Satou Y, Matsuoka M. HTLV-1 bZIP factor (HBZ) induces functionally impaired regulatory T-cells in vivo: 第73回日本血液学会学術集会、名古屋、2011年10月14-16日

7. 安永純一朗、馬広勇、范珺、柳川伸一、松岡雅雄：HTLV-1 bZIP factor による non-canonical Wnt 活性化機構とその意義：第5回HTLV-1研究会・シンポジウム、東京大学医科学研究所（東京）、2012年8月25日

8. 三浦未知、田邉順子、菅田謙治、趙鉄軍、安永純一朗、松岡雅雄：HTLV-1感染動物モデルとしてのサルT細胞白血病ウイルス1型感染ニホンザルの解析：第5回HTLV-1研究会、東京大学医科学研究所（東京）、2012年8月26日

9. 安永純一朗、柳川伸一、松岡雅雄：Dysregulation of the Wnt pathways by HTLV-1 bZIP factor is involved in leukemogenesis of adult T-cell leukemia：第71回日本癌学会学術総会、ロイトン札幌（札幌）、2012年9月19-21日

10. 園直希、萩屋啓太、安永純一朗、松岡雅雄：F-box and leucine-rich repeat protein 11 enhances the activity of two HTLV-1 proteins, HBZ and Tax: 第71回日本癌学会学術総会、ロイトン札幌（札幌）、2012年9月19-21日

11. 水戸部悠一、安永純一朗、佐藤賢文、中西梓、松岡雅雄：5' UTR of HTLV-1 bZIP fator gene is important for increased CD4+ T-cells in vivo: 第71回日本癌学会学術総会、ロイトン札幌（札幌）、2012年9月19-21日

12. Jun-ichiro Yasunaga, Azusa Tanaka-Nakanishi, Ken Takai and Masao Matsuoka:HTLV -1 bZIP factor downregulates proapoptotic genes through perturbation of FoxO3a function. 第74回日本血液学会学術集会、国立京都国際会館（京都）、2012年10月19-21日

13. 三浦未知、田邉順子、菅田謙治、Zhao Tiejun、齊藤暁、安永純一朗、明里宏文、松岡雅雄：サルT細胞白血病ウイルス1型のウイルス学的解析と病原性：第60回日本ウイルス学会学術集会、グランキューブ大阪（大阪）、2012年11月13-15日

14. 三田上侑生、安永純一朗、松岡雅雄：ヒトT細胞白血病ウイルス1型感染細胞の増殖とウイルス複製機構における脱ユビキチン化酵素USP20の役割：第60回日本ウイルス学会学術集会、グランキューブ大阪（大阪）、2012年11月13-15日

15. Junichiro Yasunaga, Guangyong Ma, Jun Fan, Shin-ichi Yanagawa and Masao Matsuoka. Noncanonical Wnt5a is induced by HTLV-1 bZIP factor, and supports proliferation and migration of adult T-cell leukemia cells :54th American Society of Hematology (ASH) Annual Meeting and Exposition, Geogia World Congress Center, Atlanta, U.S.A, December 7-11, 2012.

16. 川月章弘、安永純一朗、松岡雅雄：HTLV-1 bZIP Factor(HBZ) Interacts with Enhancer of Polycomb Homolog1(EPC1), and Suppresses c-fos Transcription：第35回日本分子生物学会年会、福岡国際会議場・マリンメッセ福岡（福岡）、2012年12月11-14日

17. Miura Michi, Junko Tanabe, Kenji Sugata, Tiejun Zhao, Guangyong Ma, Paola Miyazato, Jun-ichiro Yasunaga, Masao Matsuoka: STLV-1 infected Japanese macaque as a model of HTLV-1 infection: 16th International Conference on Human Retrovirology: HTLV and Related Viruses 2013, Holiday Inn Montreal-Midtown, Montreal, Canada, June26-30, 2013.

18. Azusa Tanaka-Nakanishi, Jun-ichirou Yasunaga, Ken Takai, Masao Matsuoka: Molecular mechanisms of apoptosis suppression by HTLV-1 bZIP factor in HTLV-1 infected cells: 16th International Conference on Human Retrovirology: HTLV and Related Viruses 2013, Holiday Inn Montreal-Midtown, Montreal, Canada, June26-30, 2013.

19. Guanyong Ma, Jun-ichiro Yasunaga, Jun fan, Shin-ichi Yanagawa, Masao Matsuoka: HTLV-1 mediated dysregulation of the Wnt pathways: Roles of Tax and HBZ: 16th International Conference on Human Retrovirology: HTLV and Related Viruses 2013, Holiday Inn Montreal-Midtown, Montreal, Canada, June26-30, 2013.

20. Akihiro Kawatsuki, Jun-ichiro Yasunaga, Masao Matsuoka: HTLV-1 bZIP factor suppresses c-Fos transcription and impairs T cell activation: HTLV and Related Viruses 2013, Holiday Inn Montreal-Midtown, Montreal, Canada, June26-30, 2013.

21. 松岡雅雄、安永純一朗：ヒトT細胞白血病ウイルス1型による発がん：TaxとHBZの拮抗と協調：第61回日本ウイルス学会学術集会、神戸国際会議場（兵庫県）、2013年11月10-12日

22. 安永純一朗：ヒト T 細胞白血病ウイルス 1 型による病原性発現メカニズム：第 4 回ナノバイオ創薬研究シンポジウム、京都大学杉浦ホール、2013 年 3 月 9 日

23. 安永純一朗：HTLV-1 がコードする二つのがん遺伝子 tax と HTLV-1 bZIP：第 15 回白馬シンポジウム in 名古屋、（独）国立病院機構名古屋医療センター（愛知）、2013 年 7 月 19-20 日

24. 田中梓、安永純一朗、高井健、松岡雅雄：HTLV-1 bZIP factor(HBZ)は転写因子 FoxO3a の機能を阻害することによりアポトーシスを抑制する：第 6 回 HTLV-1 研究会・シンポジウム（東京医科学研究所講堂）、2013 年 8 月 25-26 日

25. 田中梓、安永純一朗、松岡雅雄：HTLV-1 bZIP factor suppresses intrinsic and extrinsic apoptotic pathways by targeting FoxO3a:第 72 回日本癌学会学術総会、パシフィコ横浜（神奈川）、2013 年 10 月 3-5 日

26. 三浦未知、趙鉄軍、馬広勇、安永純一朗、松岡雅雄：Simian T-cell leukemia virus type 1-infected Japanese Macaques as a model for HTLV-1 research: 第 72 回日本癌学会学術総会、パシフィコ横浜（神奈川）、2013 年 10 月 3-5 日

27. Jun-Ichiro Yasunaga, Guangyong Ma, Jun Fan, Shin-Ichi Yanagawa, Masao Matsuoka: Perturbation of the Wnt pathway by HTLV-1 is importanted in viral replication and cell proliferation: 第 75 回日本血液学会学術集会、ロイトン札幌・さっぽろ芸文館・札幌市教育文化会館（北海道）、2013 年 10 月 11-13 日

28. 菅田謙治、安永純一朗、三浦未知、明里宏文、小柳義夫、小原道法、松岡雅雄：組換えウイルスを用いた抗 HTLV-1 ワクチンの作製と Macaque 属での応用：第 61 回日本ウイルス学会学術集会、神戸国際会議場（兵庫県）、2013 年 11 月 10-12 日

29. 園直希、馬広勇、萩屋啓太、安永純一朗、松岡雅雄：FBXL11 は HTLV-1 bZIP factor と Tax の機能を共に増強し ATL 細胞の増殖を促進する：第 61 回日本ウイルス学会学術集会、神戸国際会議場（兵庫県）、2013 年 11 月 10-12 日

30. 紀ノ定明香、安永純一朗、伊豫田智典、稲葉カヨ、松岡雅雄：HBZ による CD4 陽性 T 細胞増殖促進の免疫学的機序：第 61 回日本ウイルス学会学術集会、神戸国際会議場（兵

庫県）、2013 年 11 月 10-12 日

H．知的財産権の出願・登録状況
1．特許取得

2．実用新案登録

3．その他

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研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kai H, Akamatsu E, Torii E, Kodama H, Yukizaki C, Akagi I, Ino H, Sakakibara Y, Suiko M, Yamamoto I, Okayama A, Morishita K, Kataoka H, Matsuno K.	Identification of a bioactive compound against adult T-cell leukemia from bitter gourd seeds.	Plants	*3*	18-26	2014
Nakahata S, Ichikawa T, Maneesaay P, Saito Y, Nagai K, Tamura T, Manachai N, Yamakawa N, Hamasaki M, Kitabayashi I, Arai Y, Kanai Y, Taki T, Abe T, Kiyonari H, Shimoda K, Ohshima K, Horii A, Shima H, Taniwaki M, Yamaguchi R, Morishita K.	Loss of NDRG2 expression activates PI3K-AKT signalling via PTEN phosphorylation in ATLL and other cancers.	Nature Comm.	5	3393	2014
Saito Y, Kaneda K, Suekane A, Ichihara E, Nakahata S, Yamakawa N, Nagai K, Mizuno N, Kogawa K, Miura I, Itoh H, Morishita K.	Maintenance of the hematopoietic stem cell pool in bone marrow niches by EVI1-regulated GPR56.	Leukemia	27	1637-1649	2013
Yamasaki M, Mine Y, Nishimura M, Fujita S, Sakakibara Y, Suiko M, Morishita K, Nishiyama K.	Genistein induces apoptotic cell death associated with inhibition of the NF-κB pathway in adult T-cell leukemia cells.	Cell Biol Int.	37	742-747	2013
Sugie S, Mukai S, Tsukino H, Toda Y, Yamauchi T, Nishikata I, Kuroda Y, Morishita K, Kamoto T.	Increased plasma caveolin-1 levels are associated with progression of prostate cancer among Japanese men.	Anticancer Res.	33	1893-1897	2013
Ikebe E, Kawaguchi A, Tezuka K, Taguchi S, Hirose S, Matsumoto T, Mitsui T, Senba K, Nishizono A, Hori M, Hasegawa H, Yamada Y, Ueno T, Tanaka Y, Sawa H, Hall W, Minami Y, Jeang KT, Ogata M, Morishita K, Hasegawa H, Fujisawa J, Iha H.	Oral administration of an HSP90 inhibitor, 17-DMAG, intervenes tumor-cell infiltration into multiple organs and improves survival period for ATL model mice.	Blood Cancer J.	3	e132	2013
Yamamura A, Miura K, Karasawa H, Morishita K, Abe K, Mizuguchi Y, Saiki Y, Fukushige S, Kaneko N, Sase T, Nagase H, Sunamura M, Motoi F, Egawa S, Shibata C, Unno M, Sasaki I, Horii A.	Suppressed expression of NDRG2 correlates with poor prognosis in pancreatic cancer.	Biochem Biophys Res Commun.	441	102-107	2013

Kondo Y, Nagai K, Nakahata S, Saito Y, Ichikawa T, Suekane A, Taki T, Iwakawa R, Enari M, Taniwaki M, Yokota J, Sakoda S, <u>Morishita K</u>.	Overexpression of the DNA sensor proteins, absent in melanoma 2 and interferon-inducible 16, contributes to tumorigenesis of oral squamous cell carcinoma with p53 inactivation.	Cancer Sci.	103	782-790	2012
Nakahata S, Saito Y, Marutsuka K, Hidaka T, Maeda K, Hatakeyama K, Shiraga T, Goto A, Takamatsu N, Asada Y, Utsunomiya A, Okayama A, Kubuki Y, Shimoda K, Ukai Y, Kurosawa G, <u>Morishita K</u>.	Clinical significance of CADM1/TSLC1/IgSF4 expression in adult T-cell leukemia/lymphoma.	Leukemia	26	1238-1246	2012
Nakahata S, <u>Morishita K</u>.	CADM1/TSLC1 is a novel cell surface marker for adult T-cell leukemia/lymphoma.	J Clin Exp Hematopathol.	52	17-22	2012
Ueno S, Umeki K, Takajo I, Nagatomo Y, Kusumoto N, Umekita K, <u>Morishita K</u>, Okayama A.	Proviral loads of human T-lymphotropic virus Type 1 in asymptomatic carriers with different infection routes	Int J Cancer.	130	2318-2326	2012
Takenouchi H, Umeki K, Sasaki D, Yamamoto I, Nomura H, Takajo I, Ueno S, Umekita K, Kamihira S, <u>Morishita K</u>, Okayama A.	Defective human T-lymphotropic virus type 1 provirus in asymptomatic carriers.	Int J Cancer	128	1335-1343	2011
Saito Y, Nakahata S, Yamakawa N, Kaneda K, Ichihara E, Suekane A, <u>Morishita K</u>.	CD52 as a molecular target for immunotherapy to treat acute myeloid leukemia with high EVI1 expression.	Leukemia	25	921-931	2011
Nishikata I, Nakahata S, Saito Y, Kaneda K, Ichihara E, Yamakawa N, <u>Morishita K</u>.	Sumoylation of MEL1S at lysine 568 and its interaction with CtBP facilitates its repressor activity and the blockade of G-CSF-induced myeloid differentiation.	Oncogene	30	4194-4207	2011

Ichihara E, Kaneda K, Saito Y, Yamakawa N, Morishita K.	Angiopoietin1 contributes to the maintenance of cell quiescence in EVI1 ^{high} leukemia cells.	Biochem Biophys Res Commun	416	239–245	2011
Yamakawa N, Kaneda K, Saito Y, Ichihara E, Morishita K.	The increased expression of integrin α6 (ITGA6) Enhances drug resistance in EVI1^{high} leukemia.	PLoS One	7	e30706	2012
Tanaka-Nakanishi A, Yasunaga J, Takai K, Matsuoka M.	HTLV-1 bZIP Factor Suppresses Apoptosis by Attenuating the Function of FoxO3a and Altering Its Localization.	Cancer Res	74	188-200	2014
Miura M, Yasunaga J, Tanabe J, Sugata K, Zhao T, Ma G, Miyazato P, Ohshima K, Kaneko A, Watanabe A, Saito A, Akari H, Matsuoka M.	Characterization of simian T-cell leukemia virus type 1 in naturally infected Japanese macaques as a model of HTLV-1 infection.	Retrovirology	10	118	2013
Matsuoka M and Yasunaga J.	Human T-cell leukemia virus type 1: replication, proliferation and propagation by Tax and HTLV-1 bZIP factor.	Curr Opin Virol	3	684-691	2013
Zane L, Yasunaga J, Mitagami Y, Yedavalli V, Tang SW, Chen CY, Ratner L, Lu X, Jeang KT.	Wip1 and p53 contribute to HTLV-1 Tax-induced tumorigenesis.	Retrovirology	9	114	2012
Ma G, Yasunaga J, Fan J, Yanagawa S, Matsuoka M.	HTLV-1 bZIP factor dysregulates the Wnt pathways to support proliferation and migration of adult T-cell leukemia cells.	Oncogene	32	4222-4230	2013

Sugata K, Satou Y, Yasunaga JI, Hara H, Ohshima K, Utsunomiya A, Mitsuyama M, Matsuoka M.	HTLV-1 bZIP factor impairs cell-mediated immunity by suppressing production of Th1 cytokines.	Blood	119	434-444	2012
Yasunaga J and Matsuoka M.	Molecular mechanisms of HTLV-1 infection and pathogenesis.	Int. J. Hematol	94	435-442	2011
Yasunaga J, Lin FC, Lu X, Jeang KT.	Ubiquitin-specific peptidase 20 (USP20) targets TRAF6 and HTLV-1 Tax to negatively regulate NF-κB signaling.	J Virol	85	6212-6219	2011
Hagiya K, Yasunaga JI, Satou Y, Oshima K, Matsuoka M.	ATF3, an HTLV-1 bZip factor binding protein, promotes proliferation of adult T-cell leukemia cells.	Retrovirology	8	19	2011

Article

Identification of a Bioactive Compound against Adult T-cell Leukaemia from Bitter Gourd Seeds

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Abstract: In our previous report, an 80% ethanol bitter gourd seed extract (BGSE) was found to suppress proliferation of adult T-cell leukemia (ATL) cell lines. The present study aimed to identify the bioactive compounds from BGSE specific against ATL. From the result of an HPLC-MS analysis, α-eleostearic acid (α-ESA) was present in BGSE at $0.68\% \pm 0.0022\%$ ($\pm$SD, $n = 5$). In the cell proliferation test, α-ESA potently suppressed proliferation of two ATL cell lines (ED and Su9T01; $IC_{50} = 8.9$ and 29.3 μM, respectively) more than several other octadecanoic acids. However, α-ESA moderately inhibited phytohemagglutinin-activated human peripheral blood mononuclear cells (PBMC; $IC_{50} = 31.0$ μM). These results suggest that BGSE-derived α-ESA has potential as a functional food constituent because of its activity against ATL, particularly against ED cells. Moreover, α-ESA might be effective for the prevention of moderate adverse effects of ATL on normal T cells.

Keywords: adult T-cell leukemia; bitter gourd seed extract; α-eleostearic acid; phytohemagglutinin-activated human peripheral blood mononuclear cell

1. Introduction

Adult T-cell leukemia (ATL) occurs in a small population of human T-cell leukemia virus type I (HTLV-I) infected individuals. After transmission of HTLV-I, 2%–5% of carriers are likely to develop ATL after a long latency period (30–50 years) [1]. These patients have been frequently identified as being from a restricted area of tropical regions [2]. It is currently very difficult to effectively treat patients with ATL using existing therapeutic methods, and most clinical trials focus on chemotherapeutic treatment and allogeneic hematopoietic stem cell transplantation. Therefore, it is important to find appropriate therapeutic methods to prevent the development of ATL or to prolong survival after its occurrence.

In our previous report, we screened 52 agricultural plant samples for their ability to inhibit proliferation in seven kinds of ATL related cell lines to start structure of a study for finding potential drug candidates with the prevention of ATL. We found that an 80% ethanol bitter gourd (*Momordica charantia* L.) seed extract (BGSE) showed an inhibitory effect on the proliferation of ATL-related human leukemia cells [3].

Bitter gourd belongs to the Cucurbitaceae family and is cultivated worldwide as a vegetable crop. The fruit is not only used as a food, but also for its medicinal properties, such as anti-microbial, anti-diabetic, anti-HIV and anti-tumor activities, which were described in a recent review [4]. BGSE has also been reported to have anti-leukemic potential on human acute myelogenous leukemia cells (HL-60) [5]. However, HL-60 and ATL cell lines comprise different types of leukemia cells, and as there have been no reports about bioactive compounds from BGSE active against ATL, the effect of

BGSE on ATL cell proliferation requires elucidation. The aim of the present study was to identify bioactive compounds in BGSE exhibiting activity against ATL.

2. Results and Discussion

2.1. Identification of Active Compounds in BGSE

Figure 1a,b show the HPLC-DAD chromatogram (a) and HPLC-MS-total ion chromatogram (TIC) (b) of BGSE. As shown in Figure 1a, a major peak (peak 1: retention time (RT) = 8.570 min, λ = 270 nm) was detected for BGSE. The TIC showed several peaks (Figure 1b). Figure 1c shows the MS spectrum of the peak with the RT: 8.570 min. in Figure 1b, which shows a deprotonated molecular ion signal at m/z 277. This compound was estimated to be $C_{18}H_{29}O_2$ and was proposed to be α-ESA on the basis of reference data [6]. α-ESA was analyzed using the same method. Figure 1d–f shows the HPLC-DAD chromatogram (d), TIC (e) and MS spectrum (f) of α-ESA. α-ESA (50 µg/mL) showed a clear peak at 270 nm (Figure 1d, peak 2) and the same RT of peak 1 in Figure 1a (BGSE). The RT (8.570 min.) of the highest peak in TIC for α-ESA (Figure 1e, peak 2) was the same as peak 1 in Figure 1b. The MS spectrum of α-ESA (Figure 1f) showed the same result as the analysis of BGSE (Figure 1c). From these results, peak 1 in Figure 1 was determined to be α-ESA, and α-ESA is the main compound in BGSE.

We also attempted to quantitatively determine α-ESA in BGSE. Figure 2 shows the α-ESA calibration curve. The calibration curve showed good linearity (R^2 = 0.9977). α-ESA was contained in the concentration range of 34.0 and 34.2 µg/5 mg of freeze-dried bitter gourd seeds (0.68% ± 0.0022%, ±SD, n = 5). In our previous report, BGSE suppressed the proliferation of ATL cell lines [3]. The current study shows the presence of α-ESA in BGSE. Tsuzuki *et al.* reported that α-ESA accounted for about 60% of the total fatty acid composition of bitter gourd seed oil [7]. Therefore, α-ESA might have greatly contributed to suppressing the proliferation of ATL cell lines.

2.2. The Inhibitory Effects of Octadecanoic Acid Analogs on ATL Cell Lines

Of the octadecanoic acids, conjugated linoleic acids (CLA), which includes α-ESA, has been acknowledged to have numerous biological activities, such as anti-obesity, anti-diabetes, anti-cancer, anti-arthritis, anti-asthma, and anti-cardiovascular disease effects [8]. However, no study has yet reported the anti-leukemic effects of α-ESA on ATL cell lines. We examined the inhibitory effects of related compounds, seven octadecanoic acid groups, on the proliferation of two types of ATL cell lines (ED and Su9T01). As shown in Table 1, α-ESA (C18:3, n-5), γ-linolenic acid (C18:3, n-6), and α-linolenic acid (C18:3, n-3), which belong to the triunsaturated fatty acid group, substantially inhibited ED cell growth (IC_{50} values of 8.9, 61.3 and 129.9 µM, respectively) and Su9T01 cell growth (IC_{50} values of 29.3, 174.3 and 167.4 µM, respectively). Linoleic acid (C18:2, n-6), which belongs to the diunsaturated fatty acid group, inhibited ED and Su9T01 cell growth (IC_{50} values of 100.7 and 180.1 µM, respectively). In ED cells, these compounds exhibited higher activity than EGCG, which was used as the positive control [9] (IC_{50} = 152.7 µM). On the other hand, in Su9T01 cells, only α-ESA exhibited higher inhibitory activity than EGCG (IC_{50} = 166.0 µM). We cannot calculate the exactly IC_{50} values of monounsaturated fatty acid group (oleic acid (C18:1, n-9) and elaidic acid

(C18:1, n-9)) and saturated fatty acid (stearic acid (C18:0)) in both cell lines. This assay was employed to compare the effects of octadecanoic acids on ED and Su9T01 cell growth; α-ESA showed the highest inhibitory activity. IC_{50} values decreased roughly in proportion to the number of double bonds; therefore, the number of double bonds was an important determinant of anti-proliferation activity. Indeed, the triunsaturated fatty acid group was more potent than EGCG.

Figure 1. HPLC-DAD and MS chromatograms of BGSE and α-ESA. (**a,d**) HPLC-DAD chromatograms (270 nm) of BGSE (**a**) and α-ESA (**d**). (**b,e**) Total ion chromatograms (TIC) of BGSE (**b**) and α-ESA (**e**). (**c**) MS spectrum (negative-ion spectra) of peak 1 of BGSE in Figure 1b. (**f**) MS spectrum (negative-ion spectra) of peak 2 of α-ESA in Figure 1e.
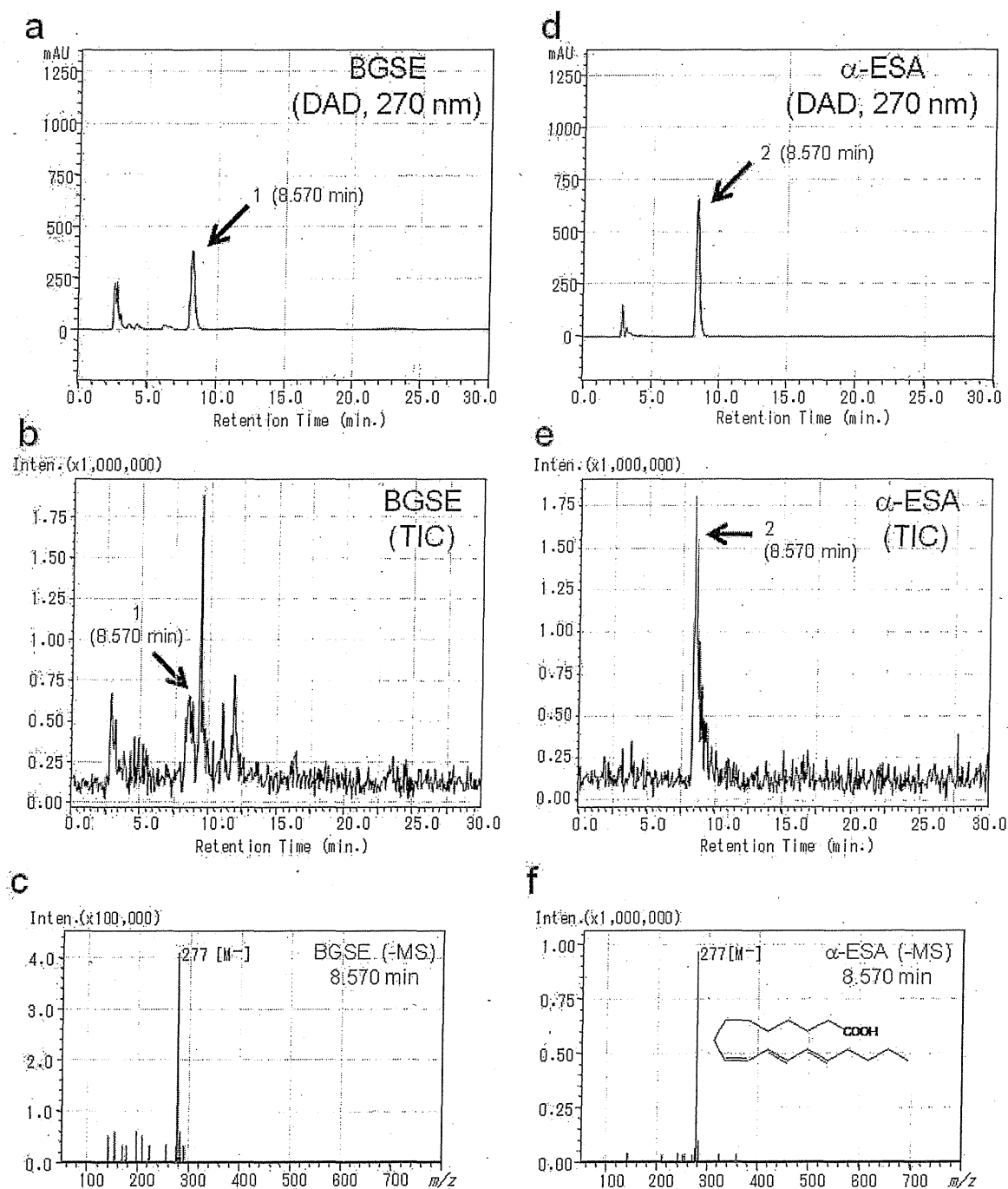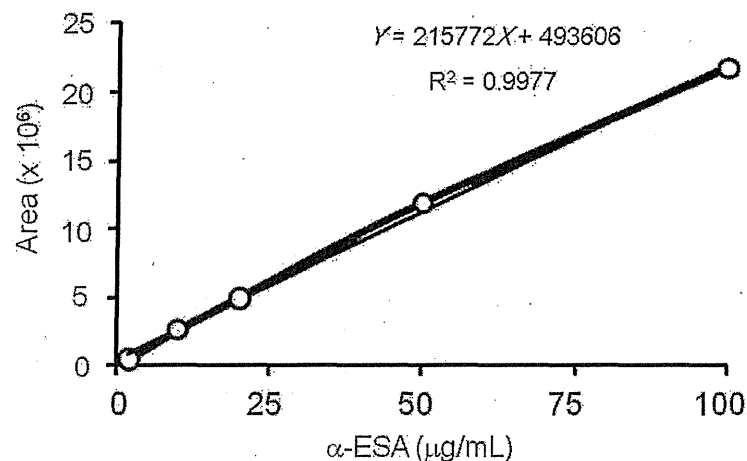
Figure 2. Calibration curve of α-ESA.

Table 1. Relationship between octadecanoic acid structure and inhibition of adult T-cell leukemia (ATL) cell line proliferation.

Compounds		IC$_{50}$ (µM)	
		ED	Su9T01
α-ESA	(C18:3, n-5)	8.9	29.3
γ-linolenic acid	(C18:3, n-6)	61.3	174.3
α-linolenic acid	(C18:3, n-3)	129.9	167.4
linoleic acid	(C18:2, n-6)	100.7	180.1
oleic acid	(C18:1, n-9)	500.0–166.7	500.0–166.7
elaidic acid	(C18:1, n-9)	>500.0	>500.0
stearic acid	(C18:0)	500.0–166.7	>500.0
EGCG	(Positive Control)	152.7	166.0

ATL cells (ED and Su9T01) were incubated for 72 h in RPMI-1640 medium containing each compound. Viable cells were detected using a WST-8 assay kit. The concentration at which cell proliferation is inhibited by 50% compared to untreated control is expressed as IC$_{50}$.

2.3. The Effect of α-ESA on ATL Cell Line and Phytohemagglutinin-Activated Human Peripheral Blood Mononuclear Cell (PBMC) Proliferation

As shown in Figure 3, we compared the suppressive effect of α-ESA on ED, Su9T01 cells and PBMCs. PBMCs are commonly used as the healthy/normal cell model in comparison to cancer cell lines. Significant differences were observed between ED cells and PBMCs treated at 6, 19 and 56 µM α-ESA ($p < 0.05$). Su9T01 cells and PBMCs treated with between 1 and 500 µM α-ESA were not significantly different. These results confirmed that the α-ESA strongly and selectively inhibited ED cells and moderately inhibited PBMCs, which are healthy normal T cells. α-ESA at 166 and 500 µM significantly decreased proliferation of all cell types. α-ESA showed inhibitory effects on ED, Su9T01 cells and PBMCs (IC$_{50}$ of 8.9, 29.3 and 31.0 µM, respectively).

Figure 3. Effect of α-ESA on ATL cell and PBMC proliferation.
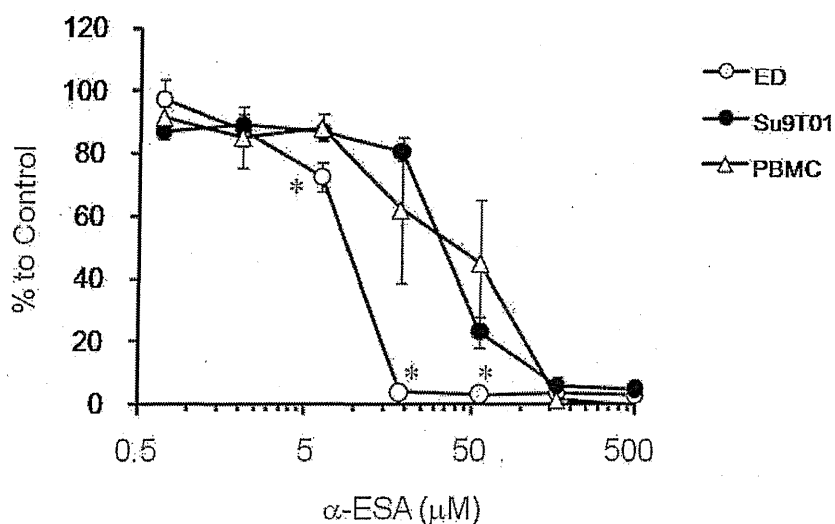
A comparison of the dose-dependent effects of α-ESA on ED, Su9T01 cells and PBMCs. Each mark represents the mean ± SD of three independent tests (Student's *t*-test). Significantly different between ED cells and PBMCs: $p < 0.05$ (*).

Tsuzuki *et al.* and Kobori *et al.* reported that bitter gourd seed oil and its constituent α-ESA had anti-leukemic potential in HL-60 cells [5,10]. While HL-60 and ATL (ED and Su9T01) are leukemia cell lines, they differ in origin and whether they are a result of viral infection. The antiproliferative properties of unsaturated fatty acids are well known. For example, Wendel *et al.* reported that the unsaturated fatty acids (mainly omega-3 fatty acids and derivatives) like conjugated eicosapentaenoic acid as important nutritional adjuvant therapeutics in the management of various human cancer diseases and the impact of nutritional omega-3 fatty acids on cancer prevention [11]. α-ESA also may have similar potential with nutritional function for cancer prevention. The present study is the first to show the inhibitory effects of α-ESA on ATL cells *in vitro*. Specifically, α-ESA showed an inhibitory effect in the rank order: ED cells > Su9T01 cells ≥ PBMCs. Sasaki *et al.* reported that tumor suppressor in lung cancer 1 (*TSLC1*) gene expression was different between ED and Su9T01 cells [12,13]. Therefore, using gene expression analysis, future studies should investigate the regulatory mechanism of *TSLC1* and its DNA methylation, as well as the possible role of α-ESA in the inhibition of ED and Su9T01 proliferation and *TSLC1* expression.

3. Experimental

3.1. Chemicals

α-Eleostearic acid (α-ESA) was obtained from Larodan Fine Chemicals AB, Malmö, Sweden and Cayman Chemical, Ann Arbor, MI, USA. γ-Linolenic acid, linoleic acid, α-linolenic acid, and elaidic acid were purchased from Cayman Chemical. Stearic acid and oleic acid were purchased from Wako, Osaka, Japan. Epigallocatechin-3-gallate (EGCG) was purchased from Nagara Science Co., Gifu, Japan. Ficoll was purchased from GE Healthcare, Uppsala, Sweden. Phytohemagglutinin (M Form) was purchased from Invitrogen, Carlsbad, CA, USA. IL-2 was purchased from R&D Systems,

Minneapolis, MN, USA. A 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-8) assay kit was purchased from Dojindo, Kumamoto, Japan.

3.2. Identification of Compounds in BGSE

The freeze-dried powder of bitter gourd seeds (5 mg) was extracted with 80% EtOH (0.5 mL) by vortexing for 30 s, followed by centrifugation at 1,500 rpm for 3 min. The supernatant was used for high performance liquid chromatography-diode array detector (HPLC-DAD) and mass spectrometry (MS) analysis. The α-ESA HPLC analysis method was a modification of the methods of Amakura *et al.* and Řezanka *et al.* [6,14]. The HPLC-DAD and MS analysis consisted of a Shimadzu HPLC System (LC-20A Prominence, Shimadzu, Kyoto, Japan) coupled to a SPD-20A (DAD; Shimadzu, Kyoto, Japan) and an LC/MS-ion trap-time of flight (LC/MS-IT-TOF, Shimadzu, Kyoto, Japan) fitted with an atmospheric pressure chemical ionization (APCI) source. HPLC separation was performed on a reverse-phase column (Atlantis T3, 2.1 mm I.D. φ100 mm, 3 μm; Waters, Milford, MA, USA). The column was maintained at 40 °C. The mobile phase consisted of eluent A (0.1% acetic acid and MeOH)/eluent B (0.1% acetic acid and 10% MeOH aq.) = 90:10 at a flow rate of 0.10 mL/min. The injection volume was 10 μL. APCI conditions were recorded from m/z = 50 to 400 in negative ion mode. The other MS conditions were as follows: nebulizer N_2 gas, 2.5 L/min; APCI interface temperature, 400.0 °C; curved desolvation line (CDL) temperature, 250.0 °C; heat block temperature, 200.0 °C; detector voltage, 1.80 kV.

3.3. α-ESA Calibration Curve

The α-ESA standard was dissolved in 80% ethanol and serial dilutions were analyzed by HPLC-DAD. α-ESA content was calculated using the following linear equation based on the calibration curve: $Y = 215772X + 493606$, $R^2 = 0.9977$. Y is the area detected by DAD (270 nm), and X is the α-ESA content in μg/mL.

3.4. ATL Cell Proliferation Assay

We used two ATL cell lines (ED and Su9T01) that are highly sensitive to inhibition of cell proliferation, as determined in our previous study [3]. ED cells were kindly provided by Dr. M. Maeda (Kyoto University, Kyoto, Japan) and Su9T01 cells were kindly provided by Dr. N. Arima (Kagoshima University, Kagoshima, Japan). The test compounds were dissolved in dimethyl sulfoxide and subjected to assay screening. The method of ATL assay is described in a previous report [3]. IC_{50} calculation was some curve fitted onto the determined proliferation inhibition points.

3.5. Isolation and Culture of PBMCs

The method of isolation and culture of PBMCs is as follows. Heparinised blood (5 mL) was diluted by adding 5 mL of PBS. The diluted blood samples were divided into four equal parts, loaded on 4 mL of Ficoll and centrifuged at 400 × g for 30 min. The PBMC layer was located within the interphase between the Ficoll and plasma. The Ficoll contained the erythrocytes and most of the granulocytes. The plasma was removed using a pipette until ~5 mL above the PBMC interphase. The cells were

washed three times with PBS (centrifuged at 200 × *g* for 15 min) and resuspended in RPMI 1640 medium supplemented with 10% foetal bovine serum containing 100 U/mL penicillin G, 100 μg/mL streptomycin, 2 ng/mL IL-2 and 128-fold dilution of phytohemagglutinin to a final cell density of 1×10^6 cells/mL. The PBMC proliferation assay was conducted using the same method as for the ATL proliferation assay [3].

3.6. Statistics

Each experiment was conducted at least three times. All data are expressed as the mean ± standard deviation (SD) of three independent experiments. Statistically significant differences were calculated by Student's *t*-test.

4. Conclusions

α-ESA was shown to be the main bioactive compound in BGSE, and contributes to the inhibition of ED cell differentiation and proliferation without damaging normal cells, leading to the disruption of ATL pathogenesis.

Acknowledgments

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Arisawa, K.; Soda, M.; Endo, S.; Kurokawa, K.; Katamine, S.; Shimokawa, I.; Koba, T.; Takahashi, T.; Saito, H.; Doi, H.; *et al.* Evaluation of adult T-cell leukemia/lymphoma incidence and its impact on non-Hodgkin lymphoma incidence in southwestern Japan. *Int. J. Cancer* **2000,** *85,* 319–324.
2. Proietti, F.A.; Carneiro-Proietti, A.B.; Catalan-Soares, B.C.; Murphy, E.L. Global epidemiology of HTLV-I infection and associated diseases. *Oncogene* **2005,** *24,* 6058–6068.
3. Kai, H.; Akamatsu, E.; Torii, E.; Kodama, H.; Yukizaki, C.; Sakakibara, Y.; Suiko, M.; Morishita, K.; Kataoka, H.; Matsuno, K. Inhibition of proliferation by agricultural plant extracts in seven human adult T-cell leukaemia (ATL)-related cell lines. *J. Nat. Med.* **2011,** *65,* 651–655.
4. Fang, E.F.; Ng, T.B. Bitter gourd (*Momordica charantia*) is a cornucopia of health: A review of its credited antidiabetic, anti-HIV, and antitumor properties. *Curr. Mol. Med.* **2011,** *11,* 417–436.

5. Soundararajan, R.; Prabha, P.; Rai, U.; Dixit, A. Antileukemic Potential of *Momordica charantia* Seed Extracts on Human Myeloid Leukemic HL60 Cells. *J. Evid.-Based Complement. Alternat. Med.* **2012**, doi:10.1155/2012/732404.

6. Amakura, Y.; Kondo, K.; Akiyama, H.; Ito, H.; Hatano, T.; Yoshida, T.; Maitani, T. Characteristic long-chain fatty acid of *Pleurocybella porrigens*. *Shokuhin Eiseigaku Zasshi* **2006**, *47*, 178–181.

7. Tsuzuki, T.; Tokuyama, Y.; Igarashi, M.; Miyazawa, T. Tumor growth suppression by alpha-eleostearic acid, a linolenic acid isomer with a conjugated triene system, via lipid peroxidation. *Carcinogenesis* **2004**, *25*, 1417–1425.

8. Ruiz, R.A.; Reglero, G.; Ibañez, E. Recent trends in the advanced analysis of bioactive fatty acids. *J. Pharm. Biomed. Anal.* **2012**, *51*, 305–326.

9. Li, H.C.; Yashiki, S.; Sonoda, J.; Lou, H.; Ghosh, S.K.; Byrnes, J.J.; Lema, C.; Fujiyoshi, T.; Karasuyama, M.; Sonoda, S. Green tea polyphenols induce apotosis *in vitro* in peripheral blood T lymphocytes of adult T-cell leukemia patients. *Jpn. J. Cancer Res.* **2000**, *91*, 34–40.

10. Kobori, M.; Ohnishi-Kameyama, M.; Akimoto, Y.; Yukizaki, C.; Yoshida, M. α-eleostearic acid and its dihydroxy derivative are major apoptosis-inducing components of bitter gourd. *J. Agric. Food Chem.* **2008**, *56*, 10515–10520.

11. Wendel, M.; Heller, A.R. Anticancer actions of omega-3 fatty acids—Current state and future perspectives. *Anticancer Agents Med. Chem.* **2009**, *9*, 457–470.

12. Sasaki, H.; Nishikata, I.; Shiraga, T.; Akamatsu, E.; Fukami, T.; Hidaka, T.; Kubuki, Y.; Okayama, A.; Hamada, K.; Okabe, H.; *et al.* Overexpression of a cell adhesion molecule, TSLC1, as a possible molecular marker for acute-type adult T-cell leukemia. *Blood* **2005**, *105*, 1204–1213.

13. Watanabe, M.; Nakahata, S.; Hamasaki, M.; Saito, Y.; Kawano, Y.; Hidaka, T.; Yamashita, K.; Umeki, K.; Taki, T.; Taniwaki, M.; *et al.* Downregulation of CDKN1A in adult T-cell leukemia/lymphoma despite overexpression of CDKN1A in human T-lymphotropic virus 1-infected cell lines. *J. Virol.* **2010**, *84*, 6966–6977.

14. Řezanka, T.; Votruba, J. Analysis of Fatty Acids by APCI-MS. In *Modern Methods for Lipid Analysis by Liquid Chromatography: Mass Spectrometry and Related Techniques*; William, C.B., Ed.; AOCS Press: Urbana, IL, USA, 2005; pp. 242–275.

nature COMMUNICATIONS

ARTICLE

Loss of NDRG2 expression activates PI3K-AKT signalling via PTEN phosphorylation in ATLL and other cancers

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Constitutive phosphatidylinositol 3-kinase (PI3K)-AKT activation has a causal role in adult T-cell leukaemia-lymphoma (ATLL) and other cancers. ATLL cells do not harbour genetic alterations in PTEN and PI3KCA but express high levels of PTEN that is highly phosphorylated at its C-terminal tail. Here we report a mechanism for the N-myc downstream-regulated gene 2 (NDRG2)-dependent regulation of PTEN phosphatase activity via the dephosphorylation of PTEN at the Ser380, Thr382 and Thr383 cluster within the C-terminal tail. We show that NDRG2 is a PTEN-binding protein that recruits protein phosphatase 2A (PP2A) to PTEN. The expression of NDRG2 is frequently downregulated in ATLL, resulting in enhanced phosphorylation of PTEN at the Ser380/Thr382/Thr383 cluster and enhanced activation of the PI3K-AKT pathway. Given the high incidence of T-cell lymphoma and other cancers in NDRG2-deficient mice, PI3K-AKT activation via enhanced PTEN phosphorylation may be critical for the development of cancer.

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A dult T-cell leukaemia-lymphoma (ATLL) is an aggressive malignant disease of CD4 $^+$ T lymphocytes caused by infection with human T-cell leukaemia virus type 1 (HTLV-1)[1]. HTLV-1 is endemic in many regions, including Japan, the Caribbean and parts of South America, and more than 20 million people are infected with this virus worldwide[2]. ATLL develops in ~5% of infected individuals after a long latency period. Although the viral transactivating protein Tax has a crucial role in the proliferation and transformation of the infected T cells, ATLL cells frequently lose their expression of Tax due to genetic and epigenetic changes in the proviral genome[3]. Another viral product, HBZ, has been shown to support the proliferation of ATLL cells; however, additional cellular events are required for the development of ATLL[3]. Recent studies have shown that the phosphatidylinositol 3-kinase (PI3K)-AKT signalling pathway, one of the major oncogenic pathways, is frequently activated in ATLL and is indispensable for the proliferation of ATLL cells[4,5].

The phosphatase and tensin homologue (PTEN) deleted on the chromosome 10 (PTEN) gene was cloned by association with the human cancer susceptibility locus at 10q23 (refs 6,7) and is a lipid phosphatase that dephosphorylates phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$) to PI(4,5)P$_2$ and opposes the PI3K-AKT pathway, exerting tumour suppressor activity[8]. Although PTEN is one of the most frequently mutated genes in human cancer, the frequency of PTEN mutations varies from 1 to 45%[9]. Loss of PTEN through genetic alterations can lead to activation of the AKT pathway; however, activation without PTEN mutations is likely to occur in the majority of cancers, suggesting that different mechanisms such as PIK3CA mutations involve activation of AKT[10]. The lipid phosphatase activity and protein stability of PTEN can be regulated through multiple mechanisms, including acetylation, phosphorylation and ubiquitination[11]. For instance, E3 ubiquitin ligases, NEDD4-1 and WWP2, have been reported to decrease PTEN stability by catalysing PTEN poly-ubiquitination, and the PTEN degradation predisposes to tumourigenesis[12–14]. In addition, PTEN is subject to phosphorylation at the C-terminal serine–threonine cluster (Ser370, Ser380, Thr382, Thr383 and Ser385) that affects its phosphatase activity, subcellular localization and protein stability[15–17]. It has been proposed that phosphorylation of the C-terminal sites keeps PTEN in an inactive form in the cytoplasm; however, the dephosphorylation induces translocation of PTEN to the plasma membrane where the active PTEN antagonizes PI3K-AKT signalling[18]. PTEN inactivation through increased phosphorylation of the C-terminal serine–threonine cluster is observed in several malignancies, including T-cell acute lymphoblastic leukaemia (T-ALL)[19,20], and casein kinase II (CK2) has been proposed as a candidate protein kinase for the phosphorylation at these sites[21]. However, the molecular mechanisms responsible for this hyperphosphorylation remain poorly understood.

Here we identify N-myc downstream-regulated gene 2 (NDRG2) as a PTEN-associated protein that recruits protein phosphatase 2A (PP2A) to facilitate dephosphorylation of PTEN at the Ser380, Thr382 and Thr383 cluster. Our results indicate that genetic and epigenetic inactivation of NDRG2 in ATLL cells leads to increased phosphorylation of PTEN, resulting in decreased PTEN lipid phosphatase activity and subsequent enhanced activation of the PI3K-AKT pathway. Moreover, NDRG2-deficient mice show high levels of AKT phosphorylation in various tissues and spontaneous tumour development of various types, including T-cell lymphomas, providing the first in vivo evidence that NDRG2 functions as a tumour suppressor gene. Because downregulation of NDRG2 is reported in several types of cancers, the phosphorylation of PTEN at the Ser380/Thr382/Thr383 cluster via NDRG2 inactivation is one of the most important events leading to the dysregulation of PTEN function in cancer.

Results

NDRG2 is a candidate tumour suppressor gene in ATLL. We recently mapped 605 chromosomal breakpoints in 61 ATLL cases by spectral karyotyping and identified chromosome 14q11 as one of the most common chromosomal breakpoint regions[22]. To map the precise location of the 14q11 chromosomal breakpoints, we performed single-nucleotide polymorphism (SNP)-based comparative genomic hybridization on leukaemia cells from acute-type ATLL patients. In patients with chromosomal deletions at 14q11, the breakpoints frequently accumulated adjacent to the T-cell receptor alpha–delta chain locus (TCRα/δ), and a recurrent 0.9-Mb interstitial deletion was identified in a region including part of the TCRα/δ locus (Fig. 1a). Because tumour suppressors and oncogenes are commonly located at fragile sites[23], we analysed the expression of the 25 genes that map within the 0.9-Mb region in ATLL cells from seven acute-type ATLL patients and in CD4 $^+$ T lymphocytes from the peripheral blood of five healthy volunteers (references) using oligonucleotide microarrays (Supplementary Methods) (Affymetrix U133 Plus 2.0). NDRG2, a member of a new family of differentiation-related genes[24], and TCR delta variable 3 (TRDV3) were consistently downregulated in all seven ATLL samples compared with the five normal control samples from healthy volunteers (Fig. 1a). Furthermore, we used a genome-wide DNA methylation analysis in combination with microarray gene expression analysis to identify genes that are frequently methylated and silenced in ATLL. We identified 34 genes including NDRG2 that become methylated and transcriptionally repressed during ATLL leukaemogenesis (Supplementary Table 1, Supplementary Methods). Here, we investigated whether NDRG2 might be a 14q11 tumour suppressor candidate in ATLL. Quantitative reverse transcription-PCR (RT–PCR) analysis confirmed that NDRG2 expression was significantly reduced in a series of ATLL cell lines and primary acute-type ATLL samples when compared with HTLV-1-negative T-ALL cell lines and CD4 $^+$ lymphocytes from healthy volunteers, respectively (Fig. 1b). Treatment with an inhibitor of histone deacetylase, trichostatin A, and/or an inhibitor of DNA methylation, 5-aza-deoxycytidine, restored the expression of NDRG2 in most of the ATLL cell lines (Supplementary Fig. 1), suggesting that epigenetic modification is an important mechanism to reduce the expression of NDRG2 in ATLL cells. In accordance with DNA methylation array data, the CpG islands of the NDRG2 promoter were heavily methylated in three ATLL cell lines and primary ATLL cells from five acute-type ATLL patients, but not in three T-ALL cell lines and control CD4 $^+$ lymphocytes from five healthy volunteers, as revealed by bisulfite-sequencing analysis (Supplementary Fig. 2). Similarly, a methylation-specific PCR confirmed the hypermethylation of the NDRG2 promoter in the majority of ATLL cells (eight of eight ATLL cell lines and 34 of 34 primary samples) (Supplementary Table 2). No somatic mutations in the NDRG2 gene were observed in 34 primary ATLL samples and 42 cancer cell lines, including eight ATLL cell lines (Supplementary Table 3), except for previously reported SNPs (Supplementary Table 4). These data indicated that NDRG2 is frequently inactivated by genomic deletion and epigenetic silencing in ATLL.

The PI3K-AKT signalling pathway is activated in ATLL cells. Because the PI3K-AKT signalling pathway is constitutively activated in ATLL cells[4], we analysed PTEN and NDRG2 expression and the activation status of PI3K-AKT in primary ATLL cells from acute-type ATLL patients and in ATLL cell lines (Fig. 2a,b).
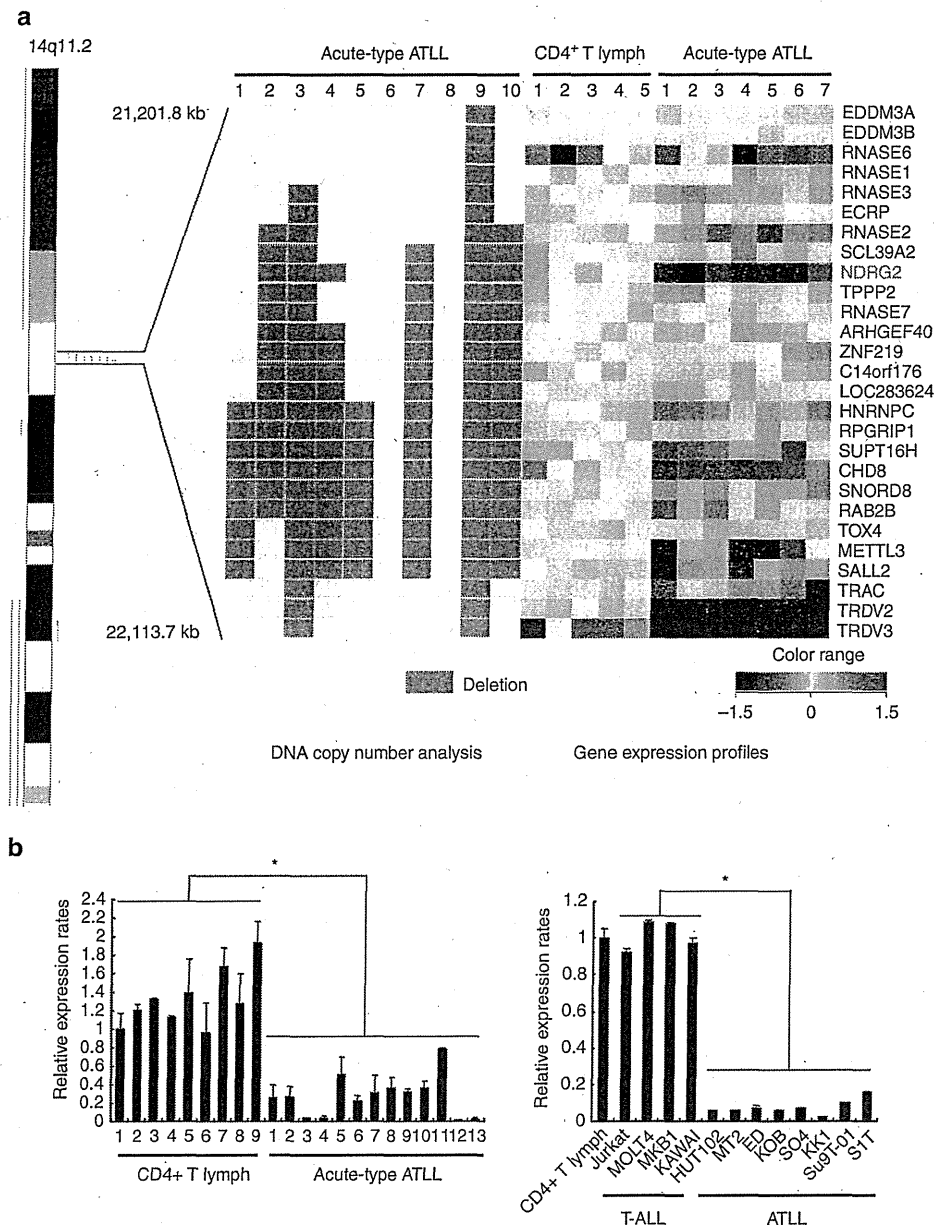
Figure 1 | NDRG2 is a candidate tumour suppressor gene in ATLL. (a) Genomic and gene expression analysis of chromosome 14q11. Results from the SNP array-based comparative genomic hybridization (CGH) analysis on chromosome 14 in ten samples from acute-type ATLL patients. Green bars represent loss of copy number and red bars represent gain of copy number. Out of the ten ATLL samples, eight had breakpoints on 14q11 clustered in a region of 0.9 Mb between genomic positions 21,201,800 and 22,113,700. A heatmap of the normalized gene expression measures for the 27 genes mapped to the recurrent breakpoint region in the CD4$^+$ T lymphocytes from five healthy volunteers and ATLL cells from seven acute-type ATLL patients is shown with the deletion map, in which columns represent samples and rows represent genes. A gradient of blue and red colours represent low- and high-relative fold changes of gene expression to the average expression in normal controls. Genes with average signal intensities less than 100 were eliminated. (b) Expression of NDRG2 mRNA in ATLL cells. Quantitative RT-PCR analysis of NDRG2 was performed with mRNA isolated from nine samples of CD4$^+$ T lymphocytes from healthy volunteers and 13 samples of ATLL cells from the patients, along with samples from four T-ALL cell lines and eight ATLL cell lines. The relative amounts of mRNA were normalized against β-actin mRNA and expressed relative to the mRNA abundance in healthy control sample 1. Mean ± s.d. is shown, *P<0.05 (Mann–Whitney U-test). More than a 50% reduction in NDRG2 mRNA expression was observed in 12 out of 13 cases (92%) of ATLL. Data are representative of two experiments.

The NDRG2 protein expression in ATLL cell lines and primary ATLL cells was significantly lower than that in T-ALL cell lines and CD4$^+$ lymphocytes from healthy volunteers, and AKT phosphorylation at Ser473 and Thr308, an indicator of AKT activation, was detected in the majority of the primary ATLL samples and ATLL cell lines. Because PTEN mRNA and protein were expressed at normal levels in all of the ATLL samples except the SO4 cell line (Fig. 2c), we performed a mutation analysis of the PTEN and PIK3CA genes. No mutations were detected within the coding and adjacent intronic regions of PTEN or in the hotspot mutation regions of PIK3CA in the ATLL samples (Supplementary Table 3). Therefore, we considered the possibility

that post-translational modifications of PTEN influence PTEN phosphatase activity in ATLL cells. The phosphorylation of PTEN at the serine–threonine cluster (Ser370, Ser380, Thr382, Thr383 and Ser385) in the C-terminal tail suppresses its phosphatase activity[17]; therefore, we evaluated PTEN phosphorylation and demonstrated that the cluster sites were highly phosphorylated in the primary ATLL cells and ATLL cell lines, whereas the

non-phosphorylated form was lower in abundance compared with controls (Fig. 2a,b; Supplementary Fig. 3). Notably, we also found that the SO4 cell line, which has a higher level of PTEN expression, contains high levels of phosphorylated PTEN and an elevated level of phosphorylated AKT (Supplementary Fig. 4). Furthermore, the expression levels of SHIP1, a different class of lipid phosphatase that catalyses PIP$_3$ to PI(3,4)P$_2$ (ref. 25),
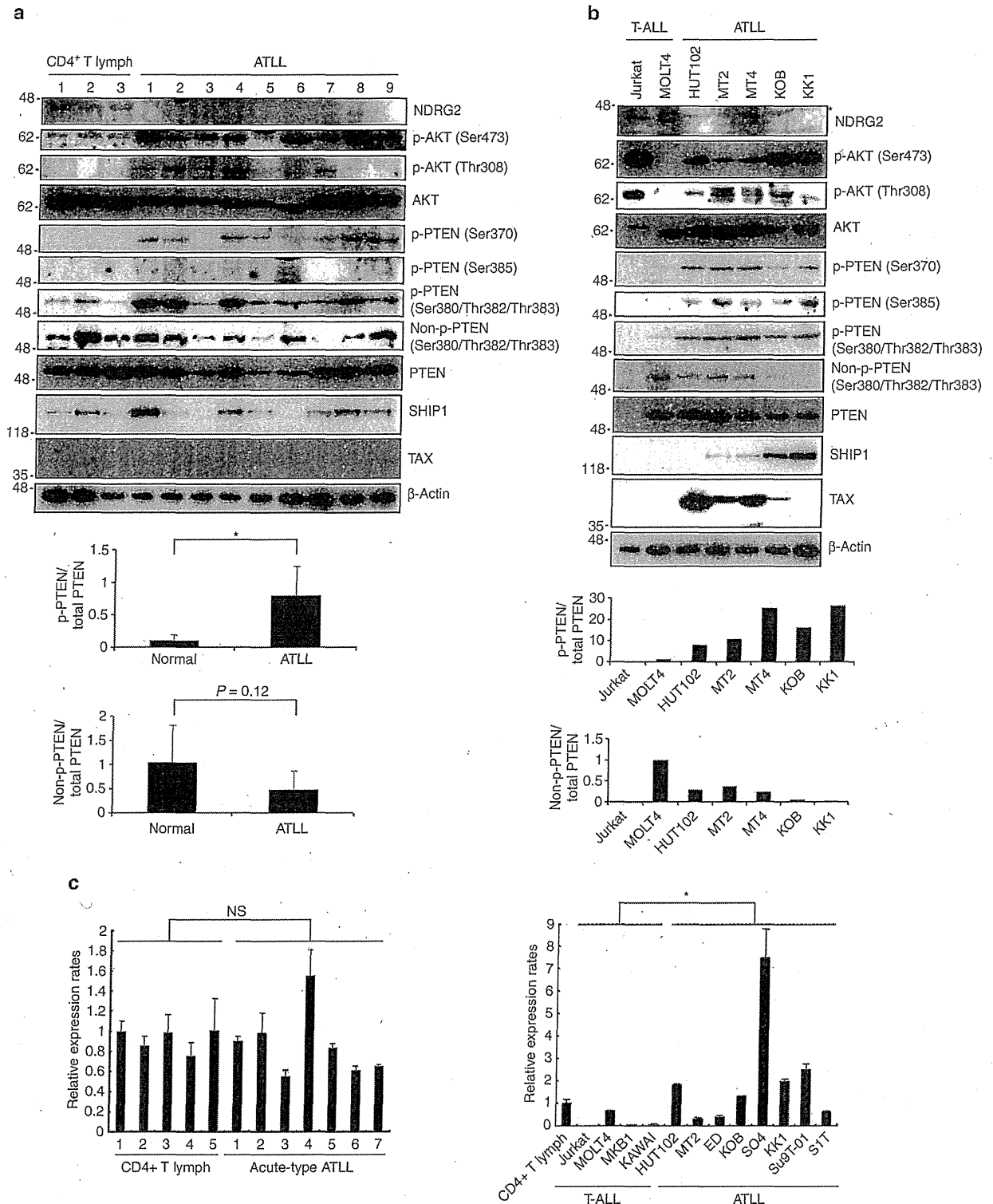
showed no significant differences between ATLL cells and controls, although a few cases showed higher levels of SHIP1 (Fig. 2a,b). Collectively, these results suggested that PI3K-AKT pathway activation in ATLL may be related to increased PTEN phosphorylation and potentially to the inactivation of NDRG2.

NDRG2 expression induces dephosphorylation of PTEN and AKT. To determine whether low NDRG2 expression was associated with increased PTEN phosphorylation and PI3K-AKT activation in ATLL cells, we introduced an NDRG2 expression vector into two ATLL cell lines (KK1 and HUT102). The phosphorylation of AKT-Ser473 and PTEN-Ser380/Thr382/Thr383, but not PTEN-Ser370 and PTEN-Ser385, was markedly decreased in both NDRG2-expressing cell lines (Fig. 3a; Supplementary Fig. 5a), although NDRG2 expression did not significantly change the basal activity of PI3K. (Supplementary Fig. 6). There was also an increase in the abundance of Ser380/Thr382/Thr383 non-phosphorylated PTEN in NDRG2-expressing cells (Supplementary Fig. 7). No change in SHIP1 expression was detected (Fig. 3a). In addition, the NDRG2-transfected cells exhibited decreased growth rates and a predominant nuclear localization of forkhead box protein O1 and O4 (FOXO1/4) (a marker of low AKT activity status) (Fig. 3b,c; Supplementary Fig. 5b). Furthermore, to determine whether NDRG2 expression may influence the tumour growth of transplanted ATLL cells in vivo, mock vector-transfected or NDRG2-transfected KK1 cells were injected intravenously into NOD/Shi-scid, interleukin (IL)-2Rg^{null} (NOG) mice. The average duration of life of 10 mice after inoculation with KK1-Mock cells was 67 days, but that of 10 mice inoculated with KK1-NDRG2 cells was extended to 207 days (Fig. 3d; Supplementary Fig. 8). Statistically significant difference in apoptotic cell rate was also found in the infiltrating neoplastic cells between KK1-Mock- and KK1-NDRG2-injected mice (Supplementary Fig. 9). To investigate these observations further, we performed the reverse experiment by inhibiting NDRG2 expression in the MOLT4 cell line. Transfection of the MOLT4 cell line with short hairpin RNA (shRNA) against NDRG2 enhanced the phosphorylation of PTEN-Ser380/Thr382/Thr383 and AKT-Ser473, which was accompanied by increased cell proliferation and a relocation of FOXO1/4 from the nucleus to the cytoplasm (Fig. 3e–g). We utilized the NIH3T3 mouse embryonic fibroblast cell line to confirm whether NDRG2 expression attenuates PTEN-Ser380/Thr382/Thr383 phosphorylation and AKT activation. After serum stimulation or transfection of a constitutively active PI3K mutant, we determined that the inhibition of NDRG2 expression resulted in increases in phosphorylated PTEN-Ser380/Thr382/Thr383 and AKT-Ser473 compared with the control cells (Supplementary Figs 10 and 11). In contrast, the forced expression of NDRG2 in NIH3T3 cells resulted in a dose-dependent decrease in PTEN-Ser380/Thr382/

Thr383 and AKT-Ser473 phosphorylation under conventional culture conditions (Supplementary Fig. 12). To establish a direct link between PTEN-Ser380/Thr382/Thr383 phosphorylation and PI3K-AKT activation, we constructed PTEN-S370A, -S380A/T382A/T383A and -S385A mutants in which Ser370, a Ser380/Thr382/Thr383 cluster and Ser385, respectively, were replaced with alanines. The forced expression of the PTEN-S380A/T382A/T383A mutant in HUT102 cells decreased AKT phosphorylation, cell growth and nuclear localization of FOXO1/4, whereas the expression of the PTEN-S370A or PTEN-S385A mutant had no significant effect (Fig. 3h–j). Treatment of ATLL cells with the CK2 inhibitors, 4,5,6,7-tetrabromobenzotriazole (TBB) and CX-4945, did not change the level of phosphorylated PTEN-Ser380/Thr382/Thr383, whereas the same treatment decreased PTEN-Ser370, a bona fide CK2 substrate[26], and AKT-Ser473 phosphorylation (Supplementary Fig. 13). These results indicate that the phosphorylation on PTEN-Ser380/Thr382/Thr383 has an important role in the activation of PI3K-AKT in ATLL cells, and that NDRG2 is able to reverse PTEN-Ser380/Thr382/Thr383 phosphorylation, leading to the suppression of PI3K-AKT activation.

NDRG2 is a novel PTEN-interacting protein. To determine whether NDRG2 physically associates with PTEN, lysates from KK1 cells stably expressing FLAG-tagged NDRG2 were immunoprecipitated with an anti-FLAG antibody, and NDRG2-associated proteins were analysed by western blotting with a PTEN-specific antibody. We found that ectopically expressed NDRG2 co-precipitated with endogenous PTEN in ATLL cells (Fig. 4a). Exogenously expressed green fluorescent protein (GFP)-tagged PTEN and FLAG-tagged NDRG2 co-precipitated in 293T cells (Fig. 4b), and endogenous NDRG2 and PTEN co-immunoprecipitated with each specific antibody and co-localized in the cytoplasm in MOLT4 cells (Fig. 4c,d), suggesting that these two proteins physically interact in T lymphocytes. By subjecting a series of deletion mutants to co-immunoprecipitation assays, a domain containing a putative α–β hydrolase fold (NDR domain) that is conserved among NDRG family members[27] was found to interact with the C-terminal half of PTEN, which contains a C2 lipid-binding domain (Supplementary Fig. 14). In addition, the S370A, S380A/T382A/T383A and S385A mutations in PTEN did not significantly interfere with the binding of NDRG2 (Supplementary Fig. 15), suggesting that NDRG2 can bind to both the phosphorylated and non-phosphorylated forms of PTEN.

NDRG2 recruits PP2A to dephosphorylate PTEN. To determine the mechanism by which NDRG2 expression induces PTEN dephosphorylation, we incubated KK1-NDRG2 cell lysates with a synthetic PTEN phosphopeptide containing the Ser380/Thr382/

Figure 2 | The PI3K-AKT signalling pathway is activated in ATLL cells. (a) A western blot analysis of NDRG2, PTEN, phosphorylated PTEN (p-PTEN) (Ser370, Ser380/Thr382/Thr383 and Ser385), non-p-PTEN (Ser380/Thr382/Thr383), SHIP1, AKT, p-AKT (Ser473 and Thr308) and Tax was performed in primary leukaemic cells from acute-type ATLL patients. The CD4$^+$ T lymphocytes from healthy volunteers (CD4$^+$ T lymph) served as the controls. No expression of Tax was detected in CD4$^+$ T lymphocytes from healthy volunteers or ATLL cells from acute-type ATLL patients. The graph shows relative band intensity of p-PTEN (Ser380/Thr382/Thr383) and non-p-PTEN (Ser380/Thr382/Thr383). Each p-PTEN or non-p-PTEN value was divided by the total PTEN value for a given sample. The data are expressed as the mean value ± s.d. (*$P<0.05$, Student's t-test). The data are representative of three experiments. (b) A western blot analysis with the same antibodies as in Fig. 2a was performed in the T-ALL and ATLL cell lines. Asterisk, nonspecific band. HTLV-1-transformed T-cell lines (HUT102, MT2 and MT4) expressed high levels of Tax. The graph shows relative band intensity of p-PTEN (Ser380/Thr382/Thr383) and non-p-PTEN (Ser380/Thr382/Thr383). Each p-PTEN or non-p-PTEN value was divided by the total PTEN value for a given sample. The data are representative of three experiments. (c) Quantitative RT-PCR analysis of PTEN in CD4$^+$ T lymphocytes from five healthy volunteers and ATLL cells from seven acute-type ATLL patients, along with four T-ALL cell lines and eight ATLL cell lines. The relative amounts of mRNA were normalized against β-actin mRNA and expressed relative to the mRNA abundance in healthy control sample 1. The mean ± s.d. is shown; *$P<0.05$; NS, not significant (Mann–Whitney U-test). The data are representative of two experiments.