Jpn. J. Cancer Res. 83, 450–457, May 1992

Human T-Cell Leukemia Virus-1-positive Cell Line Established from a Patient with Small Cell Lung Cancer

Hiromitsu Matsuzaki,[1,5] Hiroyuki Hata,[1] Norio Asou,[1] Minoru Yoshida,[1] Fumihiko Matsuno,[1] Motohiro Takeya,[2] Kazunari Yamaguchi,[3] Isao Sanada[4] and Kiyoshi Takatsuki[1]

[1]*The Second Department of Internal Medicine,* [2]*The Second Department of Pathology and* [3]*Blood Transfusion Service, Kumamoto University Medical School, Honjo 1-1-1, Kumamoto 860 and* [4]*Department of Internal Medicine, Kumamoto National Hospital, Ninomaru 1-5, Kumamoto 860*

A stable cell line, KHM-3S, was established from a patient with small cell lung cancer (SCLC), who had a high serum level of soluble interleukin 2 receptors (sIL2-R) and was seropositive for human T cell leukemia virus (HTLV)-1. KHM-3S cells were positive for IL2-R (Tac) and NKH-1, but negative for other lymphocytic markers such as OKT 11, OKT 4, OKT 8, T cell receptor (WT 31), B 1, and B 4. Moreover, the KHM-3S cells were negative for leukocyte common antigen and strongly positive for neuron-specific enolase (NSE). Secretion of sIL2-R and NSE by the KHM-3S line was detected by an enzyme-linked immunosorbent assay. Rearrangement of the T cell receptor gene and monoclonal HTLV-1 integration were found by Southern blot analysis of KHM-3S DNA. However, Northern blot analysis showed no T cell receptor mRNA. KHM-3S may be useful for studies on the role of HTLV-1 in carcinogenesis and IL2-R expression in SCLC.

Key words: Cell line — Human T cell leukemia virus-1 — Small cell lung cancer

Small cell lung cancer (SCLC)[6] is a subtype of lung cancer that differs morphologically,[1] biochemically,[2] and clinically[3] from other subtypes. Many SCLC cells have a common chromosomal abnormality, which is a partial deletion of the short arm of chromosome 3.[4–6] However, there is marked heterogeneity in the clinical behavior of SCLC and in the properties of cell lines derived from this tumor, such as the pattern of growth, the doubling time, and the expression of neuroendocrine markers[7,8] or oncogene products.[9,10] Moreover, the expression of some lymphoreticular antigens, such as NKH-1, Leu 7, Leu 11, OKT 10, OKM 1, Leu M1, Leu M2, OKT 9 and CALLA, by SCLC cells has been reported.[11–14] We recently experienced a case of SCLC in which the patient had a high serum level of soluble interleukin 2 receptors (sIL2-R). Surprisingly, rearrangement of the T cell receptor gene and monoclonal integration of proviral HTLV-1 DNA were demonstrated by Southern blot analysis of DNA isolated from the SCLC cells.[15] We also established a cell line, KHM-3S, from cells obtained from the pleural fluid of this patient. This paper reports the characterization of the KHM-3S line immunologically, pathologically, and genomically.

MATERIALS AND METHODS

Case report The patient was a 58-year-old man who was first admitted to Kumamoto University Hospital on October 4, 1988, with dyspnea because of a massive pleural effusion.[15] Chest X-ray and CT scan demonstrated hilar lymphadenopathy and a primary lesion in the left lung. Biochemical studies showed high levels of LDH (1,033 U/liter; normal:130–250), neuron-specific enolase (NSE) activity (18.8 ng/ml; normal: <7.5), and sIL2-R (19,200 U/ml; normal: 256–476). Almost pure SCLC cells were obtained from bloody pleural fluid aspirated before chemotherapy. These cells were examined for surface markers (Table I) and were cultured. Because the cells were negative for lymphocyte markers, such as OKT 11, OKT 4, OKT 8, B 1, and B 4, and because of the high level of NSE activity in the pleural fluid (237 ng/ml), he was diagnosed as having SCLC. His pleural effusion and the high serum sIL2-R level improved following treatment with cyclophosphamide, vincristine, ranimustine, pirarubicin, etoposide, cisplatin, and prednisolone. Although the patient was discharged in December 1988, he was readmitted in January 1989 because of anorexia, nausea, and vomiting. Gastric infiltration by tumor cells was demonstrated by CT scan and gastric biopsy. The tumor cells were negative for leukocyte common antigen (LCA) and the pathological diagnosis was also SCLC. Despite of intensive chemotherapy and radiation therapy, serum sIL2-R and LDH increased rapidly and he died of radiation pneumonitis in May 1989. Electron microscopy showed that tumor cells from autopsy had small numbers

[5] To whom correspondence should be addressed.

[6] The abbreviations used are: SCLC, small cell lung cancer; ATL, adult T cell leukemia; HTLV-1, human T cell leukemia virus-1; LCA, leukocyte common antigen; NSE, neuron-specific enolase; sIL2-R, soluble interleukin 2 receptors; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

of dense-cored, membrane-bound neurosecretory granules, 240 to 570 nm in diameter, which is characteristic of SCLC.

Cell culture Heparinized pleural fluid from the patient was layered onto Ficoll-Conray (specific gravity, 1.078) and was centrifuged at 400*g* for 30 min. The interphase cells, which consisted almost entirely of SCLC cells, were collected and seeded into culture plates at approximately 10^6 cells/ml after washing with complete medium. In the primary culture and the early subsequent passages, RPMI 1640 medium containing 20% fetal calf serum (FCS) was used, but when the cells began to grow steadily the medium was changed to RPMI 1640 containing 10% FCS. The cells were maintained throughout in humidified air with 5% CO_2 at 37°C. A control SCLC line, OS-2, was kindly provided by Dr. Kubota of the Department of Internal Medicine at Kinki Central Hospital.

Immunological marker studies Fresh or KHM-3S cells were reacted with the following murine monoclonal antibodies, then stained with fluorescein isothiocyanate-conjugated $F(ab')_2$ goat anti-mouse IgG (Sigma Chemical, St. Louis, MO), and counted by flow cytometry (FACStar, Becton Dickinson Monoclonal Center, Mountain View, CA). The murine monoclonal antibodies employed were: OKT 3, OKT 4, OKT 8, OKT 9, OKT 10, OKT 11, WT 31, OKDR, and OKNK (Ortho Diagnostics, Raritan, NJ); B 1, B 4, My 4, My 7, My 9, NKH 1, Tal, and Tac (Coulter Immunology, Hialeah, FL).

Cytogenetics Cytogenetic studies of KHM-3S cells were performed using conventional trypsin-Giemsa chromosome banding techniques.[16] Karyotypes were arranged according to the criteria of the international system for human cytogenetic nomenclature (ISCN).[17]

sIL2-R assay sIL2-R levels in the culture medium were determined using a sandwich enzyme immunoassay (T Cell Science, Cambridge, MA), as described by Rubin *et al.*[18] In brief, the IL2-R available in the test sample or in the standards was bound to polystyrene microtiter wells previously incubated with 100 μl of anti-Tac equivalent monoclonal antibody (1 μg/ml). A horseradish peroxidase-conjugated anti-IL2-R monoclonal antibody directed against a second epitope on the IL2-R molecule was then bound to the molecule captured by the first antibody to complete the sandwich. After washing to remove any unbound enzyme-conjugated anti-IL2-R monoclonal antibody, a substrate solution was added to the wells. The reaction was then stopped and the absorbance was determined at 490 nm. A standard curve was prepared using four IL2-R standards. The IL2-R standard used was the cell-free supernatant obtained from cultures of phytohemagglutinin (PHA)-stimulated T cells, and it was assigned a value of 1,000 IL2-R U/ml.

Southern blot analysis The KHM-3S DNA was analyzed using methods described previously.[19] In brief, 10 μg of high-molecular-weight DNA was digested with appropriate restriction enzymes, subjected to electrophoresis in 0.7% agarose gel, and transferred to nitrocellulose filters. The filters were prehybridized at 42°C in sealed plastic bags containing 5×standard saline citrate (SSC), 5× Denhardt's solution, 50% formamide, 20 m*M* sodium phosphate buffer, and heat-denatured salmon sperm DNA (200 μg/ml). Filters were then hybridized with a ^{32}P-labeled c-DNA probe at 42°C, and washed three times at room temperature with 5×SSC/0.1% sodium dodecyl sulfate (SDS). Finally, filters were exposed overnight at −70°C to X-ray film using an intensifying screen.

Northern blot analysis Total cellular RNA was extracted using the guanidine thiocyanate/cesium chloride method, as described elsewhere.[20] Ten micrograms of total RNA was denatured by heating at 60°C for 20 min in 50% (v/v) formamide, and then subjected to electrophoresis in 1% agarose gel containing 2.2 *M* formaldehyde. After transfer of the RNA to nitrocellulose filters, the filters were baked for 2 h at 80°C, followed by hybridization, washing, and autoradiography performed as for the Southern blotting.

c-DNA probes The probe consisting of a 3-kb *Eco*RI-*Hin*dIII fragment containing the Cβ1 region of the Tβ gene[21] was kindly provided by Dr. H. Sakano of the University of California (Berkeley, CA). The probe consisting of a 0.7-kb *Hin*dIII-*Eco*RI fragment containing the first J region (Jγ1) of the Tγ gene[22] was kindly provided by Dr. T. H. Rabbitts of the Medical Research Council Centre (Cambridge, UK). The probe comprising a 0.7-kb *Eco*RI-*Hin*dIII fragment containing the J region of the Tδ gene[23] and fragments containing Cα, Cγ, and Cδ of the T cell receptor gene [24] were kindly provided by Dr. T. W. Mak of the Ontario Cancer Institute (Toronto, Canada). The entire HTLV-1 genome designated as pMT-2i[25] was kindly provided by Dr. R. C. Gallo of the National Cancer Institute (Bethesda, MD). The probe consisting of a *Pst*I-*Xba*I fragment of the CD3δ gene[26] was kindly provided by Dr. C. Terhorst of the Dana-Farber Cancer Institute (Boston, MA).

RESULTS

Morphology of cultured cells SCLC cells were collected and cultured from the patient's pleural fluid before chemotherapy. During early passages, KHM-3S cells floated in the culture medium as single cells or small soft clusters. Later, they formed tighter clusters that continued to float and were easily separated to single cells by pipetting. In Wright-Giemsa-stained smears, KHM-3S cells had nuclei with nucleoli and slightly basophilic cytoplasm with vac-

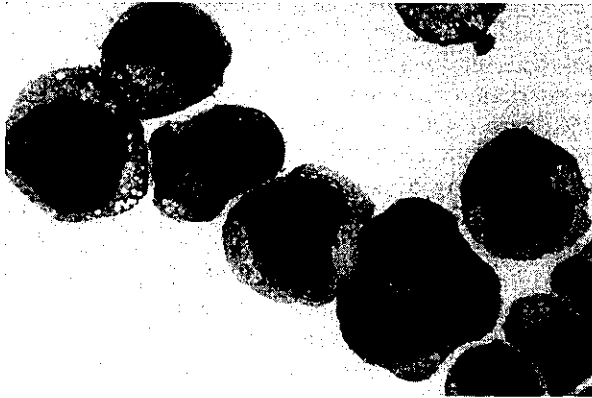

Fig. 1. Histological appearance of KHM-3S cells (Wright-Giemsa stain). KHM-3S cells were morphologically similar to lymphocytes.

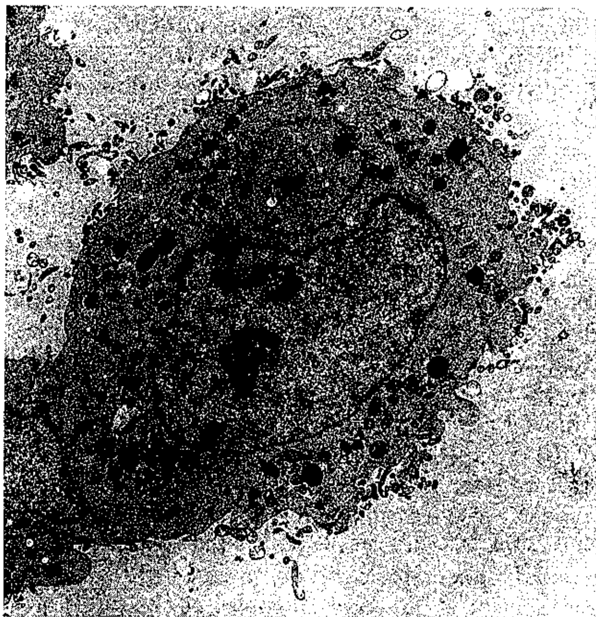

Fig. 2. Electron microscopic appearance of KHM-3S cells (×5,000). KHM-3S cells contained some granules in the cytoplasm and had non-convoluted nuclei.

Table I. Surface Markers of Fresh Tumor Cells from the Pleural Fluid and of the Cell Line

CD	MoAb	Fresh cells (%)	KHM-3S (%)	OS-2 (%)
2	OKT 11	2.0	1.3	0.5
3	OKT 3	NT	1.5	0.9
4	OKT 4	12.1	2.1	0.3
8	OKT 8	13.1	2.0	11.3
—	WT 31	NT	1.2	0.3
19	B 4	3.9	0.0	0.0
20	B 1	6.4	4.4	7.9
13	My 7	4.8	0.0	0.0
14	My 4	5.1	0.5	0.8
33	My 9	5.0	0.2	0.1
16	OKNK	5.0	7.2	47.6
—	NKH 1	53.5	28.0	98.7
25	Tac	79.7	98.3	1.0
26	Tal	2.7	0.5	0.3
38	OKT 10	4.8	1.9	0.1
—	OKDR	99.0	98.8	0.0
—	OKT 9	83.4	94.3	39.8

NT: Not tested.

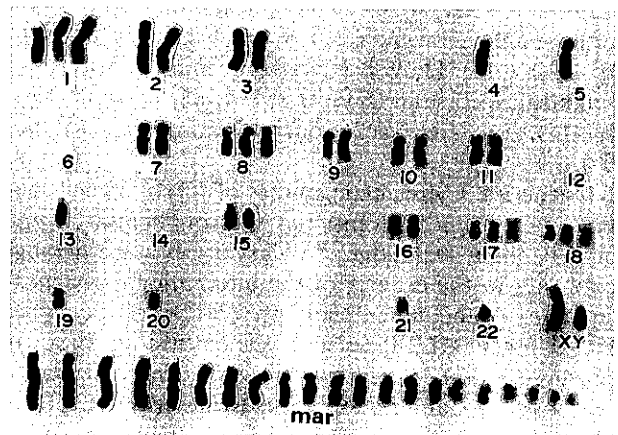

Fig. 3. Representative G-banded karyotype of KHM-3S cell showing 58,Y, −X, −1, −4, −5, −6, −6, +8, −12, −12, −13, −14, −14, +17, +18, −19, −20, −21, −22, +der(X)t(X;?)(q28;?), +der(1)t(1;?)(p13;?), +der(?)t(1;?)(q11;?),del(4)(p13), +21 mar. Losses of No. 13 and 19 are random in this cell. Other structural and numerical abnormalities are clonal.

uoles (Fig. 1). Immunoperoxidase studies showed that KHM-3S cells were negative for LCA and positive for NSE (data not shown). Electron microscopy demonstrated that the cell possessed some granules and non-convoluted nuclei. Typical neurosecretory granules, which were detected in fresh tumor cells, were not demonstrated in KHM-3S cells (Fig. 2).

Surface markers Fresh cells and KHM-3S cells were positive for HLA-DR (OKDR), transferrin receptor (OKT 9) and IL2-R (Tac), and weakly positive for NKH-1. They were negative for OKT 3, OKT 4, OKT 8, OKT 11, and even for the T cell receptor marker WT 31 (Table I) and envelope protein of HTLV-1 (data not shown), which are usually expressed by ATL cell lines.

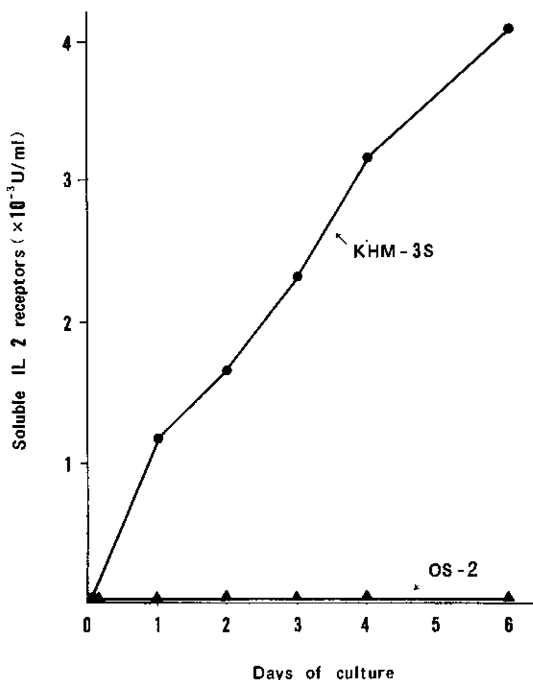

Fig. 4. Accumulation of sIL2-R per 1×10^6 KHM-3S (●) and OS-2 (▲) cells cultured in RPMI 1640 containing 10% fetal calf serum. sIL2-R levels increased almost linearly from the time of cell seeding. No medium changes were made during the experiment.

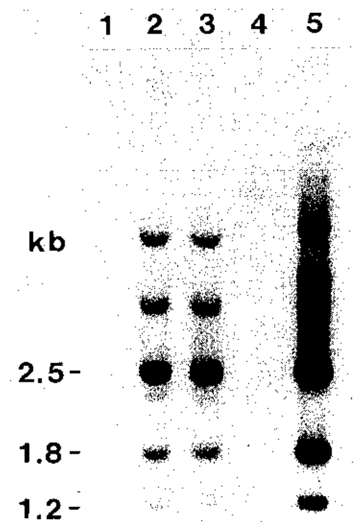

Fig. 6. Integration sites of proviral HTLV-1 DNA. DNA was digested with *Pst*I. The adult T cell leukemia cell line, SKT 1B (5) which was established in our laboratory,[27] was used as the positive control and HL60 (1) was used as the negative control. Southern blot analysis was performed using the entire HTLV-1 genome designated pMT-2i as a probe. Monoclonal integration of the HTLV-1 provirus was detected in DNA from fresh tumor cells (2) and KHM-3S cells (3), but not in DNA from the SCLC cells line, OS-2 (4).

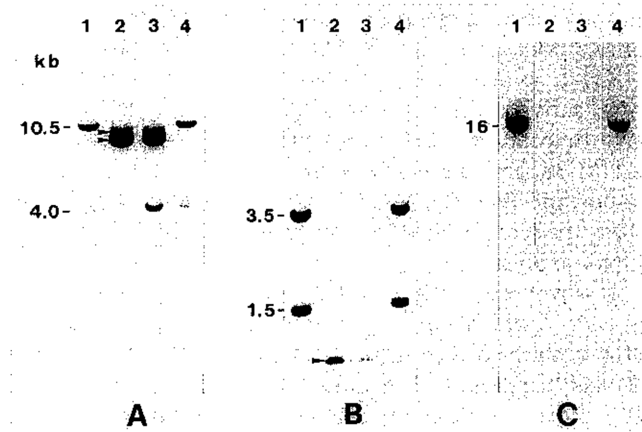

Fig. 5. Rearrangement of the T cell receptor in KHM-3S cells. DNA from HL60 cells (1) was used as a germ line control. DNA was digested with *Eco*RI. Southern blot analysis was performed using T cell receptor β (A), γ (B), and δ (C) chain gene fragments as probes. Arrowheads indicate bands representing the rearranged Tβ and Tγ receptor gene and the Tδ receptor gene deletion in fresh tumor cells (2) and KHM-3S cells (3). DNA from the SCLC cell line, OS-2 (4), showed the germ line.

Karyotypic analysis All seven metaphases examined were abnormal with karyotypic variation from cell to cell. Their chromosome numbers were between 57 and 61, and the modal number was 57. Composite karyotype was as follows: 57,Y,−X,−1,−4,−5,−6,−6,+8,−12, −12, −14, −14, +17, +18, −20, −21, −22, +der(X) t(X;?) (q28;?), + der (1) t (1;?) (p 13;?), + der (?) t (1;?) (q11;?),del(4)(p13),+18−22 variable markers (Fig. 3).

sIL2-R production by KHM-3S cells sIL2-R levels were assayed serially without changing the culture medium to determine the accumulation of sIL2-R during culture. An almost linear increase of the sIL2-R levels in the culture medium of the KHM-3S cells was observed until day 6 of cultivation, but no sIL2-R was detected in the culture medium of OS-2 cells (Fig. 4).

Southern blot analysis Despite the absence of T cell markers such as OKT 11, OKT 4 and OKT 8, the same Tβ and Tδ gene rearrangements and Tδ gene deletion were observed in both fresh tumor cells and KHM-3S cells (Fig. 5). In contrast, the DNA of the other SCLC cells line, OS-2, conformed to the germ line pattern (Fig. 5). Moreover, monoclonal integration of the HTLV-1 provirus was found in fresh tumor cells, KHM-3S cells and the ATL cell line, SKT 1B,[27] but not in OS-2 and HL 60 cells (Fig. 6).

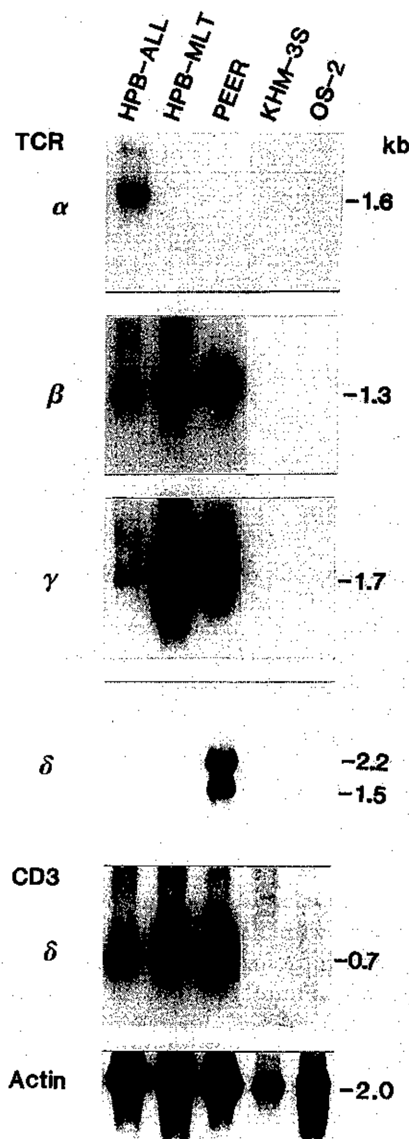

Fig. 7. Northern blot analysis of 10 μg of mRNA from KHM-3S cells. mRNA from HPB-ALL, HPB-MLT, and PEER cells was used as the positive control for T cell receptor mRNA. The filter was hybridized with T cell receptor α, β, γ, and δ chain, CD3 chain, and actin probes. Expression of T cell receptor and CD3 mRNA was not demonstrated in KHM-3S and OS-2 cells.

Northern blot analysis Southern blot analysis showed T cell receptor rearrangement, but KHM-3S cells were negative for T cell receptors (WT 31). In order to determine whether the T cell receptor was transcribed or not, Northern blot analysis of KHM-3S cells was performed. It was found that the cells contained an actin transcript similar to that seen in T cell lines, HPB-ALL, HPB-MLT, and PEER cells, but did not possess T cell receptor α, β, γ or δ, or CD3δ chain mRNA (Fig. 7).

DISCUSSION

Adult T cell leukemia (ATL) is associated with infection by the human T cell leukemia virus (HTLV)-1.[28-31] In patients with ATL, HTLV-1 sequences are integrated into the tumor cell DNA, and antibodies to viral proteins are found in the serum of these patients. Recently, HTLV-1 has also been shown to cause the non-malignant disease, HTLV-1 associated myelopathy (HAM).[32, 33] However, there have been no previous reports of direct integrated HTLV-1 infection of tumor cells of hematopoietic or non-hematopoietic origin except in ATL. We established KHM-3S, which contains genomically integrated proviral DNA, from a patient who was diagnosed as having SCLC. The karyotype of KHM-3S cell had no partial deletion of the short arm of chromosome 3, which is a common abnormality in SCLC.[4-6] KHM-3S cells also showed rearrangement of the T cell receptor gene as demonstrated by Southern blot analysis. These results indicated that KHM-3S cells are genotypically closely similar to ATL, though their surface markers differ from those of ATL cells and mimic those of SCLC. They were negative for LCA, OKT 3, OKT 4, OKT 8, OKT 11, and even for T cell receptor (WT 31) which was also not detected by Northern blot analysis. To our knowledge, there are a few reports of double negative (CD 4−, CD 8−) ATL[34, 35] but no report of ATL without T cell receptor expression. Moreover, KHM-3S cells do not express the envelope protein of HTLV-1 on their surface, unlike ATL cell lines. KHM-3S cells were positive for NKH-1, as has been reported in other cases of SCLC. NSE activity, which is one of the markers of SCLC, was also detected in KHM-3S cells. The characteristics of KHM-3S are summarized in Table II. It is clinically important that KHM-3S is phenotypically SCLC, and these patients would be diagnosed as SCLC without DNA analysis.

It is well known that IL2-R are expressed on activated T cells[36] which have been stimulated to proliferate by IL2. It has also been shown that NK cells[37] and activated B cells[38] express IL2-R and are induced to proliferate by IL2. Recently, three myeloid cell lines positive for IL2-R have been identified: KG-1,[39] a myeloblast cell line derived from erythroleukemia; EoL-1,[40] an eosinophilic cell line derived from eosinophilic leukemia; and SCC-3,[41] a monocytic cell line derived from non-Hodgkin's lymphoma. Inoue *et al.*[42] have demonstrated that the Tat protein produced by the HTLV-1 genome induces the expression of IL2-R by T cells. It is suggested that HTLV-1 induced the expression of IL2-R by KHM-3S cells. We found that 6 out of 21 SCLC patients tested had

Table II. Characterization of KHM-3S Cell Line

Characteristic	Fresh cell	KHM-3S
Morphology		
Neuron-specific enolase (NSE)	+	+
Leukocyte common antigen (LCA)	−	−
Electron microscopy: neurosecretory granules	+	−
Surface markers		
HLA-DR	+	+
T cell markers (T11, T3, T4, T8)	−	−
T cell receptor (WT 31)	NT	−
Tac	+	+
Envelope of HTLV-1	NT	−
B cell markers (B1, B4)	−	−
NK cell marker (NKH 1)	+	+
Karyotype: 3p deletion	NT	−
DNA analysis		
T cell receptor rearrangement	+	+
HTLV-1 provirus	+	+

NT: Not tested.

serum sIL2-R levels above 2,000 U/ml. However, only one person is positive for HTLV-1 antibody. These data provide evidence that HTLV-1-negative SCLC also caused high serum sIL2-R levels. Further studies are needed to define the association of HTLV-1 and SCLC in endemic areas for HTLV-1. KHM-3S may be a useful tool for developing our understanding of the process of carcinogenesis in HTLV-1-associated SCLC.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Cancer Research (61-2) from the Ministry of Health and Welfare, and a grant (03671194) from the Ministry of Education, Science and Culture of Japan.

(Received October 30, 1991/Accepted February 18, 1992)

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