determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Fifty micrograms of each cell lysate were electrophoretically separated on sodium dodecyl sulphate–polyacrylamide gel and transferred onto a nitrocellulose membrane using a semi-dry transblot system (Bio-Rad). After blocking, the membranes were incubated with the appropriate combination of primary and secondary antibodies as indicated, washed intensively, then examined using the enhanced chemiluminescence reagent system (ECL, Amersham Life Science, Buckinghamshire, UK). The results obtained from a 1-min exposure of the ECL-treated membrane to film are presented.

For the immunoprecipitation, 500 μg of the cell lysates was incubated with 1 μg of antibody and 50 μl of 50% protein-G agarose (Boehringer Mannheim Biochemica, Mannheim, Germany) for 1 hr. After intensive washing, the immunoprecipitates were separated by electrophoresis and analysed as described above.

To measure Ras activation, EZ-Detect™ Ras Activation Kits from PIERCE Biotechnology (Rockford, IL) were used according to the manufacturer's protocol.

Ca^{2+} mobilization assay

Intracellular levels of Ca^{2+} were measured by flow cytometry using Fluo 3-AM (Dojin, Kumamoto, Japan) after pre-BCR cross-linking with anti-μ antibodies. Ten million cells were washed and resuspended in 1 ml of OPTI-MEM containing 0·5% bovine serum albumin, and incubated with 1 mM of Fluo 3-AM for 30 min at 37°. After washing, the cells were resuspended in 10 ml of medium, stimulated by adding different concentrations of rabbit anti-human μ HC antibody as described in Figure 4 and the intracellular calcium concentration was measured by flow cytometry as described previously.[34] Calcium ionophore (ionomycin, Sigma-Aldrich Fine Chemicals, St Louis, MO) was used as the positive control. The data obtained were analysed using WINMDI software (distributed by Dr Joe Trotter) and presented as a kinetics line.

RESULTS

Expression of cell surface pre-BCR and BLNK in human pre-B-cell lines

It has been reported that some pre-B-cell lines express pre-BCR on their cell surface, even if not abundantly.[35] To identify cell lines that express pre-BCR on their cell surface, we tested a series of human pre-B-cell lines for surface expression of μ HC. Flow cytometry showed that all four cell lines tested, i.e. lines NALM-6, HPB-NULL, NALM-17, and P30/OHK, expressed μ HC on their cell surface, and that two of them, NALM-17 and HPB-NULL cells, expressed μ HC on their cell surface more abundantly than the others (Fig. 1a). To investigate the cell surface expression of pre-BCR, we examined reactivity to mAb against SL chains. The mAbs HSL11, HSL96 and HSL2 specifically recognize λ5, VpreB and the conformational epitope of pre-BCR, respectively.[30] When evaluated by flow cytometry, the cell lines stained positive with all three mAbs (Fig. 1a), but not with anti-κ and -λ mAb (data not shown), indicating that the pre-BCR is indeed expressed on the cell surface of some pre-B cell lines. It is noteworthy that although NALM-6 cells clearly reacted with both HSL11 (λ5) and HSL96 (VpreB), they revealed much weaker reactivity with HSL2 (conformational epitope of pre-BCR), for which the precise reason is unknown (Fig. 1a).

Since complete loss or drastic reduction of BLNK expression has also been reported in approximately 50% of childhood precursor-B ALL cases[29] we tested pre-B cell lines for expression of BLNK. Immunoblotting revealed abundant expression of BLNK by NALM-17 cells, whereas no BLNK expression was detectable in HPB-NULL cells (Fig. 1b). The NALM-6 and P30/OHK cells showed an intermediate amount of BLNK expression (Fig. 1b). We also investigated HPB-NULL and NALM-17 cells for intracellular BLNK expression by flow cytometry. When membrane-permeabilized cells were stained with fluorescein-labelled anti-BLNK mAb, clear expression of BLNK was observed only in NALM-17 cells (Fig. 1c), consistent with the results of immunoblotting. In contrast, when tested for expression of other B-cell-related signalling molecules, i.e. BTK, Syk, PLC-γ1 and PLC-γ2, by immunoblotting, all four molecules were comparably expressed in all four pre-B cell lines (Fig. 1b). Based on these findings, we decided to use NALM-17 and HPB-NULL cells in the following experiments as *in vitro* models of BLNK-positive and negative pre-B cells, respectively.

Phosphorylation of intracellular proteins induced by pre-BCR cross-linkage in both BLNK-positive and -negative pre-B cells

Next, we investigated whether cross-linking of pre-BCR with anti-μ antibodies would induce tyrosine phosphorylation of intracellular proteins in pre-B cell lines. Evaluation by immunoblotting with the anti-PY mAb, DA4.4 directed against human μ HC was found to cross-link pre-BCR strongly enough to induce tyrosine phosphorylation of intracellular proteins in HPB-NULL cells (Fig. 2a), but isotype-matched control mouse immunoglobulin did not (data not shown). Phosphorylation of the tyrosine residues peaked at 1–5 min after pre-BCR cross-linking and then decreased to a resting level by 30 min (Fig. 2a). Testing of other antibodies specifically reacting with μ HC, including the other clone of anti-μ HC mAb (AF6 and G20-127) and $F(ab')_2$ fragment of rabbit polyclonal anti-μ HC, yielded identical results (data not shown). Based on the results obtained under various conditions, incubation with 10 μg/ml of anti-μ mAb DA4.4 for 5 min was considered the optimal condition for pre-BCR cross-linking.

Next, immunoprecipitation and immunoblotting were used to identify signal transduction molecules located downstream in the pre-BCR signalling cascade in HPB-NULL cells. When Syk PTK (Fig. 2b) and Shc adapter molecules (Fig. 2c) were immunoprecipitated with specific antibodies, a pre-BCR-mediated increase in tyrosine phosphorylation was observed on anti-PY immunoblotting, and use of phospho-specific antibodies revealed that ERK MAP

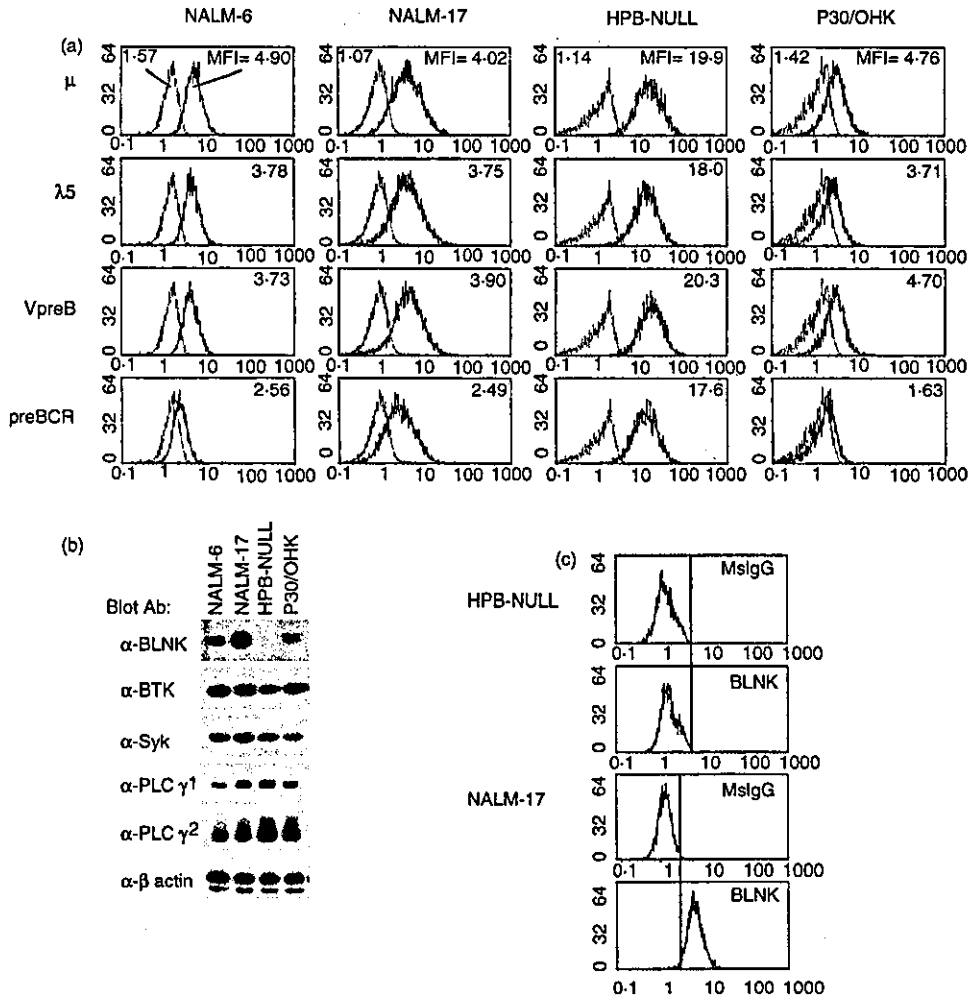

Figure 1. Expression of pre-BCR and BLNK in human pre-B-cell lines. (a) The pre-BCR-related molecules, i.e. μ heavy chain, $\lambda 5$, VpreB and conformational pre-BCR (preBCR), expressed on the cell surface of human pre-B cell lines, NALM-6, NALM-17, HPB-NULL, and P30/OHK, were stained with the specific monoclonal antibodies indicated and analysed by flow cytometry as described in the Materials and methods. The histograms obtained (solid lines) have been superimposed on those of the negative control (cells stained with isotype-matched control mouse immunoglobulin, IgG1 KOPC-31C, light broken lines). The mean fluorescence intensity (MFI) for each staining is presented in the top right hand corner of each panel. The MFI for each negative control staining is presented in the top left hand corner of the top panel of each cell line. The *x*-axis represents fluorescence intensity; the *y*-axis represents relative cell number. (b) Human pre-B-cell lines were tested for expression of BLNK and signal-transduction-related molecules by immunoblotting analysis with the specific antibodies indicated. For PLC-γ2 blotting, anti-PLC-γ2 monoclonal antibody (B-10) was used. (c) Human pre-B cell lines were tested for expression of BLNK by flow cytometry. Cells were permeabilized, stained with a combination of anti-BLNK monoclonal antibody and fluorescein isothiocyanate-conjugated secondary goat anti-mouse IgG, and analysed as described in the Materials and methods. As a negative control, cells were also stained with isotype-matched control mouse immunoglobulin (IgG2a G155-178, MsIgG).

kinase and MEK kinase were phosphorylated immediately after exposure to the anti-μ HC mAb DA4.4 (Fig. 2d). The time course of the phosphorylation state of MEK and MAP kinase showed kinetics similar to those of intracellular proteins detected with anti-PY mAb (Fig. 2a,d), and clear phosphorylation of PLC-γ1 and AKT was also detected in HPB-NULL cells after pre-BCR cross-linking (Fig. 3a). We also examined the activation of Ras, an upstream signalling molecule of ERK MAP kinase. As shown in Fig. 3(b), we observed an activation of Ras in HPB-NULL cells after pre-BCR cross-linking.

Similar testing of BLNK expressing NALM-17 cells revealed that pre-BCR cross-linking induced phosphorylation of these intracellular molecules as well as Ras activation that was as immediate and clear as we observed in HPB-NULL cells (Figs 3a,b). However, it is noteworthy that examination of phosphorylation of PLC-γ2 in the same manner showed that pre-BCR cross-linking induced phosphorylation of PLC-γ2 only in NALM-17 cells, and not in HPB-NULL cells (Fig. 3a).

A major mechanism by which BLNK has been proposed to regulate PLC-γ2 is through the juxtaposition of BTK and

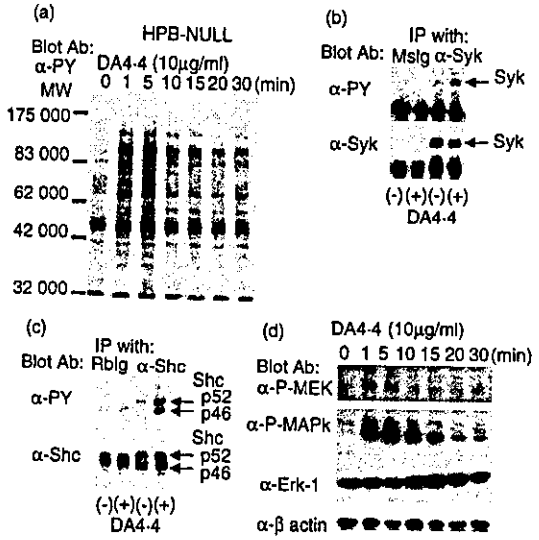

Figure 2. Increase in phosphorylation of intracellular proteins in HPB-NULL cells after exposure to anti-μ antibodies. (a) Immunoblot analysis with anti-phosphotyrosine (α-PY) monoclonal antibody was performed on cell lysates prepared from HPB-NULL pre-B cells exposed to 10 μg/ml of anti-μ mAb DA4.4 for the periods indicated. A molecular weight standard is indicated on the left. (b) Proteins immunoprecipitated with either isotype-matched control mouse immunoglobulin (MsIg, lanes 1 and 2) or mouse monoclonal antibody against Syk (α-Syk, lanes 3 and 4) from HPB-NULL lysates were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis in duplicate. After transfer onto a nitrocellulose membrane, the samples were analysed by immunoblotting with either anti-phosphotyrosine (α-PY) monoclonal antibody (upper panel) or anti-Syk monoclonal antibody (lower panel). Corresponding bands for Syk are indicated by arrows. (c) An experiment similar to that in (b) was performed using a combination of HPB-NULL lysates and rabbit polyclonal anti-Shc antibody (α-Shc). (d) The cell lysates prepared from HPB-NULL as in (a) were also tested by immunoblotting with antibodies indicated.

PLC-γ2.[3,20,21,28] Therefore we examined the complex formation between BTK and PLC-γ2 in both cell lines. As shown in Fig. 3(c), immunoprecipitation revealed that a portion of BTK was detected in anti-PLC-γ2 immunoprecipitates from the lysates prepared from both NALM-17 and HPB-NULL cells without pre-BCR cross-linking. Interestingly, the total amount of BTK protein precipitated with anti PLC-γ2 antibody was increased after pre-BCR cross-linking in NALM-17 cells, while pre-BCR cross-linking did not affect the amount of BTK protein precipitated with anti PLC-γ2 antibody in HPB-NULL cells (Fig. 3c).

Ca^{2+} influx does not occur in BLNK-negative cell lines after cross-linking of pre-BCR

Next, we investigated whether the cross-linkage of pre-BCR leads to an increase in intracellular Ca^{2+} level. Measurement of the intracellular Ca^{2+} level of HPB-NULL cells by flow cytometry with Fluo 3-AM showed no significant increase in intracellular Ca^{2+} level after exposure to anti-μ antibodies, while treatment with calcium ionophore led to a clear

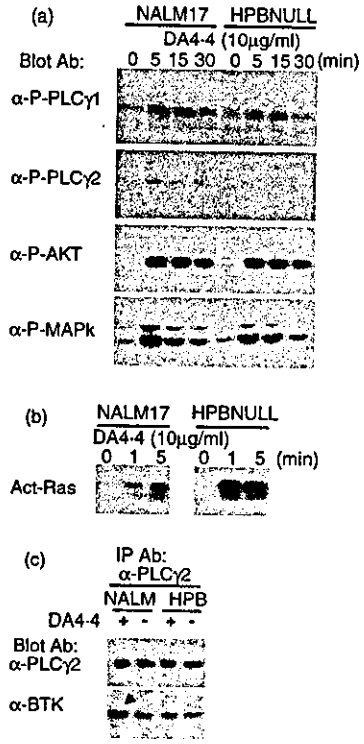

Figure 3. Pre-BCR-mediated signalling in pre-B cell lines. (a) Immunoblot analysis using phospho-specific antibodies on NALM-17 and HPB-NULL cells after exposure to anti-μ monoclonal antibody DA4.4. Cell lysates prepared from NALM-17 and HPB-NULL cells as in Fig. 2(a) were also tested by immunoblotting with the anti-phospho-specific antibodies indicated. (b) NALM-17 and HPB-NULL cells were exposed to DA4.4 and cell lysates were prepared as in (a). Active form of Ras (Ras-GTP) proteins were captured with Raf1-immobilized resin and detected by immunoblotting using anti-Ras antibody. (c) Cell lysates were prepared from NALM-17 and HPB-NULL cells treated with (+) or without (–) DA4.4 (10 μg/ml) for 5 min as in (a). Proteins were immunoprecipitated with rabbit anti-PLC-γ2 antibody from the cell lysates and were separated by SDS–PAGE in duplicate. Immunoblotting was performed with either mouse anti-PLC-γ2 monoclonal antibody (B-10, α-PLC-γ2, upper panel) or goat anti-BTK antibody (lower panel).

increase in the intracellular Ca^{2+} level (Fig. 4). By contrast, however, when NALM-17 cells, which express BLNK were examined, an increase in intracellular Ca^{2+} level after pre-BCR cross-linkage was observed under identical experimental conditions (Fig. 4). We therefore concluded that the cross-linking of pre-BCR failed to increase intracellular Ca^{2+} in pre-B cells that lack BLNK expression.

DISCUSSION

Several different groups have reported that 5–10% of BLNK-deficient mice spontaneously develop pre-B cell leukaemia/lymphomas expressing large amounts of pre-BCR on their surface.[27,28] Injection of immunodeficient mice with a $BLNK^{-/-}$ pre-B-cell line has been found to result in the development of pre-B-cell leukaemia that was

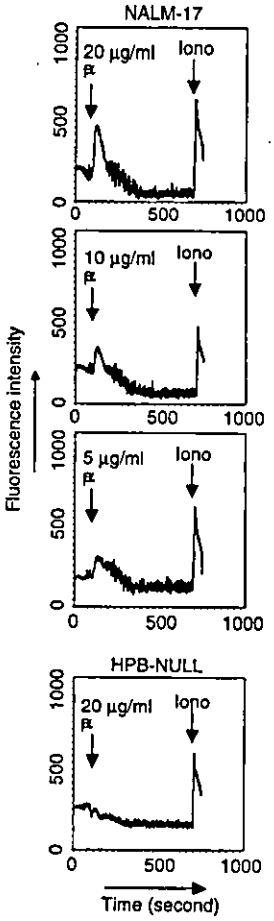

Figure 4. Flow cytometric analysis of Ca^{2+} mobilization in human B-cell lines. NALM-17 or HPB-NULL cells were loaded with Fluo 3-AM as described in the Materials and methods, and the intracellular Ca^{2+} levels were measured by flow cytometry. Rabbit anti-μ monoclonal antibody (α-μ, 5, 10, and 20 μg/ml) or the calcium ionophore ionomycin (Iono, 0·1 μg/ml) was added to the cells at the times indicated by the arrows. Other anti-μ antibodies were also used, and identical results were obtained (data not shown).

prevented by reconstitution of BLNK expression in $BLNK^{-/-}$ pre-B-cell line.[29] More important, it is also reported that 16 of 34 human childhood precursor-B ALL cases showed complete loss or drastic reduction of BLNK expression.[29] Thus, it was hypothesized that BLNK acts as a tumour suppressor and that somatic loss of BLNK and the accompanying block of pre-B-cell differentiation is one of the primary causes of childhood ALL.[27,28] Consistent with the above observations, the results of the present study demonstrated the existence of a human pre-B ALL cell line that lacks BLNK expression, and our findings indicate that the BLNK-deficiency phenotype in human precursor-B ALL cells is maintained in the cell lines established from these ALL cells.

The function of BLNK in pre-B cells is still a matter of controversy. Since, as stated above, reconstitution of BLNK expression in the $BLNK^{-/-}$ pre-B-cell line prevented the development of the leukaemia in immunodeficient mice, Jumaa *et al.* suggested that BLNK is essential to limiting pre-B-cell proliferation.[27,29] By contrast, Hayashi *et al.* observed that the pre-B cells that accumulate in BLNK-deficient mice are mostly non-cycling large pre-B cells, and they therefore concluded that BLNK is critical to pre-BCR signalling that induces proliferation of large pre-B cells.[28] Although the function of BLNK in pre-B ALL cell lines has not yet been clarified, the variable expression levels of BLNK in the pre-B ALL cell lines that we described in this study may mean that the level of expression of BLNK no longer critically affects growth and survival in established human pre-B ALL cell lines. In fact, we did not observe any significant difference in growth rate between the human pre-B ALL cell lines regardless of their BLNK expression level (data not shown).

In the present study we examined the downstream events mediated by cross-linking of pre-BCR in both BLNK-positive and -negative human pre-B-cell lines. The analysis with HPB-NULL cells that lack BLNK clearly indicated that the cross-linking of pre-BCR induces activation of Syk, Shc, Ras, ERK MAP kinase, and AKT, suggesting that the pre-BCR-mediated signalling in the MAP kinase pathway and the phosphatidyl-inositol 3 (PI3) kinase-AKT pathway do not require BLNK. In contrast, cross-linking of pre-BCR induced phosphorylation of PLC-γ2 and an increase in the intracellular Ca^{2+} level in NALM-17 cells alone, and not in HPB-NULL cells, suggesting that BLNK is essential to the pre-BCR-mediated Ca^{2+} influx via PLC-γ2 activation.

Kuwahara *et al.* analysed the downstream events of pre-BCR signalling in the human pre-B cell lines NALM-6 and 796 cells and reported a significant difference in comparison with the events mediated by the conventional BCR expressed on mature B cells, namely, less tyrosine phosphorylation of the cytoplasmic proteins, including Syk, and failure of Ca^{2+} mobilization.[35] However, the fact that the pre-B lines that they studied express only a small amount of pre-BCR on their cell surface may have been a limitation. Thus, their failure to detect signals following pre-BCR cross-linkage may have been because the signals were below the threshold of the detection system.[36] In fact, Nakamura *et al.* analysed the downstream signalling events after pre-BCR stimulation in $\mu\kappa$-transfected NALM-6 cells, which express larger amounts of the reconstituted BCR, and observed that cross-linkage of BCR on pre-B cells caused an elevation in intracellular Ca^{2+} that was qualitatively indistinguishable from the elevation following cross-linkage of BCR on mature B cells.[36]

In addition, we observed that NALM-6 cells clearly reacted with both anti-λ5 and anti-VpreB antibodies, while they revealed much weaker reactivity with antibody against conformational epitope of pre-BCR (Fig. 1a). Although the precise reason for the discrepancy is unknown, our finding may be related to the failure of Ca^{2+} mobilization by pre-BCR cross-linking in NALM-6 cells.

By contrast, our findings also indicated that cross-linkage of pre-BCR expressing on pre-B NALM-17 cells induces hyperphosphorylation of the tyrosine residues in numerous intracellular proteins as well as elevation of the intracellular Ca^{2+} level. Therefore, NALM-17 cells should

provide an *in vitro* model for investigation of the pre-BCR-mediated intracellular signalling mechanism. In addition, since HPB-NULL cells that lack BLNK also revealed similar signalling events induced by pre-BCR cross-linking, with some exceptions such as Ca^{2+} mobilization, comparison between these two cell lines could provide a means of analysing the function of BLNK in human pre-B cells.

BLNK was demonstrated to couple activated Syk to PLC-γ, Vav and Grb2, and participate in the BCR-mediated signalling in mature B cells.[19] Although BLNK has been suggested to also play important roles in pre-BCR signalling, the details remain poorly understood. Based on the molecular similarity of pre-BCR to BCR, however, BLNK is thought to play a role in the pre-BCR signalling system similar to that of BCR, and consistent with this, our results clearly indicated that BLNK is essential for pre-BCR-mediated PLC-γ2 activation and the following Ca^{2+} mobilization. By contrast, they also demonstrated that BLNK is unnecessary for the activation of MAP kinase and the PI3 kinase signalling pathway.

Based on models of transmembrane receptor tyrosine kinases,[37,38] recruitment of the Grb2 adaptor protein, which is associated with Sos GEF, to the plasma membrane was thought to be a likely mechanism for Ras and ERK activation by antigen receptor signalling. However, recent studies have shown that PLC-γ-Ras GRP plays a critical role in Ras activation in the TCR signalling context, in addition to the Grb2-Sos pathway.[39,40] It was also suggested that the latter pathway is dominant in DT40 chicken B cells, because a PLC-γ2-deficient DT40 mutant manifested a large decrease in BCR-mediated ERK activation, whereas it has less of an effect on ERK activation in Grb2-deficient cells, and ablating Sos2, a dominantly expressed Sos isoform in DT40 cells, barely affected ERK activation.[41] As we showed in this study, pre-BCR cross-linking effectively activated Ras and ERK in BLNK-deficient HPB-NULL cells in spite of the impairment of PLC-γ2 phosphorylation and the failure of an increase in complex formation between BTK and PLC-γ2. Thus, our findings may mean that PLC-γ2 activation is unnecessary for the activation of ERK MAP kinase in human pre-B cells. Alternatively, it is also possible that HPB-NULL cells possess a mechanism that partially compensates for the function of BLNK in pre-BCR-mediated signalling, thereby enabling pre-BCR cross-linking to induce ERK activation without PLC-γ2 activation. Interestingly, we observed that PLC-γ1 was expressed and phosphorylated by pre-BCR cross-linking both in BLNK-expressing and BLNK-deficient pre-B-cell lines. The data may indicate that PLC-γ1 and PLC-γ2 have distinct functions in pre-B-cell lines.

As stated above, BLNK-deficient mice have a block in B-cell development at the pre-B-cell stage, but the block is incomplete, and a small number of mature B cells are still present in the periphery.[23–26] This suggests that other signalling molecule(s) may partially neutralize the BLNK deficiency in B cells, and indeed, combined expression of adaptor proteins SLP-76 and LAT has been reported to reconstitute BCR function in $BLNK^{-/-}$ DT40 chicken B cells.[42,43] In addition, most recent reports have suggested that LAT and SLP-76 are involved in pre-BCR signalling and rescue arrested murine $BLNK^{-/-}$ pre-B cells.[44] Thus, alternative signalling molecule(s) that partially compensate for the function of BLNK may be present in HPB-NULL cells.

In conclusion, human pre-B-cell lines NALM-17 and HPB-NULL should provide an ideal *in vitro* model for analysis of pre-BCR-mediated signalling and the role of BLNK in pre-B cells. Further characterization including an analysis on the effect of transfection of the BLNK gene into HPB-NULL cells are clearly necessary. However, experiments using these cell lines will be of assistance in understanding the signalling mechanism in early B-cell development.

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Dietary bioflavonoids induce apoptosis in human leukemia cells

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Abstract

Dietary bioflavonoids are secondary metabolites of plants that are known to have a variety of bio-effects, including anti-cancer activity. In this study, we examined the effects of flavonoids on the growth of human leukemia cells and found that certain flavonoids induce apoptosis in a variety of human leukemia cells. The apoptosis induced by bioflavonoids was dose-dependent and was accompanied by a disruption of the mitochondrial transmembrane potential and the activation of caspase. Our data suggests that dietary bioflavonoids may be useful chemotherapeutic reagents for leukemia patients.

Keywords: Bioflavonoid; Apoptosis; Acute lymphoblastic leukemia; Precursor-B-cell

1. Introduction

Flavonoids are ubiquitously occurring and widely consumed secondary metabolites of plants [1,2]. Flavonoids can be divided into three main groups: Flavones, Flavonones (2,3-dihydroflavones), and isoflavones, which differ in structure and ring substitutions [3]. They have diverse pharmacological properties, including antioxidant, cytoprotective, and anti-inflammatory activities [1,2], and have also been reported to display anti-viral [4] and anti-parasitic [5] activities.

Moreover, some flavonoids are known to act as anti-cancer reagents. For example, Yoshida et al. reported that Quercetin markedly inhibited the growth of human gastric cancer cells [6]. Record et al. also described the inhibition of B16 melanoma cells by Genistein, both in vivo and in vitro [7]. Huang et al. demonstrated that Luteolin and Quercetin significantly inhibited the proliferation of epidermoid carcinoma A431 cells with an overexpression of epidermal growth factor receptor [8]. Indeed, some bioflavonoids like Quercetin and Genistein have already been used as chemotherapeutic agents in phase trials [9,10].

In an attempt to examine the effects of flavonoids on the growth of human leukemic cells, we challenged cultured human leukemic cell lines with several kinds of flavonoids. In the present study, we demonstrated that certain flavonoids can induce significant apoptosis in a variety of human leukemia cells.

2. Materials and methods

2.1. Cells and reagents

The cell line BV-173 that were established from a patient in an acute relapse who most likely had Ph1-positive chronic myelogenous leukemia [11]; the acute-phase of chronic myelogenous leukemia-derived cell lines K-562 (Japanese Cancer Research Resources Bank, JCRB, Tokyo, Japan)

Abbreviations: ALL, acute lymphoblastic leukemia; CD, cluster of differentiation; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PC-5, PE-Cy-5; PC-7, PE-Cy-7; topo, topoisomerase

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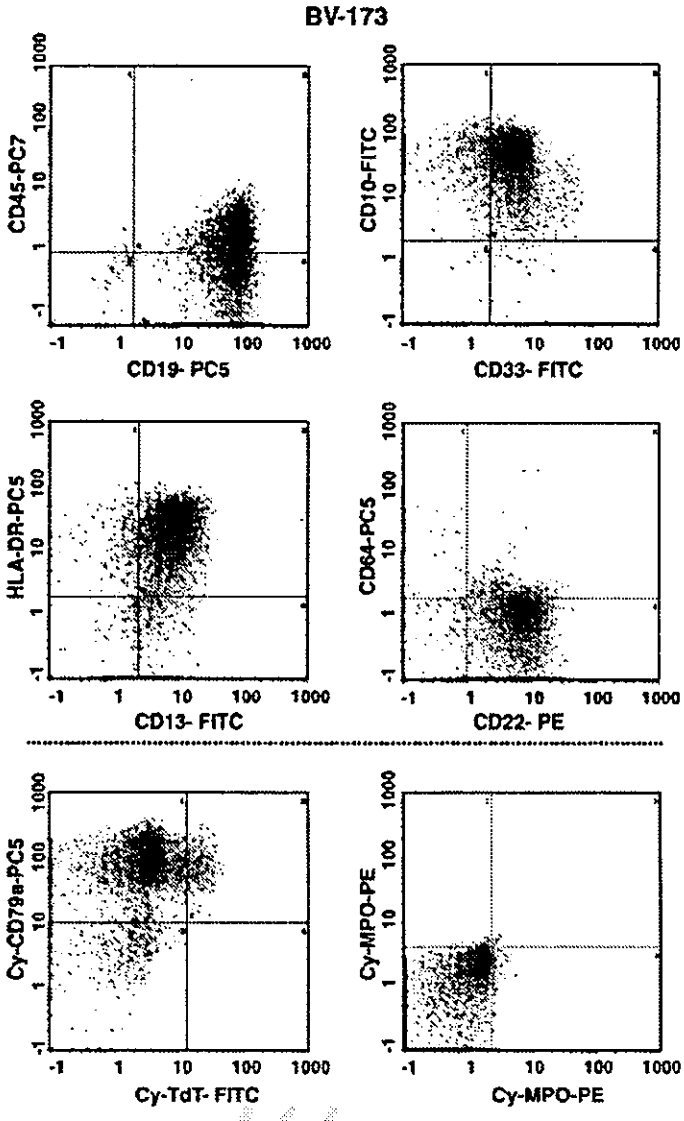

Fig. 1. Immunophenotypic analysis of BV-173 cells. The BV-173 cells were stained with fluorescence-labeled specific monoclonal antibodies against leukocyte antigens, as indicated, and analyzed by flow cytometry. The resulting histograms are shown. (Cy-) Cytoplasmic antigen stained after cell-permeabilization treatment.

and KU-812 (Institute for Fermentation, Osaka, Japan); precursor-B-acute lymphoblastic leukemia (ALL)-derived cell lines, including NALM-16, NALM-20, HPB-NULL and NALM-17 [12]; Burkitt's lymphoma-derived cell lines, Daudi and Ramos (JCRB); the histiocytic lymphoma-derived cell line U-937 (JCRB) and the acute monocytic leukemia-derived cell line THP-1 (JCRB) were used. Cells were cultured in RPMI1640 supplemented with 10% FCS at 37 °C in a humidified 5% CO_2 atmosphere.

Fluorescence-labeled monoclonal antibodies against leukocyte antigens were obtained from Beckman/Coulter Inc. (Westbrook, MA). Bioflavonoids, including Flavone, Genistein, Genistin, Quercetin, Fisetin, Luteolin, Apigenin and Rutin, and the anti-cancer drug VP-16 (which exhibits topoisomerase (topo) II-inhibitor activity) were purchased from Sigma–Aldrich (St. Louis, MO). The peptide-inhibitors for the caspases were obtained from Calbiochem-Novabiochem Co. (San Diego, CA). Bioflavonoids, VP-16 and the caspase inhibitors were desorbed in DMSO and then added to the cell cultures. All other chemical reagents were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), unless otherwise indicated.

2.2. Immunofluorescence study and detection of apoptosis

A multi-color immunofluorescence study was performed using a combination of fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy-5 (PC-5) and PE-Cy-7 (PC-7). Cells were stained with fluorescence-labeled monoclonal

antibodies and analyzed by flow cytometry (EPICS-XL, Beckman/Coulter), as described previously [13]. Staining of the cytoplasmic antigens was performed using Cytofix/Cytoperm™ Kits (Becton Dickinson), according to the manufacturer's protocol.

To quantitate the incidence of apoptotic cells, cells were stained with FITC-labeled annexin V using a MEBCYTO®-Apoptosis Kit (Medical & Biological Laboratories (MBL) Co. Ltd., Nagoya, Japan) and then analyzed by flow cytometry according to the manufacturer's protocol. Experiments were performed in triplicate, and the mean ± S.D. of the cells that bound annexin V are shown. Caspase-3 activity was assessed with a PhiPhiLUX™ G1D2 kit (MBL) and analyzed by flow cytometry according to the manufacturer's protocol. The disruption of the mitochondrial transmembrane potential was detected by the MitoCapture Apoptosis Detection Kit (MBL) and analyzed by flow cytometry according to the manufacturer's protocol.

2.3. Examination of morphological appearance

BV-173 cells were immobilized onto glass slides with Cytospin 2 (Shandon Inc., Pittsburg, PA), Giemsa-stained, and their morphological appearance was examined by light microscopy (BX-61, Olympus, Tokyo, Japan).

3. Results

3.1. Immunophenotypic analysis of BV-173 cells

First, we examined the cell surface and cytoplasmic antigens expressed in BV-173 cells originally derived from a patient with Ph1-positive acute leukemia. As shown in Fig. 1, the BV-173 cells expressed B-cell antigens, such as cluster of differentiation (CD)19, CD22 and cytoplasmic CD79a, as determined by flow cytometry. Together with the expression of CD10 and HLA-DR (Fig. 1) and the absence of surface IgM (data not shown), the cell line was thought to have originated from a precursor-B-cell. However, flow cytometric analysis also revealed that the BV-173 cells simultaneously expressed myeloid antigens, including CD13 and CD33 (Fig. 1). Therefore, BV-173 was thought to exhibit biphenotypic leukemia characteristics with both precursor-B-cell and myeloid lineages. This cell line was mainly used in the following experiments.

3.2. Dietary bioflavonoids induce apoptosis in BV-173 cells

Next, we tested whether the administration of dietary bioflavonoids induced any cytotoxic effects on BV-173 cells. When BV-173 cells were treated with 200 μM of Flavone for 24 h and then examined morphologically by light microscopy, a portion of the cells exhibited condensation (arrow-head) and cleavage (arrow) of the nuclei, findings that are typical of apoptosis (Fig. 2). No such figures were observed in untreated cells (Fig. 2). The following results clearly show that the administration of Flavone indeed induced apoptosis in BV-173 cells. First, DNA prepared from BV-173 cells treated with Flavone for 24 h showed oligonucleosomal ladder fragmentation on agarose gel electrophoresis (Fig. 3). Second, the number of cells binding to annexin V increased significantly after Flavone-treatment (Fig. 4). As shown in Fig. 4, other bioflavonoids, including Luteolin, Genistein, Quercetin, and

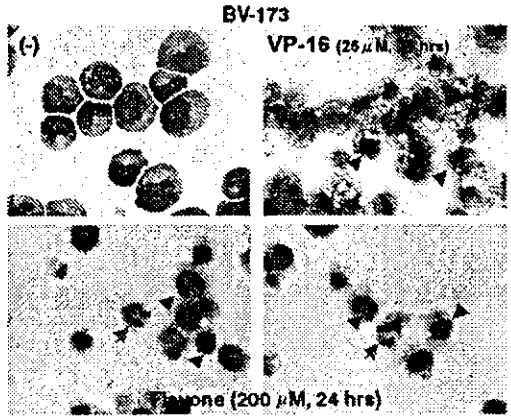

Fig. 2. Morphological examination of Flavone-treated BV-173 cells. BV-173 cells were cultured in the presence or absence of either Flavone or VP-16, as a positive control of apoptosis induction, for 24 h as indicated in the figure, then cytocentrifuged on the slide glasses. After Giemsa-staining, the morphological appearance of the cells was examined using light microscopy. The arrowheads indicate nuclear condensation. Typical apoptotic cells, characterized by cleaved nuclei, are indicated by the arrows. Magnification 400×.

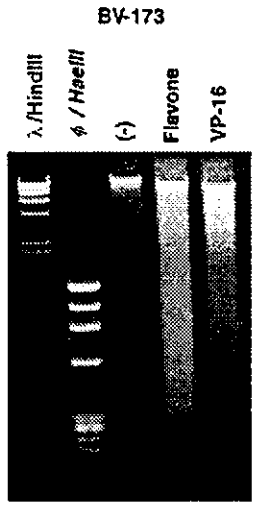

Fig. 3. DNA ladder formation in BV-173 cells after Flavone-treatment. BV-173 cells were treated with (lane 4) or without (-, lane 3) 200 μM of Flavone for 24 h. The extracted DNA (1.5 μg per lane) from each sample was separated by 1% agarose gel electrophoresis. The λ/*Hin*dIII and φ/*Hae*III DNA markers were applied to the same gel (left two lanes). As a positive control for DNA ladder formation, DNA extracted from BV-173 cells treated with 25 μM of VP-16 for 24 h was also examined (VP-16, lane 5).

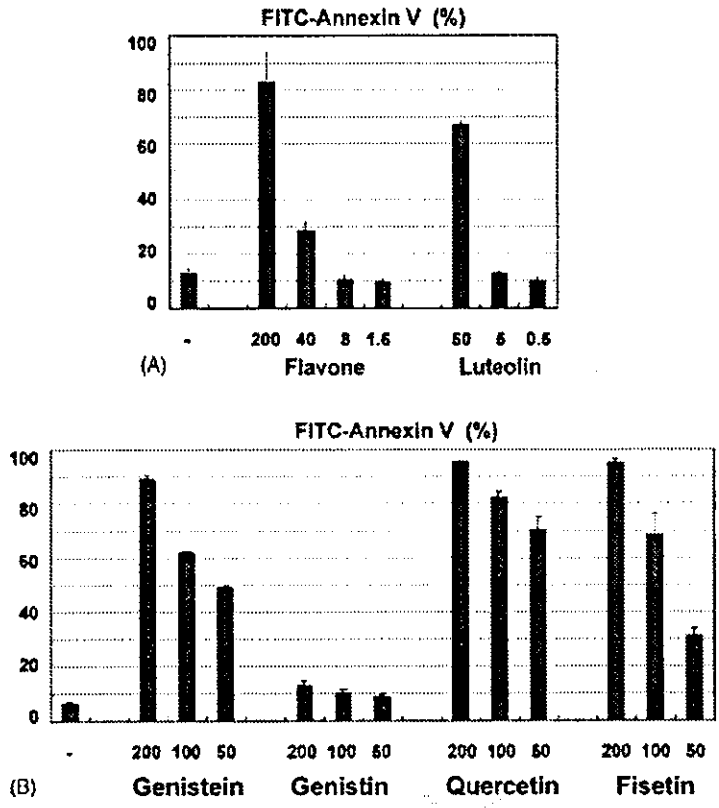

Fig. 4. Detection of annexin V binding cells after flavonoid-treatment. After culturing for 24 h in the presence of the indicated concentrations of each flavonoid, BV-173 cells were incubated with FITC-conjugated annexin V and then analyzed by flow cytometry. Experiments were performed in triplicate and the means + S.D. of the percentages of annexin V bound cells are indicated.

Fisetin, but not Genistin, also induced an increase in the number of cells binding to annexin V.

3.3. Characterization of bioflavonoid-induced apoptosis

The apoptosis induced by bioflavonoids in BV-173 cells was further characterized. In healthy cells, MitoCapture, a cationic dye, accumulates and aggregates in the mithocondria, giving off a bright red fluorescence. When the mitochondrial transmembrane potential is disrupted, however, this dye remains in the cytoplasm in its monomoer form, fluorescing green. As shown in Fig. 5, flow cytometric analysis revealed that treatment with Flavone significantly increased the number of cells with green fluorescence indicating that the mitochondrial transmembrane potential was disrupted after the induction of Flavone-induced apoptosis. In addition, Luteolin and Apigenin, but not Rutin, also disrupted the mitochondrial transmembrane potential (Fig. 5). Next, we examined the activation of caspase-3 in the process of bioflavonoid-induced apoptosis. Flow-cytometric analysis with PhiPhiLux^{TM} G1D2 indicated that treatment with Flavone significantly increased the number of cells in which caspase-3 was activated (Fig. 6). The incidence of caspase-3-activated cells induced by Flavone-treatment was much higher than that induced by VP-16-treatment (Fig. 6). Luteolin and Apigenin also increased the number of cells in which caspase-3 was activated (Fig. 6).

We further examined whether the activation of caspase was indeed involved in the bioflavonoid-induced apoptosis. As shown in Fig. 7, when BV-173 cells pretreated with either z-DEVD-fmk (a tetrapeptide inhibitor of caspase-3), z-IETD-fmk (a tetrapeptide inhibitor of caspase-8), or z-VAD-fmk (a tripeptide inhibitor of a broad range of caspases), a reduction in annexin V-positive cells after Flavone treatment was observed (Fig. 7). Of these inhibitors, z-VAD-fmk produced the most significant inhibition of Flavone-induced apoptosis in BV-173 cells.

3.4. Bioflavonoids induce apoptosis in other leukemia cell lineages

Next, we tested the effect of dietary bioflavonoids on other leukemia cell lines. As shown in Fig. 8, all of the leukemia cell lines tested in this study, including the pro-B cell lines NALM-16 and NALM-20, the pre-B cell lines HPB-NULL and NALM-17, the Burkitt's lymphoma cell lines Ramos and Daudi, the erythroleukemia cell line K-562, the basophilic leukemia cell line KU-812, the histiocytic lymphoma cell line U-937, and the acute monocytic leukemia cell line THP-1, were found to be sensitive to the apoptosis-inducing effect

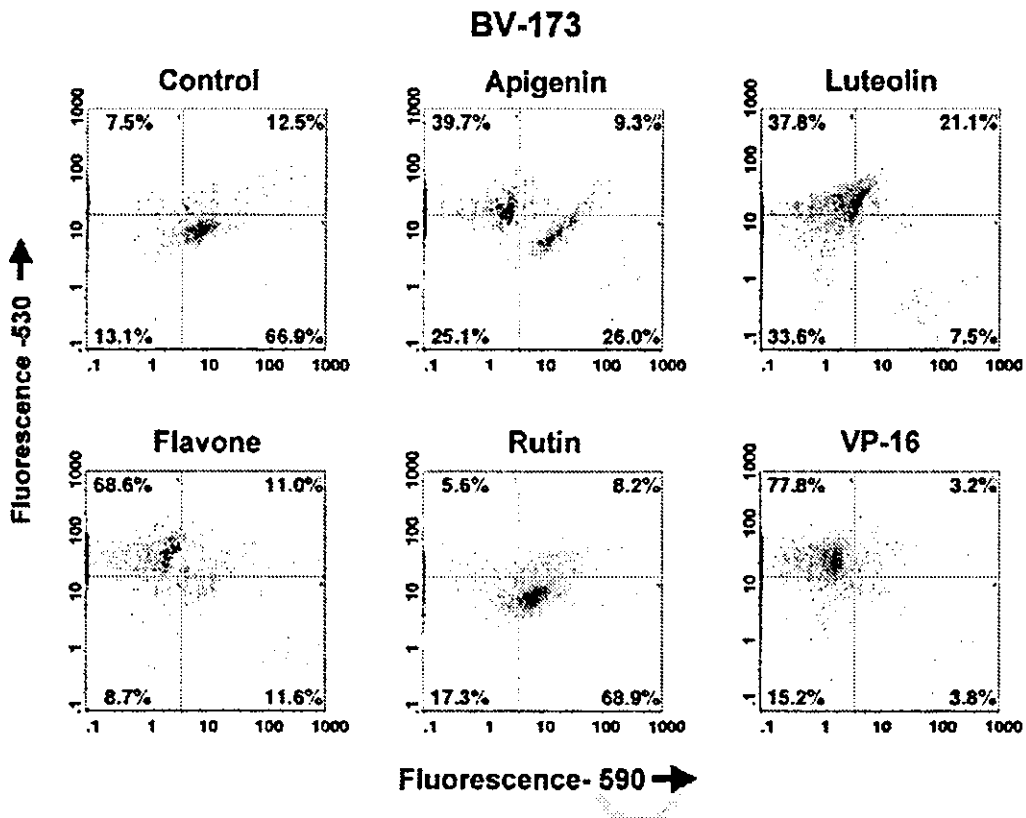

Fig. 5. Disruption of the mitochondrial transmembrane potential in BV-173 cells after bioflavonoid-treatment. BV-173 cells treated with or without flavonoids (Apigenin, 200 μM; Luteolin, 50 μM; Flavone, 200 μM; Rutin, 200 μM) or VP-16 (25 μM) for 24 h were examined using a MitoCapture Apoptosis Detection Kit and analyzed by flow cytometry. The resulting histograms are shown. *X*-axis, intensity of fluorescence -590 (red); *Y*-axis, intensity of fluorescence -530 (green). A shift in the fluorescence from red to green indicates the disruption of the mitochondrial transmembrane potential.

of dietary bioflavonoids. Among these cell lines, however, NALM-6 and K-652 exhibited a relatively lower sensitivity. Although the Burkitt's cell lines showed a limited sensitivity to VP-16 cytotoxicity, they showed a very high sensitivity to Flavone-mediated apoptosis induction.

4. Discussion

Our data clearly indicates that certain, but not all, bioflavonoids induce apoptosis in a variety of human leukemia cell types. As shown in the present study, Flavone, Luteolin, Genistein, Quercetin, and Fisetin induced significant apoptosis in BV-173 cells, while Genistin and Rutin did not. The apoptosis-inducing effect of Apigenin was intermediate. As demonstrated in the present study, all of the human leukemia cells that were tested were effectively induced to undergo apoptosis after bioflavonoid treatment. The bioflavonoid-induced apoptosis occurred in a dose-dependent manner and was accompanied by the disruption of the mitochondrial transmembrane potential and the activation of caspase-3 and perhaps caspase-8. Indeed, the apoptosis was diminished by pretreatment of the cells with anti-caspase inhibitors.

A number of studies have reported the potential ability of bioflavonoids to act as anti-cancer drugs. The precise mechanism of this phenomenon, however, remains unclear, although several effects of bioflavonoids on cell growth and cell death have been reported. For example, bioflavonoids are reported to have topo inhibitor activity. Luteolin is reported to inhibit both topo I and II and induces apoptosis in Leishmania cells [5,14]. Strick et al. [15] reported that certain bioflavonoids induce MLL gene cleavage through the inhibition of topo II.

Some topo II-inhibitors, such as VP-16 and doxorubicin, are widely used as anti-cancer reagents and have been linked with therapy-related leukemia induction due to topo II-inhibition. The effect of topo II-inhibiting substances on cells is thought to consist of two stages [16,17]. During the first stage, topo II-inhibitors stabilize topo II-cleavable complexes by forming drug:topo II:DNA ternary complexes on chromosomal DNA. This stage is reversible by DNA religation or by DNA repair. However, cellular processing of the accumulating ternary complexes triggers the initiation of apoptotic DNA cleavage, an irreversible process (secondary stage of the pathway). At this stage, it is reported that caspase-8 is activated through FADD/TRADD-dependent mechanism and plays a critical role in caspase-3 activation and apop-

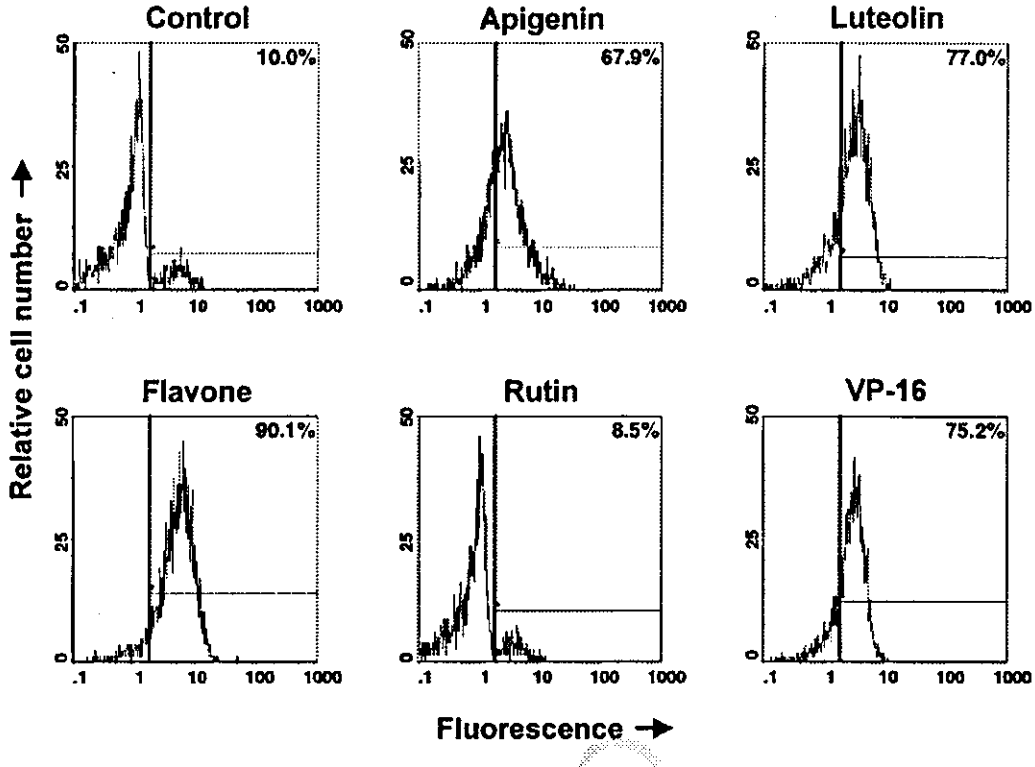

Fig. 6. Caspase-3 activity in BV-173 cells after bioflavonoid-treatment. To measure caspase-3 activity in BV-173 cells after bioflavonoid-treatment, cells prepared as in Fig. 5 were examined using PhiPhiLux™ GiD2 and analyzed by flow cytometry. The resulting histograms are shown. *X*-axis, fluorescence intensity; *Y*-axis, relative cell number.

totic cell death [18]. In parallel, caspase-9 is also activated by apoptosome-mediated mechanism as a result of mitochondrial dysfunction. However, latter pathway seems to play a much less role in caspase-3 activation [18]. Considering the above evidence, including similar activation pattern of caspase pathway (Figs. 6 and 7), it seems reasonable to assume that the anti-cancer effect of the bioflavonoids originated in their topo II-inhibitor activity.

However, as we presented in Fig. 8, Ramos Burkitt's cells, which were resistant to VP-16, were sensitive to Flavone-induced apoptosis. In addition, Strick et al. have reported that Luteolin exhibits more strong topo II-inhibitory effect than that of Flavone [15], whereas apoptosis-inducing effect of Flavone is not lower than that of Luteolin (Fig. 8). Therefore, the induction of apoptosis by flavonoids cannot be explained solely by their topo II-inhibitory effect, and additional possible anti-cancer effects may be involved.

Of note, K-562 cells have been reported to be topo II-resistant [19], whereas our data indicated that K-562 cells are sensitive to VP-16-induced apoptosis and show over than 70% annexin V-positive cells, similar to the flavonoids used. The precise reason for the discrepancy between the previous reports and our data is presently unclear. However, it is also reported that K-562 cells show delay in the VP-16-induced caspase activation in compared with HL-60 cells, leading to a long latent period before initiation of apoptosis, and once the active phase of apoptosis is initiated, a similar proportion of cells are ultimately killed in both cell lines [20]. Therefore, K-562 cells are not completely resistant to topo II and the sensitivity to VP-16-mediated apoptosis may vary among the stocks of K-562 cells in different laboratories.

On the other hand, some bioflavonoids, such as Genistein and Quercetin inhibited tyrosine kinase activity both in vitro and in vivo [21]. Since the overactivation of tyrosine kinases is thought to be involved in oncogenesis in many types of cancer, it seems reasonable that bioflavonoids

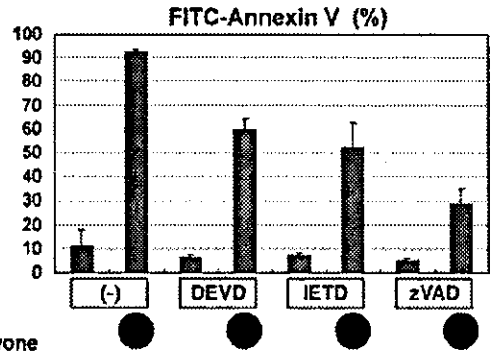

Fig. 7. Effect of caspase-inihibitors on Flavone-induced apoptosis. BV-173 cells pretreated with 50 μM of caspase-inihibitor, as indicated, were treated with 200 μM of Flavone for 24 h, as in Fig. 4. Subsequent apoptotic cells were detected by annexin V binding and analyzed by flow cytometry and are indicated as shown in Fig. 4.

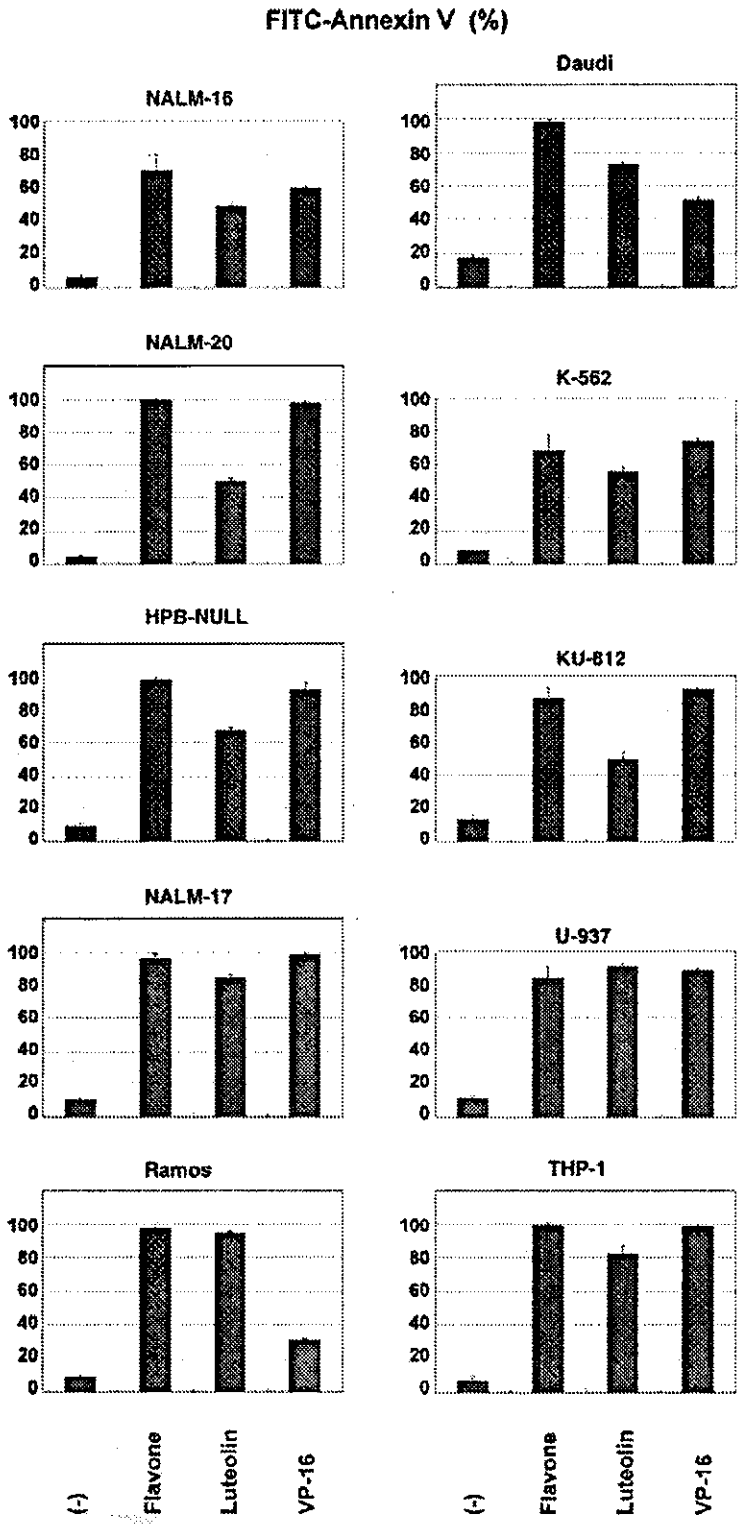

Fig. 8. Apoptosis-inducing effect of bioflavonoids in other leukemia cell lines. A variety of leukemia cell lines, as indicated in the figure, were treated with 200 μM of Flavone, 50 μM of Luteolin, or 25 μM of VP-16 (as a positive control for apoptosis induction), as in Fig. 4. Subsequent apoptotic cells were detected by annexin V binding and analyzed by flow cytometry and are indicated as shown in Fig. 4.

with anti-tyrosine kinase activity would exhibit an anti-cancer effect.

Bioflavonoids have also been reported to be involved in cell cycle regulation. For example, Quercetin was found to downregulate the expression of mutant p53 protein in human breast cancer cell lines, leading to an arrest of the cells in the G2–M phase of the cell cycle [21]. In the case of human leukemic T-cells, Quercetin was found to arrest the cells in late G1 phase. In addition, Luteolin has been reported to arrest the cell cycle in the G1 phase of human melanoma cells [22], and Genistein induces cell cycle arrest at the G2–M stage and the inhibition of cdc2 kinase activity [23]. The arrest of the cell cycle in turn reduces cell growth and results in apoptosis induction.

Another explanation for the anti-cancer activity of bioflavonoids is suggested by their ability to interact with hormone receptors [21]. Certain bioflavonoids have been reported to bind to estrogen binding sites in estrogen receptors, there by interrupting estrogen binding. Indeed, flavonoids, such as Daidzein, Genistein, Quercetin, and Luteolin, were found to suppress the induction of the proliferation-stimulating activity of environmental estrogens in human breast cancer cell lines [24]. However, whether estrogen binding induces the proliferation of leukemic cells has not been reported. Alternatively, flavonoids may interact with the binding sites of growth factors other than estrogen, thereby inhibiting the growth of leukemia cells.

In conclusion, dietary bioflavonoids exhibited an apoptosis-inducing effect in various human leukemia cells. Although further studies must be performed to elucidate the mechanism by which bioflavonoids induce apoptosis in leukemia cells, the present data indicates that dietary bioflavonoids might be useful chemotherapeutic reagents for leukemia patients.

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Contributions: J. Mat-mi contributed to the concept and design, interpreted and analyzed the data, provided drafting of the article, and gave final approval. N. Kiyokawa contributed to the concept and design, interpreted and analyzed the data, provided drafting of the article, and gave final approval, and obtained a funding source. H. Takeaouehi interpreted and analyzed the data, provided critical revisions and important intellectual content. T. Taguchi and K. Sutuki interpreted and analyzed the data. Y. Shiozawa provided administrative support, provided critical revisions and important intellectual content. M. Saito, M. Saito and W.-R. Tang provided administrative support. H. Okitu provided critical revisions and important intellectual content. J. Fuiimoto provided critical revisions and important intellectual content, and obtained a funding source.

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ORIGINAL ARTICLE

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Toshiyuki Miyashita

Identification of a novel polymorphism involving a CGG repeat in the *PTCH* gene and a genome-wide screening of CGG-containing genes

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Abstract Mutations in the human homologue of the *Drosophila patched* gene (*PTCH*) are responsible for the hereditary disorder called nevoid basal cell carcinoma syndrome (NBCCS). *PTCH* has a CGG triplet repeat located 4 bp upstream of the first methionine codon. Here we report a novel polymorphism involving the number of the CGG-repeat. The major allele (86.3%) contained a repeat size of seven, whereas the minor allele contained eight. No significant difference in the distributions of genotypes was observed between normal and NBCCS individuals. However, when the repeat was inserted between a heterologous promoter and the luciferase gene, the longer repeats tended to induce higher luciferase activities, suggesting that the repeat length potentially affects the levels of gene expression. A genome-wide screening revealed that 68 and 146 genes contained a CGG/CCG repeat in the coding region and in the 5'-untranslated region (5'-UTR), respectively. None of the genes had this repeat in 3'-UTR. Interestingly, the number of genes with a CGG repeat in the 5'-UTR was significantly higher than that with a CCG repeat in the 5'-UTR. The localization of a CGG/CCG repeat in *PTCH* is quite unique in that only four other genes have been found in which the repeat is localized up to 4 bp upstream of the first methionine.

Keywords PTCH · Nevoid basal cell carcinoma syndrome · Gorlin syndrome · Polymorphism · Triplet repeat

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Introduction

Mutations in the *PTCH* gene are responsible for the hereditary disorder called nevoid basal cell carcinoma syndrome (NBCCS; MIM# 109400) (Hahn et al., 1996; Johnson et al., 1996). NBCCS, also called Gorlin syndrome, is an autosomal dominant neurocutaneous disorder characterized by developmental abnormalities and tumorigenesis such as palmar and plantar pits, jaw cysts, calcification of the falx cerebri, skeletal anomalies, basal cell carcinoma, ovarian fibroma, and medulloblastoma (Gorlin, 1987). *PTCH* (MIM # 601309) is a human homologue of the *Drosophila* segment polarity gene *patched*. It has been mapped to 9q22.3-q31 and consists of 23 exons encoding a protein with 1,447 amino acid residues. The PTCH protein is a receptor for a secreted molecule Sonic hedgehog and has twelve transmembrane domains. At least two forms of PTCH protein are known to exist, reflecting the use of alternative exon 1a versus 1b (Hahn et al., 1996; Wicking et al. 1997a). Mutations in exon 1b have not been investigated so far due to, at least in part, the extreme GC-rich sequence (Wicking et al. 1997a; Fujii et al. 2003a). In the course of analyzing mutations in exon 1b, using a new set of primers and a PCR condition, we discovered a novel polymorphism involving a CGG trinucleotide repeat immediately upstream of the first in-frame methionine codon. We compared allele frequencies between healthy individuals and NBCCS patients. We also investigated the effect of the repeat length on the gene expression using a heterologous reporter gene. In addition, the results of a genome-wide screening of CGG/CCG-containing genes are demonstrated.

Materials and methods

DNA samples

After informed consent was obtained from 51 healthy, unrelated individuals and 14 patients with NBCCS, total genomic DNAs were isolated from peripheral leukocytes by the standard phenol/

chloroform extraction method. Patients were diagnosed as having NBCCS according to the clinical criteria (Kimonis et al. 1997). All studies were approved by the local ethnic committee. Among 14 patients with NBCCS, *PTCH* mutations were found in 11. Some of the mutations have already been reported (Fujii et al. 1999, Fujii et al. 2003a, Fujii et al. 2003b) and some will be reported elsewhere.

Polymerase chain reaction and sequencing

The genomic region of *PTCH* including the 5'-untranslated region (5'-UTR) and exon 1b was amplified by using the forward primer, 5'-CGCGCAATGTGGCAATGGAA-3', and the reverse primer, 5'-AGAGGAGGGAAGAGAAAGTG-3'. The polymerase chain reaction (PCR) was carried out in a 20 µl reaction volume by using LA Taq with GC Buffer (TaKaRa) according to the manufacturer's instruction. PCR was run for 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min on a Program Temp Control System PC-800 (ASTEC, Fukuoka, Japan). Both the sense and antisense strands of the PCR products were directly sequenced by using the same primers as described above. PCR products purified by a QIAquick PCR Purification Kit (QIAGEN) were used as the template DNA for cycle sequencing with a CEQ DTCS Quick-Start Kit (Beckman Coulter). Sequencing analysis was performed on a CEQ 8000 Genetic Analysis System (Beckman Coulter) according to the manufacturer's instructions.

Plasmid construction

Luciferase constructs containing the sequence of *PTCH* 5'-UTR were generated by a PCR-mediated method described previously (Imai et al. 1991) using pGV-P2 (Wako Chemicals, Osaka, Japan) as a template. The authenticity of all constructs was confirmed by sequencing.

Luciferase assay

The human embryonic kidney cell line 293 growing on six-well culture plates were cotransfected using Effectene reagent (QIAGEN) with 0.5 µg of luciferase plasmid and 0.5 µg of pCMVβGal. The cells were harvested at 24 h after the transfection and used for a luciferase assay. Luciferase activities were measured as described previously and normalized for transfection efficiency based on β-galactosidase activities (Shikama et al. 2001).

Real-time quantitative RT-PCR

Total RNA was extracted from the transfected cells described above using TRIzol reagent (Invitrogen). One-step RT-PCR was performed with a 7700 ABI PRISM Sequence Detector System (Perkin Elmer-Applied Biosystems) using primers 5'-TCTGGATCTACTGGTCTGCCTAA-3' and 5'-GCGCACTTTGAATCTTGTAATCCTG-3'. To normalize the expression of luciferase, the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) housekeeping gene was also amplified, using primers 5'-GAAGGTGAAGGTCGGAGT-3' and 5'-GAAGATGGTGATGGGATTTC-3'. Fluorogenic probes 5'-CAAATCATTCCGGATACTGC-3' and 5'-CAAGCTTCCCGTTCTCAGCC-3' carrying 5' 6-carboxy-fluorescein as a reporter dye and 3' 6-carboxy-tetramethyl-rhodamine as a quencher dye were used to detect the PCR product of luciferase and *GAPDH*, respectively. In every experiment, *GAPDH* was amplified using a series of dilutions of a known amount of the standard RNA supplied by Perkin Elmer to prepare a standard curve.

Computational screen

Human mRNA sequences that contain more than seven repeats of CGG were downloaded from NCBI nucleotide databases using the search program termed as "Search for short, nearly exact sequences" with $(CGG)_7$ as a query (http://www.ncbi.nlm.nih.gov/BLAST/). A full screening of the genes of interest was confirmed because genes with exact matches (bit score 42) were followed by the genes with partial matches (bit score less than 42). Genes for unidentified coding sequences were excluded from further study.

Statistical analysis

Genotype distributions and allele frequencies of CGG repeat numbers were compared between cases and controls by means of the χ^2 test. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated by Wolf's method.

Results and discussion

The *PTCH* gene has two alternative first exons—exon 1a and exon 1b (Fig. 1A). Exon 1b contains the first in-frame methionine codon, while exon 1a is a noncoding exon. We noticed a CGG trinucleotide repeat located 4 bp upstream of the first methionine codon. Although exon 1b is a coding exon, mutations in this exon have not been reported. In the course of analyzing mutations in exon 1b using samples from NBCCS individuals that do not have a mutation elsewhere in *PTCH*, we discovered a novel polymorphism involving the CGG trinucleotide repeat (Fig. 1B). The major allele contained

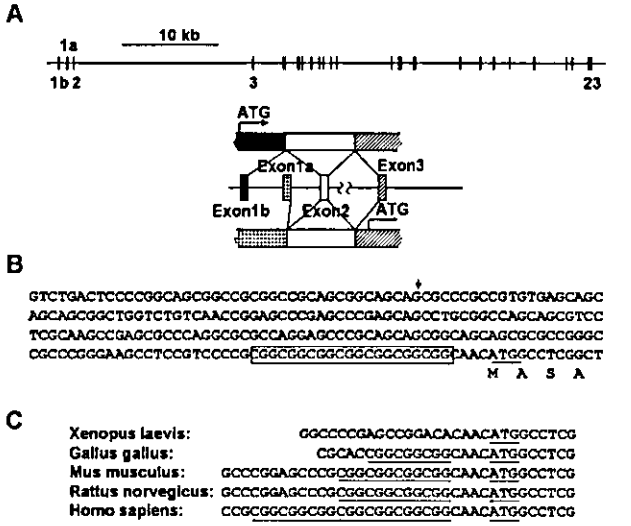

Fig. 1 A Genomic organization of human *PTCH*. The *PTCH* locus based on the sequence AL161729 is shown at the *top*. Two cDNA sequences, GenBank U43148 and U59464, are generated by alternative splicing using exon 1a and 1b, respectively, as schematically depicted at the *bottom*. **B** Nucleotide sequence of the human *PTCH* gene including 5'-UTR and exon 1b. The first methionine codon is *underlined*. Polymorphic CGG repeat is *boxed*. The putative transcription start site is indicated by an *arrowhead*. **C** Nucleotide sequence alignment of the *PTCH* genes. CGG repeat and the first methionine codon are underlined

seven repeats of CGG, while the minor one contained eight (Table 1). As far as we examined, we did not find repeat numbers other than seven or eight. The repeat is conserved among vertebrates, since chicken, mouse, and rat *PTCH* contain four or five repeats of CGG (Fig. 1C). However, the repeat has not been found in Xenopus *PTCH*, indicating it is not conserved in amphibians.

Abnormal expansion of the CGG triplet repeat in the 5'-UTR of the *fragile X mental retardation-1* (*FMR1*) gene is responsible for fragile X syndrome, in which the repeat is abnormally hypermethylated, resulting in the silence of the *FMR1* (reviewed by Jin et al. 2000). Since CGG repeat in *PTCH* is immediately upstream of the first in-frame methionine codon, the repeat number may influence the efficiency of translation as well as of transcription. To address this issue, various lengths of $(CGG)_nCAAC$ were subcloned into the luciferase plasmid pGV-P2 between the SV40 promoter and the coding sequence for luciferase (Fig. 2A), and luciferase assays were performed. Luciferase activities gradually increased with the number of CGG repeats, at least within the range we examined. The highest level of luciferase activity was obtained when cells were transfected with the plasmid pGV-$(CGG)_{19}CG(CGG)_6$, which was generated by chance during PCR reaction (Fig. 2B). These results suggest that individuals with $(CGG)_8/(CGG)_8$ have higher levels of PTCH protein expression than those with $(CGG)_7/(CGG)_7$. This is contradictory to the case of *FMR1*. However, it should be noted that in fragile X syndrome, the repeat is massively expanded over 230, and the repeat is located more than 50 bp upstream of the first methionine codon.

To address the question of whether the difference in luciferase activity is transcriptional or translational, the levels of luciferase RNA expression were quantified by a real-time RT-PCR. As shown in Fig. 2C, in contrast to the activities of luciferase, no significant difference in luciferase transcription was observed. Moreover, unexpectedly, the cells transfected with the plasmid pGV-$(CGG)_{19}CG(CGG)_6$ expressed significantly lower levels of luciferase RNA. Therefore, the increase in luciferase activities with the expansion of the CGG repeat is due to the increased efficiency of translation.

Table 1 Genotype data of a $(CGG)_n$ on the *PTCH* gene

	Controls	NBCCS
Major allele (repeat number: 7) [%]	88 [86.3]	25 [89.3]
Minor allele (repeat number: 8) [%]	14 [13.7]	3 [10.7]
Total	102 [100.00]	28 [100.00]
Major homozygous [%]	39 [76.5]	12 [85.7]
Heterozygous [%]	10 [19.6]	1 [7.1]
Minor homozygous [%]	2 [3.9]	1 [7.1]
Total	51 [100.0]	14 [100.0]
χ^2 [*P*]		
Genotype frequency (2×3 table)	1.38 [0.50]	
Allele frequency (major versus minor)	0.18 [0.68]	
Major homozygous versus others	0.56 [0.46]	
Minor homozygous versus others	0.26 [0.61]	
Odds ratio [95% CI]		
Major homozygous versus others	1.85 [0.36–9.43]	
Minor homozygous versus others	1.88 [0.16–22.44]	
Major allele versus minor allele	0.75 [0.20–2.83]	

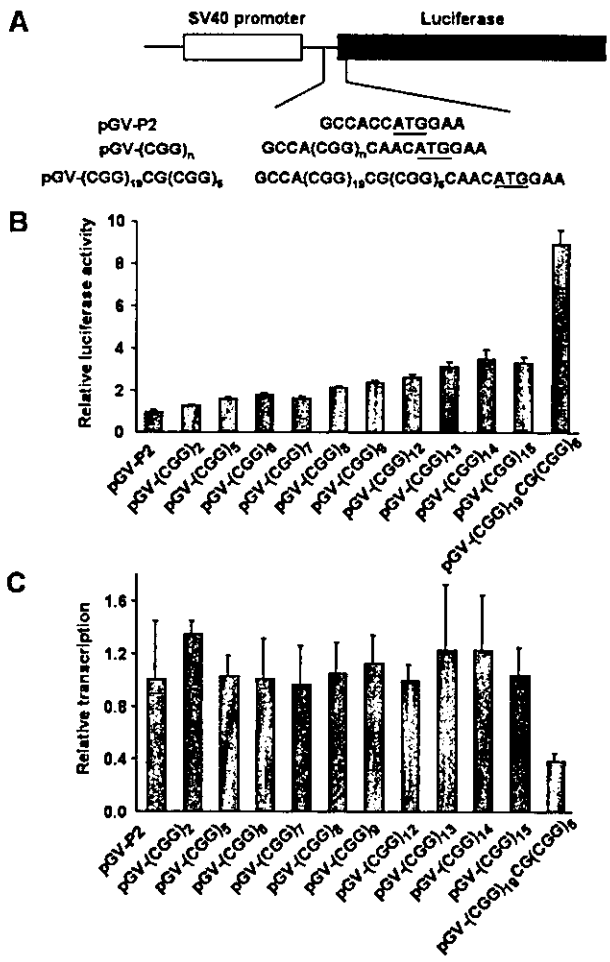

Fig. 2 A Schematic depiction of reporter gene constructs used for a luciferase assay. Nucleotide sequences inserted between SV40 promoter and the luciferase gene are indicated at the *bottom*. The first methionine codon of the luciferase gene is *underlined*. **B** The effect of the repeat length on luciferase activities; 293 cells transfected with plasmids indicated at the *bottom* were harvested 24 h after the transfection and subjected to a luciferase assay. **C** The effect of the repeat length on luciferase transcriptions. Total RNA was extracted from 293 cells transfected with plasmids indicated at the *bottom* and subjected to a real-time RT-PCR. Luciferase transcriptions were normalized by those of *GAPDH*

Table 2 CGG/CCG-containing genes. *UTR* untranslated region

	$(CGG)_n$	$(CCG)_n$	Total
CDS	39	29	68
5' UTR	95[a]	51[a]	146
Total	134	80	214

Genes with a repeat of seven or more CGG/CCG were downloaded from NCBI nucleotide databases:
[a]The number of CGG-containing genes are significantly higher than CCG-containing genes (χ^2 test, $P = 0.00027$)

Table 3 $(CGG)_n/(CCG)_n$-containing genes in which the triplet repeat is located immediately upstream of the first in-frame methionine codon

Accession no.[a]	Gene[b]	Nucleotides between $(CGG)_n/(CCG)_n$ and ATG	CGG or CCG	n
BC029158	Clone MGC:34313 IMAGE:5198758	CCCCCGGGGC	CCG	10
NM_025075	Hypothetical protein FLJ23445	CGCACGCC	CCG	8
BC015930	Clone MGC:8881 IMAGE:3920963	CGGGGGCC	CCG	8
NM_013396	Ubiquitin specific protease 25 (USP25)	CGGGGGCC	CCG	8
NM_013272	Solute carrier family 21 (organic anion transporter), member 11 (SLC21A11)	GGGAAGG	CGG	8
NM_005360	Transcription factor C-MAF (c-maf)	CAGGAGA	CGG	7
NM_004699	DNA segment on chromosome X (unique) 9928 expressed sequence (DXS9928E) (XAP-5)	CTGCC	CCG	9
NM_000264	PTCH	CAAC	CGG	7
NM_145296	TSLC1-like 2 (TSLL2)	CACC	CGG	7
NM_002958	RYK receptor-like tyrosine kinase (RYK)	CCC	CGG	7
NM_017811	Ubiquitin-conjugating enzyme E2R 2 (UBE2R2)	CG	CCG	7
NM_173054	Reelin (RELN)	C	CGG	8–10[c]

[a]One representative accession number for one gene
[b]Genes that have less than ten nucleotides between triplet repeat and the first methionine
[c]Repeat number varies depending on deposited sequences

The distributions of genotypes that we observed in NBCCS patients and controls did not differ from the expected frequencies under the assumption of Hardy-Weinberg equilibrium (data not shown), nor were significant associations with NBCCS observed (Table 1). Thus far, no genotype-phenotype correlation between the position of mutations and major clinical features of NBCCS is evident (Wicking et al. 1997b). Since developmental defects associated with the disorder are most likely due to haploinsufficiency, and the repeat length potentially alter the expression levels of PTCH, the repeat number may have an effect on the severity of the disease. It would also be interesting to examine the association of the repeat number with sporadic or non-inherited basal cell carcinoma or medulloblastoma, since PTCH acts as a tumor suppressor in these tumors (reviewed by Hunter 1997).

In order to find other genes with CGG repeats, we next performed a genome-wide screening of CGG/CCG-containing genes from NCBI nucleotide databases. A total of 214 genes having seven or more of the repeat number were downloaded. A complete list of the CGG/CCG-containing genes can be obtained from our Web site, http://genetics.nch.go.jp/supplements.htm. Of those 214 genes, 146 (68.2%) contained the repeat in the 5'-UTR (Table 2). Interestingly, significantly more genes have CGG repeats than CCG repeats (65.1% versus 34.9%, $P=0.00027$). More significantly, none of the downloaded genes contained repeats in the 3'-UTR. The genes containing CGG/CCG repeats in close proximity to their first methionine codons are listed in Table 3. Only five genes including *PTCH* have intervening sequences of up to 4 bp between $(CGG)_n/(CCG)_n$ and ATG. In this regard, *PTCH* is quite unique in terms of the location of the repeat. Considering our results, polymorphisms of the repeat number that might exist in these genes potentially affect their expression levels.

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