データベース

HTTP://WWW.I.KYUSHU-U.AC.JP/DB_ANGEL/

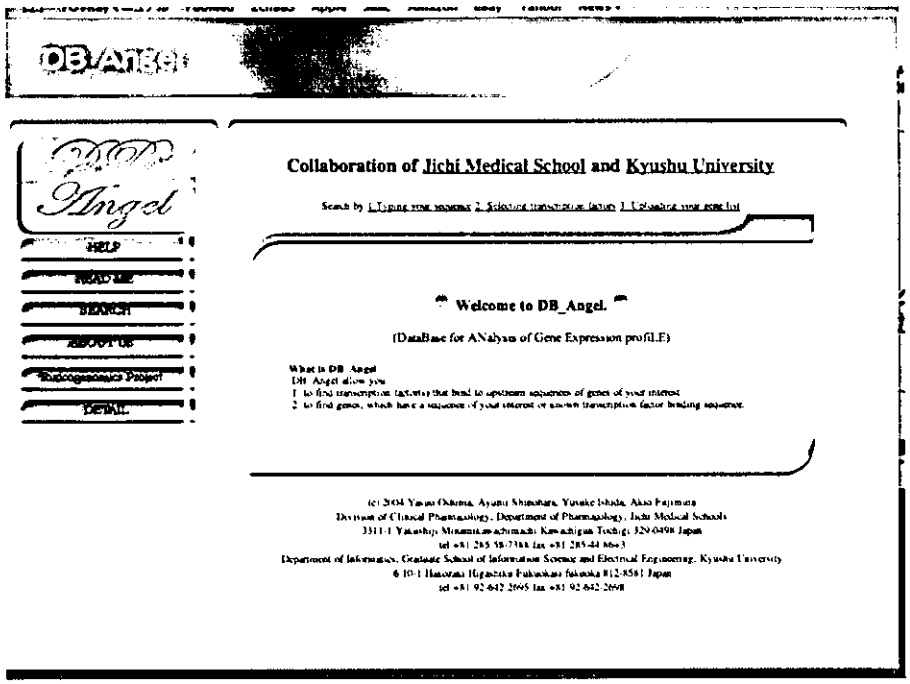

図 11　データベース

データベースのルックスアンドフィールを示す。機能は 1) 配列を自由に入力して、その配列を上流に有する遺伝子のプローブセット ID のリストを出力する、2) 遺伝子を入力して、その遺伝子の上流に、5,000 の既知の転写因子がそれぞれ結合する配列が存在するか否か、存在するならば、その転写因子のリストを出力する、3) 遺伝子のリストを入力して、その遺伝子とそのリスト以外の遺伝子を統計計算・比較し、リストにある遺伝子の上流に有意に高頻度に存在する転写因子結合配列およびその転写因子を出力する、上記 2) 3) により得た転写因子それぞれについて、報告された文献にリンクする、等である。バイオインフォマティクス部門の篠原博士の異動に伴い九州大学のサーバーでの維持が不透明となった。本報告書作成時点 (2005 年 3 月) では http://www.i.kyushu-u.ac.jp/~y-ishida/DBANGEL/
よりアクセスが可能である。

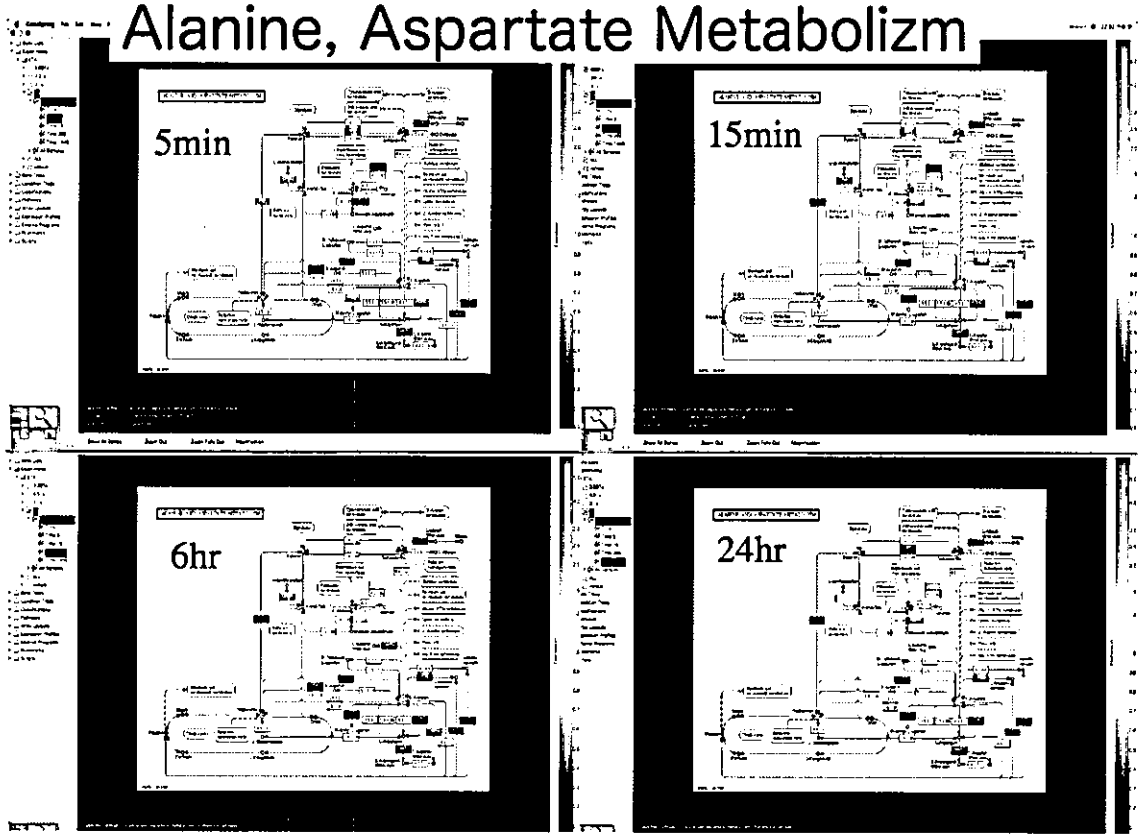

図 12　パスウェイ解析

蛋白質合成を阻害する ETA 曝露後 5 分・15分・6時間・24時間後の発現データを示す。発現量は右のカラースケールで示し、赤が発現量の高い状態を、青が発現量の低い状態を示す。アミノ酸代謝にかかわる酵素群が発現誘導・抑制を受けている様子が観察できた。

薬物曝露-遺伝子発現解析

- 解析薬物：21種類の（主に腎障害性）薬物・薬物類似化合物
  - プラチナ錯体3種
  - 抗生物質13種
  - 免疫抑制剤3種
  - 免疫抑制剤類似化学物質1種
  - ETA(リコンビナントトキシン)
- 曝露時間：pre, 10 min, 60 min, 6 hr, 24hr（5のタイムポイント）
- 各観察点につき3枚のGeneChip使用

図 13　薬物曝露条件

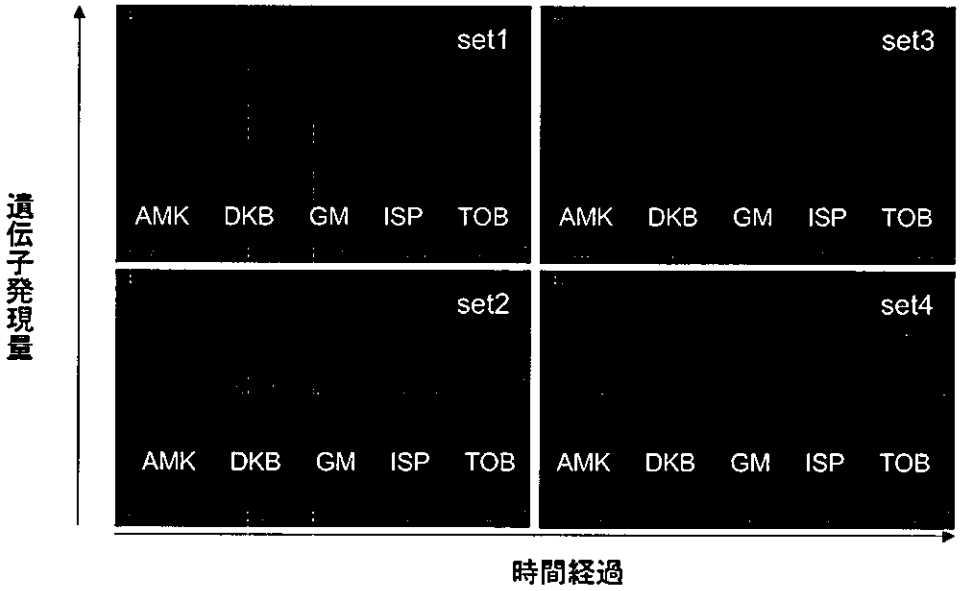

図 14　アミノグリコシド系抗生物質曝露後の遺伝子発現

横のカテゴリーは薬物の略称、横軸は時間経過、縦軸が発現量である。4 つのクラスターがそれぞれ示されている。abbrebiations: AMK; amikacin sulfate, DKB; dibekacin sulfate, GM; gentamicin sulfate, ISP; isepamicin sulfate, TOB; tobramycin

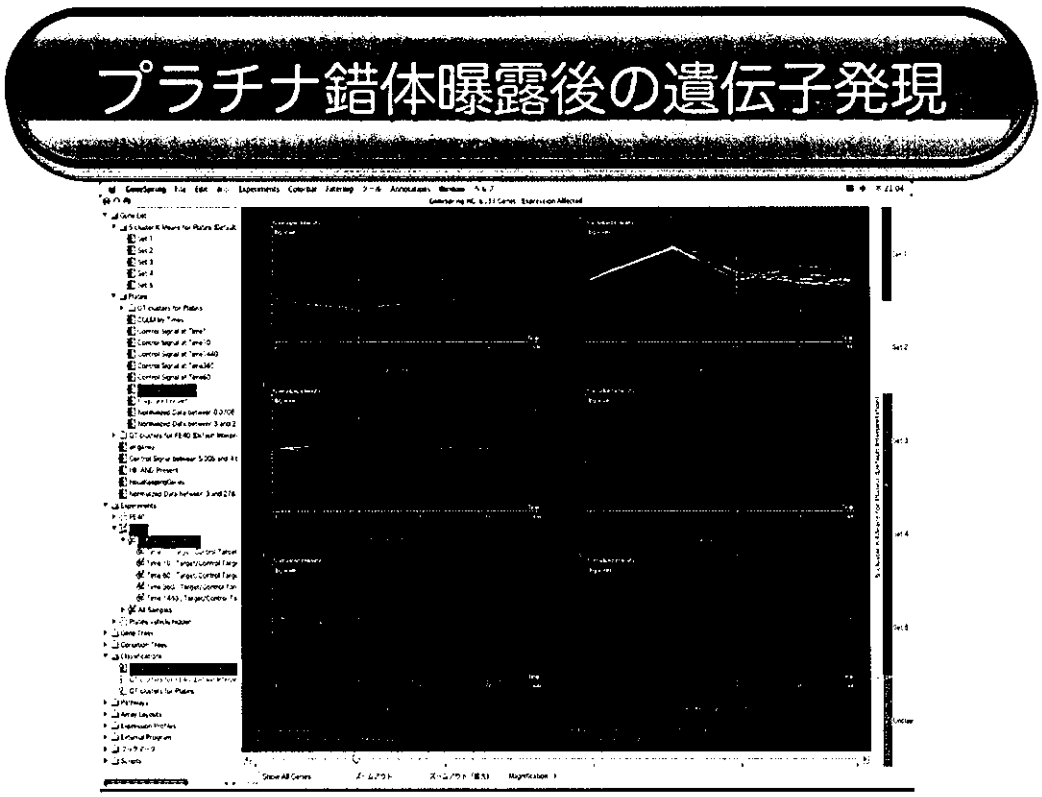

図 15　プラチナ錯体曝露後遺伝子発現

横のカテゴリーは薬物の略称、横軸は時間経過、縦軸が発現量である。図示されている発現データは 3 種類のプラチナ錯体(cisplatin, carboplatin and nedaplatin)を用いて得られたデータの平均がそれぞれの遺伝子ごとに計算されて表示されている。5 つクラスターが表示されているが、実際には 3 つのクラスター（set 2, set 3 および set 5）に分類された。残りの2つ（set 1 および set4）はコントロールとしてスパイキングした RNA などが表示されている。

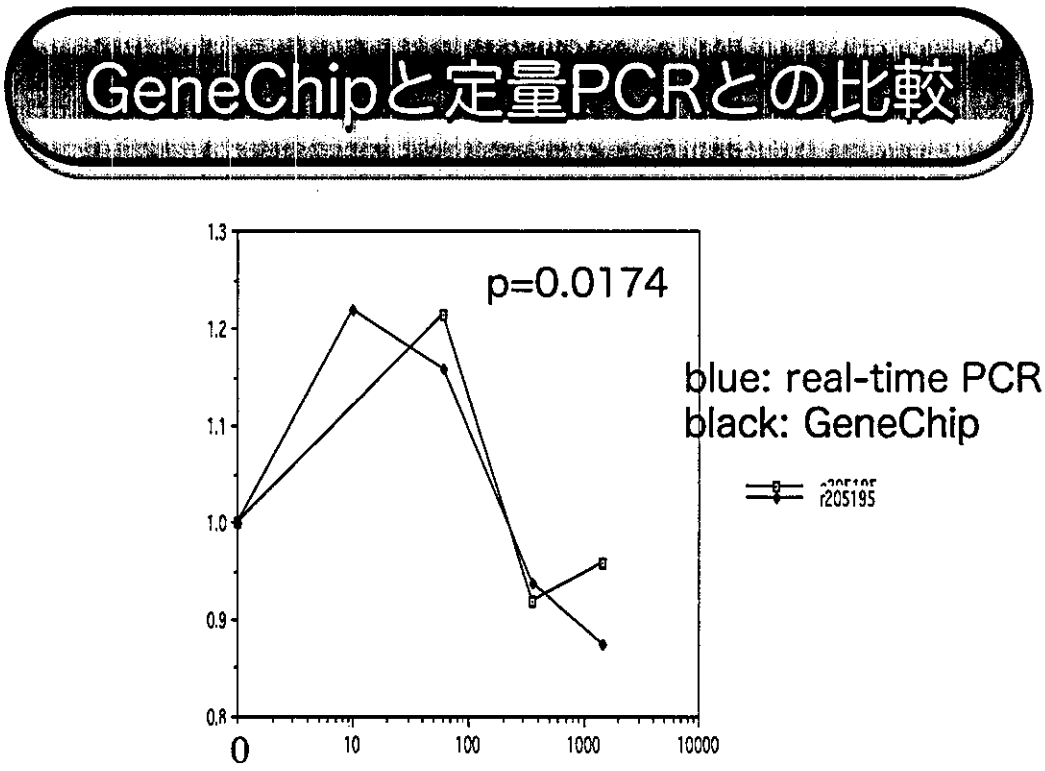

図 16　GeneChip と定量 PCR の結果の比較

横軸がシスプラチン曝露後時間(分)で、縦軸が曝露前と比較した発現量変化の割合(曝露前を1とする)を示す。青のクローズドサークルは定量 PCR の結果を黒のオープンスクエアは GeneChip の結果を示す。

研究成果の刊行に関する一覧表

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健康危険情報

なし

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Reprints from Wiley Encyclopedia of Molecular Medicine, 5 Vol. Set
ISBN 0-471-37494-6

may promote T-cell (and possibly B-cell) neoplasia through its stimulatory effects on the Akt1 signaling cascade.

GENE LOCI AND ACCESSION NUMBERS

TCL1/TCL1b locus on 14q32.1: GenBank HTG database, emb/AL139020.1/CNS01DX2 *TCL6* (*TNG1* and *TNG2*) genomic and cDNA accession numbers are listed in Refs. 12 and 13.

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TEC KINASES

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Protein-tyrosine kinases (PTKs) phosphorylate tyrosine residues on their substrate proteins. As evidenced by a number of transforming and active PTKs, it is widely believed that PTKs are directly involved in the cell growth mechanism of higher eukaryotes. PTKs can be classified into two groups; namely, receptor type PTKs and nonreceptor type PTKs. Tec kinases comprise the second largest subfamily among the latter, and Tec is the first member of this family of proteins. The mouse *Tec* gene was initially isolated in 1990 as a related gene to the v-*Fps* transforming one, followed by the discovery of four *Tec*-related genes [*Btk*, *Itk* (or *Emt* or *Tsk*), *Bmx*, and *Rlk* (or *Txk*)] in mammals (1,2). There is also one *Tec*-related gene (*Tec29*) in the fruit fly (3) (Table 1). Many of these *Tec*-related genes are abundantly expressed in hematopoietic tissues. Surprisingly and significantly, the Btk kinase was revealed to be the affected protein in, and the responsible one for, a severe immunodeficiency (X-linked agammaglobulinemia: XLA) in humans. Therefore, Btk function is indispensable for the maturation process of B-lymphocytes. Other Tec-kinases have been also shown to be implicated in the lymphocyte growth–activation process. It is, however, yet to be revealed how the Tec kinases are involved in the development and maintenance of the complexed, lymphocyte-mediated immune network.

PROTEIN STRUCTURE OF TEC KINASES

All of the Tec kinases but Txk share the same protein structure. As illustrated in Figure 1, Tec has an NH_3-terminal pleckstrin homology (PH) domain, followed by a Tec homology (TH) domain, an Src homology (SH) 3 domain, an SH2 domain, and a catalytic (or SH1) domain. The PH domain is the binding module for phosphoinositides including phosphatidyl inositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$], an in vivo product of PI3 kinase (4). Because a variety of growth stimuli activates intracellular PI3 kinase, these stimuli indirectly lead the recruitment of Tec kinases to the cell membrane in which $PI(3,4,5)P_3$ exists. Given the fact that XLA is caused by a PH-domain mutant of Btk that can no longer bind $PI(3,4,5)P_3$ molecular interaction between the PH domains and phospholipids plays an essential role for the regulation of Tec activities. Because Tec kinases are the only PTKs that possess PH domains, Tec kinases should have a unique character that they stay at the merging point of phosphotyrosine-mediated and phospholipid-mediated signaling pathways. Within the TH domain are found the Btk motif in the NH_3 terminal-half and the proline-rich region in the COOH terminal-half (5). Together with PH domain, the Btk motif forms a binding pocket for the α-subunits of heterotrimeric G-proteins. The SH3 domain functions as the binding site for polyproline helices, and the SH2 domain is the one for phosphotyrosine-containing peptides (6).

The unique member of the Tec family, Rlk, also contains SH2, SH3, and kinase domains, but lacks the PH domain, the hallmark of the Tec kinases. Rlk, instead, carries a cysteine string motif in the NH_3-terminal unique domain, which may

Table 1. Human Members of the Tec Kinase Family

Kinase	Chromosome Allocation	GenBank Accession No.
Tec	4p12	NM_003215
Btk	Xq22	NM_000061
Itk	5q31–32	NM_005546
Bmx	Xp22.2	NM_001721
Rlk	4p12	NM_003328

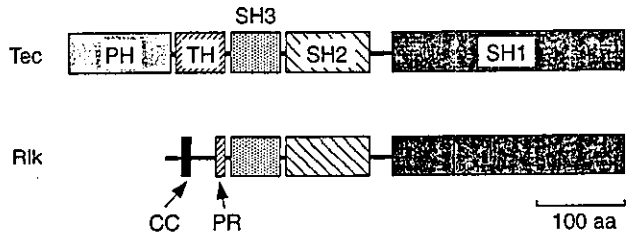

Figure 1. Protein structure of Tec kinases. All Tec kinases but Rlk contains an NH_3-terminal PH domain, followed by a TH domain, an SH3 domain, an SH2 domain, and a COOH-terminal catalytic (SH1) domain. Rlk, a unique member of Tec family, has no PH or TH domain, but instead carries a cysteine string motif (CC) and a proline-rich region.

be palmitoylated in vivo and help to constitutively target Rlk to cell membrane (7).

REGULATION OF TEC ACTIVITIES

Among the Tec kinases Tec and Btk are abundantly expressed in B-lymphocytes, whereas Tec, Itk, and Txk are expressed in T-lymphocytes. Stimulation of various lymphocyte surface antigens are capable of activating Tec kinases. In B cells, for instance, Tec and Btk become tyrosinephosphorylated in response to the engagement of B-cell antigen receptor (BCR), CD19, CD38, or CD72. In T-lymphocytes cross-linking of CD3 or CD28 engagement induces the activation of Tec and Itk (2).

A number of mechanisms are proposed to explain how such cell surface stimuli result in the activation of the Tec kinases. First, as described in the previous section the PI3-kinases and their products are involved in the activation process, which is reinforced by the fact that the reduction of $PI(3,4,5)P_3$ by an inositol polyphosphate phosphatase, SHIP, suppresses the activity of Btk in cells. Second, src-family PTKs can bind to, and activate Tec kinases. The TH domains of Tec kinases are recognized and bound by the SH3 domains of Src kinases. Src kinases phosphorylate Tec members, and thereby induce their activation (8,9). In the case of Btk, Src kinases have been shown to target the tyrosine residue (Tyr-551 in human Btk) in the activation loop of the Btk catalytic domain. Btk then autophosphorylates a tyrosine residue (Tyr-223) within its SH3 domain, leading to the full activation of Btk. This functional hierarchy between Src and Tec kinases is also seen in the fruit fly. The third mechanism is the docking proteins, which facilitate the membrane recruitment of Tec kinases. In T-cells engagement of T-cell receptor (TCR) induces the activation of ZAP-70 (Zeta chain–associated protein), a T-cell-specific nonreceptor PTK, which in turn phosphorylates a T-cell-specific scaffold protein, LAT. Phosphotyrosines on LAT are then recognized by the SH2 domain of Itk, generating the signaling complex of LAT-Itk and the proximity of Itk to its substrates also associated with LAT (10). A similar scenario is also seen in the BCR-mediated signaling system. The engagement of BCR induces the activation of Syk, a PTK relative to ZAP-70, which phosphorylates a B-cell-specific docking protein, BLNK, and leads the recruitment of Btk to BLNK.

In addition to the lymphocyte surface antigens, cytokine receptors have been also demonstrated to induce the activation of Tec kinases. For example, Tec becomes activated by stimulation with the stem cell factor (or Kit-ligand), interleukin (IL)-3, IL-6, granulocyte colony-stimulating factor, erythropoietin, granulocyte-macrophage colony-stimulating factor, or thrombopoietin. With regard to Btk, its activity is induced by the ligand stimulation of the receptor for IL-5 or IL-6 (2).

Furthermore, ligand-mediated stimulation of heterotrimeric G-protein-coupled receptor (GPCR) can activate Tec kinases. Tec, Btk, and Bmx were shown to become activated by the stimulation with thrombin, thromboxane A2, or adrenaline. Although the exact mechanism of this G-protein-mediated activation is not clear, a recent report indicates that the PH domain and the Btk motif within the TH domain form a direct binding site for the α-subunits of heterotrimeric G-proteins (11). In addition to the physical interaction between heterotrimeric G-proteins and Tec kinases, activation of the latter may be mediated also by $PI(3,4,5)P_3$ that is generated by a G-protein-specific PI3 kinase (PI3-Kγ).

Reports also indicate the involvement of Tec kinases in the integrin-driven signaling. In human platelets, stimulation of surface glycoprotein IIb-IIIa molecule with fibrinogen induces the activation of Tec, which is further enhanced by costimulation with thrombin.

In addition to the activation mechanism of Tec kinases, only a few pieces of information are available for their inactivation process. As already described, SHIP suppresses Tec activities through the dephosphorylation of $PI(3,4,5)P_3$ and the concomitant blockade of membrane recruitment of Tec proteins. The other mechanism of suppression can be mediated by SHP-1, an SH2-containing tyrosine phosphatase, which may directly dephosphorylate Tec proteins. The paired immunoglobulin-like receptor B (PIR-B) on lymphocytes evokes a negative signal to the BCR-mediated pathways. This effect is believed to be exerted through the phosphorylation of PIR-B and the resultant recruitment and activation of SHP-1. SHP-1 plays an essential role for the PIR-B-mediated dephosphorylation of Syk and Btk kinases (2).

DOWNSTREAM EFFECTORS

All Tec kinases can efficiently phosphorylate phospholipase C (PLC)-γ that, in a phosphotyrosine-dependent manner, hydrolyzes $PI(4,5)P_2$, thereby generating inositol(1,4,5)-trisphosphate (IP_3) and diacylglycerol (DA). IP_3 in vivo functions to mobilize intracellular $[Ca^{2+}]$ through the IP_3-gated $[Ca^{2+}]$, which is believed to be essential for the ontogeny of B-lymphocytes, and, therefore, failure of PLC-γ activation may be one of the main causes of XLA in which the Btk function is impaired. In fact, a targeted disruption of the *Btk* gene results in the loss of activation of PLC-γ2 in chicken B-lymphocytes. In addition, human B cells from XLA patients do not induce calcium flux in response to BCR engagement, and the ectopic expression of wild-type Btk, Tec, or Itk recovers the $[Ca^{2+}]$ mobilization in these cells (12).

Apart from PLC-γ2, a variety of intracellular signalings can be evoked by Tec kinases. Surprisingly, many of these pathways are Tec-member-specific, indicating that the Tec kinases have nonredundant functions. Dok-1 is a docking protein that can be an efficient substrate for a wide range of normal as well as transforming PTKs. Although the in vivo role of Dok-1 is still obscure, Dok-1 in B-lymphocytes becomes tyrosine-phosphorylated to a high stoichiometry by the ligation

of BCR. Tec strongly phosphorylates Dok-1 both in vitro and in vivo. However, Itk only weakly phosphorylates Dok-1, and none of the other members can do so (13,14).

Another member-specific example is BRDG1, a docking protein expressed in B-lymphocytes and hematopoietic stem cells. Although BRDG1 becomes a direct substrate of Tec kinase in cells, it is scarcely phosphorylated by the other Tec kinases. These substrate specificities should clearly affect the in vivo function of each Tec kinase (15).

In addition to docking–adaptor proteins, Tec kinases are able to directly regulate transcriptional factors. Btk has been shown to associate with (and, probably, phosphorylate) TFII-I, a ubiquitously expressed multifunctional transcriptional factor. Importantly, Btk activates, and induces the nuclear transport of, TFII-I that then acts on several promoter elements including the initiator (Inr) elements found in the promoter regions of VpreB, TdT, and λ5 (16). TFII-I was also shown to drive the expression of c-*Fos* protooncogene. This is a novel addition to the scenario in which PTKs phosphorylate and then direct the translocation of transcriptional factors, as in the case of Jak kinases and signal transducers and activators of transcription (STATs).

Other possible mediators for Tec kinase signalings include RIBP, WASP, Sab, Sam68, EWS, and Grb 10, although their Tec member specificity is not fully documented.

IN VIVO FUNCTIONS

XLA patients have a severely diminished number of peripheral mature B-lymphocytes and a reduction in serum immunoglobulin levels. The affected patients suffer from recurrent bacterial infection and often require sustained administration of antibiotics or immunoglobulins. Because XLA patients have a normal number of pro-B cells in bone marrow, the main defect in XLA should lie at the expansion process of early pre-B cells. Interestingly, in the mouse system loss-of-function of Btk does not lead to maturation arrest at the pre-B stage. The pre-B cells of *Btk*–/– mice seem to normally undergo differentiation into surface immunoglobulin-positive B cells. However, the final maturation of $IgM^{high}IgD^{low}$ immature B cells into $IgM^{low}IgD^{high}$ mature B cells is blocked, and these B cells fail to produce immunoglobulins when challenged with polysaccharide thymic-independent type II antigens (17,18). To decipher the signaling responsible for XLA, it would be valuable to address the difference in Btk requirement for pre-B expansion between human and mouse systems.

No other human diseases have yet been assigned to the mutations of any *Tec* genes. However, targeted disruption of *Tec* genes in mice have demonstrated that Tec kinases other than Btk play an essential role in the immune system.

Mice lacking Itk, a T-cell-specific member of Tec proteins, have reduced numbers of mature lymph node T-lymphocytes and reduced ratio of CD4 to CD8 single positive cells. In these cells the TCR-mediated proliferative response is impaired. Interestingly, the suppression of growth response is exaggerated when the mice also lack functional Fyn, a T-cell-specific member of Src kinases. These results indicate that Itk and Fyn independently contribute to the T-cell growth mechanism (19).

In contrast to the *Itk*–/– mice, disruption of *Rlk* gene does not produce any change in T-cell development. Additional mutation in the *Itk* gene, however, gives a profound defect in the TCR-mediated T-cell proliferation. In T cells of the double "knockout" mice, ligation of TCR generates little Ca^{2+} flux. Therefore, loss of both Itk and Rlk functions leads to a severe defect in $[Ca^{2+}]$ mobilization, which may be an important cause for the failure in TCR-mediated growth response (20).

However, as discussed in the substrate specificity of Tec kinases, Itk and Rlk display as many nonredundant functions as their redundant ones. In helper T cells, Itk is required to induce the production of IL-4 whose expression is characteristic of Th2 type of T cells. In contrast, activation of Rlk is involved in the synthesis of interferon-γ, which is specifically produced in Th1 cells. Interestingly, Tec is involved in both pathways. These data may imply that Tec kinases regulate the commitment of helper T cells to Th1–Th2 subtypes.

In addition to the specific roles in the immune system, Tec kinases have been proposed to be involved in a wide range of intracellular signaling systems. The small G-protein RhoA, a member of the Ras-superfamily, is an important molecular "switch" to control actin organization and mitotic response. Tec kinases upregulate RhoA and thereby help stress fiber formation in cells (21).

It is also known that Tec kinases are directly involved in the apoptotic pathway. In B cells Btk activity is required to induce *Bcl-xL* message, indicating that Btk is an antiapoptotic factor. In the case of DT-40, a chicken lymphoma cell line, Btk is required for the radiation-induced apoptosis, suggesting a proapoptotic function of Btk in this particular cell line. The ability of Btk to regulate JNK in cells also supports the latter hypothesis. It remains to be elucidated how these apparently "opposite" effects are determined in different cell types under different conditions.

Rapid transcriptional activation of the protooncogene c-*Fos* is a phenomenon common to many growth factor stimuli. Tec activities strongly induce transcriptional activation of

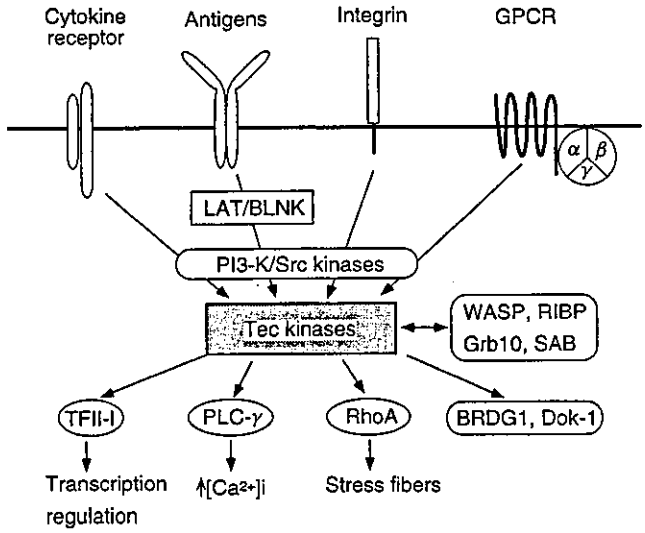

Figure 2. Signaling pathways to and from Tec. Tec kinases receive activation signals from lymphocyte surface antigens (Antigens), cytokine receptors, GPCR, and integrin molecules. Scaffolding proteins such as LAT or BLNK are required for Tec activities in certain contexts. Direct activator of Tec kinases include PI3-kinases (PI3-K) and Src kinases. Downstream effectors for Tec include PLC-γ, RhoA, Dok-1, BRDG1, and TFII-I. Other candidates for such Tec-interacting proteins may be WASP, RIBP, Grb10, and SAB.

the c-*Fos* protooncogene in many cell types. Introduction of a dominant interfering mutant of Tec suppresses the cytokine-driven activation of c-*Fos* promoter. These findings suggest that Tec kinases are involved in the regulation of c-*Fos* transcription.

CONCLUSION

The signaling mechanism to and from Tec kinases are illustrated in Figure 2. Tec members can be regulated by a wide range of cell surface proteins including lymphocyte surface antigens, cytokine receptors, GPCR, and integrin molecules. These stimuli may directly regulate Tec kinases or indirectly through P13-kinases or Src kinases. There is no doubt that scaffolding proteins, such as LAT or BLNK, play an indispensable role in the activation process of Tec kinases. Tec triggers a wide spectrum of intracellular signalings including those for $[Ca^{2+}]$ mobilization, c-*Fos* activation, actin organization, apoptosis, and direct regulation of transcriptional factors. Further elucidation of Tec member-specific messengers would provide an important clue to decipher redundant and nonredundant functions of Tec kinases.

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TELOMERES AND TELOMERASE

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This article describes the structure and function of telomeres, the physical ends of eukaryotic chromosomes, and of telomerase, the ribonucleoprotein enzyme required for the synthesis and maintenance of telomeres. The involvement of telomerase in cancer progression, its role in some of the features of human aging, and its potential role in human disease are discussed.

Every eukaryotic chromosome consists of a linear DNA molecule encoding genetic information, which is complexed with a multitude of chromosomal proteins. Telomeres, the termini of these linear chromosomes, consist of both DNA and a subset of the chromosomal proteins. Telomeres were first discovered, through cytogenetic studies of maize and fruitfly chromosomes beginning in the 1930s, as elements required to protect and stabilize the genetic material carried by eukaryotic chromosomes. Since then, telomeres have been extensively characterized both molecularly and functionally and have been found to play critical roles in chromosomal replication and maintenance. Of the three essential *cis*-acting elements required for eukaryotic chromosomal replication and maintenance—replication origins, centromeres, and telomeres—telomeres have emerged as the most conserved in structure, having function and metabolism among eukaryotes as diverse as single-celled protozoans, fungi, warm and cold-blooded animals and plants. Telomeres are quite different from the termini of linear viral, nonnuclear plasmid, mitochondrial, or various bacterial DNA genomes. Hence, it is useful to define telomeres of eukaryotic chromosomes separately from the much greater variety of terminal structures found at the ends of other linear DNA genomes.

CYTOLOGY OF TELOMERES

Telomeres do not normally move freely throughout the cell nucleus. They are often found clustered in nonrandom locations in close proximity to the nuclear envelope (1). They are frequently found toward one side of the nucleus, an arrangement called the "Rabl" configuration. Telomeric clustering is particularly noticeable in premeiotic nuclei, giving rise to the "bouquet" arrangement of chromosomes characteristic of this stage. This premeiotic telomere-positioning is affected by several genetic factors, some of which are involved in the DNA damage response and may be required for efficient homologous recombination to occur during meiosis.

TELOMERIC DNA MAINTENANCE AND LOSS

Conventional DNA polymerases are unable to fully replicate the terminal regions of linear chromosomes. Such incomplete replication is a consequence of two invariant properties of conventional replicative DNA polymerases: their ability to polymerize DNA in the 5′ to 3′ direction only, and their requirement for a primer containing a free –OH onto which new DNA is polymerized (2). Left unchecked,

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EXPERIMENTAL HEMATOLOGY

Experimental Hematology 32 (2004) 828–835

DNA microarray analysis of dysplastic morphology associated with acute myeloid leukemia

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(Received 17 February 2004; revised 17 May 2004; accepted 5 June 2004)

Objective. **Acute myeloid leukemia (AML) develops de novo or secondarily to either myelodysplastic syndrome (MDS) or anticancer treatment (therapy-related leukemia, TRL). Prominent dysplasia of blood cells is apparent in individuals with MDS-related AML as well as in some patients with TRL or even with de novo AML. The clinical entity of AML with multilineage dysplasia (AML-MLD) is likely to be an amalgamation of MDS-related AML and de novo AML-MLD. The aim of this study was to clarify, by the use of high-density oligonucleotide microarrays, whether these subcategories of AML are intrinsically distinct from each other.**

Materials and Methods. **The AC133$^+$ hematopoietic stem cell–like fractions were purified from the bone marrow of individuals with de novo AML without dysplasia (n = 15), AML-MLD (n = 11), MDS-related AML (n = 11), or TRL (n = 2), and were subjected to the synthesis of cRNA which was subsequently hybridized to microarray harboring oligonucleotide corresponding to more than 12,000 probe sets.**

Results. **We could identify many genes whose expression was specific to these various subcategories of AML. Furthermore, with the correspondence analysis/three-dimensional projection strategy, we were able to visualize the independent, yet partially overlapping, nature of current AML subcategories on the basis of their transcriptomes.**

Conclusion. **Our data indicate the possibility of subclassification of AML based on gene expression profiles of leukemic blasts.** © **2004 International Society for Experimental Hematology. Published by Elsevier Inc.**

Acute myeloid leukemia (AML) may develop de novo or as a result of either myelodysplastic syndrome (MDS) or anticancer treatment [1]. Given that MDS is characterized by dysplastic changes in differentiated blood cells, individuals with MDS-related leukemia often manifest prominent dysplasia in their blood cells. Therapy-related acute leukemia (TRL) may develop after the administration of alkylating agents, topoisomerase inhibitors, or radiotherapy. The clinical outcome of TRL is generally worse than that of de novo AML [2], and a subset of individuals with TRL also exhibit multilineage dysplasia of blood cells.

A clinical record of a preceding MDS phase is also an indicator of poor prognosis for the individuals with AML. Therefore, to predict the outcome of, and to optimize the treatment for, each AML patient, it would be important to differentiate de novo AML from MDS-related AML and TRL. However, even in the bone marrow (BM) of healthy elderly people, it is not rare to find dysplastic changes (in particular, dyserythropoiesis) in differentiated blood cells [3]. Therefore, the differential diagnosis among such AML-related disorders is not always an easy task in the clinical settings, especially if a prior record of hematopoietic parameters is not available.

Making issues further complicated, prominent dysplasia in blood cells may be found among certain cases of de novo AML, with which prior MDS phases can be excluded [4,5]. It is known that such de novo AML with dysplasia has a poor outcome with conventional chemotherapy, as does MDS-related leukemia [6]. However, Taguchi et al. have argued that the former may be a distinct clinical entity from the

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0301-472X/04 $–see front matter.
doi: 10.1016/j.exphem.2004.06.003

latter based on the finding that the former cases respond far better to allogeneic bone marrow transplantation than the latter one [7]. In the revised classification of AML by the World Health Organization (WHO) [1], an entity of AML with multilineage dysplasia (AML-MLD) has been proposed, which includes both de novo AML with dysplasia and secondary AML from MDS. Whether such amalgamation holds a clinical relevance awaits further studies on this issue.

DNA microarray has made it possible to measure the expression levels in tens of thousands of genes simultaneously, and thus should be a promising tool to discover useful and reliable molecular markers for these AML-related disorders. However, a simple comparison of BM mononuclear cells (MNCs) with DNA microarray is likely to generate a large body of pseudopositive and pseudonegative data, which may only reflect different proportions of blastic cells within BM or the different lineage commitment of leukemic cells [8]. To minimize such "population-shift effect," it should be effective to isolate and compare leukemic blasts at the same differentiation level from AML-related disorders.

Toward this goal, we started the Blast Bank project to purify and store AC133 surface marker [9]–positive hematopoietic stem cell (HSC)–like fractions from patients with a wide range of hematological disorders. Microarray analysis of these Blast Bank specimens has been highly successful in the isolation of molecular markers to differentiate de novo AML from MDS-related leukemia [8,10], and in the identification of genes that may be involved in the stage progression mechanism in chronic myeloid leukemia (CML) [11] or MDS [12]. Further, a proteomics approach with these Bank cells could identify a protein that may be associated with chromosome instability in leukemic cells [13].

We have now determined the expression intensities for more than 12,000 human probe sets in a total of 39 Blast Bank specimens, including those from 15 cases of de novo AML without dysplasia, 11 cases of MDS-related leukemia, 11 cases of AML-MLD, and 2 cases of TRL. The resulting large data set was analyzed to address whether these clinical entities are actually distinct from each other or whether they partially overlap.

Patients and methods

Purification of AC133$^+$ cells

BM aspirates were obtained from the study subjects with written informed consent. From each specimen, MNCs were isolated by Ficoll-Hypaque density gradient centrifugation, and were labeled with magnetic bead–conjugated anti-AC133 monoclonal antibody (AC133 MicroBead; Miltenyi Biotec, Auburn, CA, USA). AC133$^+$ HSC-like fractions were then purified through a miniMACS magnetic cell separation column (Miltenyi Biotec), and enrichment of the HSC-like fraction was evaluated by subjecting portions of the MNC and AC133$^+$ cell preparations either to staining with Wright-Giemsa solution or to the analysis of the expression of CD34, CD38, and AC133 by flow cytometry (FACScan; Becton-Dickinson, Mountain View, CA, USA). In most instances, the CD34^{high}CD38^{low} fraction constituted greater than 90% of the eluate of the affinity column.

DNA microarray analysis

Total RNA was extracted from the AC133$^+$ cell preparations by an RNeasy Mini column with RNase-free DNase (both from Qiagen Inc., Valencia, CA, USA), and was subjected to two rounds of amplification of mRNA fractions by T7 RNA polymerase [14]. The high fidelity of the amplification step was confirmed previously [10]. One microgram of the amplified complementary RNA (cRNA) was then converted to double-stranded cDNA by PowerScript reverse transcriptase (BD Biosciences Clontech, Palo Alto, CA, USA), which was used to prepare biotin-labeled cRNA with ENZO BioArray transcript labeling kit (Affymetrix, Santa Clara, CA, USA). Hybridization of the samples with GeneChip HGU95Av2 microarrays was conducted by the GeneChip system (Affymetrix), revealing the expression intensities of 12,625 probe sets in each sample.

The transcriptome of 10 cases each with de novo AML and MDS-related AML has been already reported separately [10], aiming at the comparison between these two clinical conditions with the same differentiation background; the M2 subtype according to the classification of the French-American-British (FAB) Cooperative Group [15].

Statistical analysis

The fluorescence intensity for each gene was normalized relative to the median fluorescence value for all human genes with a "Present" or "Marginal" call (Microarray Suite; Affymetrix) in each hybridization. Hierarchical clustering of the data set and analysis of variance (ANOVA) were performed with GeneSpring 6.0 software (Silicon Genetics, Redwood, CA, USA). Correspondence analysis [16] was performed with the ViSta software (http://www.visual-stats.org) for all genes showing a significant difference. Each sample was plotted in three dimensions based on the coordinates obtained from the correspondence analysis. All array data as well as details of the genes shown in the figures are available as supplementary information at the *Experimental Hematology* web site.

Results

Comparison of AML-MLD and MDS-related AML

Summarized in Table 1 are the clinical characteristics of 39 patients enrolled in this study, including 15 cases with de novo AML without dysplasia, 11 cases with AML-MLD, 11 cases with MDS-related AML, and 2 cases with TRL. The presence of "MLD" was determined according to the definition in the WHO classification [1], by a central review at the Department of Hematology and Molecular Medicine Unit, Nagasaki University, which is also a "central review institute" for the Japan Adult Leukaemia Study Group. It should be noted that favorable karyotypes, t(8;21) and inv(16), were found only in the cases with AML without dysplasia.

According to the WHO proposal of classification, AML-MLD is likely to be an amalgamation of bona fide de novo

Table 1. Patient characteristics

Patient ID	Disease	Age (year)	Sex	Karyotype
1	MDS	79	M	+8
2	MDS	80	M	+8
3	MDS	71	F	Other
4	MDS	44	M	Normal
5	MDS	61	M	+8
6	MDS	69	M	+8
7	AML	83	M	−7
8	MLD	61	M	Other
9	AML	85	M	−7
10	MDS	84	M	−7
11	MDS	57	M	Normal
12	AML	58	M	t(8;21)
13	AML	37	M	t(8;21)
14	AML	84	M	Normal
15	AML	43	M	Normal
16	MLD	41	M	Normal
17	AML	38	M	t(8;21)
18	MDS	69	M	+8
19	AML	49	F	t(8;21)
20	AML	61	F	t(8;21)
21	MLD*	38	M	Normal
22	MLD*	80	M	Normal
23	AML	53	F	−7
24	AML	32	F	Other
25	AML	46	F	Other
26	AML	53	M	Normal
27	MLD*	57	F	+8
28	TRL	59	M	Other
29	TRL	67	M	−7
30	MDS	70	M	Other
31	MLD*	64	M	−7
32	AML	22	F	inv(16)
33	MLD*	16	F	Normal
34	AML	67	M	t(8;21)
35	MLD*	67	M	−7
36	MDS	88	F	Other
37	MLD*	53	M	Normal
38	MLD*	46	M	Other
39	MLD*	50	M	Other

AML, de novo AML; MLD, AML with multilineage dysplasia; MDS, MDS- associated AML, TRL, therapy-related AML; M, male; F, female.
*Individuals proven not to have a prior history of MDS.

AML with dysplasia and secondary AML evolving from an undiscovered phase of MDS. Although the clinical characteristics of the former have not been fully defined, it has been reported that de novo AML-MLD may be associated with poor prognosis [17,18] and, in some cases, with an increased megakaryopoiesis in BM [5].

To clarify directly whether de novo AML-MLD is truly a clinical entity distinct from MDS-related leukemia, we searched for differences between the transcriptomes of $AC133^+$ cells derived from the individuals diagnosed with these two conditions. Among the 11 cases of AML-MLD studied, 9 were revealed not to have prior MDS records, while we could not obtain the clinical information for the other two with regard to their prior MDS history. Therefore, we could not exclude the possibility that the latter cases had evolved from MDS stages. The former nine cases were thus used to measure the difference between de novo AML-MLD and MDS-related secondary AML.

For the expression data set of these 20 subjects, we first set a condition that the expression level of a given gene should receive the "Present" call (from the Microarray Suite 4.0 software) in at least 30% (6 cases) of the samples, aiming to remove transcriptionally silent genes from the analysis. A total of 4851 genes passed this selection window. Toward such genes was then applied a Student's t-test ($p < 0.001$) to extract genes, expression level of which significantly differed between the two classes, de novo AML-MLD and MDS-related AML. However, many of the genes thus identified yet had very low absolute expression levels throughout the samples, even though the ratio of the expression levels between the two classes might be relatively large. To eliminate such "nearly silent" genes and to enrich genes whose expression levels were significantly high in at least one of the classes, we further selected those whose effect size (absolute difference in the mean expression intensities) [19] between the two classes was at least 10 arbitrary units (U).

We could finally identify a total of 56 genes significantly contrasting the two clinical conditions, expression profiles of which are shown in a "gene-tree" format (Fig. 1A). Here genes with similar expression patterns across the samples were clustered near each other. Many of the genes thus identified were preferentially expressed in de novo AML-MLD (upper two-thirds of the tree), while some were so in MDS-related AML (bottom third). Given the association of de novo AML-MLD with dysmegakaryopoiesis in BM, it was of interest to find that the gene for platelet factor 4 (PF4) was preferentially expressed in individuals with this condition. PF4 is a CXC-type chemokine secreted from platelets, and its serum level is known to reflect platelet activities [20]. High production of PF4 from MLD blasts should influence the environment within BM, and may thereby affect megakaryopoiesis.

Were the expression profiles of these 56 genes potent enough to differentiate AML-MLD from MDS-related AML? To examine this possibility, two-way clustering analysis [21] was conducted on the data set to make a "patient tree" among the subjects, based on the standard correlation values with a separation ratio of 1.0 (Fig. 1B). This tree, which reflects the similarity in the expression profiles of the 56 genes among the subjects, showed the presence of a cluster of individuals only with MDS-related AML. However, the large branch at the left contained not only most of the patients with de novo AML-MLD, but also some individuals with MDS-related AML. It was not clear whether the failure in the clear separation of the two clinical categories was due to an inadequacy of the separation power of the clustering method or to an inaccurate clinical diagnosis. Further, it has not been addressed whether de novo AML-MLD should be treated as a single clinical entity distinct

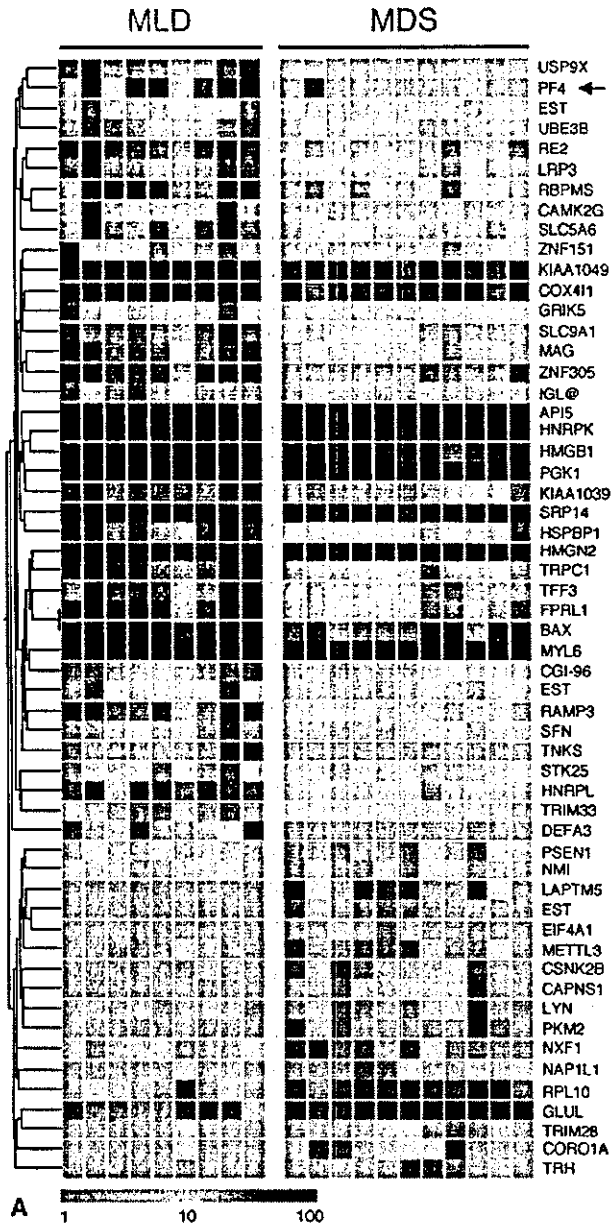

Figure 1. Comparison of gene expression profiles between individuals with de novo AML-MLD and those with MDS-related leukemia. (A): Gene tree for the expression levels (color-coded as indicated by the scale at the bottom) of 56 human genes in AC133$^+$ cells from patients with de novo AML-MLD (MLD) or MDS-related leukemia (MDS). Each row corresponds to a single gene and each column to a different patient. The gene symbols are indicated at the right. The position of the *PF4* gene is indicated by an arrow. (B): Two-way clustering analysis of the patients with de novo AML-MLD (green) or MDS-related leukemia (red) based on the similarities in the expression profiles of the 56 genes shown in (A). (C): Correspondence analysis of the 56 genes identified three major dimensions in their expression profiles. Projection of the specimens into a virtual space with these three dimensions revealed that those from de novo AML-MLD and those from MDS-related leukemia were separated from each other. The arrow indicates a nonconforming specimen (ID 35).

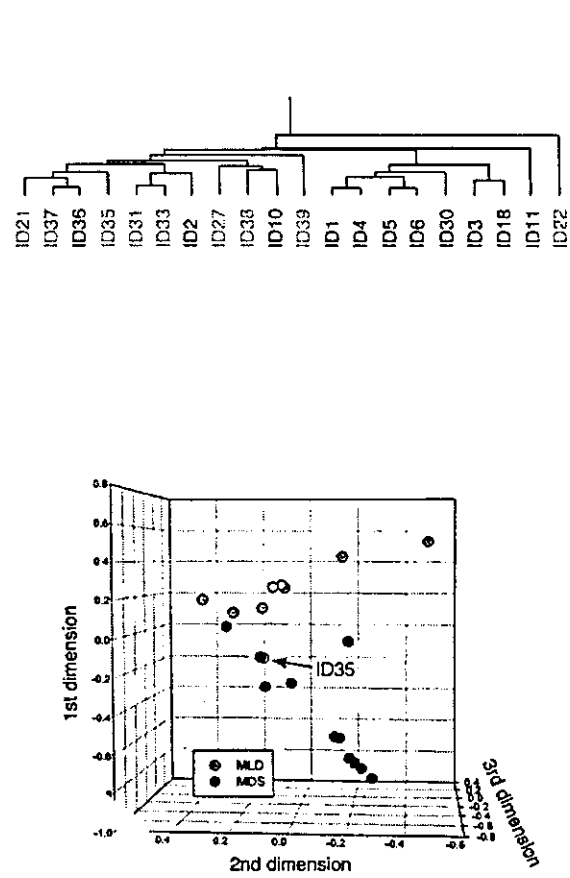

Figure 1. Continued

from MDS-related AML in, at least, the point of view of gene expression profiles.

To address these issues, we tried to visualize the similarity/difference between the two classes. Correspondence analysis is a novel method to decomposite multidimensional data [16]. It enables not only a low-dimensional projection of expression profiles for numerous genes, but measurement of the contribution of each gene to a given extracted dimension and, at the same time, measurement of the contribution of each extracted dimension to the whole complexity. Correspondence analysis was performed on the expression data of the 56 genes in Figure 1A, successfully reducing the complexity of 56 dimensions into 3. On the basis of the calculated three-dimensional (3D) coordinates for each sample, the specimens were then projected into a virtual space (Fig. 1C). It was clear from this figure that most of the samples could be separated into two diagnosis-related groups (whether the coordinate in the first dimension was greater than or equal to 0 or less than 0), supporting the feasibility to set a clinical entity "de novo AML-MLD." Figure 1C also suggests that gene expression profiling could be applied to the differential diagnosis of AML-MLD and MDS-related AML. There was, however, a single patient with AML-MLD