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INVOLVEMENT OF *KRAS* G12A MUTATION IN THE IL-2-INDEPENDENT GROWTH OF A HUMAN T-LGL LEUKEMIA CELL LINE, PLT-2

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ABSTRACT

Cytokine-dependent cell lines have been used to analyze the cytokine-induced cellular signaling and the mechanism of oncogenesis. In the current study, we analyzed MOTN-1 and PLT-2 cell lines established from different stages of a T-cell large granular lymphocyte leukemia patient (Daibata et al. 2004). MOTN-1 is IL-2-dependent derived from the chronic phase, whereas IL-2-independent PLT-2 is from the aggressive and terminal stage. They shared considerable chromosome abnormalities and the pattern of T-cell receptor rearrangement, presuming that the cytokine independence of PLT-2 was due to the additive genetic abnormality. Besides IL-2, IL-15 supported MOTN-1 cell growth, because these receptors share β- and γ-subunits. IL-2 activated ERK, AKT and STAT pathway of MOTN-1. STAT3 pathway of PLT-2 was also activated by IL-2, suggesting intact IL-2 induces signal transduction of PLT-2. However, ERK1/2 but not AKT, was continuously activated in PLT-2, consistent with the increased Ras-activity of PLT-2. Sequence analysis revealed *KRAS* G12A mutation but not *NRAS* and *HRAS* mutation of PLT-2 but not MOTN-1. Another signaling molecule affecting Ras-signaling pathway, SHP2, which has been frequently mutated in juvenile myelomonocytic leukemia (JMML), did not show mutation. Moreover, MEK inhibitor, PD98059, as well as farnesylation inhibitor inhibited PLT-2 cell growth. Using NIH3T3 and MOTN-1, ERK activation, increased cell proliferation and survival by *KRAS* G12A were shown, suggesting the important role of *KRAS* G12A in IL-2-independent growth of PLT-2. Taken together, *KRAS* G12A is important for IL-2-independent growth of PLT-2 cells and suggests the possibility of involvement of *KRAS* mutation with disease progression.

Key Words: Human LGL leukemia cell lines, MOTN-1 and PLT-2, IL-2 independent growth, Ras/MAP/ERK pathway, *KRAS* G12A mutation, Ras activity

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INTRODUCTION

Leukemogenesis is a complex process that transforms a normal hematopoietic stem cell or progenitor cell into the proliferative and non-differentiated state. Various alterations such as chromosome translocation, deletion and inversion have been reported as its causes. Gilliland[1] has proposed a multi-hit model according to which many genetic alterations play a role in the leukemogenesis process. Thus, it is important to understand the combination and/or the sequential appearance of genetic or epigenetic alterations during leukemogenesis.

Established cell lines have been extensively utilized in *in vitro* experiments to supplement the paucity of clinical samples for the analysis. Many features of primary malignant cells are recapitulated in derived cell lines and the genomic and transcriptomic aberration profiles are usually highly conserved.[2, 3] Most cell lines only require serum factors to survive and proliferate. However, some cell lines require special cytokines for their survival, proliferation and/or differentiation. These cytokine- dependent cell lines are ideal models to study cytokine signaling[4] as well as materials for the cytokine bioassay.

Stimulation with cytokines activates several intracellular post-receptor signaling pathways including Ras/MAP kinase pathway, JAK/STAT pathway, PI3K/AKT pathway and PKC pathway, depending on the target cell line and cytokine species. Cytokine-dependent cell lines are also useful for the study of apoptosis, because depletion of cytokine induces rapid cell death mostly due to apoptosis.

Special selection pressure (endogenous expression of some oncogene-related cytokine receptor or cytokine, itself) or introduction of oncogenes such as activated Ras and v-fps results in the establishment of factor-independent cell lines. The hypothesis of leukemogenesis has been proposed from the standpoint of cytokine dependence.[5] Although the second hit has been recognized, its importance in the process of oncogenesis and disease progression has not been fully disclosed.

Daibata *et al.* reported interesting cell lines, MOTN-1 and PLT-2,[6, 7] which are two human T-cell large granular lymphocyte (T-LGL) leukemia cell lines derived from the same patient at different disease stages. MOTN-1 is IL-2 dependent and was established at the indolent and chronic stage, whereas PLT-2 is IL-2 independent and was established from the aggressive and terminal stage. Karyotype analysis of the two cell lines showed that PLT-2 cells retain the basic abnormalities observed in MOTN-1 cells. Moreover, Southern blotting of TCR rearrangements demonstrated the identical origin of these cell lines.[6] Therefore, the comparison of these cell lines can elucidate the cause of disease progression and/or the cytokine-independent growth of PLT-2 cells. Microarray analysis revealed several quantitative changes of mRNA between them,[6] however, the IL-2 independence of PLT-2 cells still remains to be elucidated.

In the present study, we analyzed the signal transduction pathway of these two cell lines with or without IL-2 treatment, and found that the constitutively active ERK pathway is important for the survival of PLT-2. We detected *KRAS* mutation in parallel with the increased Ras activity of PLT-2 but not MOTN-1 cells. We further analyzed the effect of *KRAS* mutation using signaling inhibitors and mutated *KRAS* transfection into NIH3T3 and MOTN-1 cells. Our present study suggests the possibility that *KRAS* mutation occurred in the late stage of T-LGL leukemia and that this mutation is at least important for the survival and proliferation of PLT-2 cells without requiring IL-2.

MATERIALS AND METHODS

Cells and reagents

MOTN-1 and PLT-2 cells described before[6, 7] were established from a patient of LGL leukemia in different disease stages after obtaining the informed consent and the approval of the institutional board of Kochi University School of Medicine. MOTN-1 cells were cultured in 10% FCS and IL-2 (100 U/ml) in RPMI1640. IL-2 and IL-15 were purchased from Peprotech (Rocky Hill, NJ, USA). Cell signaling inhibitors: SB203580 was purchased from Wako (Osaka, Japan), JAK Inhibitor I was from Merck chemicals (Darmstadt, Germany), and LY294002, PD90859, and U0126 were from Cell Signaling Technology (Beverly, MA, USA) respectively. NIH3T3 cells and its transfectants were cultured in 5% FCS in DMEM medium.

Cell viability

Viable cell number was counted by the trypan blue dye exclusion method.

Western blotting

Western blotting was performed as described before.[8] Anti-Bcl-2 antibody (BD Transduction Laboratories, Franklin Lakes, NJ, USA), anti-Bcl-XL antibody (BD Transduction Laboratories), anti-p15 antibody (Cell Signaling), anti-p27 antibody (Cell Signaling), anti-cyclin D3 antibody (Cell Signaling), anti-cyclin dependent kinase (CDK) 4 and 6 antibodies (Cell Signaling), anti-STAT3 antibody (Sigma), anti-p-STAT3 antibody (Cell Signaling), anti-STAT5 antibody (Sigma), anti-p-STAT5 antibody (Sigma), anti-Akt antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti- p-Akt antibody (Santa Cruz), anti-ERK 1/2 antibody (Santa Cruz Biotechnology), anti-p-ERK 1/2 antibody (Santa Cruz), and β-actin antibody (Bio vision, Milpitas, CA, USA).

Ras activity assay

Ras activity was measured using an Active Ras Pull-Down and Detection kit (Thermo Scientific, Billerica, MA, USA) according to the manufacturer's instructions.

MAPK/ERK pathway activation

PathDetect Elk1 trans-reporting system (Agilent Technologies, La Jolla, CA, USA) was used to determine the activated state of the MAPK/ERK pathway according to the manufacturer's manual. This assay was based on the measurement of the luciferase activity stimulated by Elk1 stimulated via MAPK activation.

RAS and PTPN11 mutation study

N, *K* and *HRAS* and *PTPN11* mutations were analyzed by RT-PCR followed by direct sequencing. Reported hot spots of *RAS* (corresponding to the amino acid 12, 13 (exon 2) and 61 (exon 3) were surveyed. The whole coding sequence of *PTPN11* was analyzed. Primer sets used to detect mutation hot spots of *N*, *K*, and *HRAS* were as follows: *NRAS* exon 2 forward; 5'-TGATTACTGGTTTCCAACA-3', *NRAS* exon 2 reverse; 5'-TCCTCTATGGTGGGATCATATTC-3', *NRAS* exon 3 forward; 5'-ATAAAAATTGAACTTCCCTCCCTCC-3', *NRAS* exon 3 reverse; 5'-ATTGATGGCAAATACACAGAGGAAG-3', *KRAS* exon 2 forward; 5'-ACCTTATGTGTGACATGTTCTA-3', *KRAS* exon 2 reverse; 5'-ATTGTTGGATCATATTCGTC-3', *KRAS* exon 3 forward; 5'-AAGTAAAAGGTGCACTGTAA-3', *KRAS* exon 3 reverse; 5'-TTTATGGCAAATACACAAA-3', *HRAS* exon 2 forward; 5'-GGAGACCCTGTAGGAGGACCC-3', *HRAS* exon 2 reverse; 5'-GGTTCTGGATCAGCTGGATGG-3', *HRAS* exon 3 forward; 5'-GGAAGCAGGTGGTCATTGATGGGGAGACGTGCC-3', *HRAS* exon 3 reverse;

5'-TCCCTGTACTGGTGGATGTCCTCAAAAGACTTGGTGTTGTTGAT-3' RT-PCR primer set and four sequence primer sets used to detect mutations of *PTPN11* were as follows: *PTPN11* RT-PCR forward; 5'-CCAGCCCGATGTGACCGAGCC-3', *PTPN11* reverse; 5'-AGGAAATGAG-TAATTGGGAAGCAGGTATTGTTGA-3'

PTPN11 Sequence primers: *PTPN11* seq 1; 5'-TTTCATGGACATCTCTCTGG-3', *PTPN11* seq 2; 5'-AATTAGCTGAGACCACAGAT-3', *PTPN11* seq 3; 5'-GGTACTGTGCTTCTGTCTGG-3', *PTPN11* seq 4; 5'-AGTGCAACAATTCAAAGCCC-3'

Transfection of KRAS cDNA into NIH3T3 cells

KRAS expression vectors: Human *KRAS* wild-type and *KRAS* G12V were from Prof. K. Kaibuchi (Nagoya University Graduate School of Medicine). Inserts were cut from their original vectors and re-inserted into pcDNA3.1. Their DNA sequences were confirmed. *KRAS* G12A was prepared from pcDNA3.1 *KRAS* G12V using mutated primer sets as follows. Forward I; CCCACTGCT-TACTGGCTTATCGAAATTAATACGACT, Reverse I; CTTGCCTACGCCAGCAGCTCCAACTA, Forward II; GTAGTTGGAGCTGCTGGCGTAGGCAAGAGT, Reverse II; TTTCTCGAACTA-ATGTATAGAAGGCATCATCAACAC, Forward III; CCCACTGCTTACTGGCTTATCGAAAT-TAATACGACT, Reverse III; TTTCTCGAACTAATGTATAGAAGGCATCATCAACAC. Underline denotes mutated base. After obtaining PCR products using primer sets I and II, overlapping PCR was performed using the primer set III to obtain *KRAS* G12A fragment. After DNA sequence was confirmed, it was digested with *Bam*HI and *Xho*I, and ligated with pcDNA3.1-*KRAS* G12V vector pre-treated with the same enzymes.

Transfection and colony formation of stably transfected-NIH3T3 cells: NIH3T3 cells were transfected with pcDNA 3.1 expression vectors containing wild-type *KRAS*, *KRAS* G12A and *KRAS* G12V, respectively. After G418 selection, suitable subclones were selected by determining respective phosphorylated ERK level (representative data shown in Fig. 4). In order to directly demonstrate the growth advantage of NIH3T3 cells transfected with mutated *KRAS*, we performed the colony formation assay. Though preliminary experiments, the suitable initial cell density for plating and the culture period were determined. Briefly, stably transfected-NIH3T3 cells (*KRAS* wild-type (WT), *KRAS* G12A (G12A) and *KRAS* G12V (G12V)) (1.5×10^3/plate) were placed in a tissue culture plate (35 mm diameter) in triplicate. After 10 days, tissue culture plates were stained with May-Giemsa staining dye. Colony number was counted under the inverted microscope.

Retrovirus preparation of KRAS expression vectors and MOTN-1 transfection

Original MSCV-GFP vector was obtained from Dr. A. Katsumi (National Center for Geriatrics and Gerontology, Research Institute, Aichi, Japan). *KRAS* wild type, *KRAS* G12V, and *KRAS* G12A were inserted into the suitable multi-cloning site of this vector. Each sequence was confirmed by direct sequencing. After obtaining culture supernatant containing retroviruses, MOTN-1 cells were transfected with culture supernatant containing MSCV-GFP-*KRAS* (wild, G12A, and G12V) or MSCV-GFP (as the mock vector). Cells were sorted by FACS according to their GFP fluorescent intensity. Strongly positive cells were collected and used for further analysis.

RESULTS

Difference of cytokine dependence between MOTN-1 and PLT-2 cells

It has recently been reported that IL-15, IL-2 and PDGF are major factors that stimulate large

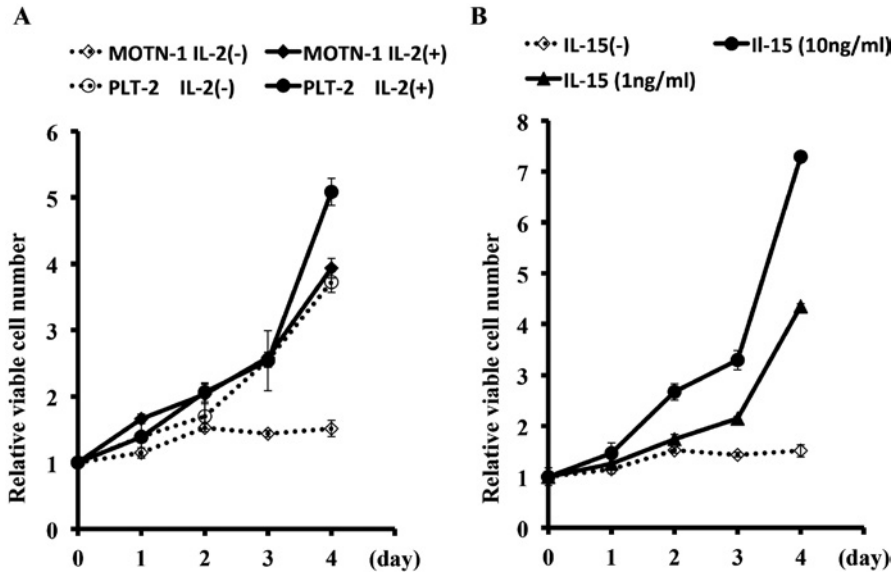

Fig. 1 Effects of IL-2 and IL-15 on cell proliferation of MOTN-1 and PLT-2 cells.
(A) MOTN-1 and PLT-2 cells were cultured with or without IL-2 (100 U/ml).
(B) IL-15 (1 or 10 ng/ml) was used to culture MOTN-1 cells. Viable cells were counted by trypan blue dye exclusion.

granular leukemia cells.[9] IL-2 and IL-15 receptor are composed of αβ and γ subunits, and IL-15 and IL-2 receptors share β and γ chains. Consistently, MOTN-1 proliferated with either IL-2 or IL-15 but stopped proliferation by depleting IL-2 or IL-15. However, PLT-2 did not require either of these cytokines for proliferation (Fig. 1). Long-term culture of MOTN-1 without IL-2 showed gradual cell death mostly due to apoptosis (data not shown).

Status of signaling pathway and Ras analysis

Examination of apoptosis- and cell cycle-related proteins revealed that p15 and p27 were increased after depletion of IL-2 in MOTN-1, whereas cyclin D3, CDK6 did not show significant changes by IL-2 depletion (Fig. 2A). Unexpectedly, Bcl-xL, Bcl-2, Bax and Bad did not show significant changes by IL-2 depletion in both cell lines (data not shown). It might be due to the fact that MOTN-1 cells could survive and did not show cell death at least during our observation period. Most cytokines in hematopoiesis are directly connected with the JAK/STAT signaling pathway. In MOTN-1, activated STAT3 and STAT5 were observed in IL-2 (+) cells (Fig. 2B left). STAT activation was also observed in IL-2 (+) but not in IL-2 (-) PLT-2, suggesting that JAK2/STAT pathway was not involved in its IL-2 independent growth.

However, activated ERK pathway was observed in PLT-2 cells as compared with that of MOTN-1 (Fig. 2B right). In these cell lines, activation of ERK2 is remarkable compared with ERK1. The similar phenomenon was reported in NK-LGL patients.[10] AKT signaling did not show significant change between MOTN-1 and PLT-2 regardless of cytokine treatment. Increased Ras activity of PLT-2 parallels ERK activation compared with MOTN-1 (Fig. 3A). Direct sequence of *N*, *K* and *HRAS* revealed *KRAS* G12A mutation in PLT-2 but not in MOTN-1, while other RAS genes exhibit no point mutation (Fig. 3B and data not shown). Our preliminary analysis using allele-specific PCR showed that this mutation was only present in a population of the original

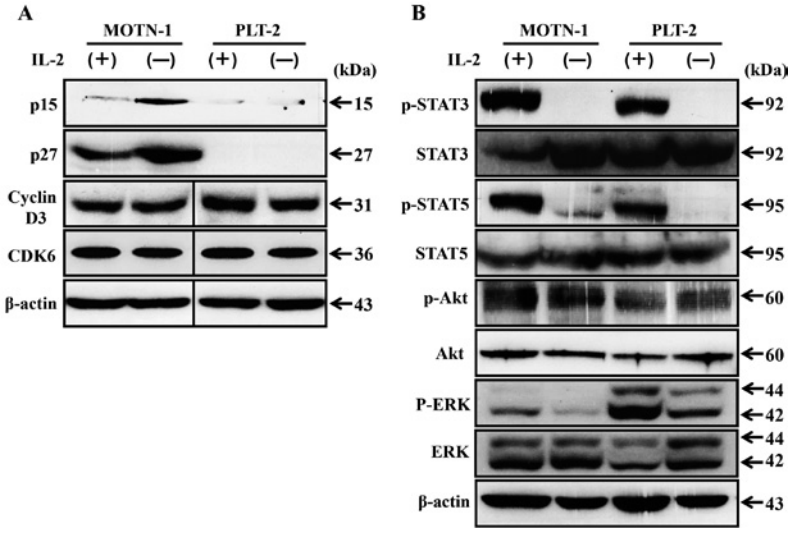

Fig. 2 Effect of IL-2 on cell cycle- and apoptosis-related proteins and cellular signaling pathways. (A) MOTN-1 and PLT-2 were cultured with or without IL-2 (50 U/ml) for 24 h. Cell cycle-related proteins were examined as described in the Materials and Methods. β-actin was shown as the internal control. (B) In similar ways shown in (A), the cellular signaling pathways were analyzed as described in the Materials and Methods.

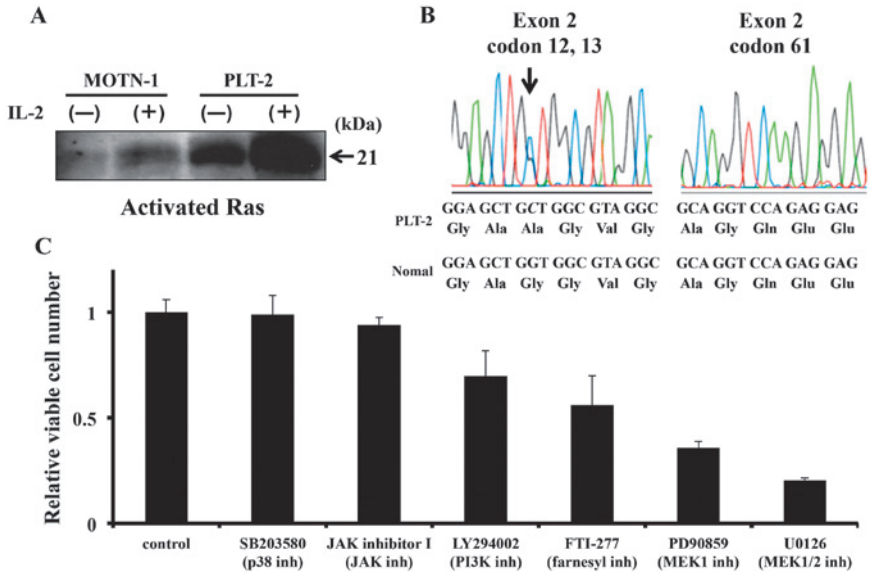

Fig. 3 Ras activity, *RAS* mutation analysis, and effect of signaling inhibitors on PLT-2 cell growth. (A) MOTN-1 and PLT-2 were cultured with or without IL-2 (50 U/ml) for 24 h. Ras activity was examined as described in the Materials and Methods. (B) Mutation analysis of *KRAS*. Mutation of *HRAS*, *KRAS*, and *NRAS* was examined according to the Materials and Methods. Mutated G12A of PLT-2 was shown in italic. (C) Viable cell number of PLT-2 cells treated with or without signaling inhibitors on day 4 was demonstrated. Initial cell concentration of the control group was determined as 1.0. Concentrations of reagent used are: SB203580, 5 μM; JAK inhibitor I, 330 nM; LY294002, 10 μM; FTI277, 2 μM; PD98059, 100 μM; U0126, 20 μM.

leukemia cell DNA in the aggressive phase (data not shown).

In Noonan syndrome and JMML, mutations of *PTPN11* that encode SHP2, an SH-2 domain containing tyrosine phosphatase, have been reported, and *PTPN11* mutation leads to the enhanced Ras/MEK signaling.[11] However, *PTPN11* mutation was not detected in PLT-2 (data not shown). Moreover, Fig. 3C illustrated that MEK inhibitor, PD98059 and U0126, and farnesylation inhibitor FTI277, inhibited cell growth of PLT-2 cells. PI3K inhibitor, LY294002, but not p38 inhibitor, SB203580 and JAK inhibitor I, showed some inhibition. These results demonstrate the importance of Ras/MAPK signaling pathway of PLT-2 cell proliferation. We could not succeed in experiments using siRNA for *KRAS* due to the low transfection efficiency of PLT-2 cells.

Overexpression of KRAS G12A to NIH3T3 cells and MOTN-1 cells

In order to examine the role of *KRAS* G12A found in PLT-2, we transfected various *KRAS* expression vectors into NIH3T3 and MOTN-1 cells. The effect of *KRAS* G12V, a *KRAS* common mutation observed in various cancer cells, was also analyzed. High ERK activation was observed in *KRAS* G12A and *KRAS* G12V transfectants of NIH3T3 as compared with mock- and wild-type *KRAS* expressed cells, respectively (Fig. 4A). Enhanced Ras activity of *KRAS* G12A and G12V leading to activation of ERK pathway was also demonstrated using Elk-1 trans-reporting system (Fig. 4B). Fig. 4C showed colony formation of respective *KRAS* transfected NIH3T3

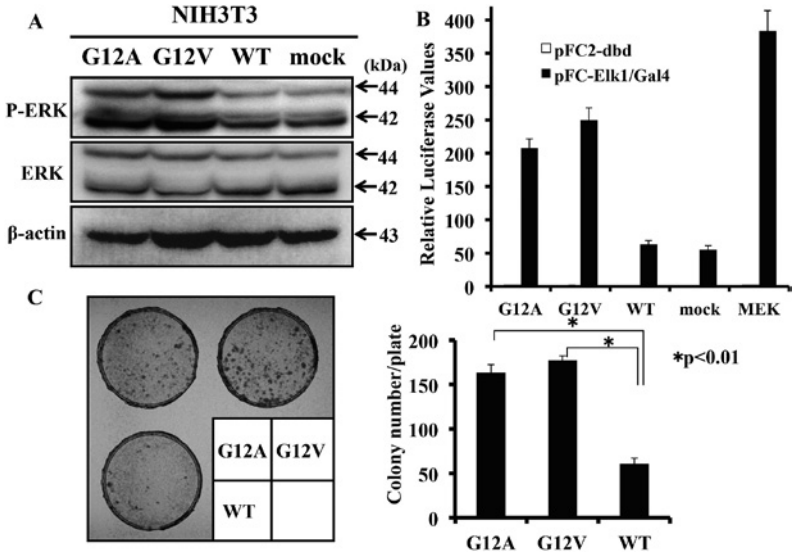

Fig. 4 Effects of overexpression of wild- or mutated-*KRAS* in NIH3T3 cells.
(A) NIH3T3 cells were transfected with mock-, *KRAS*-wild (WT), *KRAS* G12A or *KRAS* G12V, respectively according to the Materials and Methods. After G418 selection, ERK activation of established cells was determined by p-ERK protein level. β-actin was shown as the internal control. (B) Activated ERK was also demonstrated using PathDetect Elk1 Trans-Reporting System as described in the Materials and Methods. ERK activation was shown as the relative luciferase activity. MEK denotes the positive sample provided from the manufacturer. pFC2-dbd (negative control vector)-transfected Mock-NIH3T3 was regarded as the relative ratio of 1.0. The means +/- SD was calculated from the triplicate cultures. (C) Colony formation of *KRAS*-transfected cells. Colony formation assay of *KRAS*-transfected NIH3T3 cells was performed according to the Materials and Methods. Left part shows May-Giemsa staining of colonies, and the right illustrates the mean +/- SD of colony number/1.5×10^3 cells calculated from triplicate culture.

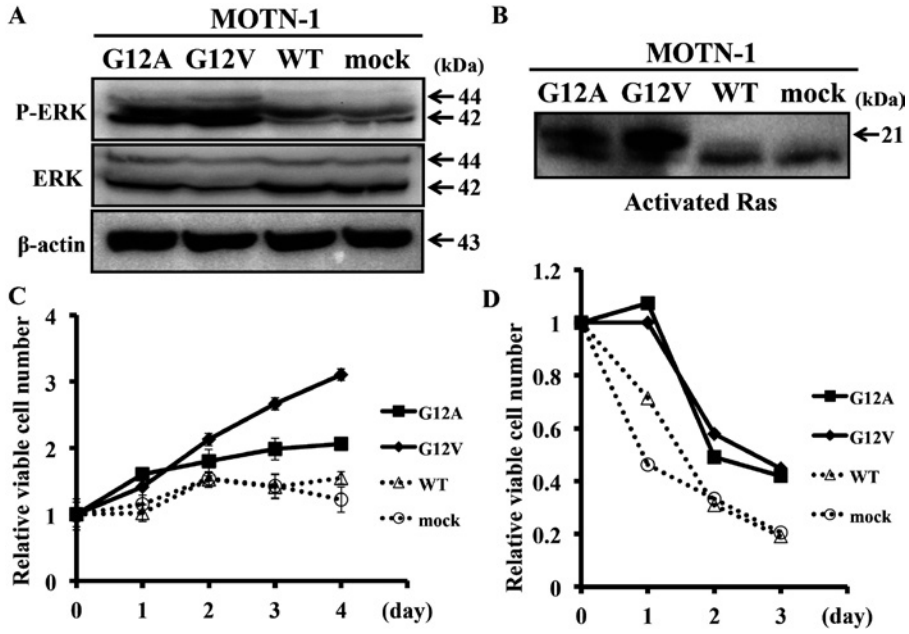

Fig. 5 Effect of overexpressed wild or mutated *KRAS* in MOTN-1. (A) MOTN-1 was transfected with retrovirus vectors containing mock-, *KRAS*-wild (WT), *KRAS* G12A or *KRAS* G12V according to the Materials and Methods. After selecting highly GFP-expressing cells with FACS (data not shown), their ERK activation status was analyzed. β-actin was shown as the internal control. (B) Various *KRAS*-transfected cells were cultured without IL-2 for 24 h. Ras activity of respective cells was examined as described in the Materials and Methods. (C) Stably transfected cells were cultured in the medium containing 2.5% FCS but not IL-2. The initial cell concentration on day 0 was regarded as the relative ratio of 1.0. The means +/- was illustrated from triplicate cultures. (D) Viable cell number was counted after both serum and IL-2 depletion. The initial cell concentration on day 0 was regarded as the relative ratio of 1.0.

cells, suggesting the significantly enhanced colony formation of *KRAS* G12V and *KRAS* G12A but not of wild-type *KRAS* NIH3T3 cells.

ERK activation by mutated *KRAS* was also observed in *KRAS* retrovirus-transfected MOTN-1 (Fig. 5A). High Ras activity was observed in mutated *KRAS*-transfectants (Fig. 5B). *KRAS* G12V and *KRAS* G12A exhibited higher proliferation in the absence of IL-2 (Fig. 5C) and resistance against both serum and IL-2 depletion during our observation period (Fig. 5D).

DISCUSSION

Large granular lymphocyte leukemia features a clonal expansion of antigen-primed, competent, cytotoxic T lymphocytes. IL-15, IL-2 and PDGF are included in the recent network model of survival signaling of this leukemia.[9] IL-2 and IL-15 share their β and γ chains, and IL-2 and IL-15 β receptor subunits are co-expressed in T cells.[12] Our results (Fig. 1) indicate that the proliferation signals of MOTN-1 utilized these common chains.

Comparison between MOTN-1 and PLT-2 cultured with or without IL-2 revealed increased levels of cell cycle regulators, p27 and p15, in MOTN-1 by IL-2 depletion, which is consistent with the growth arrest of MOTN-1. However, we could not detect the change of cyclins, CDK,

and apoptosis-related proteins of MOTN-1 under our experimental conditions. Apoptosis-related proteins such as Bcl-2, Bcl-xL, Bax and Bad also showed no significant changes after IL-2 depletion. These data might be related with the fact that IL-2-depleted MOTN-1 cells were relatively resistant to apoptosis during the short-term observation period.

Considering a similar TCR rearrangement pattern between MOTN-1 and PLT-2 and shared chromosome abnormalities,[6, 7] PLT-2 was thought to be derived from MOTN-1 or at least maintain the basic cellular characteristics. Our analysis revealed that IL-2-independent growth of PLT-2 was at least partially due to the constitutive activation of the MAPK/ERK pathway but not the activated AKT and STAT pathway (Fig. 2). MEK inhibitor or farnesylation inhibitor inhibited cell growth of PLT-2 cells (Fig. 3). The coupling of ERK and activated Ras has been reported previously in lymphoproliferative disease of granular lymphocyte.[13]

Enhanced Ras activity of PLT-2 (Fig. 3) was thought to be due to *KRAS* G12A mutation, because no *HRAS*, *NRAS* or *PTPN11* mutation was observed. The possibility of autocrine production of IL-2 or IL-15 was less likely, because culture medium of PLT-2 did not sustain cell proliferation of MOTN-1 (data not shown). Ras pathway is important for survival, proliferation, senescence and differentiation. Ras activation causes Raf recruitment and activation by phosphorylation, followed by activation of the MEK and ERK sequentially. Mutation of Ras family (HRas, NRas and KRas) induced abnormal cellular signaling, deregulation of gene expression and oncogenensis.[14] Oncogenic *KRAS* mutation initiates leukemia in hematopoietic stem cells.[15]

Among three *KRAS* mutation hot spots, *KRAS* codon 12 mutation induces higher resistance to apoptosis and predisposition to anchorage-independent growth than codon 13 mutation.[16] *KRAS* G12A is relatively rare in clinical cancer samples and cell lines,[17] and the transformation activity of *KRAS* G12A remains to be disclosed.[18] Our results showed increased Ras activity and enhanced ERK signaling by *KRAS* G12A was clearly shown using transfected NIH3T3 cells and MOTN-1 cells (Figs. 4 and 5). The analysis of relapsed childhood acute lymphoblastic leukemia revealed that *KRAS* mutation was observed only in relapsed patients.[19] Such patients with *KRAS* mutation profit from MEK inhibitors.[20] *RAS* mutation analysis of CMML patients suggests that *RAS* mutation is a secondary event that contributes to the expansion of a malignant clone with a proliferative advantage.[21] PLT-2 is assumed to be from patient leukemia cells in the aggressive phase, which are transformed from cells in the chronic phase. Our preliminary results using allele-specific PCR showed that this mutation was present in the patient blast of the aggressive phase but did not occur during the *in vitro* culture process. Thus, the *KRAS* mutation of this patient was a late event during his LGL leukemia progression.

The frequency of *KRAS* mutation in LGL leukemia remained to be determined previously. The mechanism of the cytokine-independent growth of LGL clinical samples has not been disclosed fully. Thus, the clinical significance of our observation is not clear enough at the moment and the future analysis of LGL leukemia samples is warranted. Recently, the comprehensive dynamical and structural analysis of a network model of this disease has revealed several key components important for the leukemogenesis and the value of importance of Ras as the network component in T-LGL leukemia has been shown.[9, 22]

The present study clearly showed that *KRAS* G12V as well G12A induced ERK1 and ERK2 activation and anchorage-independent growth in NIH3T3 cells and the growth advantage of mutated *KRAS*-transfected MOTN-1 cells in IL-2 depleted medium. However, *KRAS* G12A mutation has been reported in IL-2-dependent KHYG-1 cell lines.[23] Ras-overexpressed factor-dependent FDC-P1 cells exhibit resistance to apoptotic cell death by cytokine depletion,[24] and *RAS* mutation induces increased anti-apoptosis in some models.[25] Thus, *RAS* mutation is necessary but not always sufficient for cytokine-independent growth. Other factors such as c-myc cannot be ruled out to confer IL-2 independent growth to PLT-2 cells.

Taken together, we found that *KRAS* G12A mutation is important for IL-2- independent proliferation, which occurred during disease progression of the patient from whom these two cell lines were established.

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CONFLICT OF INTEREST

We have no conflict of interest.

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