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# CD56+, NKp46+ cell line (MZ93) expressing T-cell and myeloid antigens

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*Key Words:* T/NK cell precursor acute leukemia, cell line, MZ93, CD56, NKp46, chronic myelogeneous leukemia.

**Abstract** The MZ93 cell line, established from a patient with CML, expressed CD4, CD7, CD13, CD25, CD33, CD34, CD56 and NKp46. The additional karyotype abnormality of the Ph-positive leukemia cells in vivo, 6p+, was also observed in the MZ93. The early passages of MZ93 expressed CD3 in the cytoplasm, but the late passages did not. The cells did not express mature NK-markers as expected. The messenger RNAs of CD2 and NKp46 were detected and those of CD3ε and CD3ζ were absent in the cells. Therefore, the cell line has the immunophenotype likely to NK and/or T cell precursor.

*List of abbreviations:* CML, chronic myelogeneous leukemia; AML, acute myelogeneous leukemia; Ph, Philadelphia; NK, natural killer; MPO, myeloperoxidase; PPO, platelet peroxidase; TCR, T cell receptor; GPA, glycophorin A; FITC, Fluorescein isothiocyanate; PE, phycoerythrin, PCR, polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction.

## Introduction

NK malignancies are a rare disorder, and very few NK-cell lines have been reported [1 –6]. The 6 reported cell lines, including the NK-YS established by us, are characterized as intermediate to fully maturated NK-cells [7]. Most of these cell lines show azulophilic granules, express perforin and granzyme B in the cytoplasm, and have NK activity and antibody-dependent cellular cytotoxicity (ADCC). Therefore, these cell lines will prove invaluable for studies of normal and malignant NK cell biology. In contrast, there have been no reports of an NK-precursor cell line, which may help in understanding the hematopoiesis and mechanism of commitment of stem cells to the NK-lineage, by means of immunological and biological approaches.

The immunophenotypic analysis of *de novo* acute leukemia gives important information for diagnosing the disease. Although CD56 (N-CAM) is one of the NK-associated antigens of human white blood cells [8], CD56 is also expressed in the nervous system, some of the T-cell subset [9, 10], and monocytes. The CD56 gene is located on chromosome 11q23 [11], which is also known as a break-point region of acute leukemia and lymphoma to produce fusion products of the mixed leukemia lymphoma (MLL) gene as a result of balanced translocation [12]. The expression of CD56 is sometimes observed in hematopoietic malignancies, including NK-cell tumors [13 – 16], AML [17 – 19], and multiple myeloma [20]. The expression of CD56 in blast cells is usually observed in several types of AML [17 – 19, 21].

Among the CD56-positive AML, the "myeloid/NK cell precursor acute leukemia" reported by Suzuki showed the expression of CD7, CD33, CD34 and CD56 on the surface, and expression of CD3ε in the cytoplasm by flow cytometry and/or by Northern blotting analysis [22, 23]. Although this category of leukemia may define the NK-precursor in part, the hypothesis, an origin from common progenitors between the NK cell and myeloid lineages, proposed by him is unconvincing because the leukemia cells showed the character of ALL by morphology, cytochemistry, immunophenotyping and gene analysis. CD33 expression often appears in progenitor- and precursor-B ALL and its normal counterparts, and in some cases with

progenitor- and precursor-T ALL, and, therefore, does not mean that the cells arise from myeloid precursor.

We have recently established the MZ93 cell line from a patient with CML in blast crisis, and have characterized the biological features of this unique cell line at the cellular and molecular levels.

**Materials and Methods**

*Case report.*

A 42-year-old Japanese man was admitted to receive an allogeneic bone marrow transplantation from an HLA-identical sibling in April, 1993. Two years previously, he was diagnosed with CML in the chronic phase, and his blood tests revealed a white cell count of 196.0 x $10^9$/l, a red cell count of 4.26 x $10^{12}$/l and platelets of 213 x $10^9$/l. Karyotype analysis was performed on the bone marrow which showed a simple Ph abnormality. He was treated with a daily oral dose of busulfan and a monthly injection of cymerin, and the leukocyte count decreased to approximately 10 x $10^9$/l. The leukocyte count increased to 110.0 x $10^9$/l and the karyotype showed a complex abnormality of Ph: 46,XY, 6p+, t(9;22)(q34;q11), in 19 of 20 metaphases studied in April, 1993. He was diagnosed with CML in an accelerated phase, and an oral administration of hydroxyurea was added to reduce the leukocyte count. One month after his admission, he developed a high grade fever and lower limb pain. The bone marrow aspirate revealed 39.2% of the immature blasts with myelomonocytic morphology. He was then diagnosed with CML in blast crisis. The phenotype of the blasts is shown in the Table 1. He was treated with combination chemotherapy using daunorubicin, vincristine, prednisolone and/or cytosine arabinoside. He died of massive pulmonary bleeding in July, 1993.

*Establishment of the cell line.*

The mononuclear cells were isolated by the Ficoll-Hypaque method from the patient's peripheral blood in the crisis phase, and were suspended in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Dainippon Pharmaceutical, Osaka, Japan), 100 U/ml of penicillin G sodium (PCG) and 100 μg/ml of streptomycin sulfate (SM; Biowhittaker, Walkeresville, MD). The cell suspension was maintained in a humidified atmosphere and hypoxigenic condition at $37^0$C with 5% $CO_2$ and 5% $O_2$ in a 25-$cm^2$ culture flask (Falcon 3013; Becton Dickinson, Oxnard, CA). After 2 weeks of culture, half of the medium was changed weekly. A limited-dilution technique in a U-bottom 96-well plate was used for cloning the cell line.

The control cell lines, MOLT3 [24] and HT93 [25], were maintained in RPMI 1640 (GIBCO) supplemented with 10% FBS, PCG and SM at $37^0$C in 5% $CO_2$ in humidified air. MOLT3 is a cytoplasmic CD3-positive T-cell line, and HT93 is an acute promyelocytic leukemia cell line with chromosome t(15;17).

*Flow cytometric analysis.*

The leukemic cells from the patient and the cell line were analyzed, by 2-color immunofluorescence with a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA), for the expression of surface and cytoplasmic antigens. FITC- or PE-conjugated monoclonal antibodies for CD2 (Leu5b), CD3 (Leu4), CD4 (Leu3a), CD5 (Leu1), CD7 (Leu9), CD8 (Leu2), CD10 (CALLA), CD14 (LeuM3), CD15 (LeuM1), CD16 (Leu11), CD19 (Leu12), CD20 (Leu16), CD22 (Leu14), CD25 (IL-2R), CD33 (LeuM9), CD34 (HPCA-2), CD45 (Hle1), CD56 (Leu19), CD57 (Leu7), HLA-DR and control IgG1 were purchased from Becton Dickinson. FITC- or PE-conjugated anti-CD13 (MCS-2), CD117 (c-kit) and CD122 (Mik-β1) were purchased from Nichirei, Tokyo, Japan. FITC- or PE-conjugated anti-CD41, GPA, CD24, CD36, CD38, CD94, CD158a, CD158b, KAR p50.3, KIR p70, CD161, NKG2A and purified anti-CD2 were purchased from Immunotech, Westbrook, ME. PE-conjugated AC133 was purchased from Miltenyi Biotec, Auburn, CA. FITC- or PE-conjugated anti-OKT6 (CD1a) and OKB7 (CD21) were purchased from Ortho Diagnostic, Raritan, NJ. FITC-conjugated anti-MPO, PE-conjugated anti cytoplasmic-CD3 and a Fix & Perm™ cell permeabilization kit for staining intracellular antigens were purchased from Caltag Laboratories, South San Francisco, CA, and used as described previously [26]. Monoclonal antibodies, NKp46 (IgG1) and NKp44 (IgG1), were kindly provided by Dr. Alessandro Moretta [27, 28]. Mouse myeloma protein IgG1 (MOPC21) was purchased from Sigma Immuno Chemicals, St. Louis, MO, and used as a control of purified monoclonal antibodies in an indirect staining method. PE-conjugated goat

anti-mouse IgG1 was purchased from Southern Biotechnology, Birmingham, AL.

*Southern blotting analysis of T-cell receptor genes.*

The rearrangement of the T-cell receptor β and δ chain genes was evaluated according to the standard method. In brief, 5 μg DNA from the sample was extracted according to the standard methods, digested with *Ech*RI, *Bam*HI, *Hind*III or *Kpn*I, electrophoresed through a 0.6% agarose gel and transferred onto a nylon membrane filter. The filter was hybridized with a $^{32}$P-labeled TCR/Cβ1 and Cβ2 probes, or TCR/Jδ1 probe, and washed under the appropriate stringency conditions, before the bands were visualized by autoradiography.

*RT-PCR analysis for mRNA expression of CD2, CD3 and NKp46.*

The total RNA was extracted from the MZ93 cells, MOLT3 cells and HT93 cells by the guanidium thiocyanate-phenol-chloroform method using TRIZOL reagent (Life Technologies, Tokyo, Japan). Complementary DNA was synthesized from the total RNA with oligo-dT primers and reverse transcriptase in a reaction buffer. The reaction buffer containing the cDNA sample was amplified by using an RNA PCR kit (Ver.2.1, Takara, Tokyo, Japan) and specific oligonucleatide primers for CD2, CD3ε, CD3ζ, NKp46 and β-actin in a thermal cycler (Perkin-Elmar, Norwalk, CT). The specific primers were as follows: for CD3ζ; sense primer, CTCTGCCTCCCAGCCTCTTT; antisense primer, GCGTCGTAGGTGTCCTTGGT [29], for CD3ε: sense primer, GTCTCCATCTCTGGAACCACA; antisense primer, TGTTCTCCAGAGGGTCAGATC [30], for CD2: sense primer, CCGAATTAAACCTGTATCAAGA; antisense primer, AACTTCCCAACATCCTCCT, for NKp46: sense primer, CTGAGCGATGTCTTCCACAC; antisense primer, GCTGTACGT GGCTGGATCTT, for β-actin: sense primer, ATCATGTTTGAGACCTTCAA, antisense primer, CATCTCTTGCTCGAAGTCCA. The conditions for each PCR reaction were denaturation at 94℃ for 1 min, elongation at 72℃ for 1 min, and annealing at 55℃ (CD3ζ), 57℃ (CD3ε), 54℃ (CD2 and β-actin) or 60℃ (NKp46) for 1 min. The PCR cycle was repeated 30 times, and a fraction of each sample was electrophoresed on a 3% agarose gel in Howley buffer, stained with ethidium bromide, and visualized under ultraviolet illumination. Adequate sizes of CD3ζ, CD3ε, CD2, NKp46 and β-actin were 504bp., 480bp., 389bp., 503bp., and 318bp., respectively.

**Results**

*Morphological and chromosomal analysis of MZ93.*

The established MZ93 cells showed irregular nuclei and cytoplasmic vacuoles, and did not have azulophilic granules. They did not have peroxidase, alpha-naphthyl-butyrate or ASD-chloro-acetate esterase activities in the cytoplasm, and did not have antigens of terminal deoxynucleotidyl transferase. Electron-microscopic MPO and PPO [31] activities were also negative.

The G-banding analysis showed that the MZ93 cells had a complex karyotype of 48, X, -Y, add(1)(p34), +del(3)(p13), der(5)t(5;12)(q13;q13), add(6)(p25), +8, der(9)t(9;22)(q34;q11), +10, i(11)(q10), -12, add(14)(p11), -15, add(17)(p11), -19, +20, add(22)(q11) +2mar (Fig. 1).

*The surface and cytoplasmic phenotype of MZ93.*

The surface phenotype of the cells is shown in Figs. 2 and 3, and summarized in Table 1. The cells expressed CD4, CD7, CD13, CD21, CD25, CD33, CD34, CD45, CD56 and NKp46 on the surface. The cytoplasmic phenotype of the cells is shown in Fig. 3. The relatively early passages of the cells expressed cytoplasmic CD3ε in 1998, and the late passages of the cells lost the antigens in 2000. The cells expressed cytoplasmic CD2, and did not express MPO. Further analysis of mature NK-related antigens revealed the absence of CD94, CD158a, CD158b, KAR p50.3, KIR p70, CD161 and NKG2A.

*Southern blotting analysis and RT-PCR.*

The T-cell receptor β and δ chain genes of the MZ93 cells showed a germline configuration (data not shown). The cells did not express mRNA of CD3ζ or CD3ε. In contrast, the cells expressed mRNA of CD2 and NKp46 (Fig. 4). The breakpoint analysis of the Ph chromosome of the cells using RT-PCR showed a b2a2 subtype (data not shown).

**Discussion**

The morphological classification of de novo acute leukemia is mostly easy, while the

identification of cell lineage is sometimes difficult in the blasts obtained from patients with CML in a blast crisis. Hyperdiploid B-lymphoblasts appear to be large immature cells. The morphology of near-diploid CML cell line, MZ93, showed MPO-negative large myeloblastoid appearance in spite of the dim expression of intracytoplasmic CD3. TdT was negative in the cells. The majority of blast crisis from CML are non-lymphoid, and most of the cell lines established from CML are MPO-negative myeloid.

Human NK-cells have phenotypes of CD2, CD7, CD11a, CD11b, CD16, CD56, CD57, CD58-positive and CD3, CD19-negative, and also express cytotoxic molecules, and have NK activity [9, 32]. Moretta recently reported an NK-cell marker NKp46, which was only expressed in the NK-cells in the human peripheral white blood cells, and has an important role in NK-cell activation [27, 28]. Therefore, the presence of this molecule may help to define the NK-precursor cells.

NK cells lack CD3ε, yet contain CD3ζ [33]. The cells are derived from bone marrow CD33+/CD34+ cells in the presence of IL-2 [33, 34], and the CD33+/CD34+ cells do not mean purely myeloid-committed progenitor. Even though the maturation passages of the NK cells from the CD33+/CD34+ precursors are not fully understood, the "myeloid/NK cell precursor acute leukemia" is proposed to be the malignant counterparts of the common progenitors between the NK cell and myeloid lineages by Suzuki [16, 22, 23]. This type of acute leukemia proposed by him shows morphology of ALL (L2), and expresses CD7, CD56, CD33, CD34, HLA-DR and cytoplasmic CD3ε (cCD3ε) in most cases, and CD13 in some cases. The blast cells do not express MPO. Hence, those leukemias apparently appear to be T cell-related CD56+ ALL. Reuss-Borst have also reported CD7/CD56/CD33-positive acute undifferentiated leukemia as the "NK-precursor leukemia" [35]. In contrast, Scott has reported another type of "myeloid/NK cell acute leukemia", potentially misdiagnosed as acute promyelocytic leukemia [36]. The blast cells were MPO+, HLA-DR-, CD33+, CD56+ and CD16-, and the disease is, therefore, a subclass of MPO-positive AML. In conclusion, despite there being several proposals of NK-precursor acute leukemia including so called "myeloid/NK cell precursor acute leukemia", the disease entity is still complex and confusing.

The MZ93 cells expressed the immature hematopoietic precursor marker CD34, myeloid markers CD13 and CD33, T-cell markers CD7 and cytoplasmic CD2, and NK markers CD56 and NKp46. Therefore, the cells looked like T cell-related NK progenitor coexpressing myeloid antigens. In contrast, they did not express CD3ε or CD3ζ. The mature NK cell specific molecules NKp46 are combined with cytoplasmic CD3ζ in the cellular membrane of mature NK cells [28, 33]. Mature NK cells do not express CD3ε, while fetal NK cells express cytoplasmic CD3ε and CD3δ proteins [37]. CD34+, CD2+, CD7+ progenitor cells in human bone marrow express CD3γ, CD3δ and CD3ζ [38]. The progenitor cells lose CD3γ and CD3δ according to the progress of maturation. The reason for the absence of CD3ζ in the MZ93 cells is unclear. Although the expression form of NKp46 in the immature hematopoietic precursors is not known, it is possible that the expression of CD7 and NKp46 proteins appears before the expression of CD3 molecules in the possible common precursors of NK and T cells. Although potential cross-contamination of another cell line [39, 40] into the MZ93 culture in vitro is not fully denied by means of DNA fingerprinting, the MZ93 is estimated to have originated in the patients, because the additional karyotype abnormality of the Ph-positive leukemia cells in vivo, 6p+, was also observed in the MZ93.

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Table 1 The phenotypic analysis of freshly isolated leukemic cells and the cell line MZ93

| | fresh leukemic cells | MZ93 |
|---|---|---|
| **CD1a** | - | - |
| **CD2** | - | - |
| **CD3** | - | - |
| **CD4** | + | + |
| **CD5** | n.d | - |
| **CD7** | + | + |
| **CD8** | - | - |
| **CD10** | - | - |
| **CD13** | + | + |
| **CD14** | - | - |
| **CD15** | - | - |
| **CD16** | n.d | - |
| **CD19** | - | - |
| **CD20** | - | - |
| **CD21** | - | + |
| **CD22** | - | - |
| **CD24** | n.d | - |
| **CD25** | + | + |
| **CD33** | + | + |
| **CD34** | + | + |
| **CD36** | - | - |
| **CD38** | + | - |
| **CD41** | - | - |
| **CD45** | + | + |
| **CD56** | - | + |
| **CD57** | n.d | - |
| **CD117** | n.d | - |
| **CD122** | - | - |
| **AC133** | n.d | - |
| **GPA** | - | - |
| **HLA-DR** | + | - |
| **NKp46** | n.d | + |
| **NKp44** | n.d | - |

n.d., not determined; GPA, glycophorin A.

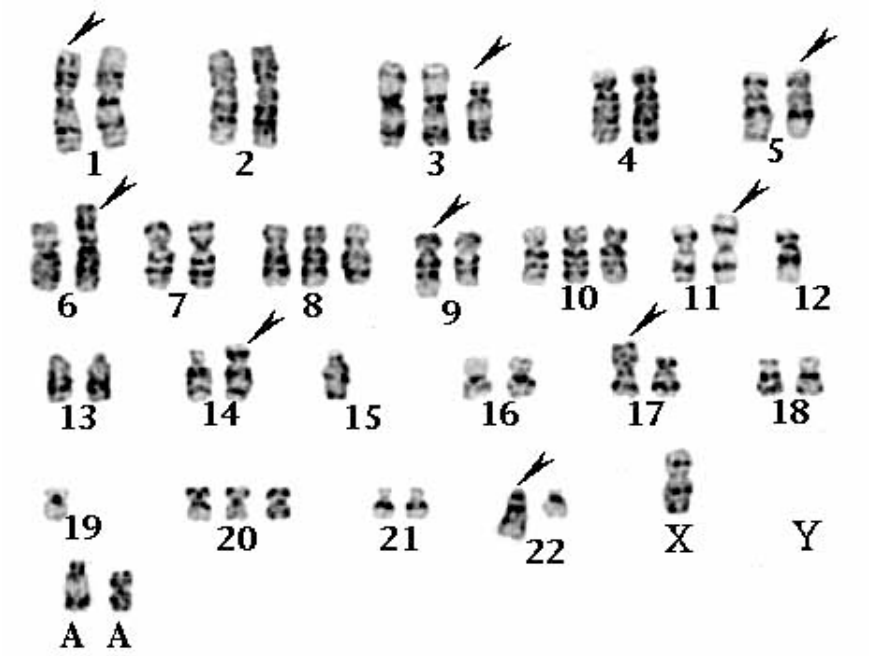


Fig 1. The karyotype of the MZ93 cells. The chromosome analysis of the cells showed 48, X, -Y, add(1)(p34), +del(3)(p13), der(5)t(5;12)(q13;q13), add(6)(p25), +8, der(9)t(9;22)(q34;q11), +10, i(11)(q10), -12, add(14)(p11), -15, add(17)(p11), -19, +20, add(22)(q11) +2mar. The arrowheads indicate the abnormal chromosomes. A, the marker chromosomes.

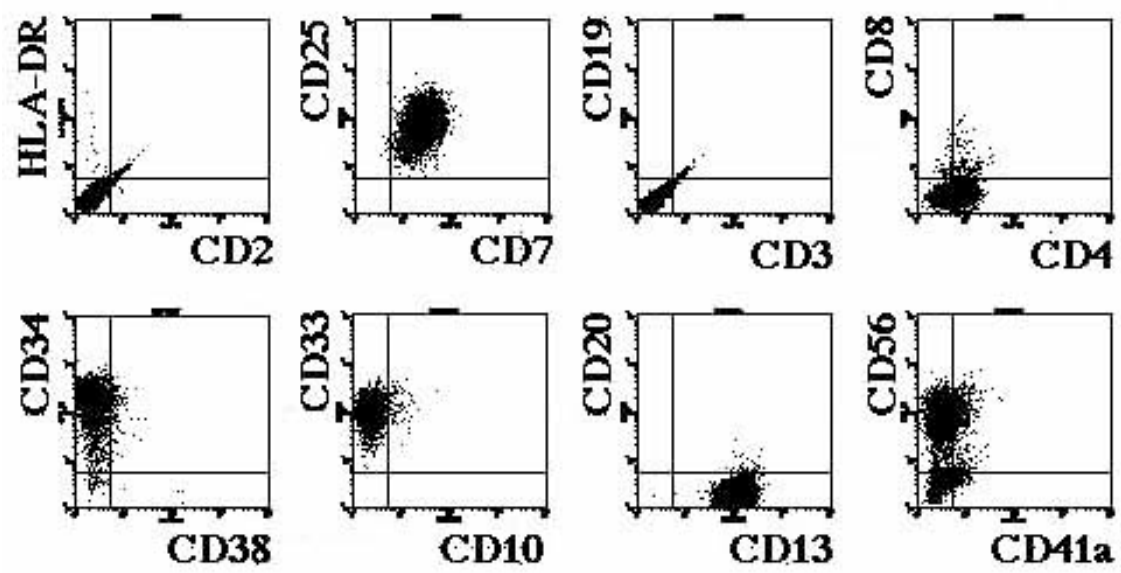


Fig 2. The surface phenotype of the MZ93 cells, analyzed by flow cytometry and FITC- and PE-conjugated monoclonal antibodies. The horizontal and vertical axes show FITC- and PE-fluorescence, respectively.

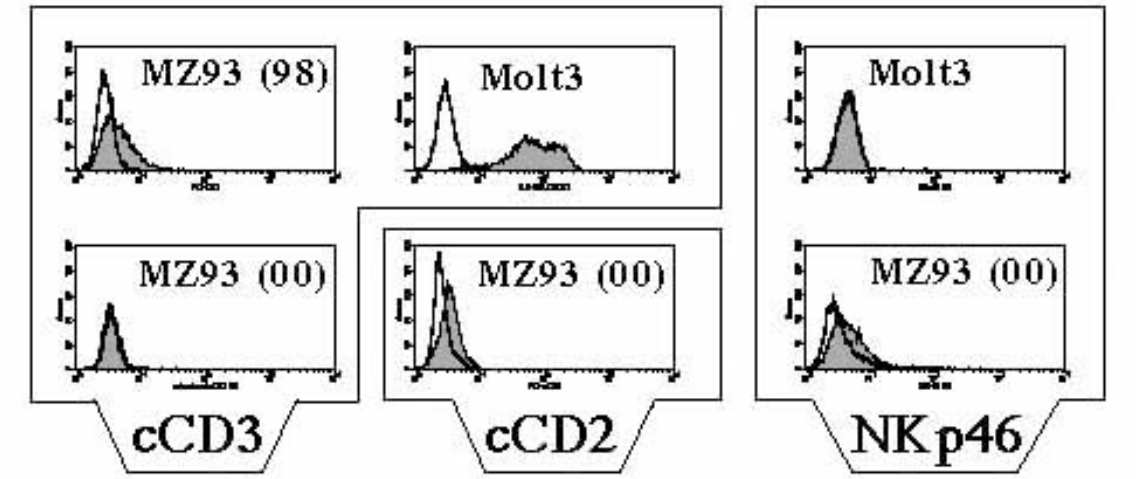


Fig 3. The surface (NKp46) and cytoplasmic (CD2 and CD3) antigen expression of the MZ93 cells, analyzed by flow cytometry and an indirect staining method using monoclonal antibodies and a PE-conjugated second antibody.

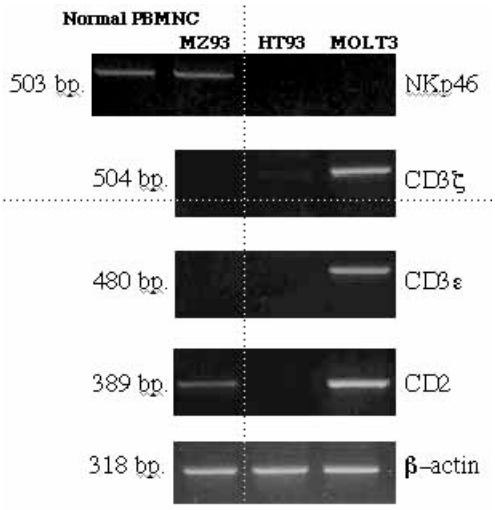


Fig 4. The RT-PCR analysis of NKp46, CD2, CD3ζ and CD3ε mRNA expression in the MZ93 cells, HT93 cells and MOLT3 cells. The MZ93 cells expressed both NKp46 and CD2 mRNA. The t(15;17) cell line HT93 expressed none of these mRNAs. The T-cell line MOLT3 expressed CD2, CD3ε and CD3ζ, but not NKp46 mRNA.