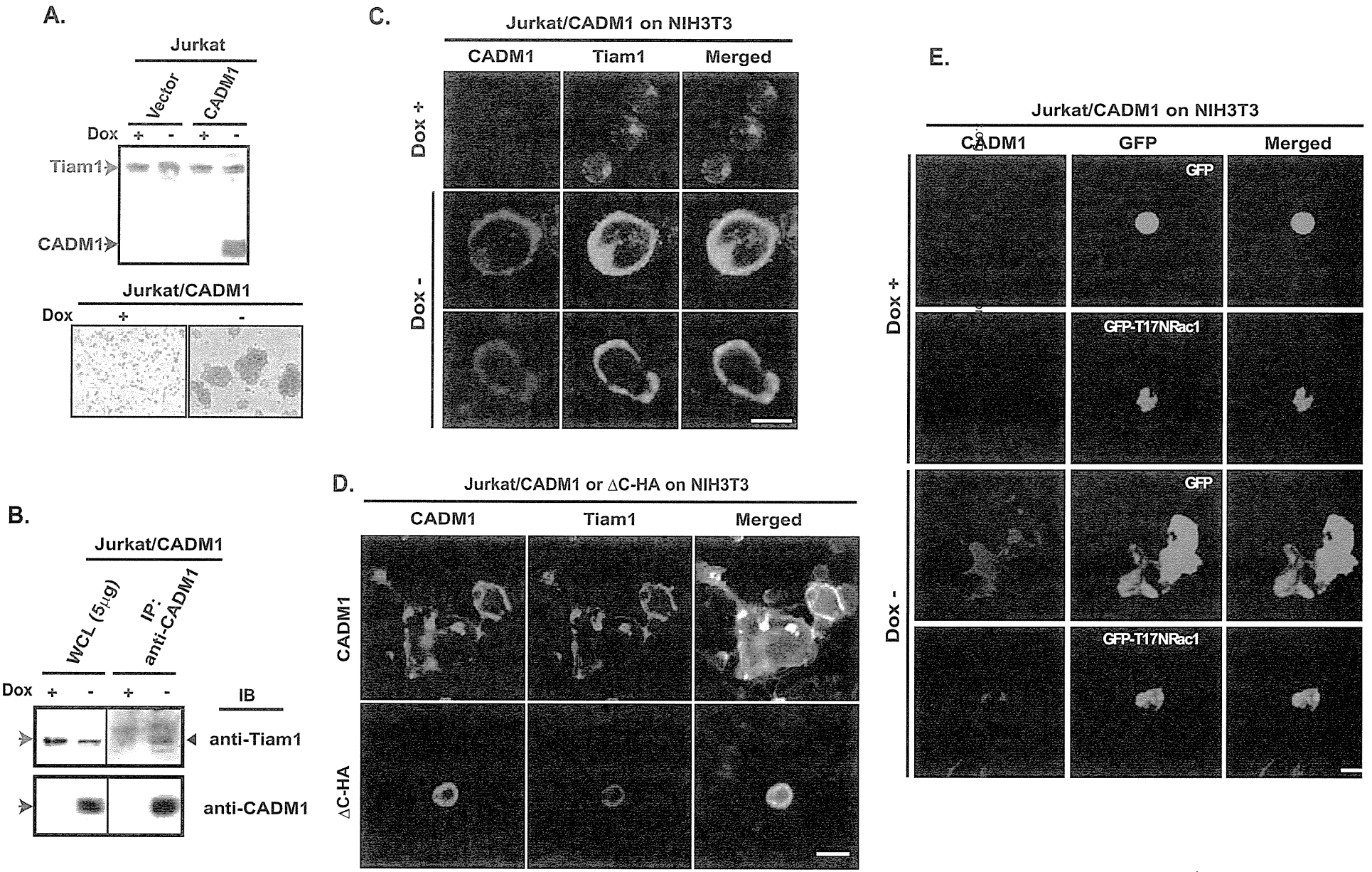
A.
Jurkat
Vector
CADM1
Dox + - + -
Tiam1
CADM1
Jurkat/CADM1
Dox + -
B.
Jurkat/CADM1
WCL (5μg)
IP: anti-CADM1
Dox + - + -
IB
anti-Tiam1
anti-CADM1
C.
Jurkat/CADM1 on NIH3T3
CADM1
Tiam1
Merged
Dox +
Dox -
D.
Jurkat/CADM1 or ΔC-HA on NIH3T3
CADM1
Tiam1
Merged
CADM1
ΔC-HA
E.
Jurkat/CADM1 on NIH3T3
CADM1
GFP
Merged
Dox +
Dox -
GFP
GFP-T17NRac1
GFP
GFP-T17NRac1

Figure 3.

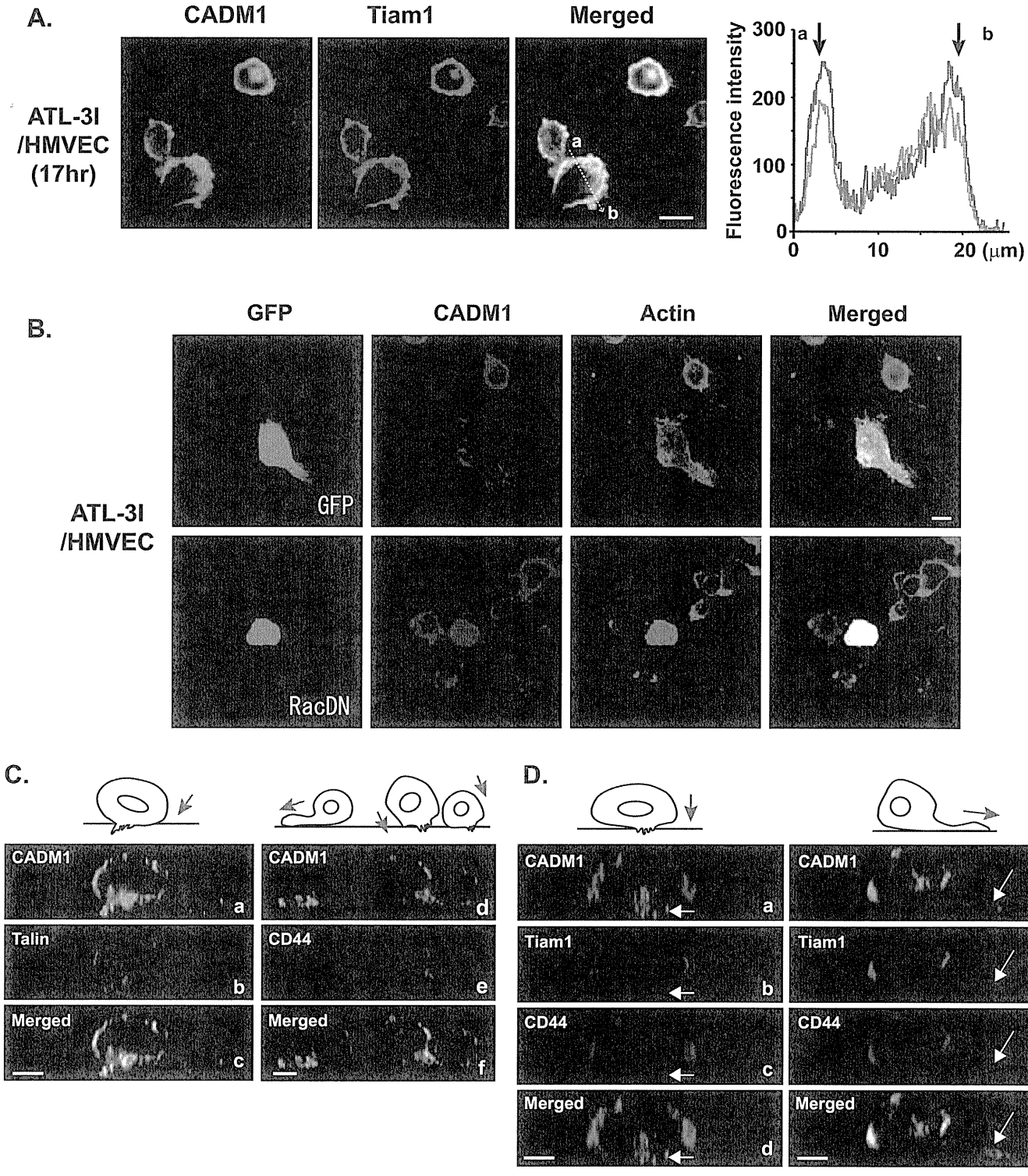
A.
CADM1
Tiam1
Merged
ATL-3I /HMVEC (17hr)
a
b
Fluorescence intensity
300
200
100
0
0
10
20 (μm)
B.
GFP
CADM1
Actin
Merged
ATL-3I /HMVEC
GFP
RacDN
C.
D.
CADM1
Talin
Merged
CD44
Tiam1
a
b
c
d
e
f
g
h

Figure 4.

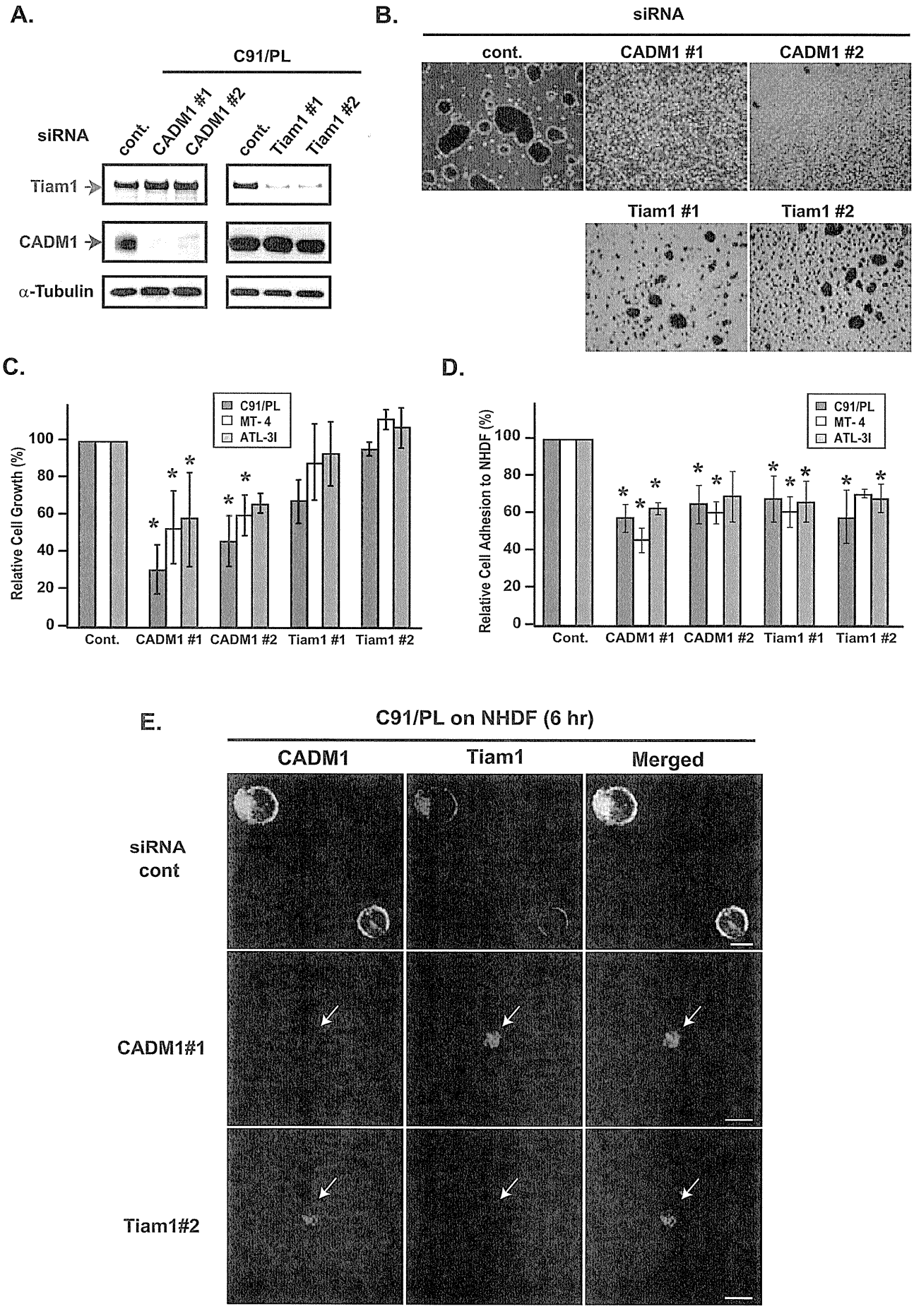
A.
C91/PL
siRNA
cont.
CADM1 #1
CADM1 #2
cont.
Tiam1 #1
Tiam1 #2
Tiam1
CADM1
α-Tubulin
B.
siRNA
cont.
CADM1 #1
CADM1 #2
Tiam1 #1
Tiam1 #2
C.
C91/PL
MT-4
ATL-3I
Relative Cell Growth (%)
100
80
60
40
20
0
Cont.
CADM1 #1
CADM1 #2
Tiam1 #1
Tiam1 #2
D.
C91/PL
MT-4
ATL-3I
Relative Cell Adhesion to NHDF (%)
100
80
60
40
20
0
Cont.
CADM1 #1
CADM1 #2
Tiam1 #1
Tiam1 #2
E.
C91/PL on NHDF (6 hr)
CADM1
Tiam1
Merged
siRNA cont
CADM1#1
Tiam1#2

Figure 5.

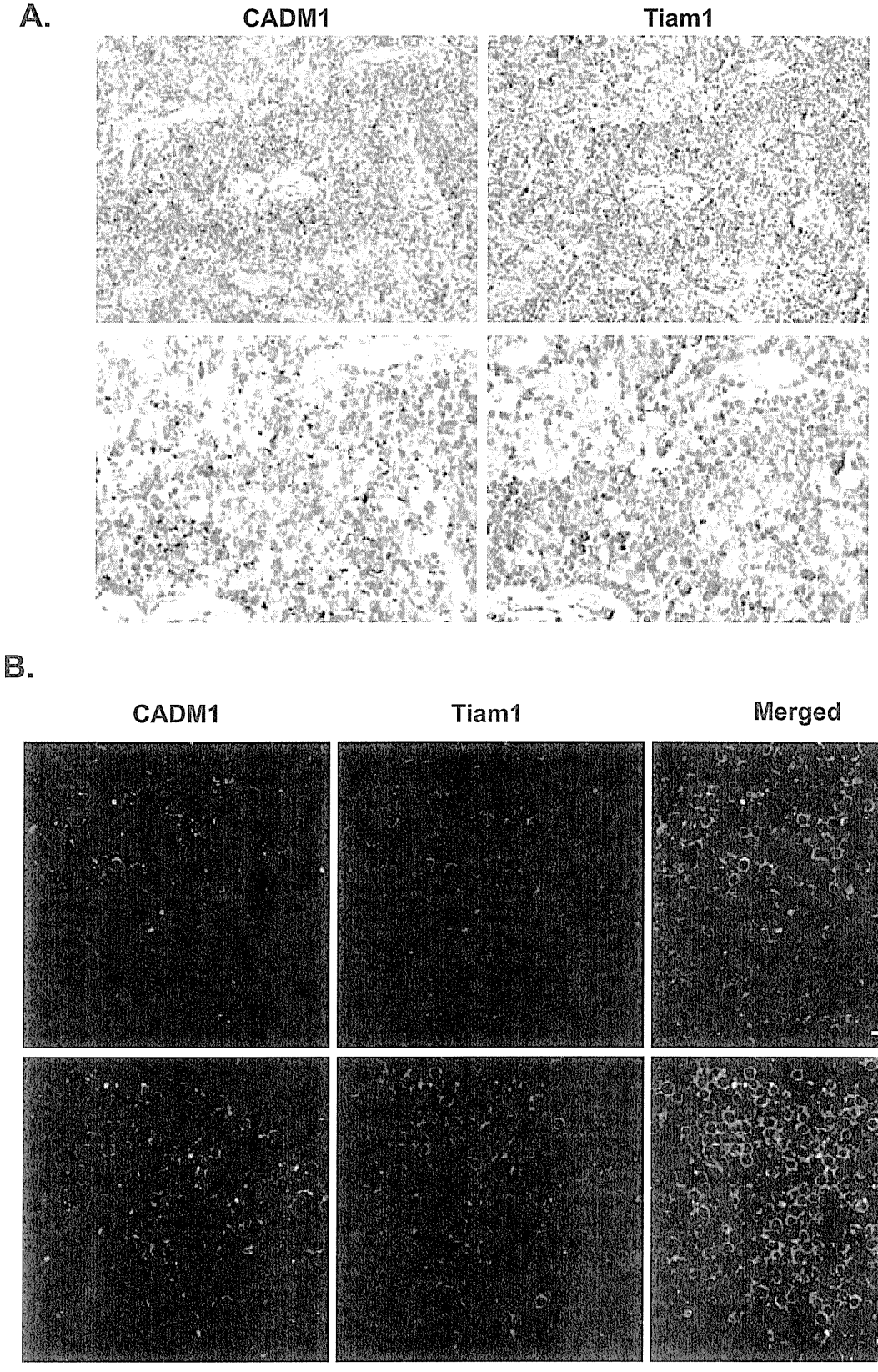

Figure 6.

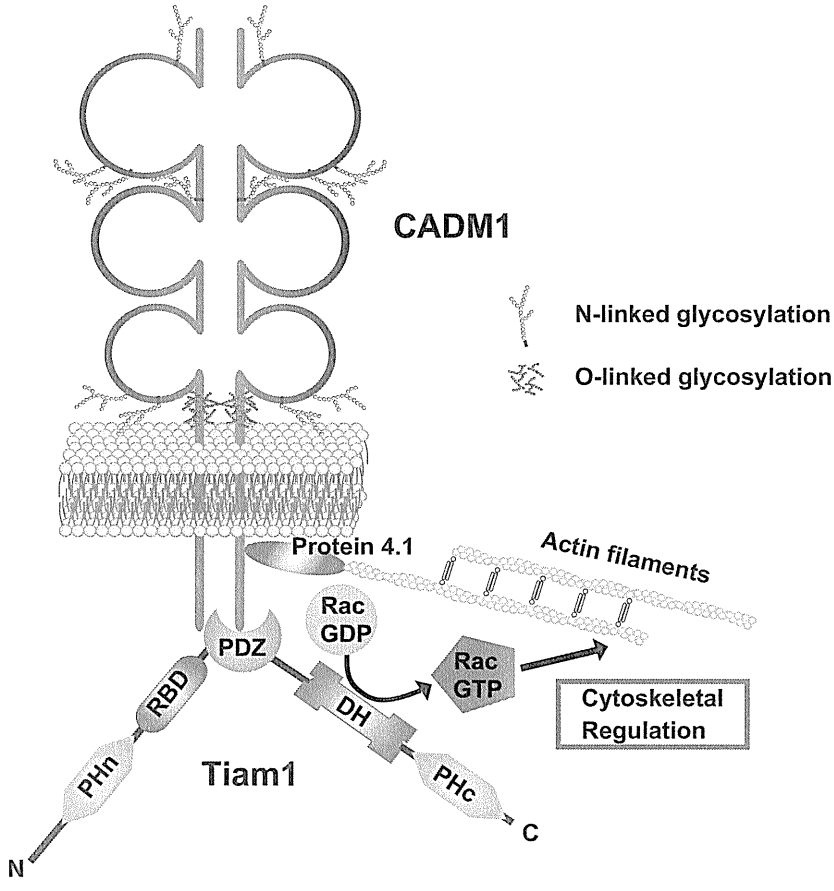
CADM1
N-linked glycosylation
O-linked glycosylation
Protein 4.1
Actin filaments
Rac
GDP
PDZ
RBD
DH
Rac
GTP
Cytoskeletal
Regulation
PHn
Tiam1
PHc
N
C

Figure 7.

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AP-1-Dependent miR-21 Expression Contributes to Chemoresistance in Cancer Stem Cell-Like SP Cells

Aya Misawa,*‡ Ryohei Katayama,* Sumie Koike,*
Akihiro Tomida,† Toshiki Watanabe,‡ and Naoya Fujita*

*Division of Experimental Chemotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan
†Division of Genome Research, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan
‡Laboratory of Tumor Cell Biology, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Tokyo, Japan

The side population (SP) of cancer cells is a minor population of cells that has been identified in a variety of cancers and harbors many cancer stem cell (CSC)-like properties, such as self-renewal potential, tumor-forming capacity, and chemoresistant phenotype. CSCs are regarded as the root of cancer origin and recurrence. Thus, new therapeutic approaches targeting these malignant cells have become the topic of ongoing research. However, the chemoresistant phenotype of CSCs makes it difficult to increase their sensitivity to anticancer drugs and to decrease the rate of cancer recurrence in patients. In this study, we analyzed the chemoresistant phenotype of SP cells derived from various cancer cell lines. Microarray analysis discriminated differential gene expression profiles between SP and non-SP cells. MicroRNA-21 (miR-21) and its upstream regulator activator protein-1 (AP-1), composed of c-Jun and c-Fos family transcription factors, were found to be frequently upregulated in SP cells. Downregulation of tumor suppressor programmed cell death 4, one of the miR-21 target gene products, confirmed miR-21 overexpression in SP cells. Treatment of the cells with the AP-1 inhibitor SP600125 attenuated miR-21 levels and increased topotecan sensitivity. Furthermore, specific inhibition of miR-21 by an anti-miR-21 locked nucleic acid increased drug sensitivity and decreased colony forming ability. These findings define the critical role of miR-21 in maintenance of the chemoresistant phenotype of SP cells. Targeting miR-21 may provide a new strategy for cancer therapy by impairing resistance to chemotherapy in CSCs.

Key words: MicroRNA (miRNA); Side population (SP); Cancer stem cell (CSC); Chemoresistance

INTRODUCTION

Cancer stem cells (CSCs) or tumor-initiating cells are tumorigenic cells that constitute a minor population of tumor cells (1). According to the cancer stem cell hypothesis, these cells have unlimited self-renewal potential, ability to initiate tumors in xenograft transplants, and chemotherapy resistance (2,3). CSCs are regarded as the root of cancer origin and recurrence. Therefore, it is important to elucidate the characteristics of CSCs to develop therapeutic strategies targeting CSCs.

Side population (SP) cells, which have many CSC-like properties, are isolated using purification protocols based on their capacity to exclude the fluorescent dye Hoechst 33342 through an ATP-binding cassette (ABC) membrane transporter ABCG2 (4,5). SP cell analysis and sorting were initially used for isolation of hematopoietic stem cells from the bone marrow (4,6); however, today they are widely used for the enrichment of normal stem cells from various tissues and organs (5,7,8). Recent in vitro clonogenicity and in vivo tumorigenicity studies indicate that SP cells isolated from diverse cancer cell lines harbor CSC-like properties such as tumorigenicity and stemness (9–12). Most researchers consider SP cells to be an enriched as well as an alternative source of stem cells that are particularly useful in situations where stem cell molecular markers are unknown.

Recent studies have clarified that microRNAs (miRNAs) are a class of endogenous, small non-protein-coding RNA molecules composed of 21–25 nucleotides that are generated from larger RNA precursors. One strand of mature double-stranded miRNA downregulates target mRNAs by inhibiting their translation or through their degradation. Many studies have indicated that miRNAs participate in a wide range of biological functions, such as cellular proliferation, differentiation, cell cycle regulation, and apoptosis (13,14). Aberrant miRNA expression is strongly implicated in various human dis-

Address correspondence to Naoya Fujita, Division of Experimental Chemotherapy, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan. Tel: +81-3-3570-0468; Fax: +81-3-3570-0484; E-mail: naoya.fujita@jfcr.or.jp

eases, including cancer (13,14). Computational prediction of miRNA targets suggests that up to 30% of human protein-coding genes may be regulated by miRNAs, which makes them one of the most abundant class of posttranscriptional regulators of gene expression. Many studies have been conducted to describe important roles played by miRNAs in cancer (15–18) and in stem cells (19); however, there are very few original studies regarding miRNAs in CSCs (20). Therefore, further investigation of miRNA expression profiles and functional studies of specific miRNAs within CSCs will be crucial for the development of novel therapeutic methods to target CSCs and eradicate cancer.

miR-21 is a tumor-associated miRNA for which aberrant expression was first identified in glioblastomas (21–26). It is overexpressed in a variety of cancers, including breast, colon, lung, pancreas, prostate, stomach, hepatocellular, gastric, ovarian, cervical, multiple head and neck, papillary thyroid, and other solid tumors. Tumor suppressor programmed cell death 4 (PDCD4) is one of the principal miR-21 targets that has a highly conserved miR-21 target site within the 3′ UTR region (27,28). The regulation of PDCD4 by miR-21 has been reported in a number of human cancer cells, and reduced PDCD4 expression has been reported in at least six human tumor types or cell lines overexpressing miR-21. Downregulation of PDCD4 expression in human cancers is associated with decreased drug sensitivity (29), and epidermal expression of PDCD4 suppresses tumorigenesis in vivo (30). An analysis of consensus sequences within the miR-21 promoter region identified several conserved enhancer elements including binding sites for activator protein-1 (AP-1), which is composed of c-Fos and c-Jun family transcription factors (31,32). Experiments with luciferase reporters revealed that AP-1 triggers pri-miR-21 transcription, and that c-Fos and c-Jun are the main contributors among AP-1 components. Oncogenic transformation is frequently associated with enhanced endogenous AP-1 activity, and AP-1 activation strongly contributes to oncogenic potential. Therefore, up-regulated miR-21 expression in many cancers may reflect elevated AP-1 activity.

We previously reported that SP cells isolated from HeLa, HBC5, and BSY-1 cell lines were resistant to chemotherapeutic drugs and exhibited tumorigenic properties in xenograft transplants (33). In the present study, we isolated and analyzed SP cells as a drug-resistant cell model for elucidating chemoresistance mechanisms. Using microarray and quantitative real-time PCR analysis on several cell lines, we identified that miR-21, *c-jun*, and *c-fos* genes are upregulated in SP and not in non-SP (NSP) cells. PDCD4 was found to be repressed in HeLa SP cells. Suppression of AP-1 activity decreased miR-21 expression and increased PDCD4 expression. Furthermore, AP-1 inhibition increased chemosensitivity and abrogated the chemoresistant nature of HeLa SP cells. miR-21 knockdown decreased colony forming ability of SP cells treated with topotecan, suggesting that inhibition of miR-21 by a combination of drugs may be a potential therapeutic strategy to eradicate chemoresistant CSCs.

MATERIALS AND METHODS

Cell Culture Conditions

Cells were maintained in RPMI-1640 (Nissui, Tokyo, Japan; for BSY-1, HBC-5, HT1080, and MCF-7 cell lines) or Dulbecco's modified Eagle's medium (Nissui; for HeLa cell line) supplemented with 10% heat-inactivated fetal bovine serum.

Reagents

SP600125, an anthrapyrazolone Jun N-terminal kinase inhibitor (Sigma, St. Louis, MO, USA), was prepared as a 2.5 mM stock solution in dimethyl sulfoxide. The mercury™ locked nucleic acid (LNA) knockdown probes (Hsa-miR-21 LNA and scrambled miR LNA oligoribonucleotides) for miR-21 inhibition experiments were purchased from Exiqon (Vedbaek, Denmark). Fumitremorgin C (FTC) was purchased from Alexix Corp. (Lausen, Switzerland) and topotecan [10-hydroxy-9-dimethylaminomethyl-(*S*)-camptothecin], a water-soluble camptothecin analogue, was provided by GlaxoSmithKline (King of Prussia, PA, USA).

Flow Cytometry

Cells (1×10^6 cells/ml) were suspended in HBSS (pH 7.5) supplemented with 2% FBS and incubated at 37°C for 60 min with 6 μg/ml (for HeLa cell line) or 2.5 μg/ml (for MCF-7 cell line) of Hoechst 33342 (Molecular Probe, Eugene, OR, USA), either alone or in the presence of 1 μM FTC as a control. After incubation, cells were collected and resuspended in ice-cold HBSS at 3×10^6 cells/ml with 5 μg/ml of propidium iodide (Sigma). Cell analysis and sorting were performed using a FACS Vantage SE flow cytometer (BD Bioscience, Sparks, MD, USA) using methods described previously (33).

Microarray Analysis

Total RNA from cultured SP and NSP cells was isolated using the RNeasy RNA purification kit (Qiagen, Valencia, CA, USA). The quality of total RNA was analyzed using the RNA 6000 Nano LabChip kit on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). cRNA targets for hybridization to GeneChip were prepared by reverse transcription (RT) of 3 μg of total RNA. Targets were then labeled with biotin before

fragmentation according to standard Affymetrix protocols. Hybridization to GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix, Sunnyvale, CA, USA) was performed using the Fluidics Station 450 and GeneChip Scanner 3000 (Affymetrix).

Isolation of miRNA and mRNA

mRNA and miRNA (mature miRNA and pre-miRNA) were isolated using the RNeasy Mini kit (Qiagen) or mirVana miRNA isolation kit (Ambion, Austin, TX, USA) by following the total RNA isolation procedure or enrichment procedure for small RNAs. mRNA and miRNA were quantified with Nano-Drop (Thermo Fisher Scientific Inc., Rockford, IL, USA).

TaqMan Real-Time PCR

TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) were used to detect mature miR-21 expression levels. For RT, 10 ng of total RNA was used in each reaction (5 μl) and mixed with the RT primer (3 μl). RT was conducted under the following conditions: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and then held at 4°C. cDNA products obtained were then diluted 10-fold, and 1 μl of diluted cDNA along with TaqMan primers (0.75 μl) were used for the PCR reaction. This reaction was conducted at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 60 s in the PCR LightCycler 480 System (Roche Diagnostics, Basel, Switzerland). The results of real-time PCR were analyzed and expressed relative to the CT (threshold cycle) miRNA expression value, which was then converted to fold changes. RNU6B was used for normalization.

Quantitative Real-Time PCR

RT for precursor miR-21 (pre-miR-21) and mRNAs was performed using SuperScript III (Invitrogen, Carlsbad, CA, USA) following previously described methods (33) or using the miScript RT kit (Qiagen). For the later method, 30 ng of small RNAs or 200 ng of large RNAs were used in each reaction (total volume 15 μl). RT was performed under the following conditions: 37°C for 60 min and 95°C for 5 min. Following this, cDNA products obtained were diluted to 0.5 ng/μl, and 2 ng of this product was used for the PCR reaction. RNA levels were detected using the LightCycler 480 DNA SYBR Green I Master (Roche Applied Science, Indianapolis, IN, USA) or the miScript SYBR Green PCR kit (Qiagen), according to the manufacturer's protocol. The primers used were 5′-GCTTATCAGACTGATGTTGACTG-3′ and 5′-CAGCCCATCGACTGGTG-3′ for pre-miR-21, 5′-CCAAAGGATAGTGCGATGTTT-3′ and 5′-CTGTCCCTCTCCACTGCAAC-3′ for *c-jun*, 5′-CTACCACTCACCCGCAGACT-3′ and 5′-AGGTCCGTGCAGAAGTCCT-3′ for *c-fos*, and 5′-AGCCACATCGCTCAGACAC-3′ and 5′-GCCCAATACGACCAAATCC-3′ for *GAPDH*. RNA amounts were expressed as relative mRNA expression of the CT value, which was then converted to fold changes. *RNU6B* or *GAPDH* was used for normalization. PCR primers were purchased from Operon Biotechnology (Tokyo, Japan).

Luciferase Assay

Cells were seeded (2.5×10^5 cells/well) into six-well plates and transiently transfected with 2 μg of firefly luciferase reporter plasmids AP-1 Luc (Clontech, Mountain View, CA, USA) or pGL3 Basic (Promega, Madison, WI, USA) and with 50 ng of Renilla luciferase phRL-TK (Promega; internal control) using the FuGEN EHD transfection reagent (Roche Applied Science), according to the manufacturer's protocol. Cells were incubated for 24 h and reseeded into a 96-well plate (4×10^3 cells/well), cultured overnight, and treated for 24 h with 1 μM SP600125. Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Luciferase activities were measured with an LB 960 Microplate Luminometer Centro (Berthold, Wildbad, Germany). Firefly luciferase activity for each transfected well was normalized to Renilla luciferase activity. Each transfection was performed in triplicate.

Western Blotting and Antibodies

Western blot analysis was performed using methods described previously (33). We used anti-PDCD4 (rabbit; Rockland Immunochemicals, Rockland, PA, USA) and monoclonal anti-β-actin (clone AC-15; Sigma) antibodies. The specific signals were detected with appropriate horseradish peroxidase-conjugated secondary antibodies and an enhanced chemoluminescence system (GE Healthcare, Piscataway, NJ, USA). Blots were scanned using an Image Reader LAS-3000 mini (Fuji Film, Tokyo, Japan).

Cell Proliferation Assay

HeLa cells were cultured overnight in the 96-well plates (2.5×10^3 cells/well for unsorted cells or 2×10^3 cells/well for sorted SP and NSP cells) and then treated with SP600125 (1, 1.5, or 2 μM). The following day, cells were treated with various concentrations of topotecan and SP600125 and incubated for additional 3 days. To assess the change in cell viability, we used the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega). The culture medium was replaced with 100 μl of fresh medium containing 20 μl of reagent, and

the plates were incubated at 37°C for 1 h. Optical density was measured at 490 nm with a reference at 690 nm using a microplate-spectrophotometer (Benchmark Plus, BioRad, Richmond, CA, USA). Relative cell survival (mean ± SD of triplicate determinations) was calculated by standardizing the nontreated cell survival to 100%.

Colony Formation Assay

Sorted HeLa (2×10^5) or MCF-7 cells (1×10^5) were seeded into six-well plates and then transfected with Anti-miR-21 LNA or control LNA (50 nM) using Lipofectamine RNAi Max (Invitrogen). After a 24-h incubation, cells were reseeded into six-well plates (2×10^4 HeLa cells or 2×10^4 MCF-7 cells per well). The following day, cells were treated with 125 nM (for HeLa cells) or 250 nM (for MCF-7 cells) topotecan and cultured for 3 days. The medium was then changed, and cells were cultured for an additional 10 days in topotecan-free medium with medium changes every 2–3 days. Colonies obtained were fixed in methanol for 30 min and stained with 0.1% crystal violet for 1 h. All colonies present in each well were counted with a colony analyzer system CA-7II (System Science, Tokyo, Japan).

RESULTS

Identification of Deregulated Genes in SP Cells by Microarray Analysis

We previously reported that SP cells from various cancer cell lines exhibit chemoresistant properties (33). As part of our continued efforts to investigate CSC-like properties of SP cells, we performed microarray analysis using total RNA of SP and NSP cells derived from BSY-1, HBC5, MCF-7, and HeLa cell lines, which resulted in a differential gene expression profile between SP and NSP cells. Analysis was restricted to genes commonly upregulated or downregulated in the four cell lines. We identified 43 probes corresponding to 34 upregulated genes (SP/NSP > 1.5) and 26 probes corresponding to 24 downregulated genes (SP/NSP < .75) (Table 1). A higher *ABCG2* expression in SP cells confirmed the proper enrichment of the SP cell fraction (Tables 1 and 2). Several transcription factors, including the AP-1 components *c-jun* and *c-fos*, were overexpressed in SP compared to NSP cells (Table 2). Several probes targeting pre-microRNAs are spotted in our used GeneChip Human Genome U133 Plus 2.0 array. Interestingly, a gene encoding for miR-21, miR-21 primary transcript (pri-miR-21), was also upregulated in the SP cell population (Tables 1 and 2). miR-21 has been reported to be overexpressed in a variety of tumors and was surprising to find out that its coding gene was specifically upregulated in SP cells. Overexpression of this

Table 1. Average of SP/NSP Ratio of Upregulated and Downregulated Genes in BSY-1, HBC-5, MCF-7, and HeLa Cells

Gene Symbol	Average Ratio SN/NSP
Upregulated genes	
SFRS3	10.75
MIRN21	**5.37**
HNRPDL	5.15
ABCG2	**4.69**
JUN	**3.46**
EPC1	3.14
KLHL15	2.80
PPP1R10	2.66
MYC	2.65
TNFAIP3	2.56
DUSP1	2.56
ERG1	2.55
PCF11	2.47
ATF3	2.47
FLJ44342	2.43
IFRD1	2.39
FOS	**2.31**
CCNL1	2.29
UFM1	2.23
MORF4L2	2.19
AXUD1	2.08
RND3	2.01
ZNF529	1.87
ISG20L1	1.85
NFKBIA	1.85
PTP4A1	1.85
ZBTB43	1.84
HIST1H4C	1.82
KIAA1530	1.71
MXD1	1.70
LOC440944	1.70
FRMD4A	1.69
USP38	1.69
CD55	1.55
Downregulated genes	
IGFBP5	0.28
DKFZP761M1551	0.52
KIFC1	0.52
CDKN2C	0.55
CKAP2L	0.56
CDC2	0.58
RNASE4	0.58
KIF14	0.59
MK167	0.60
FAM91A2	0.61
LMNB1	0.61
TOP2A	0.62
ERGIC1	0.65
RAB5C	0.65
HJURP	0.65
ARSD	0.69
SGOL2	0.69
WDR41	0.69
LOC401397	0.70
IER3IP1	0.71
CYP4B1	0.72
GMCL1	0.73
POLR2C	0.74
CNIH4	0.74

Table 2. SP/NSP Ratio of miR-21, ABCG2, c-Jun, and c-Fos in BSY-1, HBC-5, MCF-7, and HeLa Cells

Gene Title	Gene Symbol	Average SP/NSP	SP/NSP	Cell Line
microRNA-21 primary transcript	MIRN21	5.37	7.30	BSY1
			5.58	HBC5
			6.67	MCF7
			1.92	HeLa
ATP-binding cassette G2	ABCG2	4.69	2.47	BSY1
			3.83	HBC5
			10.90	MCF7
			1.55	HeLa
Jun oncogene	JUN	3.46	4.66	BSY1
			1.51	HBC5
			2.20	MCF7
			5.47	HeLa
v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	2.31	2.02	BSY1
			3.56	HBC5
			1.63	MCF7
			3.04	HeLa

gene suggests that mature miR-21 is processed from pri-miR-21 and, therefore, miR-21 is active in SP cells.

miR-21 Inhibits PDCD4 Expression

To verify the above results, we measured mRNA and miRNA levels in the four cell lines tested using microarray analysis. We also measured the mRNA and miRNA levels in HCC1395 cells. The real-time PCR analysis confirmed that pre-miR-21, *c-fos* mRNA, and *c-jun* mRNA levels were higher in SP than in NSP cells in most of the cell lines (Fig. 1). As previously reported (31,32), AP-1 mediates transcriptional activation of the miR-21 promoter (Fig. 2A). The generated miR-21 primary transcripts (pri-miR-21) were processed into pre-miR-21 following the generation of mature miR-21 (Fig. 2A). To confirm the high expression of miR-21, we isolated small RNAs from HeLa, MCF-7 SP, and NSP cells and performed real-time PCR to detect mature and pre-miR-21. As shown in Figure 2B, we observed high expression of mature miR-21 and pre-miR-21 in SP cells derived from both HeLa and MCF-7 cells. We then performed Western blot analysis using HeLa SP and NSP cell lysates to confirm the activity of miR-21 in SP cells. We examined expression levels of PDCD4 (27,28). PDCD4 expression levels in HeLa SP cells were lower than those in HeLa NSP cells (Fig. 2C), supporting that there was higher miR-21 activity in SP cells. Taken together, we hypothesized that the SP cell fraction harbors a population of cells with high AP-1 activity that consequently enhances miR-21 expression (Fig. 2A).

AP-1 Inhibition Reverses Chemoresistance in SP Cells

Several studies have reported that AP-1 transcriptionally regulates miR-21 expression (Fig. 2A) (31,32). To study the roles of higher miR-21 expression in SP cells, we investigated miR-21 expression after treatment of HeLa SP cells with SP600125. Real-time PCR analysis revealed a 10-fold downregulation of pre-miR-21 expression in SP600125-treated HeLa SP cells (Fig. 3A). Western blot analysis of PDCD4 revealed that inhibition of AP-1 increased PDCD4 expression in HeLa SP cells but not in HeLa NSP cells (Fig. 3B), indicating the higher activity of AP-1-regulated miR-21 in HeLa SP cells. These results indicate that upregulation of miR-21 expression is mediated by overexpression of AP-1 components c-Jun and c-Fos in SP cells.

As indicated in our previous study (33), HeLa SP cells showed chemoresistance compared to HeLa NSP cells. When sorted HeLa SP and NSP cells were treated with 250 nM topotecan, there were more viable HeLa SP cells than HeLa NSP cells (Fig. 3C). Although 1.5 μM SP600125 alone did not affect cell viability, treatment with topotecan and SP600125 almost completely suppressed viability of both HeLa SP and NSP cells (Fig. 3C), suggesting that AP-1 activity plays a crucial role in maintaining chemoresistance in SP cells.

To exclude effects of cell sorting, we investigated the role of AP-1 in parental HeLa cells. We first checked AP-1 activity using HeLa cells that had been transfected with the AP-1 Luc plasmid and confirmed that treatment of cells with 1 μM SP600125 suppressed AP-1 activity

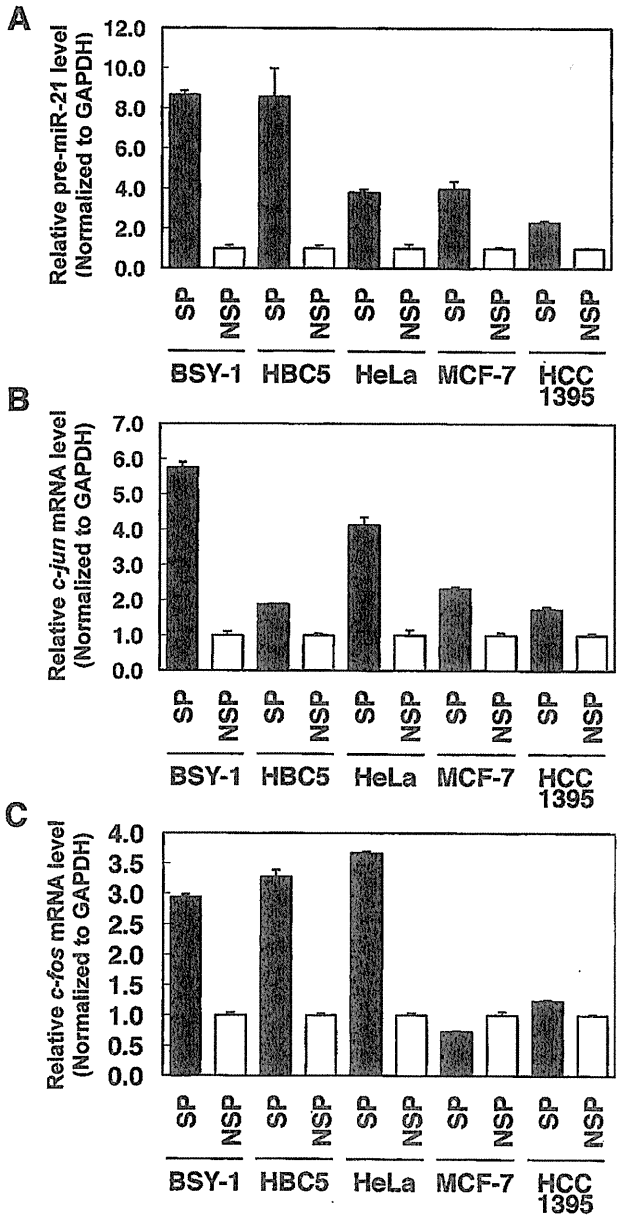

Figure 1. Validation of deregulated genes identified by microarray analysis. mRNA was isolated from sorted SP and NSP cells derived from the indicated cell lines using the RNeasy Mini kit. RNA (1 μg) was reverse transcribed using SuperScript III, according to the manufacturer's instructions. Real-time PCR for pre-miR-21 and mRNA was performed using LightCycler 480 Probe Master. The amount of pre-miR-21 (A), *c-jun* (B), and *c-fos* (C) was expressed as relative mRNA expression of the CT (threshold cycle) value, which was then converted to fold changes. GAPDH was used for normalization.

(Fig. 4A). To clarify the effects of AP-1 inhibition on the chemosensitivity to topotecan, HeLa cells were treated with SP600125 combined with various topotecan concentrations. As depicted in Figure 4B, treatment of cells with SP600125 enhanced sensitivity to topotecan in a dose-dependent fashion, indicating a close association between AP-1 activity and chemoresistance.

miR-21 Inhibition Decreases SP Cell Colony Formation

miR-21 was overexpressed in a variety of cancers and was thought to be involved in tumor development. Oncogenic transformation is frequently associated with the enhancement of endogenous AP-1 activity, and AP-1 activation strongly contributes to the oncogenic potential. In addition, miR-21 overexpression might be involved in the AP-1-dependent chemoresistance. Therefore, upregulated miR-21 expression in many cancers may reflect the elevated AP-1 activity in these carcinomas (Fig. 2A). To evaluate the effects of miR-21 on drug sensitivity, we conducted transfection experiments to determine whether direct inhibition of miR-21 could affect chemosensitivity of SP cells. We altered cellular levels of miR-21 by transfection of HeLa and MCF-7 cells with anti-miR-21 LNA and verified its effects by assessing PDCD4 protein levels. The immunoblot analysis showed stronger bands in anti-miR-21 LNA transfected cells compared to control cells, suggesting that anti-miR-21 LNA increased PDCD4 protein expression in both HeLa and MCF-7 cells (Fig. 5A).

Next, we performed colony formation assays. Although anti-miR-21 LNA treatment alone exhibited marginal or moderate inhibitory effects on colony-forming ability of HeLa and MCF-7 SP cells, respectively, transfection with anti-miR-21 LNA drastically increased the sensitivity to topotecan in HeLa and MCF-7 SP cells (Fig. 5B). Consistent with cell viability data (Fig. 3C), HeLa NSP cells were more sensitive to topotecan than were SP cells (Fig. 5B). Anti-miR-21 LNA treatment did not affect the sensitivity to topotecan in both HeLa and MCF-7 NSP cells. Inhibition of colony forming ability by anti-miR-21 was stronger in SP cells than in NSP cells, indicating that the chemoresistant nature of SP cells depends on miR-21 function.

DISCUSSION

Drug resistance remains a major cause of chemotherapy failure and relapse. More evidence has supported the idea that cancer may arise from a rare population of cells with CSC-like properties, such as the capacity to self-renew, to differentiate, to cause heterogeneous lineages of cancer cells that comprise the tumor, and to resist conventional chemotherapy. Disease relapse may

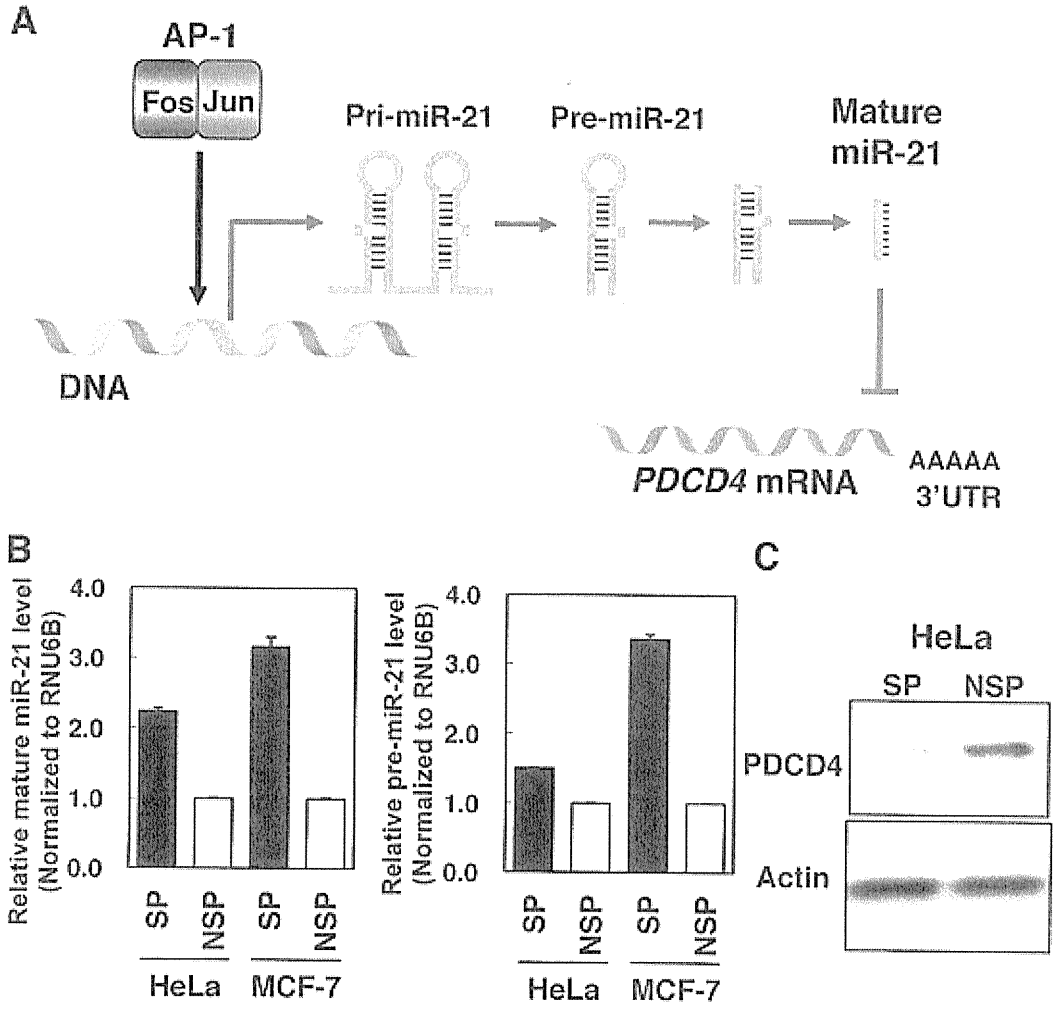

Figure 2. Increase in the expression and activity of miR-21 in SP cells. (A) Schematic representation of miR-21 transcription, processing, and targeting. (B) miRNA was isolated from sorted SP and NSP cells derived from HeLa or MCF-7 cells using the mirVana miRNA isolation kit following the enrichment procedure for small RNAs. TaqMan MicroRNA assays were used to detect expression levels of mature miR-21 (left panel). Real-time PCR for pre-miR-21 was performed using the miScript RT kit and the miScript SYBR Green PCR kit (right panel). Results of real-time PCR were analyzed and expressed as relative miRNA expression of the CT (threshold cycle) value, which was then converted to fold changes. *RNU6B* was used for normalization. (C) Sorted HeLa SP and NSP cell lysates were electrophoresed and immunoblotted with an anti-PDCD4 antibody or an anti-β-actin antibody.

occur because therapies eliminate proliferating cells that constitute the bulk of the tumor but fail to eradicate CSCs that can reinitiate malignancy after a period of latency. We and others have demonstrated that SP cells, which cannot accumulate Hoechst 33342 fluorescent dye because of active efflux via ABC-transporters, harbor many CSC-like properties (4–12,33). As a result of differential gene expression profiles between SP and NSP cells from various cancer cell lines, we identified one of the main miRNAs deregulated in cancer (miR-21) and also transcription factors, such as c-Fos and c-Jun, reportedly involved in the transcriptional control of this miRNA (31,32). Because this miRNA is abundant in a variety of cancer cells, it is being studied as a biomarker. This encouraged us to further investigate the role of miR-21 in the maintenance of the SP phenotype of cancer cells, and we hypothesized that miR-21 may modulate sensitivity and resistance to anticancer drugs.

Overexpression of AP-1 components and miR-21 was confirmed by quantitative RT-PCR (Figs. 1 and 2). Several reports have indicated that AP-1 mediates transcriptional activation of miR-21, which in turn is capable of inducing AP-1 activity (31,32). These feedback mechanisms might contribute to high levels of miR-21 expression in SP cells and suggest a self-sustained machinery of miR-21 expression.

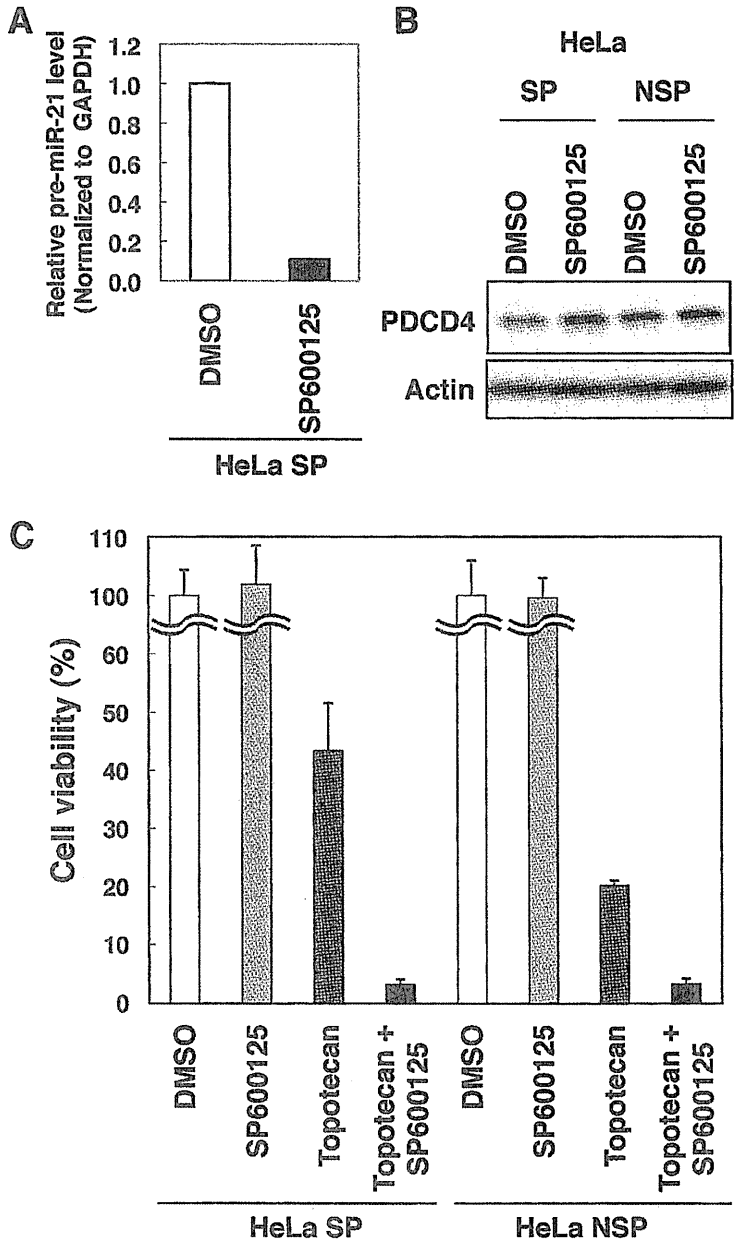

Figure 3. Inhibition of AP-1 activity decreases miR-21 level and reverses chemoresistance of SP cells. (A) Sorted HeLa SP cells were treated with vehicle control (DMSO) or SP600125 (1.5 μM) for 24 h. mRNA was isolated using the mirVana miRNA Isolation kit, following the enrichment procedure for total RNA. Real-time PCR was performed using the miScript RT kit. RNA levels were detected using the miScript SYBR Green PCR kit. The amounts of pre-miR-21 were expressed as relative mRNA expression of the CT (threshold cycle) value, which was then converted to fold changes. *GAPDH* was used for normalization. (B) Sorted HeLa SP and NSP cells were treated as in (A). Each cell lysate was electrophoresed and immunoblotted with an anti-PDCD4 antibody or an anti-β-actin antibody. (C) Sorted HeLa SP and NSP cells were treated with DMSO, SP600125 (1.5 μM), topotecan (250 nM), or topotecan plus SP600125. Cell viability was estimated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit. Cell viability was calculated by setting cell survival of the vehicle control to 100%. Each vertical bar represents the mean ± SD of triplicate determinations.

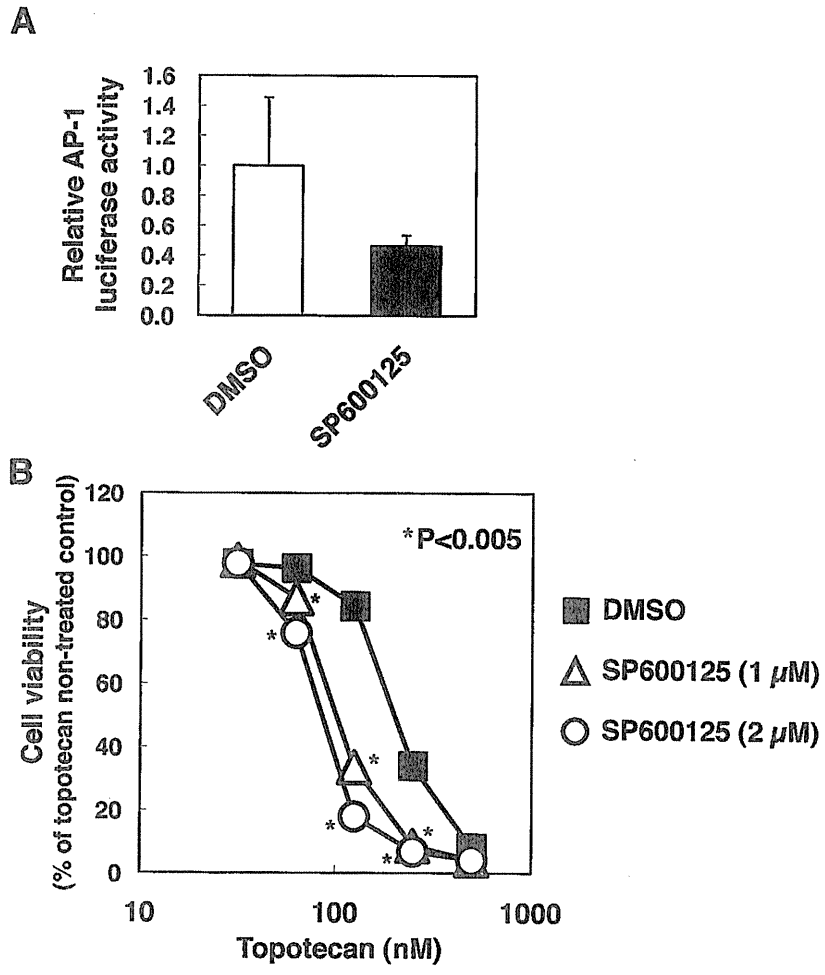

Figure 4. SP600125 sensitized HeLa cells to topotecan. (A) HeLa cells, which had been transfected with reporter constructs, were treated with vehicle control DMSO or SP600125 (1.0 μM) for 24 h, and relative AP-1 luciferase activity was measured. (B) HeLa cells were treated with vehicle control DMSO, SP600125 (1.0 μM), or SP600125 (2.0 μM) for 24 h. Then, cells were treated with various concentrations of topotecan and SP600125 and incubated for additional 3 days. Cell viability was estimated by the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit. Cell viability was calculated by setting the cell survival of the topotecan-free control to 100%. Each vertical bar represents the mean ± SD of triplicate determinations.

Activation of miR-21 in SP cells was demonstrated by Western blot analysis of PDCD4 which was repressed in SP cells (Fig. 2C). PDCD4 is one of the principal miR-21 targets validated independently by several groups (27,28). While PDCD4 was initially discovered as a gene that is upregulated during apoptosis, it has been further characterized as a potent tumor suppressor. PDCD4 inhibits PMA-induced neoplastic transformation as well as invasion and intravasation (34). In an in vivo model, it was found to inhibit both translation initiation and AP-1 activation while decreasing benign tumor development and malignant progression (30). PDCD4 has also been characterized as an enhancer of drug sensitivity, suggesting its use in new therapeutic approaches through the upregulation of PDCD4 in combination with geldanamycin (29).

As expected, suppression of AP-1 activity sensitized HeLa cells to topotecan and blocked the chemoresistant nature of SP cells (Fig. 3). Furthermore, miR-21 inhibition resulted in decreased colony forming ability of drug-treated HeLa and MCF-7 cells (Fig. 5). These findings define the critical role of miR-21 in the maintenance of the drug resistant phenotype of SP cells, probably through the involvement of AP-1 and PDCD4.

Understanding the biology of drug resistant cells offers a promising advancement in the field of cancer ther-

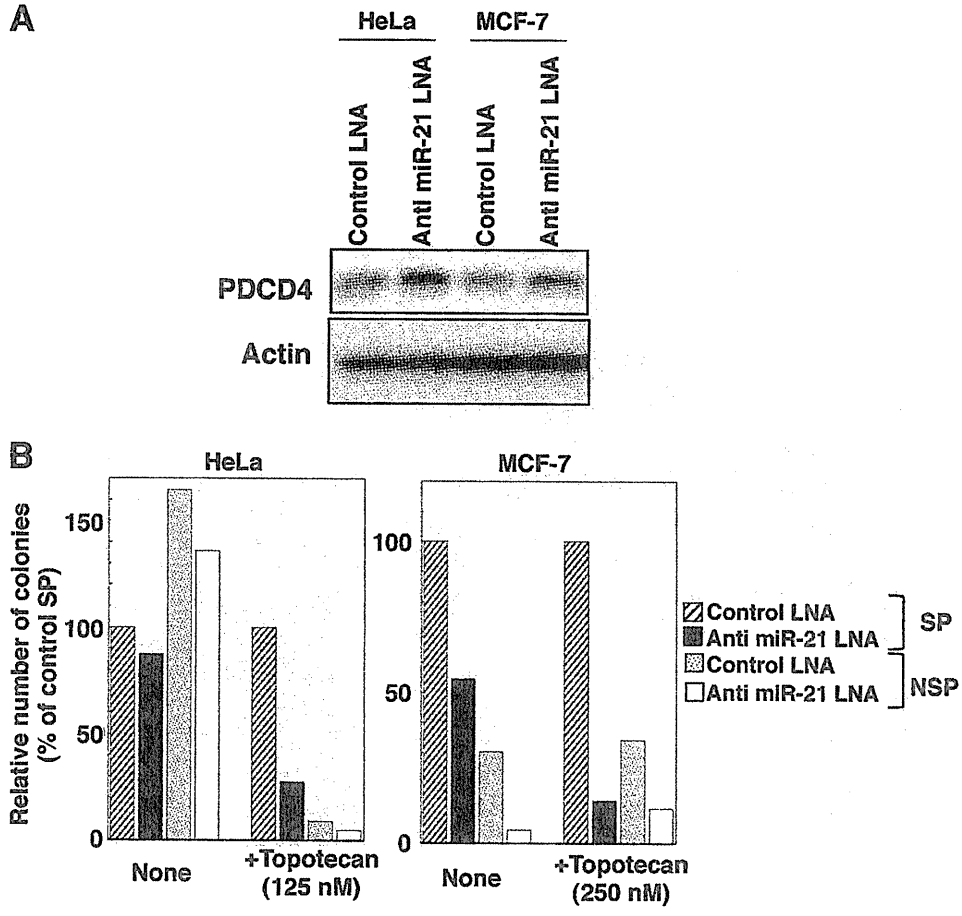

Figure 5. miR-21 inhibition sensitized SP cells to topotecan. (A) HeLa and MCF-7 cells were transfected with 50 nM anti-miR-21 LNA or control LNA. After incubation for 48 h, each cell lysate was electrophoresed and immunoblotted with an anti-PDCD4 antibody or an anti-β-actin antibody. (B) Sorted SP and NSP cells from HeLa and MCF-7 cells were transfected with 50 nM anti-miR-21 LNA or control LNA. After reseeding, the cells were treated with the indicated concentrations of topotecan for 3 days following cultivation in topotecan-free medium for an additional 10 days. All colonies present in each well were counted.

apy (35–37). miRNAs, as a class of posttranscriptional regulators, have important roles in carcinogenesis and stem cells. Analysis of miRNA expression signatures may contribute to cancer diagnosis, while miRNAs themselves are potential targets for cancer therapy. We hypothesized that miR-21, which is strongly associated with many human cancers, could play an important role in maintaining chemoresistance of SP cells and may offer novel therapeutic approaches in the field of CSCs and eradication of cancer.

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Long-term study of indolent adult T-cell leukemia-lymphoma

Yumi Takasaki,[1,2] Masako Iwanaga,[2,3] Yoshitaka Imaizumi,[2] Masayuki Tawara,[1,2] Tatsuro Joh,[1,2] Tomoko Kohno,[4] Yasuaki Yamada,[5] Shimeru Kamihira,[5] Schuichi Ikeda,[6] Yasushi Miyazaki,[2] Masao Tomonaga,[1] and Kunihiro Tsukasaki[2]

[1]Third Department of Internal Medicine, Japanese Red Cross Nagasaki Genbaku Hospital, Nagasaki; [2]Department of Molecular Medicine and Hematology, Molecular Medicine Unit, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki; [3]Department of Nutritional Health, Faculty of Wellness Studies, Kwassui Women's College, Nagasaki; [4]Division of Cytokine Signaling, Department of Molecular Microbiology and Immunology and [5]Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki; and [6]Department of Hematology, Hirado Municipal Hospital, Nagasaki, Japan

The long-term prognosis of indolent adult T-cell leukemia-lymphoma (ATL) is not clearly elucidated. From 1974 to 2003, newly diagnosed indolent ATL in 90 patients (65 chronic type and 25 smoldering type) was analyzed. The median survival time was 4.1 years; 12 patients remained alive for more than 10 years, 44 progressed to acute ATL, and 63 patients died. The estimated 5-, 10-, and 15-year survival rates were 47.2%, 25.4%, and 14.1%, respectively, with no plateau in the survival curve. Although most patients were treated with watchful waiting, 12 patients were treated with chemotherapy. Kaplan-Meier analyses showed that advanced performance status (PS), neutrophilia, high concentration of lactate dehydrogenase, more than 3 extranodal lesions, more than 4 total involved lesions, and receiving chemotherapy were unfavorable prognostic factors for survival. Multivariate Cox analysis showed that advanced PS was a borderline significant independent factor in poor survival (hazard ratio, 2.1, 95% confidence interval, 1.0-4.6; $P = .06$), but it was not a factor when analysis was limited to patients who had not received chemotherapy. The prognosis of indolent ATL in this study was poorer than expected. These findings suggest that even patients with indolent ATL should be carefully observed in clinical practice. Further studies are required to develop treatments for indolent ATL. (*Blood.* 2010;115(22):4337-4343)

Introduction

Adult T-cell leukemia-lymphoma (ATL) is a peripheral T-lymphocytic malignancy associated with human T-cell lymphotropic virus type 1 (HTLV-1).[1] ATL has been classified into 4 clinical subtypes: acute, lymphoma, chronic, and smoldering.[2] In general, acute and lymphoma types of ATL have a extremely poor prognosis despite advances in chemotherapy and allogenic hematopoietic stem cell transplantation[3-5] because of multidrug resistance, a large tumor burden with multiorgan failure, hypercalcemia, and/or frequent infectious complications associated with a T-cell immunodeficiency. A previous study, in which Japanese patients with ATL were followed for a maximum duration of 7 years, reported that the 4-year survival rates for acute, lymphoma, chronic, and smoldering type were 5.0%, 5.7%, 26.9%, and 62.8%, respectively, with the median survival time (MST) of 6.2 months, 10.2 months, 24.3 months, and not yet reached, respectively.[2] Therefore, the chronic and smoldering subtypes of ATL are considered indolent and are usually managed with watchful waiting until disease progression to acute crisis, similar to the management of chronic lymphoid leukemia or smoldering myeloma. However, the follow-up duration of the previous Japanese study was too short for indolent ATL to evaluate the overall risk of progression to acute or lymphoma types (ie, aggressive ATL). A recent Brazilian study, in which patients with ATL were followed for a maximum duration of 14 years, reported that the MST of chronic and smoldering types were 18 months and 58 months, respectively, and the overall survival (OS) rates were less than 20% in both types.[6] Their results suggest that the long-term prognosis of indolent ATL might be worse than expected.

The long-term prognosis of Japanese patients with indolent ATL has not been well evaluated so far. Prognostic factors for patients with indolent ATL are also unclear. In the present study, we investigated the long-term outcome of 90 patients with indolent ATL. The purposes of this study were to estimate the 5-, 10-, and 15-year survival rates for indolent ATL and to evaluate the prognostic factors.

Methods

Patients

We evaluated a total of 90 patients with indolent ATL (25 smoldering type and 65 chronic type) who were newly diagnosed at the Nagasaki University Hospital between July 1974 and December 2003. The distribution of patients by year of diagnosis in decades (1974-1983, 1984-1993, and 1994-2003) are presented in Table 1. The cutoff date for analysis was December 2008. The diagnosis of ATL was based on clinical features, histologically and/or cytologically proven mature T-cell malignancy, the presence of anti–HTLV-1 antibody, and monoclonal integration of HTLV-1 proviral DNA into tumor cells as described previously.[2,7-9] The subtypes of ATL were classified according to criteria established by the Lymphoma Study Group of Japan Clinical Oncology Group.[2] Clinical data included date of diagnosis, complications at diagnosis, therapy regimens if applicable, date of death, cause of death, and date of latest contact. This retrospective, nonrandomized, observational study that used existing data

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Table 1. Distribution of patients in 3 decades from 1974 to 2003

Year	Total no. of patients	No. of smoldering type (% of total)	No. of chronic type (% of total)
1974-1983	19	2 (10.5)	17 (89.5)
1984-1993	35	7 (20.0)	28 (80.0)
1994-2003	36	16 (44.4)	20 (55.6)
Total for all years	90	25 (27.8)	65 (72.2)

was granted an exemption from the institutional review board and waived the requirement for written informed consent.

Clinical factors and definitions

Age was dichotomized into 2 groups: 60 years or older and younger than 60 years. Performance status (PS) was based on the 5-grade scale of the World Health Organization. Complications at diagnosis were dichotomized into present and absent. Leukocytosis was defined as white blood cell count of 12×10^9/L or greater with the median value as cutoff level. Lymphocytosis was defined as a total lymphocyte count of 6.5×10^9/L or greater with the median value as cutoff level. Neutrophilia was defined as a neutrophil count of 7.5×10^9/L or greater.[10] Eosinophilia was defined as an eosinophil count of 0.4×10^9/L greater.[11] Lactate dehydrogenase (LDH) and blood urea nitrogen (BUN) were dichotomized into normal and elevated concentrations.[12] Albumin was dichotomized into concentrations of 40.0 g/L (4.0 g/dL) or greater and less than 40.0 g/L (4.0 g/dL).[2] Potential prognostic factors (PPFs) for chronic ATL were defined as those with at least one of the following 3 factors: low serum albumin, high LDH, or high BUN according to previous reports.[13,14] Tumor lesions were evaluated as the number of lymph node lesions, number of extranodal lesions, and number of total involved lesions. Extranodal lesions were defined as follows: bone marrow (BM) involvement as the presence of more than 5% typical ATL cells on a BM smear or detection of their infiltration in a BM biopsy specimen; skin involvement as the presence of ATL infiltration in a skin biopsy specimen or as the clinically presence of typical types of skin lesions such as tumors, nodules, erythema, and papules, if biopsy was impossible; lung involvement as lesions with ATL cell infiltration in a transbronchial lung biopsy specimen or in bronchoalveolar lavage fluid; liver involvement as hepatomegaly determined by any imaging tests or liver biopsy if done; spleen involvement as splenomegaly on any imaging test. All patients had peripheral blood involvement. Both lymph node and extranodal tumor lesions were determined according to Ann Arbor classification.[2] The number of total involved lesions was defined as the sum of lymph node lesions and extranodal lesions.[2] Factors used in analyses were listed in Table 2.

Statistical analysis

OS was defined as the time from the date of first diagnosis to the date of death or the latest contact with the patient. Survival curves were estimated using the Kaplan-Meier method and were compared using the generalized Wilcoxon test. MST was estimated as the time point at which the Kaplan-Meier survival curves crossed 50%. Time to transformation was calculated as the time from the date of the first diagnosis to the date of transformation into the aggressive type (acute or lymphoma type). Univariate and multivariate Cox regression analyses were applied to evaluate prognostic factors for survival. The effects of clinical parameters were evaluated as hazard ratios (HRs) and their 95% confidence intervals (95% CIs). All statistical analyses were performed using SAS software (Version 9.1; SAS Japan Institute). All tests were 2-tailed, and the statistical significance level was set at .05.

Results

Baseline characteristics

The median value of white blood cell count, lymphocyte count, neutrophil count, and eosinophil count was 11.5×10^9/L (range, $3.9\text{-}94.4 \times 10^9$/L), 6.5×10^9/L (range, $0.9\text{-}80.2 \times 10^9$/L), 4.9×10^9/L (range, $1.5\text{-}25.5 \times 10^9$/L), and 0.06×10^9/L (range, $0\text{-}3.0 \times 10^9$/L), respectively. Frequencies of the patients at baseline are summarized in Table 2. Fifty-eight percent of the patients were male, 52% were 60 years or older, and 22% had an advanced PS (2 or more grade). Regarding complications, 35 patients (39%) had some complications at the time of diagnosis, including 13 with chronic pulmonary diseases (10 chronic bronchitis, 2 diffuse panbronchiolitis, and 1 bronchial asthma with chronic bronchitis), 9 with opportunistic infections (3 pneumocystis pneumonia, 2 cryptococcal meningitis, 2 aspergillus pneumonia, 1 cytomegalovirus infection, and 1 pulmonary tuberculosis), 7 with malignancies other than ATL (2 lung cancer, 1 larynx cancer, 1 pharynx cancer, 1 colon cancer, 1 hepatic cell carcinoma, and 1 lip cancer), and 6 with autoimmune diseases (2 infective dermatitis, 1 primary biliary cirrhosis, 1 autoimmune hemolytic anemia, 1 dermatomyositis, and 1 ulcerative colitis). The 6 patients with autoimmune diseases had received a variety of medications as follows: antibiotics for infective dermatitis, ursodeoxycholic acid for primary biliary cirrhosis, prednisolone for autoimmune hemolytic anemia and dermatomyositis, and sulfasalazine for ulcerative colitis. Concerning the hematologic factors, 43 patients (48%) had leukocytosis, 45 (50%) had lymphocytosis, 17 (19%) had neutrophilia, and 17 (19%) had eosinophilia. Regarding the laboratory factors, 28 patients (31%) had a high LDH level (greater than the normal limit). Only 5 of 87 patients (6%) had an abnormal BUN level; 34 of 88 patients (39%) had a low albumin level. Forty-seven patients (55%) had more than 1 of the 3 unfavorable prognostic factors.

Twenty-four patients (27%) had more than 2 involved lymph node lesions. Regarding the extranodal lesions, skin involvement was observed in 46 patients (51%), liver involvement in 15 (17%), spleen involvement in 6 (7%), and pulmonary involvement in 1 (1%). Of the 64 patients who had BM examined, the involvement was observed in 16 patients (25%; data not shown). Twenty percent of the patients (n = 18) had more than 3 extranodal lesions. Regarding the number of total involved lesions (extranodal lesions plus lymph node lesions), more than 4 involved lesions were observed in 24 patients (27%), 2 or 3 involved lesions in 42 patients (46%), and only 1 involved lesion in 24 patients (27%).

Prognosis

Among 90 patients with indolent ATL, 63 (70%) died, with a median duration of follow-up of 4.1 years (range, 8 days to 17.6 years). The estimated 5-, 10-, and 15-year survival rates were 47.2% (95% CI, 36.1%-57.5%), 25.4% (95% CI, 15.3%-36.8%), and 14.1% (95% CI, 6.2%-25.3%), respectively, with an MST of 4.1 years (95% CI, 2.9-6.3 years; Figure 1A). No plateaus were observed in the survival curves for OS. Of the 27 survivors, 12 were alive for more than 10 years. Of the 63 patients who died, 41 (65.1%) died of acute ATL after transformation, 5 (7.9%) died of severe chronic ATL, 11 (17.5%) died of other diseases (3 malignancies other than ATL, 2 chronic pulmonary diseases, 2 opportunistic infections, 2 autoimmune diseases, 1 cardiac failure, and 1 myocardial infarction), 2 died of transplantation-related complications, and 4 died of unknown cause. No significant difference in OS was observed between patients who died of ATL and patients who died of other causes (data not shown). Among 90 patients, 44 (49%) progressed to aggressive ATL (all were acute types), among those, 41 (93%) died. The median time to transformation was 18.8 months (range, 0.3 months to 17.6 years).

Table 2. Survival by baseline clinical factors

Factors	No. of evaluated (% of total)	No. of deaths (%)*	MST, y	Cumulative probability of survival†		
				5-y survival, % (95% CI)	10-y survival, % (95% CI)	P†
Total	90	63 (70)	4.1	47.2 (36.1-57.5)	25.4 (15.3-36.8)	
Clinical subtype						
Smoldering	25 (28)	17 (68)	2.9	39.4 (19.8-58.6)	25.3 (8.2-47.0)	.36
Chronic	65 (72)	46 (71)	5.3	50.2 (37.0-62.0)	26.3 (14.6-39.5)	
Patient-related factors (n = 90)						
Sex						
Male	52 (58)	34 (65)	4.3	48.1 (33.4-61.3)	24.9 (11.8-40.5)	.99
Female	38 (42)	29 (76)	4.1	46.4 (29.5-61.6)	26.5 (12.0-43.4)	
Age						
60 y or older	46 (52)	32 (70)	3.7	45.5 (30.4-59.4)	29.5 (14.8-45.8)	.18
Younger than 60 y	44 (48)	31 (70)	4.5	49.2 (32.9-63.6)	24.0 (11.2-39.3)	
PS						
0	22 (24)	15 (68)	8.4	75.9 (51.4-89.1)	38.9 (16.8-60.7)	.006
1	49 (54)	33 (67)	3.4	41.5 (26.9-55.5)	22.5 (9.7-38.5)	
2 or 3	19 (22)	15 (79)	1.3	27.9 (10.2-49.0)	13.9 (1.3-41.1)	
Complications at diagnosis (n = 90)						
Absent	55 (61)	37 (67)	5.7	54.1 (39.4-66.7)	25.4 (12.9-40.1)	
Present	35 (39)	26 (74)	3.4	36.6 (20.7-52.8)	28.3 (13.5-45.1)	.06
Malignancies other than ATL	7 (8)	6 (86)	0.8	28.6 (4.1-61.2)	28.6 (4.1-61.2)	
Opportunistic infection	9 (10)	7 (78)	1.2	0	0	
Chronic pulmonary disease	13 (14)	10 (77)	4.1	38.5 (14.1-62.8)	25.6 (5.2-53.4)	
Autoimmune disease	6 (7)	3 (50)	11.4	62.5 (14.2-89.3)	62.5 (14.2-89.3)	
Hematologic factors						
WBC count (n = 90)						
At least 12.0 × 10^9/L	43 (48)	32 (74)	3.4	43.0 (27.6-57.5)	22.3 (9.9-37.8)	.24
Less than 12.0 × 10^9/L	47 (52)	31 (66)	5.3	51.0 (35.1-64.8)	28.5 (13.6-45.2)	
Total lymphocyte count (n = 90)						
At least 6.5 × 10^9/L	45 (50)	35 (78)	3.7	43.3 (28.2-57.5)	17.4 (6.8-32.0)	.34
Less than 6.5 × 10^9/L	45 (50)	28 (62)	5.3	51.4 (35.2-65.4)	36.8 (20.9-52.9)	
Neutrophil counts (n = 89)						
At least 7.5 × 10^9/L	17 (19)	14 (82)	2.3	29.4 (10.7-51.1)	14.7 (1.3-42.9)	.05
Less than 7.5 × 10^9/L	72 (81)	48 (67)	5.3	51.0 (38.3-62.4)	28.4 (16.6-41.3)	
Eosinophil count (n = 89)						
At least 0.4 × 10^9/L	17 (19)	11 (65)	4.0	34.9 (13.0-58.0)	23.2 (4.9-49.4)	.47
Less than 0.4 × 10^9/L	72 (81)	51 (71)	4.5	49.2 (36.8-60.5)	27.4 (16.0-40.1)	
Laboratory factors						
LDH (n = 90)						
Greater than NI	28 (31)	23 (82)	1.5	34.8 (17.3-53.0)	14.9 (3.9-32.7)	.004
Less than or equal to NI	62 (69)	40 (65)	5.4	52.9 (39.2-64.8)	31.8 (18.5-45.9)	
BUN (n = 87)						
Greater than NI	5 (6)	5 (100)	2.0	20.0 (0.8-58.2)	0	.18
Less than or equal to NI	82 (94)	56 (68)	4.5	48.9 (37.2-59.6)	28.4 (17.3-40.6)	
Albumin (n = 88)						
Less than 40.0 g/L	34 (39)	22 (65)	3.4	39.9 (22.4-56.8)	25.6 (8.9-46.4)	.22
At least 40.0 g/L	54 (61)	40 (74)	5.3	52.2 (37.9-64.7)	26.6 (14.3-40.6)	
Potential prognostic factors (n = 87)‡						
At least 1	47 (55)	34 (72)	2.9	38.7 (24.1-53.1)	18.1 (6.5-34.3)	.05
None	40 (45)	27 (68)	5.4	56.1 (39.2-70.0)	35.2 (19.3-51.6)	
Tumor lesions (n = 90)						
No. of lymph node lesions						
2 or more	24 (27)	16 (67)	2.1	37.5 (19.0-56.0)	30.0 (12.1-50.4)	.09
0 or 1	66 (73)	47 (71)	5.3	50.9 (37.5-62.8)	23.6 (12.2-37.2)	
No. of extranodal lesions						
3 or more	18 (20)	14 (78)	1.1	29.4 (10.7-51.1)	19.6 (4.2-43.3)	.005
1 or 2	72 (80)	49 (68)	5.3	51.6 (38.9-62.9)	26.8 (15.2-39.7)	
No. of total involved lesions						
4 or more	24 (27)	16 (67)	1.3	34.8 (16.6-53.7)	26.1 (8.8-47.6)	.03
2 or 3	42 (46)	30 (71)	4.5	49.5 (32.7-64.3)	13.1 (3.5-29.1)	
1	24 (27)	17 (71)	5.4	54.5 (32.1-72.4)	44.1 (22.8-63.5)	
Chemotherapy						
Received	12 (13)	12 (100)	1.4	25.0 (6.0-50.5)	0	.01
Not received	78 (87)	51 (65)	5.3	50.8 (38.6-61.8)	31.3 (19.3-44.0)	

WBC indocates white blood cell count; MST, median survival time (years); and NI, normal index.

*Rate of death in evaluated cases.

†Cumulative probability of survival rate was estimated with the Kaplan-Meier method, and the *P* value was calculated with the generalized Wilcoxon test.

‡PPFs indicate at least 1 of the following 3 factors: low serum albumin, high LDH, or high BUN.[13,14]

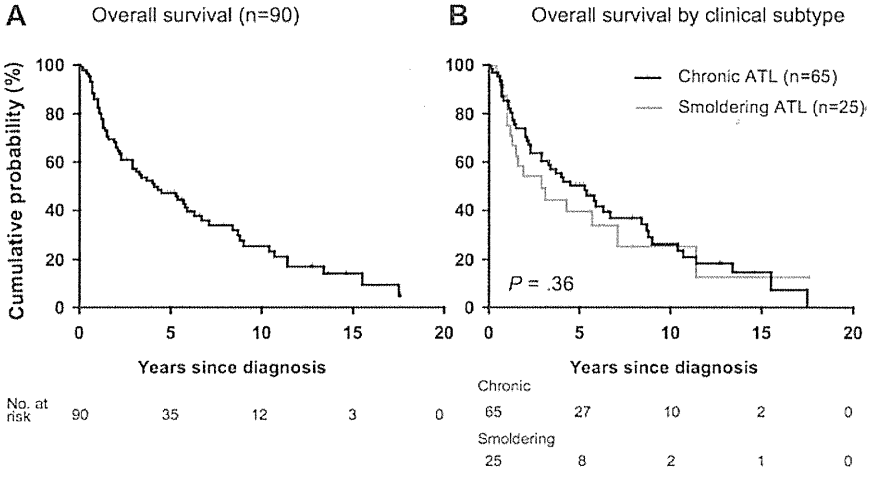

Figure 1. Survival of patients with indolent ATL. (A) For OS (n = 90), the median survival time was 4.1 years (95% CI, 2.9-6.3 years). No plateau was observed in the survival curves for OS. The estimated 5-, 10-, and 15-year survival rates were 47.2% (95% CI, 36.1%-57.5%), 25.4% (95% CI, 15.3%-36.8%), and 14.1% (95% CI, 6.2%-25.3%), respectively. (B) OS by clinical subtype (smoldering type vs chronic type). The estimated 15-year survival rate was 12.7% (95% CI, 1.1%-38.8%) with an MST of 2.9 years for smoldering type and 14.7% (95% CI, 5.7%-27.8%) with an MST of 5.3 years. There was no statistically significant difference ($P = .36$).

Among 25 patients with smoldering ATL, 17 patients (68%) died, and the estimated 15-year survival rate was 12.7% (95% CI, 1.1%-38.8%) with an MST of 2.9 years (95% CI, 1.3-7.1 years). Of the 17 patients who died, 15 died of acute ATL after transformation. Among 65 patients with chronic ATL, 46 (71%) died, and the estimated 15-year survival rate was 14.7% (95% CI, 5.7%-27.8%) with an MST of 5.3 years (95% CI, 2.9-6.7 years). Of the 46 patients who died, 29 died of acute ATL after transformation and 5 died of the disease severity. No statistically significant difference was observed in OS between subtypes ($P = .36$; Figure 1B). The overall estimated 5- and 10-year survival rates of both subtypes are shown in Table 2.

Effects of clinical factors on prognosis

Effects of clinical factors on prognosis were analyzed with the use of all the 90 patients together. Results of prognostic analyses (estimated 5- and 10-year OS rates and MST) with the use of Kaplan-Meier methods are summarized in Table 2. The survival rate was poor for patients with advanced PS ($P = .006$; Figure 2A), neutrophilia ($P = .05$; Figure 2B), and a higher LDH level ($P = .004$; Figure 2C). Patients with at least 1 of 3 PPFs for chronic ATL (a high level of LDH and BUN and a low level of albumin)[13,14] showed a poor survival rate compared with patients without ($P = .05$; Figure 2D). The difference in survival rates between patients with any complications and patients without was marginally significant ($P = .06$). Among patients with any complications, those with malignancies other than ATL or opportunistic infections at diagnosis showed a tendency of poor prognosis, although the number of patients in each category was too small (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Although no difference was observed in survival rates between patients with involvement of more than 2 lymph node lesions and patients with less involvement ($P = .09$; Table 2), the survival rate of patients

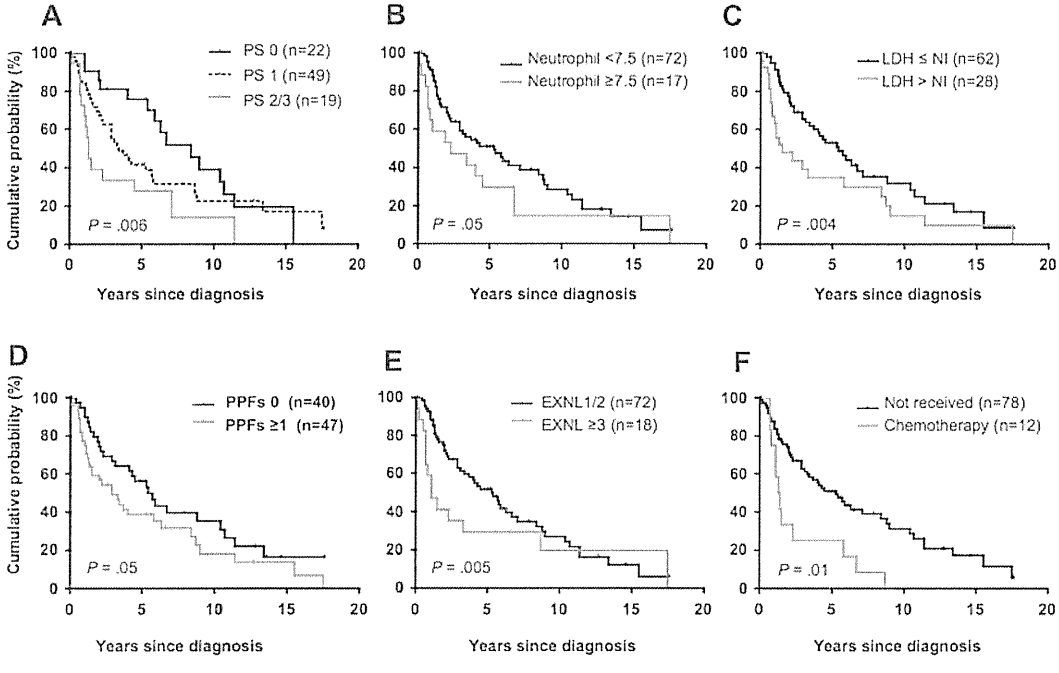

Figure 2. OS by clinical parameters. (A) OS by PS ($P = .006$). (B) OS by neutrophil count ($P = .05$). The unit is $\times 10^9$/L. (C) OS by LDH level ($P = .004$). NI indicates normal index. (D) OS by PPFs for chronic ATL that were defined based on low serum albumin, high LDH, or high BUN according to previous reports[13,14] ($P = .05$). (E) OS by the number of extranodal lesions (EXNL; $P = .005$). (F) OS by treatment states ($P = .01$).