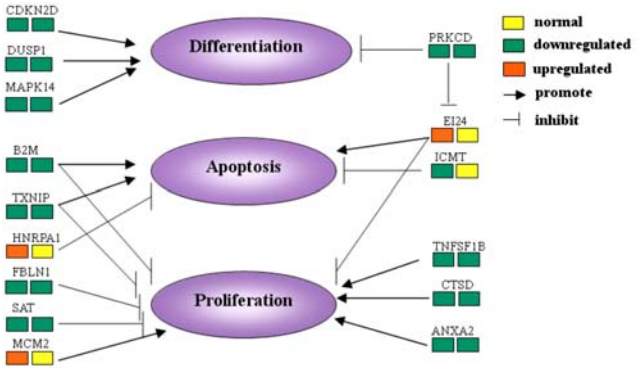

Figure 2. Genes differentially expressed and their effects on cellular processes: Each pair of small colored blocks represents gene expression in which refractory samples are represented by the small block on the left, and responsive samples are represented by the small block on the right under each gene. The red, green, and yellow blocks represent upregulation, downregulation and normal average expressions respectively. The lines connecting the small blocks to the ovals represent the effect the gene has in normal expression where the arrows mean promoting a cell process and the lines with the "T-shaped" ends mean inhibiting a cell process. Thus, when the cell has normal expression of the gene, the effect will be as shown but when the gene is upregulated, the effect will be amplified. Conversely, when the gene is downregulatated, the specific effect will be attenuated.

apoptosis. RPSA, MCM2, HNRPA1 may play important roles in refractoriness of acute leukemia and potentially can be used as biomarkers for prognosis of acute leukemia.

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Abbreviations: RPSA: Ribosomal protein SA; MCM2: minichromosome maintenance deficient 2; HNRPA1: heterogeneous nuclear ribonucleoprotein A1; AL: acute leukemia; LPL: lipoprotein lipase; HCV: hepatitis c virus; PCR: polymerase chain reaction; CR: complete remission; ADAM29: a disintegrin and metalloproteinase domain 29; FTI: farnesyl protein transferase inhibitors; GO: gene ontology; S100A11: S100 calcium binding protein A11; MFH: malignant fibrous histiocytoma; sAPP: secreted form of amyloid precursor protein; PRKCD: Protein kinase C delta; RTN4: reticulon 4; MAPK14: mitogen-activated protein kinase 14; ANXA2: annexin A2; NUP214: nucleoporin 214kDa; CDKN2D: cyclin-dependent kinase inhibitor 2D; CLU: clusterin; B2M: beta-2-microglobulin; TXNIP: thioredoxin interacting protein; SAT: spermidine/spermine N1-acetyltransferase; TNFSF1B: tumor necrosis factor receptor superfamily, member 1B; CTSD: cathepsin D; EI24: etoposide induced 2.4 mRNA

Key words: leukemia, cDNA microarray, responsive, refractory

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