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Lack of c-kit (CD117) expression in CD30+ lymphomas and lymphomatoid papulosis

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c-Kit receptor (CD117) is expressed by erythroid, megakaryocytic, and myeloid precursors and mature mast cells and has been reported to be expressed in CD30+ lymphomas such as Hodgkin's disease and anaplastic large-cell lymphoma. Imatinib mesylate, a well-established inhibitor of bcr-abl tyrosine kinase, and currently used for the treatment of patients with chronic myeloid leukemia, also inhibits c-kit receptor kinase activity. In view of the possible use of imatinib as experimental therapy for patients with c-kit-positive tumors, we assessed c-kit expression in CD30+ cell lines and lymphomas. The cell lines were assessed using multiple methods (RT-PCR, flow cytometry, and Western blot). c-Kit expression was also immunohistochemically assessed in 168 CD30+ lymphomas including 87 classical Hodgkin's disease, 63 anaplastic large-cell lymphoma, and 15 cutaneous anaplastic large-cell lymphoma. We also studied 18 cases of lymphomatoid papulosis, a CD30+ lesion closely related to cutaneous anaplastic large-cell lymphoma. Neither c-kit mRNA nor protein was detected in any of the cell lines assessed. Furthermore, treatment with imatinib did not inhibit proliferation of cell lines *in vitro*. Using immunohistochemistry, only one of 183 (0.5%) lesions was positive for c-kit, the positive case being an ALK-negative anaplastic large-cell lymphoma. Our data demonstrate that expression of c-kit receptor is exceedingly rare among CD30+ lymphomas and lymphomatoid papulosis, suggesting that c-kit receptor is unlikely to be an appropriate target for therapeutic options such as imatinib in patients with these tumors.
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The c-*kit* gene, first identified as the human analog of v-*kit*, the oncogene of HZ4 feline sarcoma virus, encodes a 145 kDa transmembrane glycoprotein capable of autophosphorylation on tyrosine residues. c-Kit receptor (CD117), henceforth referred to as c-kit in this study, is structurally related to the receptors for macrophage growth factor and platelet-derived growth factor.[1] c-Kit ligand, also known as stem cell factor (SCF), is capable of promoting cell proliferation of both myeloid and lymphoid hematopoietic progenitors in bone marrow cultures,[2] mainly by ligand-binding-dependent phosphorylation of tyrosine residues located at the cytoplasmic portion of c-kit, thereby initiating signal transduction pathways.[3]

In normal bone marrow, c-kit is expressed by stem cells, most erythroid and megakaryocytic precursors, a subset of granulocytic and monocytic cells and mature mast cells.[4–6] In 1994, Pinto *et al*[7] reported that c-kit is expressed in CD30+ lymphomas, including most cases of anaplastic large-cell lymphoma (ALCL) and approximately 50% of Hodgkin's disease (HD), but not in other non-Hodgkin's lymphomas. Subsequently, c-kit was reported to be expressed and functional in most HD cell lines.[8,9] However, others[10,11] have not confirmed these results. In addition, in a preliminary study published as a letter, we could not identify c-kit-positive cases of HD and ALK+ ALCL.[12] Thus, expression and functional status of c-kit in lymphomas remains a controversial issue. Furthermore, expression of c-kit in other CD30-positive lymphomas such as ALK-negative ALCL and cutaneous ALCL, as well as lymphomatoid papulosis, the latter closely related to cutaneous ALCL, is unknown.

Imatinib mesylate (imatinib, STI571, Gleevec®, Novartis Pharma AG, Basel, Switzerland) was first developed as a specific inhibitor of the bcr-abl protein tyrosine kinase[13] and is clinically used for the treatment of patients with chronic myeloid

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leukemia.[14,15] Subsequently, imatinib was found to inhibit other receptor tyrosine kinases, including c-kit,[16,17] and was shown to be active against c-kit-positive gastrointestinal stromal tumors.[18] With previous reports of c-kit expression in HD and ALCL having obvious therapeutic implications for imatinib or similar chemotherapeutic agents, and yet at variance with our preliminary results,[12] we decided to assess rigorously for c-kit expression in HD and ALCL cell lines and a large number of CD30+ lymphomas including HD, systemic ALCL and cutaneous ALCL. We also assessed cases of lymphomatoid papulosis. Since, the effect of imatinib in ALCL cells is unknown, we also assessed viability and proliferation of ALCL cell lines after treatment with imatinib.

Our data show that c-kit is not expressed in HD or ALCL cell lines and is rarely expressed in CD30+ lymphomas. Only one lymphoma, an ALK-negative case of ALCL, was c-kit-positive. All cases of lymphomatoid papulosis were negative. Treatment of HD and ALCL cell lines with imatinib did not affect viability or proliferation. These findings suggest that c-kit does not appear to be an appropriate target for investigational therapies in patients with HD or ALCL.

Materials and methods

Cell Lines

The panel of cell lines used included five known classical HD cell lines (HD-MYZ, HDLM2, L-428, KM-H2, and L-1236) (purchased from DSMZ, Braunschweig, Germany), two novel classical HD cell lines recently established at our institution (MDA-V and MDA-E), and five ALK-positive ALCL cell lines (Karpas 299, SR-786, SU-DHL-1, JB-6, and TS-G1). The gastrointestinal stromal tumor cell line ST-882 (a gift from Dr J Trent, Houston, TX, USA) and the megakaryoblastic leukemia cell line MO7e (a gift from Dr M Andreeff, Houston, TX, USA) served as positive controls for c-kit expression. The cell lines were maintained in RPMI 1640 medium supplemented with 1% nonessential amino acids, 10% fetal calf serum (Invitrogen Corporation, Grand Island, NY, USA), and 1% streptomycin–penicillin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Paraffin-embedded cell blocks of cell pellets fixed in 10% buffered formalin were also prepared.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

RNA was extracted using the RNAqueous® kit (Ambion Inc, Austin, TX, USA) according to the manufacturer's instructions. cDNA was synthesized using the Superscript™ First Strand Synthesis System for reverse transcription (RT)–polymerase chain reaction (PCR) (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of cDNA was tested by PCR using primers specific for the 18S cDNA. For RT-PCR amplification of c-kit, we used the following primers: 5′-AACGACACGCTGGTCCGCTG-3′ (forward), and 5′-GTACACAGAACTAGACACATC-3′ (reverse). We also tested another set of primers and conditions as reported by Aldinucci *et al*.[8]

Southern Blot Analysis of the RT-PCR Products

Following amplification, 10 μl of RT-PCR products were run on a 1.5%. agarose gel, transferred to Hybond N+ nylon membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) and hybridized with an internal oligonucleotide, 5′-CCCAGAAGTGACCAATTATTCCCT-3′. This probe was labeled for 2 h at 37°C according to the manufacturer's instructions (Amersham Pharmacia Biotech). After hybridization, the membrane was incubated with detection reagent for 5 min at room temperature, drained, and placed in a film cassette for autoradiography.

Western Blot Analysis

Cells in log-phase growth were collected and lysed at 4°C in lysis buffer with appropriate protease and phosphatase inhibitors. Western blot analysis was performed using standard methods and 50 μg of total protein from each cell line. Two commercially available polyclonal antibodies specific for c-kit were used: A4502 (DAKO, Carpinteria, CA, USA), and C-19 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Both antibodies were raised against a peptide from the cytoplasmic C-terminal part of human c-kit.

Flow Cytometry

Cells from all seven HD and one ALCL cell lines (Karpas 299) were analyzed for c-kit expression by flow cytometry with a phycoerythrin-conjugated monoclonal antibody specific for c-kit (clone 104D2, BD Biosciences Pharmingen, San Diego, CA, USA) using standard protocols and a FACScan instrument (Becton Dickinson, San Jose, CA, USA). The MO7e cell line served as a positive control for c-kit expression in these experiments.

Annexin V/propidium iodide (PI) staining was also performed using flow cytometry according to the manufacturer's guidelines. Briefly, 0.5×10^6 cells were washed in ice-cold PBS without Ca^{2+} or Mg^{2+} (Life Technologies). The cells were then resuspended in 100 μl of binding buffer and incubated with 5.0 μl of PI and 2.0 μl of annexin V-fluorescein isothiocynate for 15 min in the dark at room temperature. Flow cytometric analysis was

immediately performed using a FACSCalibur Instrument (Becton Dickinson).

Immunohistochemistry

c-Kit expression was assessed immunohistochemically in 87 classical HD (66 nodular sclerosis, 21 mixed cellularity), 63 ALCL (30 ALK-positive, 33 ALK-negative), and 15 cutaneous ALCL. We also assessed 18 cases of lymphomatoid papulosis, a disorder closely related to cutaneous ALCL. All lesions were diagnosed at The University of Texas MD Anderson Cancer Center. The histologic diagnoses of the lymphomas were based on criteria specified in the World Health Organization (WHO) classification.[19] The distinction between cutaneous ALCL and lymphomatoid papulosis was based on clinical data.

Full tissue sections and tissue microarrays[20] that included three or four tumor cores were assessed using an immunohistochemical method as described elsewhere.[21] Of the most widely used commercially available antibodies against c-kit, we used the A4502 polyclonal antibody from DAKO, since a recent validation study reported optimal immunohistochemical results and high specificity using this reagent.[22,23] Full tissue sections obtained from a gastrointestinal stromal tumor were used as a positive control. In addition, occasional monocytes and mast cells seen in many HD and ALCL tumors served as internal positive controls for c-kit expression.

Imatinib Treatment and Proliferation Assay

A panel of cell lines including Karpas 299, SUDHL-1, L-428, HD-LM2, and HD-MyZ were treated in 12-well plates with imatinib using different concentrations of drug (0.05, 0.5, and 5 μM).[11] At 24 and 48 h a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS] was added to each well using the CellTiter 96® AQ_{ueous} kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and light absorbance was measured using a μ Quant spectrophotometer (BIO-TEK Instruments Inc., Winooski, VT, USA). The MTS assay was performed twice and the mean numbers of proliferating and viable cells were calculated at 24 and 48 h after treatment with imatinib.

Results

c-kit RNA Levels in HD and ALCL Cell Lines

Table 1 summarizes the results for c-kit expression in HD and ALCL cell lines at the RNA level. Using RT-PCR and a set of primers we designed, all HD and ALCL cell lines were found to be negative for *c-kit* RNA (Figure 1). Southern blot transfer and hybridization using a probe specific for c-kit revealed no bands observed in HD or ALCL cell lines. Only the gastrointestinal stromal tumor cell line (positive control) showed hybridization of appropriately sized products.

Table 1 Expression of c-kit protein in HD and ALCL cell lines and tumors

Cell lines	*RNA level*	*Protein level*		
	RT-PCR	*FC*	*WB*	*IHC*
HD cell lines				
HD-MYZ	–	–	–	–
HDLM2	–	–	–	–
L-428	–	–	–	–
KM-H2	–	–	–	–
L-1236	–	–	–	–
MDA-V	–	–	–	–
MDA-E	–	–	–	–
ALCL cell lines				
Karpas 299	–	–	–	–
SR-786	–	ND	–	–
SU-DHL-1	–	ND	–	–
JB-6	ND	ND	–	–
TS-G1	ND	ND	–	–
Control cell lines				
ST-882	+	+	+	+
MO7e	+	+	+	+

Tumors	*IHC*	
	c-Kit positive	*%*
Classical HD ($n=87$)	0/87	0
ALK+ALCL ($n=30$)	0/30	0
ALK−ALCL ($n=33$)	1/33	3
Cutaneous ALCL ($n=15$)	0/15	0
Lymphomatoid papulosis ($n=18$)	0/18	0

FC, flow cytometry; WB, Western blot analysis; IHC, immunohistochemistry; HD, Hodgkin's disease; ALK, anaplastic lymphoma kinase; ALCL, anaplastic large-cell lymphoma.

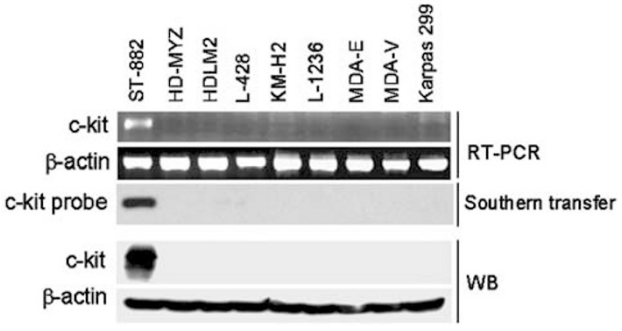

Figure 1 *Top panel*: Expression of c-kit mRNA in HD and ALCL cell lines as detected by RT-PCR using a set of primers we designed. cDNA from the gastrointestinal stromal tumor cell line, ST-882, served as a positive control. Southern blot transfer of RT-PCR products and hybridization with a c-kit-specific probe showed hybridization only for the control ST-882 cells. *Bottom panel*: Immunoblots showing expression of c-kit protein in HD and ALCL cell lines. All HD and ALCL cell lines tested were negative for c-kit. A 145 kDa band corresponding to c-kit receptor was detected only in the ST-882 control cells. β-Actin served as a control of protein load and integrity.

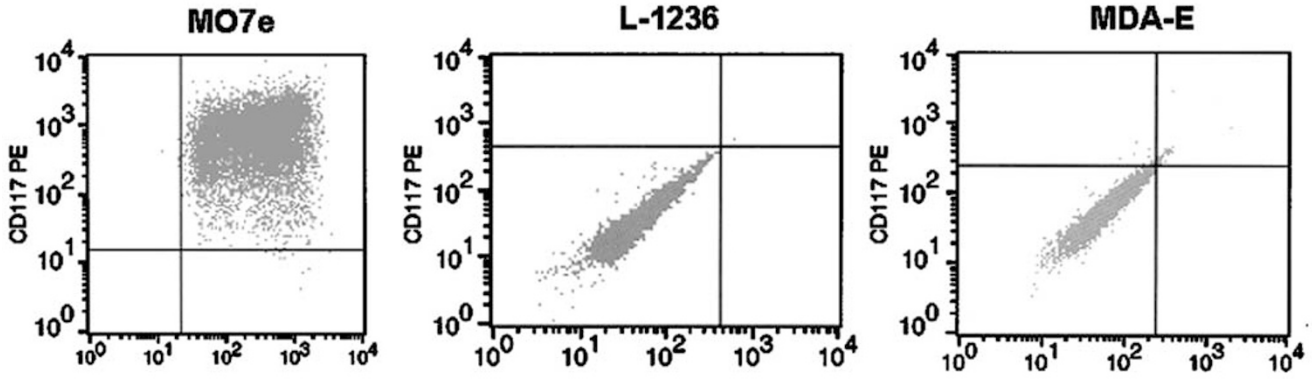

Figure 2 Representative examples of the flow cytometry immunophenotypic analysis of c-kit expression using a phycoerythrin-conjugated monoclonal antibody specific for c-kit. Results from two HD cell lines, L-1236 and MDA-V, are shown; both cell lines are negative for c-kit. The MO7e cell line served as a positive control for c-kit expression in these experiments.

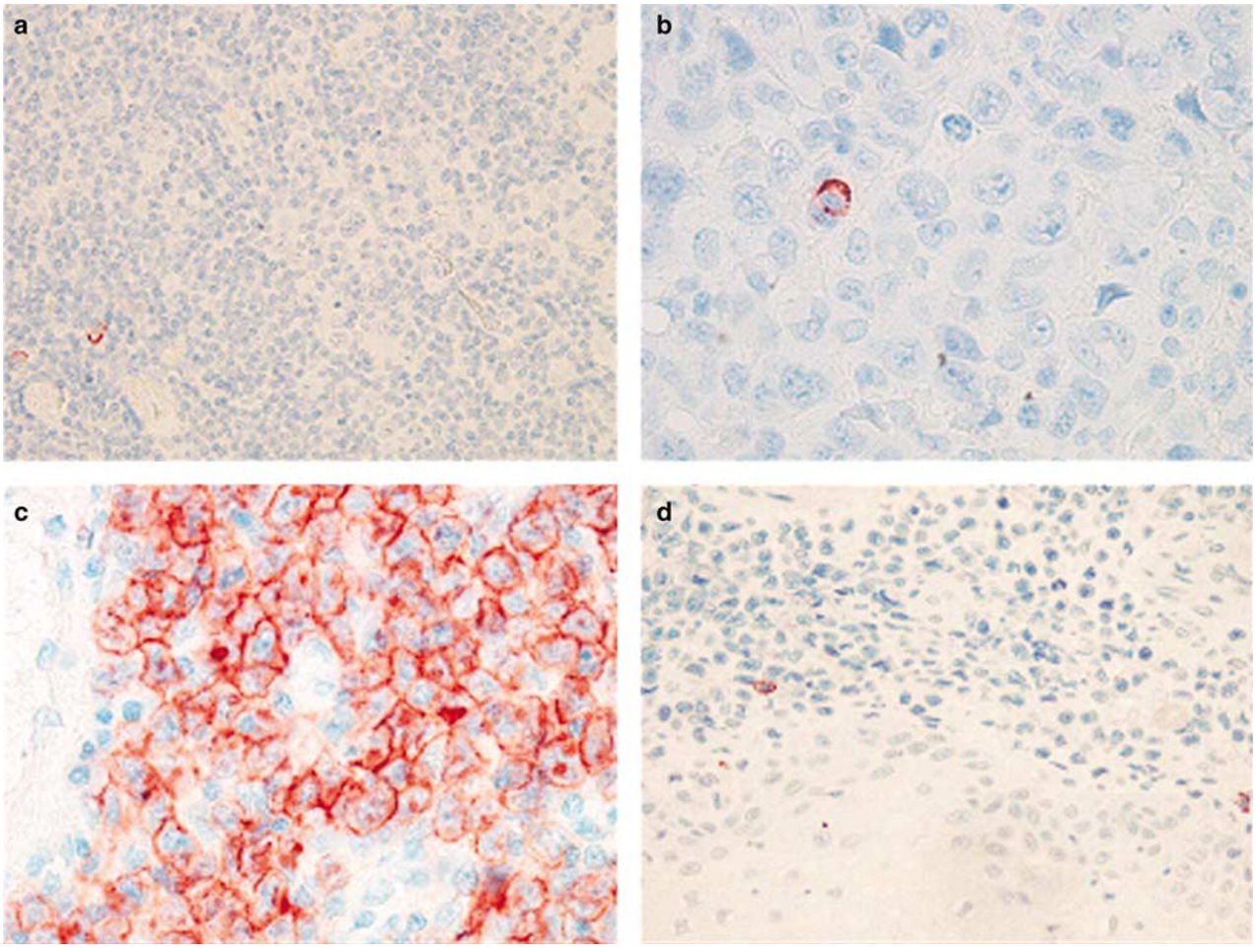

Figure 3 (**a**, **b**) Representative cases of classical HD (**a**), and ALK-positive ALCL (**b**) showing no evidence of c-kit immunoreactivity in tumor cells. Occasional monocytes or mast cells are positive for c-kit receptor and served as internal positive controls. (**c**) The only c-kit-positive ALK-negative ALCL tumor is shown. The tumor cells have a predominantly membranous staining pattern. (**d**) A representative case of primary cutaneous ALCL negative for c-kit receptor (**a**, **d**: $\times 200$; **b**, **c**: $\times 400$, immunoperoxidase with hematoxylin counterstain).

c-kit Protein Expression in HD and ALCL Cell Lines

Using Western blot analysis, no evidence of c-kit protein was detected in all seven HD and five ALCL cell lines tested (Table 1, Figure 1). These results were further confirmed by immunohistochemistry using cell blocks prepared from these cell lines and a polyclonal antibody reactive with c-kit [22,23]

(Table 1). We also studied seven HD and one ALCL (Karpas 299) cell lines using a monoclonal antibody specific for c-kit and flow cytometry immunophenotypic methods; all cell lines were negative (Table 1, Figure 2).

c-kit Protein Expression in CD30+ Lymphomas

Using immunohistochemical methods, c-kit was not expressed in the Hodgkin and Reed-Sternberg (HRS) cells of all 87 classical HD tumors (Figure 3a). Similarly, c-kit was negative in all 30 ALK-positive ALCL tumors (Figures 3b) and all cases of cutaneous ALCL ($n=15$) and lymphomatoid papulosis ($n=18$) (Figures 3d). One of 33 (3%) ALK-negative ALCL tumors expressed c-kit with a predominantly membranous pattern of staining (Figure 3c). In summary, only one of 183 (0.5%) lesions was positive.

Effect of Imatinib Treatment on ALCL Cells

Treatment of two ALCL cell lines, Karpas 299 and SUDHL-1, with different concentrations of imatinib did not affect cell proliferation or viability using the MTS assay as shown in Figure 4. The graph showing the response of ALCL cell lines to different concentrations of imatinib was generated with the results obtained at 24 and 48 h after treatment in two different experiments. Untreated Karpas 299 and SUDHL-1 cells served as a control for the baseline levels of cell proliferation and viability. Similarly, treatment of three HD cell lines, L-428, HD-LM2, and HD-MyZ, did not affect cell proliferation or viability assessed using the MTS assay (data not shown). The MO7e cell line was used as a positive control, as shown previously by others.[11]

Annexin V/PI staining revealed no difference in apoptosis of ALCL and HD cells treated with different concentrations of imatinib compared with control cells (Figure 5).

Discussion

This report provides *in vivo* and *in vitro* evidence that c-kit is not expressed at the RNA or protein level in HD and ALK-positive ALCL cell lines and tumors. In cell lines, we used an RT-PCR method to amplify c-kit mRNA and confirmed the results by Southern blot transfer and hybridization with a c-kit specific probe. We also utilized a variety of methods to assess c-kit protein including flow cytometry, Western blot analysis and immunohistochemical techniques. In 165 CD30+ lymphomas and 18 cases of lymphomatoid papulosis, we showed c-kit expression using immunohistochemical methods in only one (0.5%) case, an ALK-negative ALCL tumor that showed a typical membranous staining pattern for c-kit (Figure 3c).

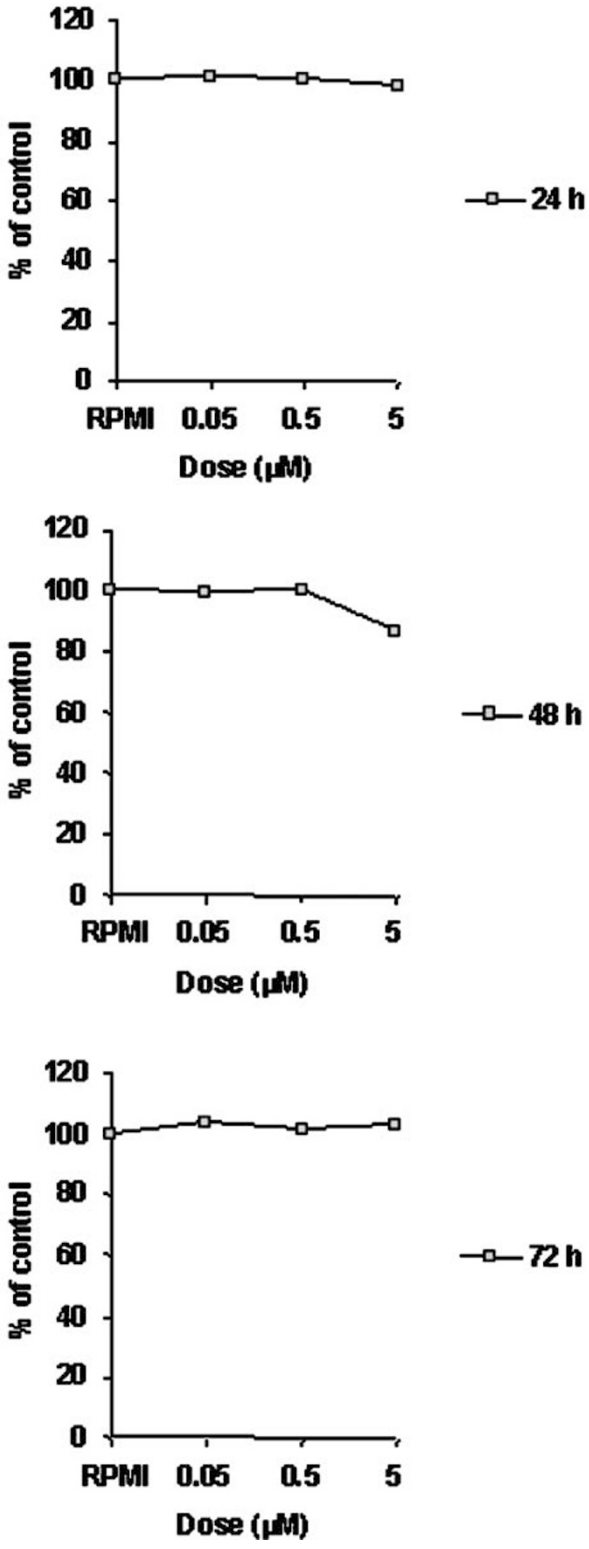

Figure 4 Proliferation of SUDHL-1 cells was determined using the MTS assay over a 72-h time course. The graphs represent the proliferation of viable SUDHL-1 cells treated with different concentrations of imatinib (0.05, 0.5 and 5 μM) compared with untreated cells. MTS-positive cells were counted at 24, 48 and 72 h. The curves show the percentage of viable cells (*y*-axis) and doses (*x*-axis). Untreated SUDHL-1 cells are designated as RPMI only.

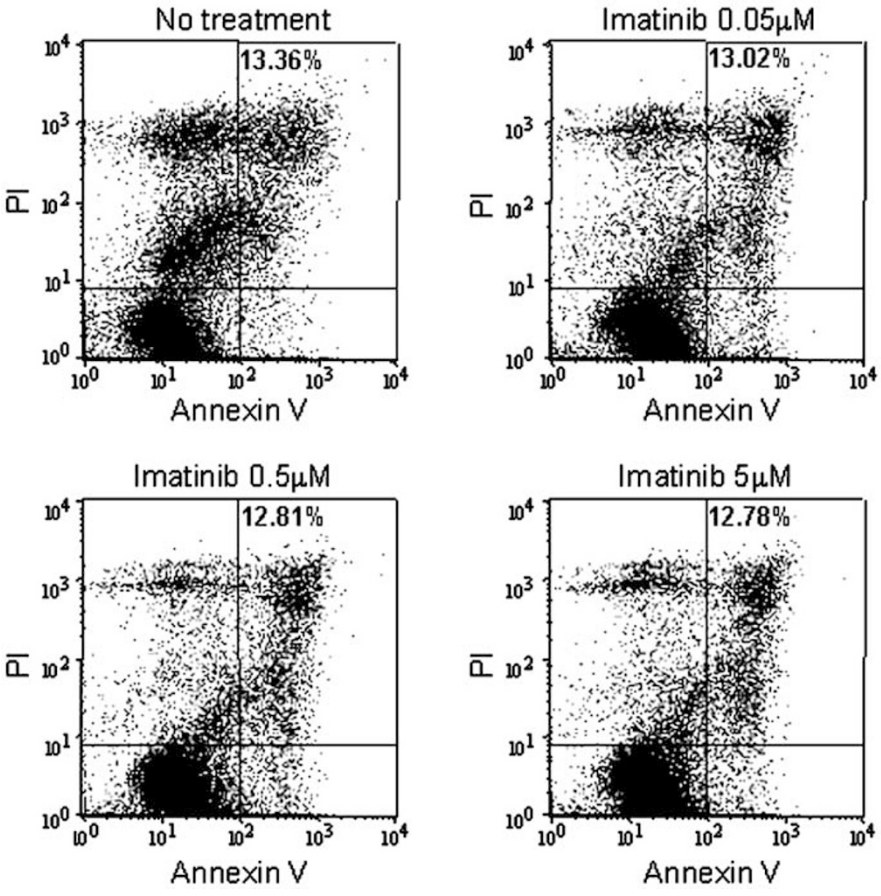

Figure 5 Apoptosis of SUDHL-1 cells treated with different concentrations of imatinib was assessed using annexin V/PI staining and flow cytometry. The fraction of annexin V-positive SUDHL-1 cells was 13.36% before treatment and 13.02, 12.81, and 12.78% after treatment with imatinib at concentrations of 0.05, 0.5 and 5 μM, respectively.

The data presented here extend the results of a preliminary study from our institution that included only ALK-positive ALCL tumors and a smaller number of HD tumors.[12] Our findings are also in agreement with two recent reports that demonstrated no evidence of c-kit immunoreactivity in a total of 32 HD tumors.[10,11] These results and our data are contrary to those of Pinto *et al*[7] who found c-kit expression in fresh-frozen sections of 11 of 16 ALCL and 11 of 21 HD tumors. Subsequently, the same group reported that five of six HD cell lines expressed functional c-kit receptor.[8] In both studies, the authors used the 17F11 monoclonal antibody that recognizes an extracellular domain of c-kit. The 17F11 antibody was raised by immunization of a Balb/c mouse with leukemic blasts from a patient with acute non-lymphocytic leukemia and was reported to react positively with most leukemic blasts of myeloid, but not lymphoid, lineage. The authors reported that 17F11 specifically recognized the c-*kit* gene product in NIH-3T3 fibroblasts transfected with the human c-*kit* gene.[24] However, subsequent functional studies showed that the corresponding epitope of 17F11 was not competing for stem cell factor (SCF), the c-kit ligand-binding site.[25] The 17F11 antibody is no longer commercially available and we could not test its immunoreactivity in this study.

Our data, showing absence of c-kit expression in HD and ALCL cell lines, are supported, in part, by a recent preclinical study by Re *et al*.[11] They reported that only one of six HD cell lines tested, the L-1236 cell line, was positive for c-kit by flow cytometry. We also demonstrate here that treatment with different doses of imatinib did not affect proliferation or viability of two ALCL and three HD cell lines (Figure 4). Annexin V/7-AAD staining also confirmed that HD and ALCL cells treated with imatinib do not undergo chemotherapy-induced apoptosis (Figure 5). Our data are supported by the results of two other studies. Re *et al*[11] showed that the proliferation of L-1236 cells was not influenced by stimulation with SCF, anti-SCF antibody, or imatinib. Ergin *et al*[26] demonstrated that imatinib had only a minimal effect on apoptosis of three ALCL cell lines (Karpas 299, SUPM2, and SU-DHL-1).

The explanation for the remarkable discrepancy between the studies of Pinto *et al*[7] and Aldinucci and colleagues[8,9] with our data regarding c-kit expression in HD and ALCL is uncertain. However, a cross reaction of the 17F11 antibody with an unrelated epitope cannot be excluded, since the

results obtained using this antibody have not been confirmed using other commercially available antibodies. In addition, independent work from others using immunohistochemical methods with widely used antibodies failed to show c-kit expression in the HRS cells of HD.[10,11] At the RNA level, Aldinucci *et al*[8] have reported that c-kit RNA is detected in HD-derived cell lines. We also tested the primers and RT-PCR conditions as described in their study.[8] Although we observed multiple RT-PCR products in ethidium-stained gels using the HD and ALCL cell lines we tested, following Southern blot transfer and hybridization with a specific c-kit probe there was no hybridization (data not shown). Thus, we believe that these RT-PCR products are likely to be nonspecific.

We conclude that c-kit RNA and protein are consistently not expressed in HD and ALK-positive ALCL cell lines and tumors or in cutaneous ALCL tumors and that c-kit is only rarely (<5%) expressed in ALK-negative ALCL tumors. All cases of lymphomatoid papulosis were also negative for c-kit. In addition, imatinib does not seem to be active in HD and ALCL *in vitro*. These findings suggest that c-kit is probably not an appropriate target for therapeutic agents such as imatinib in the treatment of patients with HD and ALCL.

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