Analysis of Gene Expression in the K562-n High Tumorigenitic Human Leukemia Cell Line

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This work was supported by the National Natural Science Foundation of China (No. 39770330).

OBJECTIVE The human leukemia K562-n cell line displays much higher tumorigenic actively in nude mice compared with its parental K562 cell line. The molecular mechanism of the differences in tumorigenicity between K562-n and K562 in nude mice was examined.

METHODS The differences in gene expression between K562 and K562-n cells were analyzed by using cDNA microarrays.

RESULTS Among the12,800 genes examined, there was a significant difference in expression of 139 genes between K562-n and K562 cells. Eighty-five of these genes have been registered in the GeneBank and 54 are unknown. The genes accessible from the GeneBank include:1) oncogenes and tumor-supressor genes; 2) genes related to transcription regulation, the cell cycle and apoptosis; 3) genes related to the cytoskeleton and cytokinetics; 4) genes related to metabolism and transport; 5) genes related to immune function. There were also some differently expressed genes with mixed functions.

CONCLUSION There are many genes differentially expressed between K562-n and K562 cells .The high tumorigenicity of the human leukemia K562-n cell line in nude mice might be related to its specific gene-expression profile.

KEYWORDS: leukemia, DNA microarrays, gene expression.

The human leukemia cell line K562-n, which was derived from the K562 cell line by repeated in vitro and in vivo passages, is highly tumorigenic in nude mice. This highly tumorigenic cell line is very useful in experimental studies of human leukemia in vivo. Compared with the original K562 cell line, K562-n cells have some unique biologic manifestations: such as a high colony forming efficiency in soft agar culture medium, an increased proportion of S phase cells, high resistance to NK cells of nude mice and the presence of complex karyotype changes.[1-3] To study the molecular mechanism of the high tumorigenicity of K562-n cells, we utilized cDNA microarrays to analyze the difference in genetic expression between K562 and K562-n cells.

MATERIALS AND METHODS

Materials

The K562 cell line was provided by the Cell Biology Institution of

Received September 10, 2004; accepted November 8, 2004.

Chinese Journal of Clinical Oncology
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the Chinese Academy of Science. The K562-n cell line was established in our laboratory. The siliconized slides and poly-lysine slides were products of the Sigma Co., and the siliconized slides and silane slides were purchased from the TeleChem Co.. An oligotex mRNA Mini Kit was obtained from the Qiagen Co. and Trizol was a product of the GIBCO Co. The primers were synthesized by the Shanghai Biotech Co. Lauryl sodium sulfonates (SDS) were obtained from the SERVA Co. and subpackaged by the Huamei Co. Superscript II inversion Kit and *Taq* polymerase were purchased from the GIBCO Co. and Cy3-dUTP and Cy5-dUTP from the Amersham Pharmacia Biotech Co.

Construction of microarrays

The 12,848 target-gene cDNA clones were provide by Shanghai United Gene Holdings, Ltd Co. (gene technology conglomerate company). The cDNA inserts were amplified by a PCR method using universal primers followed by purification as described previously.[4] The quality of PCR products was monitored using agarose gel electrophoresis. The purified PCR products were dissolved in a buffer containing 3 × SSC solution [0.45 mol/L NaCl, 0.045 mol/L sodium citrate] and then spotted onto siliconized slides using a Cartesian Pixsys 7500 motion control robot (Cartesian Technologies, Irvine, CA) fitted with the ChipMaker Micro-Spotting Technology (TeleChem International, Sunnyvale, CA). After spotting, the slides were hydrated for 2 h, dried for 0.5 h at room temperature, and crosslinked with ultraviolet light (65 mJ/cm). The slides were then processed at room temperature by soaking in 0.2% sodium dodecyl sulfate (SDS) for 10 min, in distilled H_2O for 10 min, and 0.2% soldium borohydride ($NaBH_4$) for 10 min. The slides were dried again and ready for use.

Extraction of total RNA

Total RNA was extracted according to the original single step extraction procedure of Tsujimoto, et al.[5] with slight modification. K562 and K562-n cells in exponential growth (1×10^8)were homogenized in Solution D -mercaptoethanol. After centrifugation, the supernatant was extracted twice with an equal volume of phenol: chloroform (1:1) and once with an equal volume of acidic phenol: chloroform (5:1). The aqueous phase was then precipitated by an equal volume of isopropanol at 4℃, centrifuged to pellet the RNA, which was dissolved in deionized (Milli-Q) H_2O and analyzed with ultraviolet light.

Purification of mRNA

Messenger RNAs were purified using an Oligotex-dT mRNA Midi Kit (Qiagen) (please refer to introdution).

Labelling of probes

The fluorescent cDNA probes were prepared through reverse transcription and then purified according to the methods of Schena, et al.[4] The mRNA from K562 cells was labeled with Cy3-dUTP and that from K562-n cells with Cy5-dUTP. The two-color probes were then mixed, precipitated with ethanol and dissolved in 20μl hybridization solution [5 × SSC (0.75 mol/L NaCl and 0.075 mol/L sodium citrate), 0.4% SDS, 50% formamide and 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone and 0.1% BSA)]. Hybridization and washing microarrays were pre-hybridized with hybridization solution containing 0.5 mg/ml denatured salmon sperm DNA at 42℃ for 6 h. Fluorescent probe mixtures were denatured at 95℃ for 5 min, and the denatured probe mixtures were applied onto the pre-hybridized chip under a cover glass. Chips were hybridized at 42℃ for 15-17 h. The hybridized chips were then sequentially washed at 60℃ for 10 min in solutions of 2 × SSC and 0.2% SDS, 0.1 × SSC and 0.2% SDS and 0.1× SSC, then dried at room temperature.

Detection and analysis

Microarrays were scanned with a ScanArray 3,000 (General Scanning GSI Lumonics, Bellerica, MA) at 2 wavelengths to detect emission form both Cy3 and Cy5. The intensities of Cy3 and Cy5 images and ratios of Cy3 to Cy5 were analyzed using ImaGene 3.0 software (BioDiscovery, Inc., Los Angeles, CA).

Overall intensities were normalized with a correction coefficient obtained using the ratios of 40 housekeeping genes. Genes were identified as differentially expressed only if the ratio of Cy3 to Cy5 was >2.5 or each of the Cy3 and Cy5 were >800.

Verification of gene differential expression using hybridization in situ

K562 and K562-n cells were immobilized on glass slides, dried and immersed into 4% formaldehyde /0.1 mol/l PBS (pH 7.2) for fixation. After treatment with 5 μg/ml protease K/0.1mol/L PBS in a 37℃ water bath, mRNA in the K562 and K562-n cells wa force-hybridized with a cardiox-RNA probe for 1 h and hybridized at 40℃ - 45℃ for 15 h. Alkaline phosphatase labeling anti-cardiox antibody was added onto the slides after SSC gradient elution. The reaction system was incubated for 4 h at 25℃, then a disclosing solution was added and the slides kept in the dark at room temperature for 1 h The reaction was stopped and the slides were observed under a light microscope. Black granules were considered as positive.

RESULTS

Gene expression in K562-n and K562 cells

The DNA microarray included 12,848 dots, among which there were 6,200 known genes, 6,600 unknown genes and 48 negative contrast spots. These 48 negative spots included paddy U2RNA genes (8 spots), HCV coat protein genes (8 spots) and spotting solution alone without DNA (32 spots). As expected, the results of hybridization showed there were no or very low signals at the negative spots. Between K562 and K562-n cells there were 139 genes differently expressed. Among them 42 genes were up-regulated in K562-n cells and 97 genes down-regulated. Eighty-five of these genes have been registered in the GeneBank and 54 were unknown genes.

The genes accessible from the GeneBank include: 1) oncogenes and tumor-supressor genes (Table 1); 2) genes related to transcription regulation, cell cycle and apoptosis (Table 2); 3) genes related to cytoskeleton and cytokinetics (Table 3); 4) genes related to metabolism and transport (Table 4); 5) genes related to immune function (Table 5). There were also some differently expressed genes with mixed functions (Table 6) and some unknown genes.

In situ hybridization of genes differently expressed

In situ hybridization was used to verify the results of the microarray results. The genes used for hybridization included up- and down-regulated genes in K562-n cells. The results coincided with microarray results.

DISCUSSION

Tsujimoto [5] reported that, in hybridoma cell lines originating from Hela cells with or without tumorigenicity in nude mouse and in human fibroblast cells, genes were differently expressed in encoding

Table 1. Oncogenes and tumor-supressor genes differently expressed between K562-n and K562 cells

GeneBank No.	Name of gene product	cy5\cy3
X64229	Homo sapiens dek mRNA	2.904
M73547	Human polyposis locus (DP1 gene) mRNA	2.811
NM-002634	Homo sapiens prohibitin (PHB) mRNA	0.399
U41315	Human ring zinc-finger protein (ZNF127-Xp) gene and 5' flanking sequence	0.395
J03068	Human DNF15s2 (lung) mRNA	0.388
AJ224901	Homo sapiens mRNA for ZNF198 protein	0.380
U47924	Human chromosome 12p13 sequence	0.362
NM-000251	Homo sapiens mutS homolog 2 (MSH2) mRNA	0.316
F042385	Homo sapiens cyclophilin-33A (CYP-33) mRNA	0.355

Table 2. Genes related to apoptosis differently expressed between K562–n and K562 cells

GeneBank No.	Name of gene product	cy5\cy3
M69043	Homo sapiens MAD-3 mRNA encoding IkB-like activity	3.903
AF038955	Homo sapiens G protein gamma 5 subunit mRNA	3.492
U14193	Human TFIIA gamma subunit mRNA	2.927
U72209	Human YY1-associated factor 2 (YAF2) mRNA	2.836
NM-001667	Homo sapiens ADP-ribosylation factor-like 2 (ARL2) mRNA	2.729
M14218	Human argininosuccinate lyase mRNA	0.394
U88966	Human protein rapamycin associated protein (FRAP2) gene	0.334
M37033	Human CD53 glycoprotein mRNA, complete cds	0.314
AB015856	Homo sapiens mRNA for ATF6	0.291
AJ012008	Homo sapiens genes encoding RNCC protein, DDAH protein, Ly6-C protein, Ly6-D protein and immunoglobulin receptor	0.248
AF026166	Homo sapiens chaperonin-containing TCP-1 beta subunit homolog mRNA	0.227
X77278	HYL human hematopoietic consus tyrosine-lacking kinase	0.225
AJ271408	Homo sapiens mRNA for Fas-associated factor, FAF1 (Faf1 gene)	0.167

Table 3. Genes related to the cytoskeleton differently expressed between K562–n and K562 cells

GeneBank No.	Name of gene product	cy5\cy3
J04621	Human heparan sulfate proteoglycan (HSPG) core protein	4.086
M94345	Homo sapiens macrophage capping protein mRNA	3.591
D38583	Human mRNA for calgizzarin	2.754
NM-001667	Homo sapiens ADP-ribosylation factor-like 2(ARL2) mRNA	2.729

Table 4. Genes related to metabolism and transport differently expressed between K562–n and K562 cells

GeneBank No.	Name of gene product	cy5\cy3
AF103796	Homo sapiens placenta-specific ATP-binding cassette transporter (ABCP) mRNA	3.644
U05598	Human dihydrodiol dehydrogenase mRNA	3.206
U05861(M86609)	Human hepatic dihydrodiol dehydrogenase mRNA	2.965
X16396	Human mRNA for NAD-dependent methylene tetrahydrofolate dehydrogenase cyclohydrolase (EC 1.5.1.15)	2.757
NM-006411	Homo sapiens 1-acylglycerol-3-phosphate O-acyltrans ferase 1 (lysophosphatidic acid acyltransferase, alpha) (AGPAT1), mRNA	0.398
M14218	Human argininosuccinate lyase mRNA	0.394
X69433	Homo sapiens mRNA for mitochondrial isocitrate dehydrogenase(NADP$^+$)	0.341
D14697	Human mRNA for KIAA1293 gene	0.278
M33374	Human cell adhesion protein (SQM1) mRNA	0.250
AJ012008	Homo sapiens genes encoding RNCC protein, DDAH protein, Ly6-C protein, Ly6-D protein and immunoglobulin receptor	0.248
X59543	Human mRNA for M1 subunit of ribonucleotide reductase	0.223

Table 5. Genes related to immune function differently expressed between K562–n and K562cells

GeneBank No.	Name of gene product	cy5\cy3
NM-000061	Human sapiens Bruton agammaglobulinemia tyrosine kinase (BTK) mRNA	0.305
X57809	Homo rearranged immunoglobulin lambda light chain mRNA	0.215
AJ249377	Human sapiens partial mRNA for humanIg lambda light chain variable region, clone MB91(331 bp)	0.185
X57812	Homo rearranged immunoglobulin lambda light chain mRNA	0.068

Table 6. Genes with mixed functions differently expressed between K562-n and K562 cells

GeneBank No.	Name of gene product	cy5\cy3
AF068846	Homo sapiens scaffold attachment factor A (SAF-A) mRNA	0.395
X04526	Human liver mRNA for beta-subunit signal transducing proteins Gs/Gi (beta-G)	0.376
U32315	Human syntaxin 3 mRNA	0.374
AW965934	EST378007 Homo sapiens cDNA	0.334
U09278	Human fibroblast activation protein mRNA	0.321
S75168	Human megakaryocyte-associated tyrosine kinase	0.298

cell signal-transduction molecules, extracellular matrix protein, cellular structural protein and enzymes on the surface of the cell membrane. In our study, we have compared the gene-expression profile of the highly tumorigenic K562-n cell line with its original cell line K562. Significant differences in expression were found in tumor oncogenes, apoptosis-related genes, cell structural genes, genes concerned with cell metabolism and transport, genes associated with immune status and some genes with mixed or unknown function.

Several oncogenes were up-regulated and tumor suppressor genes were down-regulated in K562-n leukemia cells compared with K562 cells. For example, the leukemia-related oncogene dek was up-regulated and the 12p13 sequence containing tumor suppressor gene ETV6 was down-regulated. Notably, these genes are associated with tumor subtypes with poor prognosis. For example, rearrangement of the dek and ETV6 genes are associated with blast crisis of chronic myeloid leukemia (CML), myelodysplasia (MDS) and atypical CML;[6,7] deletion of DNF15s2 of 3p21 is associated with small-cell lung carcinoma;[8] deletion of an allele of Cyp is related to grade II to III aggressive breast ductal cancinoma.[9] We suggest that it is the abnormal expression of these genes that increase the carcinogenicity of the K562-n cell line which enables it to escape from the surveillance of the immune system, and enhances progression of leukemia. Absence of expression of hMSH2 can lead to the destabilization of micro-satellites and increase the possibility of mutations of other genes. We also observed that this gene was down-regulated in K562-n cell lines, which may contribute to mutations of other genes.

In comparison to K562 cells, the highly carcinogenic K562-n cells showed significantly different expression of the genes associated with regulation of transcription, the cell cycle and apoptotic-associated genes. These genes are involved in cell proliferation, differentiation and apoptosis and play an important role in the formation and progression of tumors. At the same time, apoptosis inducing genes also were down-regulated in the K562-n cell line, while apoptosis-inhibiting genes were up-regulated. A difference in expression of genes associated with NF-κ B activity was also observed. All these noted differences contributed to apoptosis resistance and an increase in the tumorigenicity of the K562-n line. For example, the up-regulated MAD-3 gene encodes a protein possessing Iκ B activity, which can inhibit NF-κ B activity and regulate transcription. Human Fas associated factor (hFAF-1) gene exerts positive effects on the signals induced by Fas,[10] and the latter is an important pathway for apoptosis induced by NK cells. In K562-n cells, the hFAF-1 gene was down-regulated, which resulted in resistance to the apoptotic signals induced by NK cells. The G6A gene encoding DDAH (dimethyl arginine dimethylaminohydrolase) and arginine succinate lyase were down-regulated, which are related to the regulation of NO synthesis. NO can inhibit DNA combining activity of NF-κ B and induce apoptosis, and it is associated with the sensitivity of tumor cells to NK cells and the intrinsic activity of NK cells.[11] The NO synthase system was down-regulated in K562-n cells, which also contributes to the resistance of apoptosis induced by NK cells. The

pamycin-related protein (FRAD) gene can block cell movement from the G1 phase into S phase. This gene is down-regulated in the K562-n cell line, resulting in the enhanced passage of cells into the S phase, which is consistent with the phenomenon of an increased percentage of S phase cells in the K562-n cell line.[3]

The genes encoding the enzymes of cellular metabolism were up-regulated in K562-n cells. Dihydrodiol dehydrogenase (DDH) is associated with the carcinogenic action of aromatic compounds, a finding which is supported by the high expression of DDH in the human liver cancer cell line HePG2 and human lung cancer cell line NCI-H322.[12] The DDH gene and liver DDH are up-regulated in the highly tumorigenic K562-n cells, indicating that some carcinogenic chemicals may play an important role in the occurrence and development of leukemia. Besides DDAH and arginine succinate lyase which encode the NO synthase system, some other genes associated with metabolism were also down-regulated, such as lysophosphatidic acid acyltransferase (LPAAT). These genes participate in signal transduction, transcription and regulation of apoptosis. Some other genes associated with cellular transport were also differently expressed in K562-n cells: human placenta specific ATP-binding cassette transporter (ABCP) was up-regulated, encoding a transport protein related to multi-drug resistance;[13] the human cell adhensive protein SQM1 gene was down-regulated, encoding the SQM1 protein functions in cell differentiation, adhesion and drug transportat,[14] such as MTX. The difference in expression of ABCP and SQM1 in K562-n cells indicates that K562-n cells may be resistant to some drugs.

A series of genes associated with the structural proteins of the cell were up-regulated in K562-n cells, including the genes encoding calgizzarin, ADP glycosylized protein 2 (Arl2) and macrophage capping protein (CAPG). Calgizzarin can inhibit myosin Mg^{2+}-ATPase activated by actin, and is related to the fixation of the cell structure and the kinetics of cell motility, providing malignant and metastatic behavior to the cancer cells. Arl2 can prevent myotube protein and microtubules from being degenerated by D factor. High expression of CAPG could enhance the response of the cells to platelet derived growth factor. Under electron microscopy, one sees that microfilaments are increased in number and arranged in a disordered way, reflecting the change of expression of actin and its related genes, which are associated with intracellular signal transduction. Through these alterations, K562-n cells are more sensitive to the stimulation of growth factors, and infiltrate more easily into the peripheral tissues.[3] The HSPG gene is not only related with the reconstitution of the cellular structure proteins, but also the functions in cell adhesion, proliferation and resistance to apoptosis,[15] which are highly expressed in K562-n cells.

The MHC III antigen genes are down-regulated in the K562-n cell line, such as DDAH encoded by G6A. As mentioned above , DDAH is involved in the regulation of NO synthesis, and is associated with cell apoptosis; G6C and G6D encode Ly6-C and Ly6-D of the leukocyte antigen 6 superfamily (Ly6 protein, a kind of mature leukocyte antigen, is a GPI anchor protein); the G6 gene encodes RNCC protein; G6B encodes a new Ig sumperfamily member, which contains an IgD-like region and a cytoplasmic ending peptide involved with signal transduction (2 micro-satellite markers 82-2 and D6S273 which are associated with some autoimmune diseases are located in this region). The down-regulation of genes in this region is considered to be related to inhibition of apoptosis of K562-n cells.

Some other genes directly or indirectly associated with the immune function are down- regulated in K562 cells, such as the variable region of Ig light chain (Vλ) gene, rearranged Igλ light chain gene sequence (contains a 7-variable region and a 7 junction-constant region) and the Btk (Bruton's tyrosine kinase) gene. The IgCλ (light chain constant region) and IgVλ are located in the long arm of chromosome 22. The IgCλ is located near the breaking point of the Ph chromosome, neighboring the c-abl gene which is translocated from chromosome 9 and is amplified; IgVλ is located in 22q11, and also is amplified in K562.

In addition, a macrophage-associated kinase gene and the fibroblast-activated protein gene (U09278) in

K562-n cells were also down-regulated. The latter is up-regulated in the hybridized cell line of tumorigenic Hela cells and human fibroblasts, which is related to the carcinogenicity of K562-n cells in nude mice. The SAF-A gene, LDLC gene, signal-transduction protein beta-G gene, and P16-Arc gene in an Arp2/3 protein complex are also down-regulated in K562 cells. The function of SAF-A is associated with structure of nuclear chromatin and RNA metabolism; the LDLC gene encodes Golgi body proteins and maintains the normal function of the Golgi complex. The difference in expression of other unknown genes needs further investigation.

In conclusion, the mechanism of the carcinogenic activity of K562-n cells in nude mice is complex, involving a series of changes of gene expression, including the up-regulation of oncogenes and down-regulation of tumor suppressor genes. Genes concerning transcription, apoptosis, the cell cycle, cellular structural proteins and metabolic enzymes are all involved. These differently expressed genes represent a specific profile associated with tumorigenicity of K562-n cells in nude mice, and result in features such as the high colony formation rate in agar culture, an increased percent of cells in S phase, a micro-structure abnormality of increased microfilaments, and a tolerance to host immune cells such as NK cells. These characteristics result in an enhanced tumorigenicity of the K562-m cells in nude mice.

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