Expression of the Candidate MCT-1 Oncogene in B- and T-Cell Lymphoid Malignancies.


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Abstract
Our laboratory has recently discovered a novel candidate oncogene, MCT-1, amplified in a human T-cell lymphoma and mapped to chromosome Xq22-24. This region is amplified in a subset of primary B-cell NHL’s suggesting that increased copy number of a gene(s) located in this region confer a growth advantage to some primary human lymphomas. We examined a diverse panel of lymphoid malignancies for expression of MCT-1. We demonstrated that there are significantly increased levels of MCT-1 protein in a panel of T-cell lymphoid cell lines as well as in non-Hodgkin’s lymphoma cell lines. Furthermore, we identified a subset of primary diffuse large B-cell lymphomas that exhibited elevated levels of MCT-1 protein. Interestingly, all transformed follicular lymphomas in our study demonstrated elevated protein levels of MCT-1. There was no detectable MCT-1 protein in leukemic cells from patients with chronic lymphocytic leukemia or in any normal lymphoid tissue examined. Lymphoid cell lines over-expressing MCT-1 exhibited increased growth rates and also displayed increased protection against serum starvation induced apoptosis when compared to matched controls. We found that MCT-1 over-expressing cells show constitutively higher levels of phosphorylated-PKB/Akt protein, especially under serum starvation conditions. Activation of survival pathways may be an additional function of the MCT-1 gene. Our data suggest that high levels of MCT-1 protein may be associated with a high risk subset of lymphoid neoplasms and further support the potential role of MCT-1 in promoting human lymphoid tumor development.
Introduction

Random genomic instability, as seen in many epithelial cancers, is not a characteristic of the more stable lymphoma genome. Furthermore, defects in DNA mismatch repair that manifest as genomic microsatellite instability, commonly found in various hereditary solid tumor syndromes and rare sporadic cancers are less recognized in lymphoma\(^1\)\(^2\). Genetic alterations frequently identified in lymphomagenesis include chromosome rearrangements, disruption of tumor suppressor genes and an increase in copy number of genes (gene amplification). We have previously identified an amplified DNA sequence in the Hut 78 T-cell lymphoma cell line that was subsequently shown to represent a novel candidate oncogene mapped to chromosome Xq22-24\(^3\). This region is amplified in a subset of primary B-cell NHL’s\(^4\)\(^6\) suggesting that increased copy number of a gene(s) located in this region confer a growth advantage to a subset of primary human lymphomas. Recently, analysis of the cell cycle regulation of MCT-1 protein levels displayed little variation of MCT-1 protein during the cell cycle progression\(^7\). However, levels of MCT-1 protein are rapidly induced in irradiated human lymphoid cells through a post-translational mechanism\(^7\) consistent with MCT-1 being a DNA damage response gene. In this report we examined the expression profile of MCT-1 protein along a spectrum of human lymphoid tumors. Our results demonstrated that MCT-1 protein expression was elevated in some samples of aggressive lymphoma including diffuse large B-cell lymphoma (DLBCL) and IL-2 independent T-cell lymphoid tumors. Moreover, we also observed that MCT-1 expression levels are strongly associated with the cell
proliferation marker, proliferating cell nuclear antigen (PCNA). In addition, lymphoid cells that are stably transfected with an MCT-1 retroviral construct exhibited increased proliferative rates and increased protection against serum starvation induced apoptosis when compared with matched controls. These data suggest that high levels of MCT-1 protein may be associated with a high-risk subset of lymphoid neoplasms and support a possible role for MCT-1 in human lymphomagenesis.

Materials and Methods

Cell Lines, Normal Tissues and tumor specimens

Six T-cell lines; 3 which are IL-2 dependent; EC155 (Advanced Biotechnologies, Columbia, MD) N1186 and N1186 and 3 which are IL-2 independent; Hut 78, MT-2 and Jurkat (Advanced Biotechnologies, Columbia, Md) and seven non-Hodgkin’s lymphoma cell lines SU-DHL 4, SU-DHL 6, SU-DHL 7, SU-DHL-8, SU-DHL-10, Namalwa and Ly3(ATCC, Manassas, VA) were chosen for this study. All T-cell lines were cultured in complete RPMI 1640 with 40U/ml of recombinant IL-2 (Invitrogen, CA) added to the IL-2 dependent lines. Normal donor peripheral blood lymphocytes (PBL) were isolated by centrifugation over Ficoll-Hypaque as previously described (Pharmacia, Pleasant Hill, CA). After PHA stimulation these lymphocytes were cultured in a similar manner as the IL-2 dependent T-cell lines as above. Leukemic cells from patients with chronic lymphocytic leukemia (CLL) were obtained after informed consent by centrifugation over Ficoll-Hypaque (Pharmacia, Pleasant Hill, CA) as above. The
white blood count ranged from 10-90k with > 80% lymphocytes in all samples. No patient was exposed to chemotherapy for at least three months prior to sampling. Lymph node biopsy specimens from patients with primary DLBCL as well as normal lymph nodes were used in this study. All primary tumor samples examined were obtained at diagnosis prior to treatment and were stored as fresh frozen biopsy specimens and preserved at –80 °C in the Pathology core laboratory of the Robert H. Lurie Comprehensive Cancer Center at Northwestern University Feinberg School of Medicine.

Western Blot Analysis

Cell lines (2x10^7 cells) were washed twice in PBS, lysed in 300µl of RIPA buffer containing 20µl of Protease inhibitor cocktail (Sigma) and incubated on ice for 30 min. Primary frozen tumor tissue samples were homogenized with Dounce homogenizers in RIPA buffer. Cell line and tissue samples were further disrupted by passing them through 21 gauge needles. The supernatant fluid of total cell lysate was taken after 10,000g's centrifugation for 10 min. In general, 40µg of protein was separated on 10% SDS-PAGE at room temperature. The proteins were transferred to Hybond nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) by using a semidry electroblotter (BioRad, Hercules, CA, USA). The membranes were blocked in 5% non-fat milk TBST and subsequently incubated with the following primary antibodies; rabbit polyclonal anti-human MCT-1 (Research Genetics), mouse monoclonal anti-human B-Actin (Sigma, St. Louis, MO), mouse monoclonal anti-human PCNA (Santa Cruz,
Santa Cruz, CA), mouse monoclonal anti-V5 (Invitrogen), rabbit polyclonal anti-phospho-AKT (Ser473) (Cell Signaling), goat polyclonal anti-AKT (Cell Signaling), and an HRP-conjugated secondary antibody. The specific proteins were detected by ECL (Amersham) and film exposure.

RT-PCR SSCP analysis for mutation detection
Reverse Transcriptional PCR was performed for SSCP. PCR primer sets used for the 5’ end portion of MCT-1 were: forward AACCGGTTGCCTAAAAGGAG, reverse TGTTTCATGGCATCGGACTATTT (product of 410bp); for the 3’ end portion of MCT-1 were: forward AAATAGTCCGATGCCATGAACA; reverse ACACAGACACAAACACACAGTACAG (product of 465 bp). PCR cycling was carried out as follows: 94 °C x 1 min followed by 34 cycles of denaturation at 94 °C X 1min, annealing at 58 °C for 45s, extension at 72 °C for 1 min; final chain elongation at 72 °C for 5 min. 10µl PCR product mixed with 2µl 10X Blue Juice loading buffer (Gibco) was denatured at 95 °C for 4min and then ice cold for 2min. The mixture was separated by 6% polyacrylamide, 5% glycerol gel and silver stained according to the procedures described10. The gel was dried at 80 °C for 30 min and then scanned into computer by a Microteck Scanner.

Establishment of stable MCT-1 over-expressing lymphoid cell line
The cDNA fragment containing the coding region of MCT-1 was amplified by RT-PCR using total RNA from normal donor PBL cells. This sequence was subcloned into the TOPO cloning site of pcDNA3.1/V5-Histidine tag vector.
(Invitrogen). The pcDNA3.1-MCT-1-V5 plasmid was extracted from the vector and used to transform Top 10 bacteria (Invitrogen). The new sequence containing the MCT-1 coding region was fused with a V5- histidine tag in C-terminal and was PCR amplified from pcDNA3.1-MCT-1-V5 plasmid by the forward primer of 5'-TAGAATTCCACCATGTTCAAGAAATTTGAT-3' and reverse primer 5'-GGTTAACAGCGGGTTAAACTCAAT-3'. This fragment was then digested with EcoRI and HpaI, and ligated to EcoRI and HpaI sites of the retroviral expression vector pLXSN (Clontech, CA). pLXSN vector or pLXSN-MCT-1-V5 vector were transfected into the packaging cell line, PT67 and recombinant retroviral RNAs were packaged into infectious, replication-incompetent particles. The culture media containing viral particles was collected. The viral-containing medium was added to EC155 cell culture medium. Infected EC155 cells were selected after growing in 10% serum RPMI 1640 supplemented with 300 µg/ml geneticin (G418) for three weeks. Individual clones of stably infected with pLXSN vector or pLXSN-MCT-1-V5 were obtained by limiting dilution. The following independently isolated clones were used for our experiments, ECI55-Vector(4), ECI55-Vector(5), ECI55-MCT-1(4), ECI55-MCT-1(5) and ECI55-MCT-1(7).

Confocal immunofluorescence

EC155-Vector and EC155-MCT-1 cells were washed once with PBS in 15 ml tubes. The cells were fixed in 2ml of absolute methanol for 5 min at room temperature (RT). After fixation, cells were washed with 5ml of PBS 3 times.
Cells were incubated in 2ml of block solution (PBS containing 10% fetal bovine serum) for 20 min at RT. The block solution was replaced by 0.5ml of PBS/10% containing primary antibody, mouse monoclonal anti-V5 (1:250 dilution, Invitrogen, Co) or mouse monoclonal anti-PCNA (1:500, Santa Cruz, CA). The cells were then incubated with primary antibody at 40 °C overnight. Then cells were washed with 5 ml of PBS x3 and incubated with 0.5ml of PBS/10% containing secondary antibody, anti-mouse IgG2a-Fluorescein (1:250 dilution, Roche, Germany) for 2 hours. Following 3 washes with PBS, the cells were spread on slides and mounted on cover glass with mounting medium. The slides were then viewed using a Zeiss LSM510 confocal scanning laser microscope. Green fluorescence was detected using an excitation of 395 nm and emission of 505 nm. The fluorescence images were saved on a computer and the results were printed out on a Fujix Pictography 3000 digital printer.

Cell Growth, viability and apoptosis assays:

Cell lines used were either EC155-Vector or EC155-MCT-1 clones. Cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS) (Life Technologies, Inc) and 40 U/ml of recombinant IL-2 (Life Technologies, Inc).

EC155-Vector or EC155-MCT-1 were seeded at 0.5x10^6 cells/ml in triplicate T-25 flasks with 10ml RPMI 1640 complete supplemented with 40 U/ml of recombinant IL-2. IL-2 was replenished every three days. Cells were counted every other day for 8 days and viability measured using the trypan blue exclusion
method. The mean cell number and its standard deviation for each time point were calculated using standard methodology as previously performed\textsuperscript{3}.

To investigate a possible role of MCT-1 in cell survival, the effect of serum deprivation was investigated. We seeded 2\times 10^6 cells/ml of either EC155-Vector or EC155-MCT-1 clones in duplicate T-25 flasks with serum reduced culture medium that containing 0.2% FCS and 40U/ml IL-2 in RPMI 1640 medium. After 24, 48 and 72 hrs culture, cell viabilities of both cell lines were analyzed using the trypan blue dye exclusion method.

In order to determine whether cell death was due to apoptosis, both annexin V binding assays and mitochondrial membrane potential assays were performed. We used an immunofluorescence assay to simultaneously detect mitochondrial membrane potential (MMP) and Annexin V binding of the cells. In brief, cell pellets were collected and resuspended with culture medium to reach a final cell concentration of 1\times 10^6 cells/ml. Fifty µl of CMX Rosamine (Beckman Coulter, Netherlands) was added to 0.5 ml of cell suspension and vortexed gently. Cell-containing tubes were placed in a CO\textsubscript{2} incubator at 37 °C for 15 min. Each tube containing 1ml PBS was spun at 1200 RPM for 5min. Supernatant was discarded and cells were resuspended in 500 µl of cold 1x binding buffer from the Annexin V FITC kit (Beckman Coulter). 5 µl of Annexin V FITC was added to the cell suspension and the tube was vortexed gently and put at 40 °C for 10min. Stained cells were immediately analyzed by flow cytometry.
Results

Elevated MCT-1 protein levels in exponentially growing T-lymphoid cell lines are not associated with point mutations.

All IL-2 independent T-cell lines exhibited elevated MCT-1 protein levels. By contrast, the IL-2 dependent T-cell lines including IL-2 stimulated peripheral blood lymphocytes (PBL) demonstrated low to absent MCT-1 protein levels (Fig. 1). There was no amplification of the MCT-1 gene detected in any of these cell lines (data not shown). If the MCT-1 gene is not found to be amplified, there is still the potential for it to be activated through point mutations. The RT-PCR SSCP assay was used to screen for point mutations that may exist in the MCT-1 gene. There was no altered migration of bands in any of the T-cell lines compared with the normal PBL samples (data not shown). The separation of the amplified coding region into 2 fragments less than 500bp each greatly diminished the likelihood that we failed to detect point mutations under our assay conditions.\textsuperscript{11}
Steady state MCT-1 protein levels in a panel of transformed B-cell lines derived from patients with non-Hodgkin's lymphomas.

This striking association of growth factor independence with elevated MCT-1 protein levels in T-cell lymphoid tumor lines prompted us to examine the expression of MCT-1 in other lymphoid malignancies. We next examined a panel of lymphoma cell lines derived from patients with non-Hodgkin's lymphoma. The majority expressed high levels of MCT-1 protein when standardized to levels of β-actin protein (Fig. 2).

MCT-1 protein levels are increased in a subset of patients with DLBCL.
Based on the results obtained from non-Hodgkin's lymphoma cell lines we proceeded to analyze primary lymphoma samples. We examined lymph-node biopsy specimens obtained at initial diagnosis from patients with DLBCL. As shown in figure 3a, seven specimens displayed readily detectable MCT-1 protein. Positive findings were found in 7 of 17 NHL patients (41%) and in none of 10 control subjects (0 %) (Fig. 3b). This difference was statistically significant using Fisher's exact test (41% vs. 0%, p=.026). The four transformed lymphomas in our group all (4/4) demonstrated strong signals for MCT-1 protein. Elevated MCT-1 protein levels were also shown to correlate with increased proliferating cell nuclear antigen (PCNA) levels (Fig. 3a-b).
Figure 3

MCT-1 protein is not expressed in chronic lymphocytic leukemia:
In primary uncultured samples (n=10) of B-cell chronic lymphocytic leukemia (CLL) we found low to undetectable levels of MCT-1 protein in the peripheral lymphocytes from untreated patients (Fig. 4). These results are not unexpected as leukemic cells in this indolent lymphoid malignancy are primarily arrested in G0/G1\textsuperscript{12,13}. 

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Over-expression of MCT-1 promotes cell proliferation:

To examine the effect of constitutive over expression of MCT-1 on cell cycle progression of stably-transfected lymphoid cell lines we performed confocal microscopy on EC155-Vector(5) or EC155-MCT-1(7) cell lines. The EC155-MCT-1(7) cells showed a marked increase in the proportion of cells staining positive for PCNA, both by confocal analysis and Western blot (Figure 5).
We also observed a significant difference in the growth rate of MCT-1 over-expressing cells. The growth curve demonstrates the increased growth rate in three independent clones over-expressing MCT-1 compared with vector controls (p<0.001), figure 6a. MCT-1 over-expressing cells also demonstrate a higher percentage of cells in S phase compared with matched vector control cells as shown by the increased PCNA protein staining, figure 6b. These aggregate data support the role of MCT-1 in cell proliferation.
EC155-MCT-1 cells survive in serum starvation conditions.

To investigate a possible role of MCT-1 in cell survival, the effect of serum deprivation was investigated. Serum deprivation resulted in significantly increased death in the vector controls compared to the MCT-1 over expressing clones. The viability of EC155-MCT-1 after 72 hours serum starvation is significantly higher than that of EC155-vector control (p<0.01) as determined by trypan blue dye exclusion (figure 7).
MCT-1 protein protects against apoptosis:

To prove that cell death occurred due to apoptosis, annexin V binding assays were performed. Annexin V binds to phosphatidylserine, a phospholipid that normally is present at the inner leaflet of the cell membrane but during apoptosis is exposed at the outer leaflet. In the presence of serum, neither cell line significantly bound annexin V. However, after 48-72 hrs of serum depletion the EC155-Vector (5) cells heavily stained with annexin V-FITC, and EC155-MCT-1(7) cultures stained significantly less (Figure 8). The data presented is representative of the other clones as well. Another hallmark of early apoptosis is
mitochondrial membrane permeabilization (MMP). MMP is regulated by numerous effectors, including the proteins from the Bcl-2/Bax family. We have observed the increase in mitochondrial membrane permeabilization after serum starvation occurring more frequently in the EC155-Vector(5) cell line compared with the EC155-MCT-1(7) cell line (data not shown).

MCT-1 enhances AKT phosphorylation at residue of Serine 473

AKT plays a critical role in controlling the balance between cell survival and apoptosis. This protein kinase is activated by the growth/survival factors, and is involved in PI3 kinase signalling transduction. Akt is activated by PDK1-
dependent phosphorylation at Thr308 and Ser473 residues\textsuperscript{15,16}. The specific antibody against phospho-Ser473 on AKT was used to test EC155-Vector and EC155-MCT-1 cells (Fig. 9). Our data reveal that phosphorylation level of AKT on Ser473 residue is significantly increased in MCT-1 over-expressing clones. Thus, MCT-1 may enhance AKT kinase activation to promote cell survival by inhibiting apoptosis.

\begin{table}
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 & EC155-Vector & EC155-MCT-1(4) & EC155-MCT-1(5) & EC155-MCT-1(7) \\
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\caption{Figure 9}
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Discussion

The MCT-1 candidate oncogene product transforms fibroblasts and pushes them through the G1/S phase of the cell cycle\textsuperscript{3,17}. In the present study, we examined the expression status of the recently discovered candidate oncogene, MCT-1, in a diverse panel of primary lymphoid tumors and lymphoid cell lines. We also
investigated the role of MCT-1 on cell growth and in protecting lymphoid cells against apoptosis. Our results demonstrated that MCT-1 protein expression was significantly elevated in some aggressive lymphoma samples including diffuse large B-cell lymphoma (DLBCL) and IL-2 independent T-cell lymphoid tumors. By contrast, chronic lymphocytic leukemia (CLL), an indolent lymphoproliferative disorder \cite{12,13}, and the IL-2 dependent T-cell lines exhibited low to undetectable amounts of MCT-1 protein. We also observed a significant difference in the growth rate of MCT-1 over-expressing cells. Elevated MCT-1 protein levels were shown to strongly correlate with increased proliferating cell nuclear antigen (PCNA) levels. We showed that MCT-1 protein levels are associated with increased cell proliferation in human primary lymphoid tumors. Furthermore, we demonstrate that forced expression in lymphoid cell lines increased their proliferative rates using two independent assays, growth curve and PCNA staining. We also detected no evidence of point mutations in the coding region of MCT-1 in those tumors with elevated levels of MCT-1 protein. The finding that elevated MCT-1 protein expression is more frequent in transformed lymphomas is consistent with our hypothesis that this candidate oncogene provides a growth advantage to lymphoma cells and may be associated with a more aggressive clinical course. It is possible that a minor population of normal lymphocytes express MCT-1 protein below our detection limit and that the primary DLBCL shown here may represent the in vivo transformation of relatively rare MCT-1 expressing normal B-cells. Once MCT-1 antibodies that work on paraffin-embedded tissues are available, we will be able to more critically address this
issue and evaluate the relationship between MCT-1 protein levels and clinical parameters in a large study of DLBCL. The mechanism(s) of increased MCT-1 protein levels in a subset of human lymphoid tumors is currently unknown and is an area of active research in our laboratory.

The impact of decreased/increased MCT-1 protein levels on cell cycle progression and protection against apoptosis in lymphoid tumors may shed new light on the physiological relevance of MCT-1. Since, normally, cells that are deprived of essential growth factors will undergo apoptosis, inhibition of apoptosis is thought to be a very important requirement in the process of oncogenesis. In view of the biological relevance of apoptosis we investigated whether the increased protection against cell death by MCT-1 in vitro was due to an inhibition of apoptosis. Inhibition of apoptosis by MCT-1 would provide a physiological selective advantage in vivo for MCT-1 transformed cells. The trypan blue dye assay measures the number of surviving cells but does not distinguish between death by necrosis or apoptosis. We have shown by both annexin V staining and mitochondrial membrane permeabilization that increased MCT-1 protein expression in lymphoid cells results in protection against apoptosis induced by serum starvation. The best described anti-apoptotic pathway is the PI 3-kinase/Akt pathway. Generally, PKB/Akt activity is correlated with phosphorylation of Ser-473 and Thr-308. Under our experimental conditions, serum starved EC155-MCT-1 cells and EC155-Vector cells contain similar amounts of total PKB/Akt protein. However, we found that upon starvation the SER(P) 473 PKB levels in EC155-MCT-1 cells are increased as compared with
EC155-Vector cells. Thus, the PKB/Akt phosphorylation levels in these cells correspond well with the differential resistance that we observed against serum starvation induced apoptosis. MCT-1 may function as a positive up-stream regulator of AKT, or MCT-1 could modulate Akt activity by inhibiting a phosphatase activity, such as a tumor suppressor PTEN. The molecular mechanism(s) underlying the anti-apoptotic activity of MCT-1 and its putative involvement in PI3/AKT signaling pathway are currently under investigation. Our results suggest that inhibition of apoptosis may be an important mechanism of MCT-1.

The demonstration that MCT-1 possesses in vitro transforming and anti-apoptotic functions does not yet prove a primary role in lymphomagenesis, additional experiments demonstrating transformation in whole animal studies are ongoing and of critical importance in order to address this important issue. While the underlying mechanisms are still not yet clear, others have found that molecular determinants will predict for outcome more accurately than clinical parameters\textsuperscript{18,19} and that the identification of novel genes may result in models which can predict outcome. Indeed, investigators have shown that molecular profiling can segregate otherwise identical presentations of DLBCL into prognostic groups\textsuperscript{20-22}. Molecular profiling may be able to predict outcome and drive therapeutic decisions in these patients and may prove to be complementary to the clinical prognostic factors in use today. The identification of novel genes that encode proteins, which confer a growth advantage to lymphoma cells, will help to clarify molecular profiling data. Further, as the function of the MCT-1 gene
in regulating cell growth and apoptosis is further characterized, its putative role in lymphomagenesis will be elucidated. Ultimately, over expressed or modified proteins may serve as targets for protein specific therapy.
REFERENCES


FIGURE LEGENDS

Fig. 1: Steady state MCT-1 protein levels in exponentially growing T-lymphoid cell lines. 40 µg of total proteins from IL-2 dependent cell line PBL, ECL55, NII85 and N1186 and IL-2 independent cell line C10MJ, MT-2 and Jurkat were SDS-PAGE analysis and blotted on the membrane. Western blotting was performed with MCT-1 polyclonal antibody. Control immunoblotting was carried out with β-actin. All IL-2 independent T-cell lines exhibited elevated MCT-1 protein levels in contrast to the IL-2 dependent T-cell lines, including IL-2 stimulated PBL which has low to undetectable MCT-1 protein.

Fig. 2: Steady state MCT-1 protein levels in a panel of transformed B-cell lines derived from patients with non-Hodgkin's lymphomas. Western blotting was performed with MCT-1 polyclonal antibody on 40 µg total protein from seven of transformed B-cell lines. The results showed that the majority of the B-cell lines expressed high levels of MCT-1 protein and the MCT-1 levels are closely correlated with PCNA expressions in these cell lines. Jurkat and PBL cells served as positive and negative controls for MCT-1 signal, respectively.

Fig. 3: MCT-1 protein levels are increased in primary samples from a subset of patients. A. Western blotting was carried out to detect MCT-1 and PCNA proteins from grossly involved lymph nodes of newly diagnosed patients with
DLBCL. Seven out of thirteen specimens displayed elevated MCT-1 protein levels and that were also shown to correlate with increased proliferating cell nuclear antigen (PCNA) levels. The four transformed lymphomas in our group all (4/4) demonstrated strong signals for MCT-1 protein, samples 3, 8, 9 and 11. B. Ten of normal lymph nodes examined had no detectable MCT-1 and PCNA. The Jurkat cell line serves both as a positive control for MCT-1 and PCNA protein expression.

Fig. 4: MCT-1 protein is not expressed in chronic lymphocytic leukemia. Western blotting was performed on 40 μg of whole cell lysate with MCT-1 polyclonal antibody. All samples were isolated from peripheral blood of untreated patients (with >80% lymphocytes). Protein from Jurkat cells was served as MCT-1 positive control. Control immunoblotting with β-actin verified equal loading. Bcl-2 protein was universally expressed in the cases that we analyzed. The peripheral lymphocytes from untreated patients, ten of primary uncultured samples from B-cell chronic lymphocytic leukemia (CLL), demonstrated low to undetectable levels of MCT-1 protein.

Fig 5. Confocal immunofluorescence microscopy of EC155 cell lines. EC155-Vector(5) and EC155-MCT-1(7) cells were fixed with methanol and incubated with blocking solution and primary antibody, mouse monoclonal anti-V5 or anti-PCNA. Next, secondary anti-mouse IgG2a-Fluorescein was used for hybridization. The cells were spread on slides and mounted cover glasses with
mounting medium. The slides were examined by a Zeiss LSM510 confocal scanning laser microscope. The results showed that MCT-1 over-expressing cells had elevated PCNA protein level compared to the control.

Fig 6. MCT-1 over-expression increases the growth rate of lymphoid cells. A. EC155-Vector or EC155-MCT-1 cells were seeded at 0.5x10^6 cells/ml in triplicate T-25 flasks. Cells were counted every other day by using the trypan blue exclusion method for 8 days. The growth curve demonstrates the significantly increased growth rate in EC155-MCT-1 clones. Data are derived from three independent experiments with each clone. B. The EC155-MCT-1 cells showed a marked increase in PCNA level.

Fig. 7. MCT-1 promotes cell survival in serum starvation culturing condition. EC155-Vector or EC155-MCT-1 cells (2x10^5 cells /ml) were seeded in triplicate wells in 12 well plates with serum starved culture condition (0.2% FBS). Cell viability was analyzed by using the trypan blue exclusion method at certain time points. Data are presented as percentage of viable cells (means±SD) and are derived from three independent experiments with each clone. The viability of EC155-MCT-1 after 72 hours serum starvation is significantly higher than that of EC155-vector control.
Fig. 8 MCT-1 protein protects against apoptosis. Annexin V binding assay by flow cytometry method was performed to compare cell apoptosis induction in EC155-Vector(5) and EC155-MCT-1(7) cell lines. After 72 hours serum starvation, total annexin V positive cells of EC155-MCT-1(7) line significantly less than that of EC155-Vector(5) (20.1% verses 33.8%). Similar results were obtained with other MCT-1 over-expressing clones. The data suggest a protective function of MCT-1 against serum starvation induced apoptosis.

Fig. 9 MCT-1 over-expression enhances AKT phosphorylation at residue of Serine 473. Western analysis of extracts (150 µg) were from EC155-Vector and EC155-MCT-1 cell lines. The NC membranes were immuno-blotted by anti-V5 following by re-hybridization with anti-phospho-AKT (Ser473), total AKT, and actin antibodies after stripping overnight with 200 mM Glycine (pH2.5). The phosphorylation level of AKT on Ser473 residue is increased significantly in MCT-1 over-expressing cells compared with the vector control.
Expression of the candidate MCT-1 oncogene in B- and T-cell lymphoid malignancies

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