Table I. Characteristics and results of analysed cases.

No	WHO classification	At diagnosis or relapse	Clinical stage	Previous treatment	Prognostic index	Cytotoxicity (%)	CD22 (MFI)	P-gp MRK (%)	P-gp function PSC833 (%)	P-gp function MS209 (%)	Attenuation of fluorescence (%)
1	CLL	Recurrent	Rai (III) Binet(C)	FLU	NA	32	6·4	0·3	0·7	0·5	74
2	CLL	Recurrent	Rai (III) Binet(C)	FLU	NA	15·6	3·6	3·1	2·7	2·5	60
3	CLL	Recurrent	Rai (IV) Binet(C)	FLU, VCR, CPM,	NA	36·9	10·6	0·3	0·1	0	43
4	CLL	Recurrent	Rai (III) Binet(C)	FLU	NA	8·4	3·4	0·1	0	0	18
5	CLL	Diagnosis	Rai (I) Binet(A)	–	NA	32·8	8·9	0·2	0·5	0·3	59
6	DLBCL	Diagnosis	Ia	–	Low intermediate	18·9	1·8	0·6	0·8	0·7	47
7	DLBCL	Diagnosis	IIIa	–	High intermediate	6·3	2·4	7·8	10·3	13·9	41
8	DLBCL	Diagnosis	IA	–	Low intermediate	12·7	5·9	5·6	5·8	4·5	52
9	DLBCL	Diagnosis	Iia	–	Low intermediate	26·6	12·9	1·6	0·9	1·1	35
10	DLBCL	Diagnosis	IIIa	–	Low intermediate	18·9	3·8	0·7	0·5	0·2	41
11	DLBCL	Diagnosis	IIIa	–	High	15·7	4·4	0·9	1·7	2·5	57
12	DLBCL	Diagnosis	IVa	–	High	28·9	5·3	5·7	0·2	0·3	81
13	DLBCL	Relapse	IVa	R-CHOP	High intermediate	4·8	5·7	10·2	14·7	18·2	63
14	DLBCL	Relapse	IIIb	R-CHOP	High intermediate	11·1	1·8	0·7	1·5	1·1	72
15	DLBCL	Relapse	Ivb	R-CHOP	High	6·2	5·5	7·1	12·3	15·7	41
16	FL	Diagnosis	IIIa	–	High	21·0	1·7	1·3	1·1	0·3	17
17	FL	Diagnosis	II	–	Low	35·5	4·9	0·6	0·2	0·1	69
18	FL	Diagnosis	IVa	–	Intermediate	20·5	3·9	1·9	1·4	1·1	61
19	FL	Relapse	IVa	R-CHOP	High	5·6	1	12·3	17·4	9·5	50

WHO, World Health Organisation; CLL, chronic lymphocytic leukaemia; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; FLU, fludarabine; VCR, vincristine; CPM, cyclophosphamide; R, rituximab; CHOP, rituximab, adriamycin, VCR, CPM and predonisolone; MFI, mean fluorescence intensity; NA, not available.
Prognosis was determined according to the International Prognostic Index (IPI) or the Follicular Lymphoma International Prognostic Index (FLIPI).
Amount of P-gp was determined by the fluorescence of reacted MRK16 mAb compared to that of control mAb.
P-gp function was determined by the Rh123 accumulation in the presence or absence of MDR modifiers, PSC833 or MS209.
Attenuation of fluorescence, a marker of internalisation, was measured by the comparison of CMC-544 conjugated fluorescence before and after the incubation with CMC-544-free medium for 2 h. Low percentage indicates low internalisation activity.

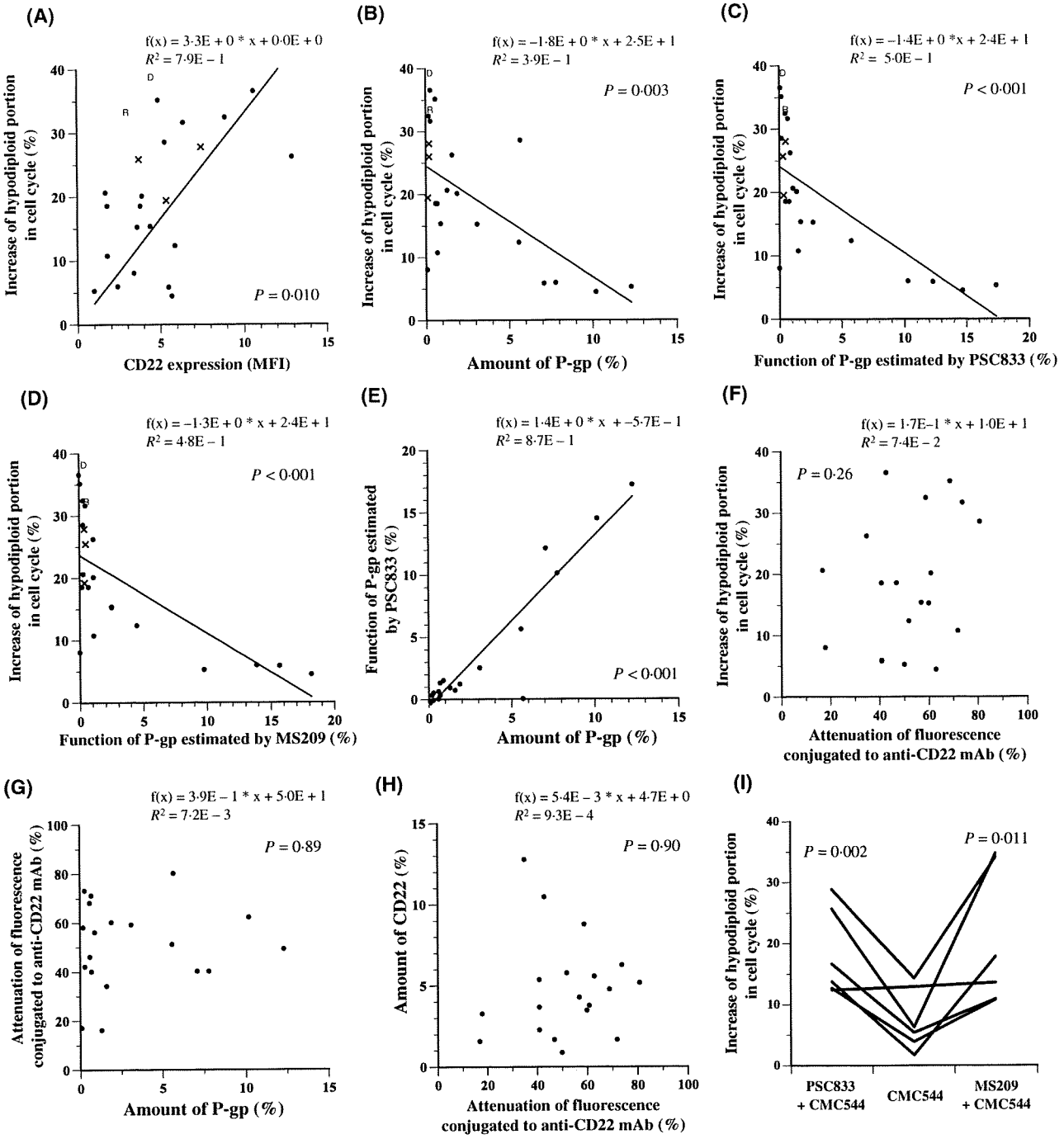

Fig 3. (A) Relationship between the *in vitro* effect of CMC-544 and the CD22 level, (B) the *in vitro* effect of CMC-544 and the amount of P-gp, (C, D) the *in vitro* effect of CMC-544 and the function of P-glycoprotein (P-gp) estimated by Rh123 accumulation with PSC833 (C) or MS209 (D). (F) The relationship between *in vitro* effect of CMC-544 and internalisation. The relationships between these factors were analysed by different combinations (E–H). Each dot represents the data summarized in Table I. The results of normal peripheral B-cells, Daudi and Raji cells were shown as cross dots, D and R respectively. (i) The *in vitro* effect of CMC-544 in the six P-gp positive samples was analysed in the presence of MDR modifiers, PSC833 or MS209, which restored the effect of CMC-544 significantly (P = 0·002 and P = 0·011 respectively). (ii) The *in vitro* effect of CMC-544 was determined by the increased hypodiploid portion (%) 72 h after incubation with CMC-544 and compared to that of G5/44.

pathways as well as oxidative stress defence through glutathione peroxidase and ATP-dependent drug efflux pumps (Friedenberg *et al*, 1999; Ohsawa *et al*, 2005; Matthews *et al*, 2006; Montserrat *et al*, 2006; Andreadis *et al*, 2007). The importance of these mechanisms will depend on agents adopted in the treatment. Although P-gp has played a limited role in the drug resistance of B-CLL and NHL, its level is closely related to the intracellular levels of calicheamicin, as

shown in the present study. Therefore, in case of using CMC-544 or other P-gp-dependent drugs, such as doxorubicin and vincristine, it will be important to analyse the level of P-gp and try to overcome its resistant mechanism.

In this study, we also tried to clarify the relationship between P-gp and the effect of CMC-544 using clinical samples from patients with B-CLL and NHL. CMC-544 was less effective on malignant cells from patients with a high amount of P-gp expression. CMC-544 is mostly used for the treatment of refractory or relapsed cases in earlier clinical studies. It should be understood that such patients often express more P-gp. Therefore, CMC-544 treatment alone may have only a limited effect on such patients. In order to achieve maximum effectiveness with this agent, treatments that utilize combination therapy with MDR modifiers or other mAbs may be a promising approach. Moreover, CMC-544 may be more effective when used as a front-line drug for CD22-expressing tumours, before the emergence of P-gp related resistance.

We have also observed the positive relationship between CD22 and the effect of CMC-544 in a study using clinical samples from patients with B-CLL and NHL. While the effect of CMC-544 related to the amount of CD22, the relationship was less prominent than that with P-gp. The cytotoxic effect of CMC-544 in P-gp negative samples varies considerably. Several factors, such as CD22 level, internalisation and other mechanisms might be related to the variation (Goemans *et al*, 2008). In fact, some of the cells that had low sensitivity for CMC-544, despite low P-gp levels, had a relatively low CD22 level or low internalisation. It is difficult to explain the variation in relation to these factors only. Samples from patients in the actual clinical study may clarify this point.

The present study has demonstrated that the effect of CMC-544 depends on the amounts of CD22 and P-gp. Thus, it will be possible to predict the clinical effect of this drug by measuring these parameters. Additionally, *in vitro* analysis of cell cycle effects and observation of apoptotic cells can support the analysis of relevant parameters. Phase I and II studies of CMC-544 are now in progress. This new agent will bring considerable therapeutic benefits to patients with CD22-positive B-CLL and NHL. Possible advantages of concomitant use with an MDR modifier should be discussed in clinical trials. Such an approach might bring an increase in efficacy with or without the worsening of associated adverse events.

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ORIGINAL ARTICLE

CMC-544 (inotuzumab ozogamicin), an anti-CD22 immuno-conjugate of calicheamicin, alters the levels of target molecules of malignant B-cells

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We studied the effect of CMC-544, the calicheamicin-conjugated anti-CD22 monoclonal antibody, used alone and in combination with rituximab, analyzing the quantitative alteration of target molecules, that is, CD20, CD22, CD55 and CD59, in Daudi and Raji cells as well as in cells obtained from patients with B-cell malignancies (BCM). Antibody inducing direct antiproliferative and apoptotic effect, complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) were tested separately. In Daudi and Raji cells, the CDC effect of rituximab significantly increased within 12 h following incubation with CMC-544. The levels of CD22 and CD55 were significantly reduced ($P<0.001$ in both cells) after incubation with CMC-544, but CD20 level remained constant or increased for 12 h. Similar results were obtained in cells from 12 patients with BCM. The antiproliferative and apoptotic effect of CMC-544 were greater than that of rituximab. The ADCC of rituximab was not enhanced by CMC-544. Thus, the combination of CMC-544 and rituximab increased the *in vitro* cytotoxic effect in BCM cells, and sequential administration for 12 h proceeded by CMC-544 was more effective. The reduction of CD55 and the preservation of CD20 after incubation with CMC-544 support the rationale for the combined use of CMC-544 and rituximab.
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Introduction

Rituximab, a chimeric monoclonal antibody (mAb) that binds to CD20, has greatly improved therapy for B-cell malignancies (BCM) including non-Hodgkin's lymphoma and chronic lymphoblastic leukemia.[1–4] Nevertheless, a considerable percentage of patients are refractory to treatment with rituximab and relapse after an initial response. Several resistant mechanisms have been proposed including escape into CD20-negative cells by limited surface antigen renewal, cell membrane drug efflux pumps, escape into the resting phase of the cell cycle, enhancement of complement inhibitory factors, alterations in intracellular signaling or cell death pathways, FcγRIIIA polymorphism and reduction of effector cells.[5] Above all, downregulation of CD20 and enhancement of complement inhibitory factors plays an important role in acquired resistance to rituximab. Investigation of complement inhibitory factors demonstrated that CD55 plays an important role as a regulator of complement-dependent cytotoxicity (CDC) in malignant B-cells, and that its expression correlated with resistance to CDC,[6,7] which is one of the main mechanisms of action in the treatment of rituximab.

To overcome resistance to rituximab, several new agents have been developed, including radioimmunotherapy and mAbs against targets other than CD20.[2,8,9] Among them, CMC-544 has been introduced as a promising agent to treat refractory/resistant BCM. CMC-544 is a conjugate of *N*-acetyl γ-calicheamicin dimethyl hydrazide (NAc γ-calicheamicin DMH) and a recombinant humanized antibody (IgG_4) directed against the CD22 antigen.[10] Calicheamicin, a very potent antitumor antibiotic agent, binds to the minor groove of DNA in a sequence-specific manner and breaks double-stranded DNA.[11] Preliminary data from ongoing clinical trials reveal that CMC-544 is efficacious against recurrent/refractory B-cell lymphomas with manageable thrombocytopenia reported as the most significant toxicity.[12]

Concomitant use of CMC-544 and rituximab is an ideal therapeutic method because these agents have a different target molecule and mechanism of action. In fact, the additive combination efficacy of CMC-544 and rituximab was shown against xenogenic BCM in severe combined immunodeficient mice.[13] However, the mechanism of the combination efficacy and the best administration schedule have not yet been elucidated. In this study, we attempted to clarify them from the viewpoint of the alteration of target molecules, that is, CD20, CD22, CD55 and CD59, which are essential for the action of these mAbs.

Materials and methods

Cells

CD22-positive cell lines used were: human lymphoma cell lines, Daudi and Raji, and their mdr-1 DNA-transduced sublines, Daudi/MDR and Raji/MDR.[14] Daudi/MDR and Raji/MDR had detectable mdr-1 messenger RNA (mRNA) and P-glycoprotein. The CD22-negative cell lines used were K562 (Riken Cell Bank, Tsukuba, Japan), Jurkat (Riken Cell Bank) and NB4 (kindly provided by Dr M Lanotte, Hospital Saint-Louis, Paris, France). These cell lines were cultured in RPMI-1640 supplemented with L-glutamine (2 mM), antibiotics and 10% fetal calf serum (FCS) (Gibco BRL, Grand Island, NY, USA) (10% FCS-RPMI) at 37 °C in a humidified 5% CO_2 incubator.

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After informed consent, malignant cells were obtained from 12 patients with BCM. Lymphocytes were collected from the peripheral blood of eight patients with chronic lymphoblastic leukemia. Lymphoma cells were separated from the lymph nodes of four patients with large B-cell non-Hodgkin's lymphoma, and purified by density gradient with Ficoll-Paque (Pharmacia, Uppsala, Sweden).

Flow cytometry for CD20, CD22, CD55 and CD59

For the detection of CD20, CD22, CD45, CD55 and CD59, cells were stained with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated anti-CD20, anti-CD22, anti-CD55 or anti-CD59 mAbs in addition to Cy7-conjugated anti-CD45 mAb (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), according to the manufacturer's instructions.[15] Ten thousand events were counted and mean fluorescence intensities (MFIs) were calculated using an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). All measurements were performed in triplicate.

Real-time reverse-transcription polymerase chain reaction (RT-PCR) assay for CD20, CD22, CD55, CD59 and GAPDH mRNA in Daudi and Raji cells

The levels of CD20, CD22, CD55, CD59 and GAPDH mRNA in Daudi or Raji cells were measured by real-time RT-PCR. In brief, total cellular RNA was isolated using an RNeasy Plus Mini kit (Qiagen, Tokyo, Japan). The extracted RNA was reverse transcribed by random primers and Super Script III reverse transcriptase (Invitrogen Japan, Tokyo, Japan). Complementary DNA (cDNA) fragments were amplified by PCR using designed specific primers: forward (5′-CTCTCTGGGGAGGCATTATGTA-3′) and reverse (5′-GTAACAGTATTGGGTAGATGGGGAG-3′) for CD20; forward (5′-CAGAATACATTCACGCTAAACCTG-3′) and reverse (5′-AACACTGGGGTTACTGGAATTGTA-3′) for CD22; forward (5′-TTCAGGCAGCTCTGTCCAGTG-3′) and reverse (5′-GAGGCTGAAGTGGAAGGATCG-3′) for CD55; forward (5′-CTGTGGACAATCACAATGGGAATGGGA-3′) and reverse (5′-GGTGTTGACTTAGGGATGAAG-3′) for CD59; and forward (5′-AAGGTCATCCCAGAGCTGAA-3′) and reverse (5′-ATGTCA TCATACTTGGCAGGTT-3′) for GAPDH using Power SYBR Green PCR Master Mix kit (Takara Bio Inc., Kusatsu, Japan) with an automatic 7500 Fast Real-Time PCR System (Applied Biosystems, Tokyo, Japan).[16–18] The data were expressed on a log scale as the relative expression to GAPDH. All measurements were performed in triplicate.

Monoclonal antibodies and NAC-calicheamicin DMH

Humanized IgG_4 anti-CD22 mAb (G5/44) and NAc-γ-calicheamicin DMH conjugated one (CMC-544) as well as calicheamicin-conjugated humanized IgG_4 anti-CD33 mAb (GO) and unconjugated NAc-γ-calicheamicin DMH were kindly provided by Wyeth Research (Collegeville, PA, USA). Rituximab was purchased from Zenyaku Co. (Tokyo, Japan).

Cell cycle distribution analysis

Cells suspended in 1 ml hypotonic fluorochrome solution containing propidium iodide (PI) (Sigma, St Louis, MO, USA) were analyzed by flow cytometry as described earlier[15,19] after incubation with CMC-544 containing 1–100 ng/ml calichemicin DMH for 72 h.

Morphological analysis by video-microscopic technique

Cells were plated in a glass-bottomed dish (MatTec Corporation, Ashland, MA, USA) at a concentration of 10^5 cells per ml in a medium containing CMC-544 (10 ng/ml calichemicin DMH) or an equivalent amount of G5/44. After 12 and 24 h incubations at 37 °C, cells were observed under an inverted Normarski microscope (Axiovert 35; Zeiss, Oberkochen, Germany) as described previously.[19]

Laser microscopy

Cells were incubated with CMC-544 or G5/44 for 12–24 h at 37 °C before staining with fluorescence-labeled mAbs (described in *Flow cytometry for CD20, CD22, CD55 and CD59*). Cells were placed on a non-fluorescent glass slide and observed by the C1si real spectral imaging system (Nikon Instech, Kawasaki, Japan). The fluorescence of FITC and phycoerythrin were detected simultaneously.

Dye exclusion test with propidium iodide (PI) staining

After the incubation of cells with CMC-544, G5/44 or rituximab for the indicated periods of time, cells were stained with 0.1 μg/ml PI solution and counted under the microscope.[20] Viable cell counts were calculated as follows: (viable cell count) = (total cell count)−(PI-stained cell count).

Direct antiproliferative and apoptotic effect of CMC-544

Three possible mechanisms responsible for the combined effect of CMC-544 and rituximab were investigated separately: direct antiproliferative and apoptotic effects of the mAb, CDC and antibody-dependent cellular cytotoxicity (ADCC). These assays were conducted after the incubation of Daudi and Raji cells with CMC-544 either in the presence or absence of rituximab.

The antiproliferative effect of rituximab in the presence and absence of CMC-544 or G5/44 was determined by a viable cell count in triplicate. The apoptotic effect was analyzed by cell cycle distribution. In brief, 10^6 per ml viable cells were incubated in the presence or absence of CMC-544 containing 1–10 ng/ml calichemicin DMH for 2 h, washed three times and then incubated with or without 20 μg/ml rituximab for 72 h. Separately, all of the cells were incubated in the presence of 10 μl per 10^6 cells of anti-human IgG goat antibody $F(ab)_2$ (Becton Dickinson Immunocytometry Systems) to enhance the cross-linking effect of rituximab on the cell surface.

Complement-dependent cytotoxicity

The CDC effect was measured by a dye exclusion test in triplicate. After 10^6 per ml cells were incubated with or without rituximab in the presence of fresh human AB serum for 2 h at 37 °C, cells were placed on ice to stop the CDC reaction. Viable cells were counted immediately after incubation and compared with those counted before incubation.[21]

The enhancement of the CDC effect was studied in a similar way in the presence of CMC-544 or G5/44. Specifically, after cells were incubated with or without CMC-544 (5 ng/ml calichemicin DMH) or G5/44 at 37 °C for 2 h, they were washed three times to remove unbound antibodies. The viability of cells before incubation with CMC-544 was 99.8%. After the cells were re-incubated in CMC-544- and rituximab-free medium at 37 °C for 0–48 h, CDC was analyzed as described above.

After the first 30 min of the CDC assay, a part of the cells was placed on ice to stop the reaction, and analyzed the compliment deposition on the cells. This was determined by flow cytometry

after staining with FITC-anti-human C3c rabbit antibody (Dako, Glostrup, Denmark) according to the manufacturer's instructions. MFIs were compared among the groups.

Antibody-dependent cellular cytotoxicity assays

Total mononuclear cells were obtained by Ficoll-Paque centrifugation and natural killer cells were enriched to 78–91% by an Easy Sep human positive natural killer cell kit (Invitrogen). Concurrently, 10^6 per ml target cells were stained with PKH67 using a MINI67 cell linker kit (Sigma).[22] After the PKH67-stained target cells ($PKH67^+$) were incubated in the presence or absence of CMC-544 containing 5 ng/ml calichemicin DMH or equivalent amount of G5/44 for two hours and washed three times, they were co-cultured with 10^4 per ml natural killer cells in triplicate with or without 2 μg/ml rituximab for 4 h at 37 °C. Then the cells were stained with 0.1 μg/ml PI solution and analyzed by flow cytometry. Target cells damaged by ADCC were classified as $PKH67^+PI^+$ cells, whereas viable target cells were classified as $PKH67^+PI^-$ cells.

Statistical analyses

The data of real-time PCR and CDC, shown as means ± s.d., were analyzed and compared using the Student's *t*-test. MFIs from flow cytometry were analyzed by paired *t*-test.

Results

CD20, CD22, CD55 and CD59 expression on cells before and after incubation with CMC-544 as analyzed by flow cytometry

CD20 and CD22 were expressed on more than 99% of Daudi and Raji cells. CD55 was expressed on 72 and 57% of these cells, respectively. They were similarly expressed on Daudi/MDR and Raji/MDR cells (71 and 59%, respectively). However, CD20 and CD22 were not expressed on Jurkat, K562 or NB4 cells (data not shown).

To assess the effect of CMC-544 on antigen expression, cells were cultured for 2 h in a medium containing CMC-544 or G5/44, switched to an antibody-free medium and then examined at various time points thereafter to determine the levels of CD20, CD22, CD55 and CD59 on the cells. After 12–24 h, the level of CD22 on CMC-544-treated cells had decreased relative to that on G5/44-treated cells (Figure 1a) and continued to decrease, with the reduction of cell viability determined by PI staining (83 and 47% at 48 and 72 h, respectively in Daudi cells), and with apoptotic morphological changes determined by microscopic observation (38 and 64% at 48 and 72 h, respectively in Daudi cells). In contrast, the level of CD20 remained constant or increased after 24 h (Figure 1a), but decreased at 48 h (data not shown). Thereafter the level of CD55 significantly decreased after 12–24 h, and continued to decrease further. However, the level of CD59 did not change significantly. The levels of CD20, CD22, CD55 and CD59 on Daudi/MDR and Raji/MDR cells did not change after incubation with CMC-544 (data not shown). Further, the levels of these antigens did not change after incubation with G5/44 or GO (data not shown).

CD20, CD22 and CD55 expression on cells before and after incubation with CMC-544 as analyzed by laser microscopy

The levels of CD20, CD22 and CD55 on Daudi cells were also analyzed by laser microscopy. The levels of antigens before and after exposure to CMC-544 or G5/44 were compared (Figure 2a). Although the level of CD20 did not change significantly, that of CD22 and CD55 significantly decreased after 24 h when compared with the controls. These results are compatible with the data analyzed by flow cytometry. Similar results were obtained in Raji cells (data not shown).

CD20, CD22, CD55 and CD59 expression on cells before and after incubation with CMC-544 as analyzed by real-time RT-PCR

After incubating in a medium containing CMC-544 or G5/44 for 2 h, cells were cultured in an antibody-free medium and harvested at 1-, 3-, 6- and 12-h time points. The levels of CD20, CD22, CD55 and CD59 mRNA in Daudi or Raji cells were measured by real-time RT-PCR and normalized to GAPDH mRNA level (Figure 2b). The levels of CD22 and CD55 mRNA in CMC-544-treated cells began to decrease at 6-h and was significantly lower at 12-h. On the other hand, the levels of CD20 and CD59 mRNA did not change significantly. These changes observed in Daudi and Raji cells were not in Daudi/MDR and Raji/MDR cells. G5/44 had no effect on these mRNA levels.

CD20, CD22, CD55 and CD59 expression on patients' samples of BCM before and after incubation with CMC-544 as analyzed by flow cytometry

Quantitative alterations of CD20, CD22, CD55 and CD59 levels were also analyzed in samples from 12 patients with BCM. The level of CD22 decreased after incubation with CMC-544 in the samples from all patients ($P<0.001$). The level of CD20 was constant in the samples from four patients but increased in eight patients. Although the level of CD55 decreased significantly ($P<0.001$), that of CD59 did not decrease ($P=0.096$). Results derived from four representative samples are shown in Figure 1b. Cell sizes increased in all samples after 24 h incubation as determined by a two-dimensional analyses of forward and side scatter (Figure 3a). The enlargement was confirmed by morphological changes observed by microscopy (Figure 3b).

Direct antiproliferative and apoptotic effects of rituximab in combination with CMC-544

The antiproliferative effect was determined by a viable cell count after incubation with either rituximab, CMC-544 or both agents (Figure 4a). Viable cell counts after incubation with rituximab, CMC-544 or both agents compared with those before the incubation were 93, 45 and 41% in Daudi cells, and 96, 37 and 34% in Raji cells, respectively.

The apoptotic effect was investigated by cell cycle after incubation with either rituximab, CMC-544 or both agents (Figure 4a). The hypodiploid portion after incubation with rituximab, CMC-544 or both agents increased 4, 35 and 38% in Daudi cells, 8, 56 and 60% in Raji cells, respectively.

Thus, the antiproliferative and apoptotic effects of rituximab alone were significantly less than that of CMC-544 alone. The same results were obtained in BCM cells. The antiproliferative and apoptotic effects were not observed in incubation with G5/44 (data not shown).

Combination effect of CMC-544 on CDC caused by rituximab

The viable cell count significantly decreased after incubation with rituximab through CDC in Daudi and Raji cells. However,

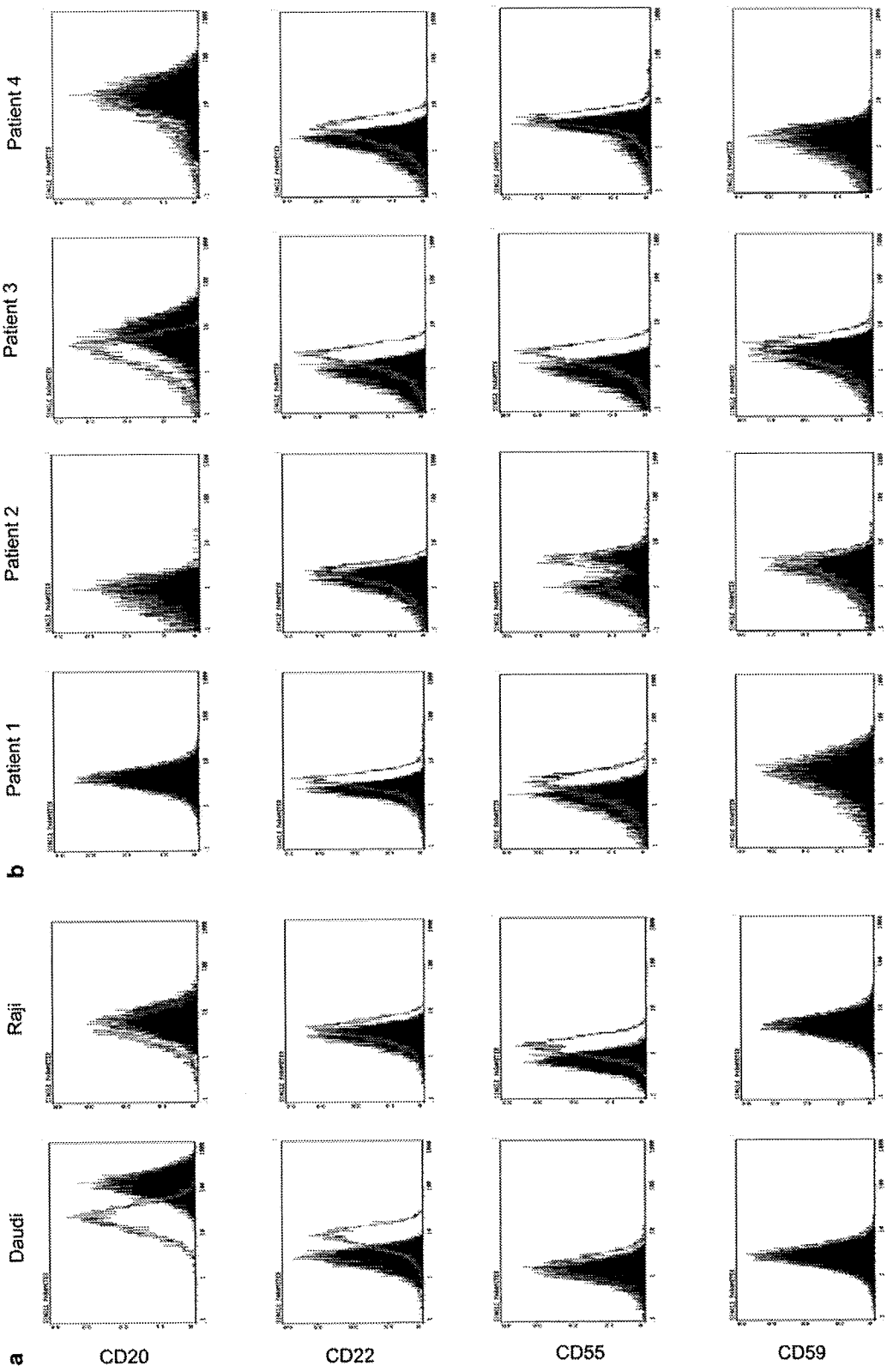

Figure 1 (**a**) The levels of CD20, CD22, CD55 and CD59 antigens on Daudi and Raji cells were analyzed by flow cytometry after exposure to a medium with or without CMC-544. The horizontal lines show the fluorescence intensity, and the vertical lines show the levels of CD20, CD22, CD55 or CD59. White and black histograms show the data obtained after cells were incubated with G5/44 or CMC-544, respectively. The levels of CD22 and CD55 decreased 12–24 h after CMC-544 exposure, whereas those of CD20 increased. The levels of CD59 did not change significantly. (**b**) Results are shown from four representative patients with BCM that contained a sufficient number of cells for analysis. The same results were obtained for the remaining samples.

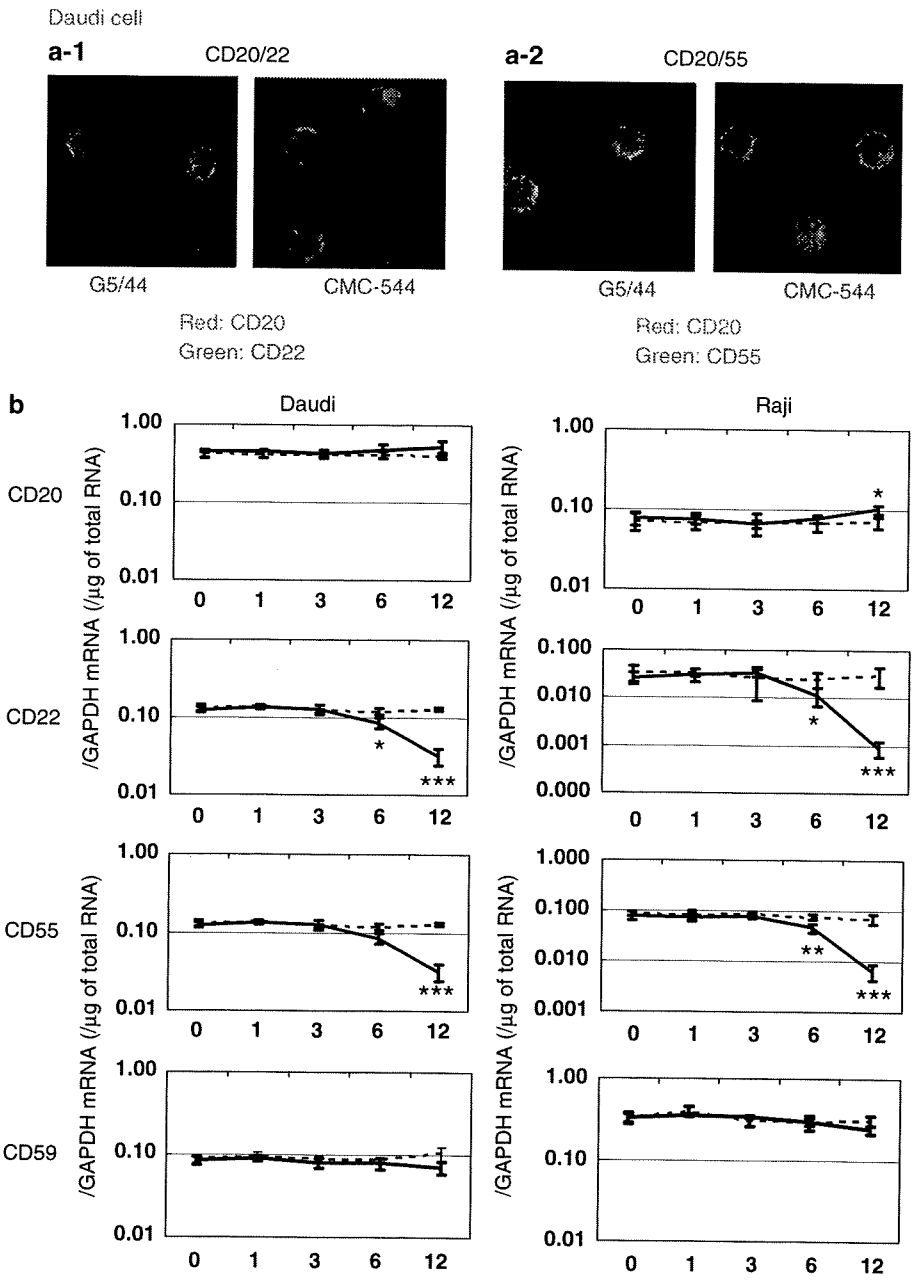

Figure 2 (**a**) Laser scanning microscope images taken 24 h after a 2 h incubation in a medium containing CMC-544 or G5/44. (**a-1**) Daudi cells were stained with PE-conjugated anti-CD20 mAb (red) and FITC-conjugated anti-CD22 mAb (green). (**a-2**) Daudi cells stained by PE-conjugated anti-CD20 mAb (red) and FITC-conjugated anti-CD55 mAb (green). The levels of CD22 and CD55 were significantly reduced, whereas the CD20 expression was constant or increased. (**b**) The levels of CD20, CD22, CD55 and CD59 mRNA in Daudi and Raji cells were analyzed by real-time RT-PCR. After incubating in a medium containing CMC-544 (straight lines) or G5/44 (dotted lines) for 2 h, cells were cultured in an antibody-free medium and harvested at 1-, 3-, 6- and 12-h time points for real-time RT-PCR. The levels of CD22 and CD55 mRNA were significantly reduced after CMC-544 exposure, whereas those of CD20 and CD59 mRNA were maintained. The quantitative data were expressed on a log scale as the relative expression to GAPDH. The data after incubation with CMC-544 were compared with that of G5/44. $*P<0.05$, $**P<0.01$ and $***P<0.001$.

no effect on CDC was observed with CMC-544 or G5/44 alone (Figure 4b). Furthermore, the CDC caused by rituximab was not enhanced by simultaneous incubation with CMC-544 or G5/44.

To determine if CDC is enhanced by the sequential incubation of rituximab and CMC-544, Daudi and Raji cells were first incubated in a medium containing CMC-544 for 2 h, incubated for 12 h in an antibody-free medium, and then CDC by rituximab was analyzed. The CDC effect of rituximab was significantly increased 12 h after incubation with CMC-544 ($P<0.001$) (Figure 4b). G5/44 did not increase the CDC effect of rituximab. Similar results were obtained from patients' samples (Figure 4c).

After the first 30 min of the CDC assay using rituximab and AB serum, compliment deposition on the cells was analyzed by flow cytometer. The level of C3 on CMC-544-treated cells increased to G5/44-treated cells significantly ($P=0.034$) (Figure 4d).

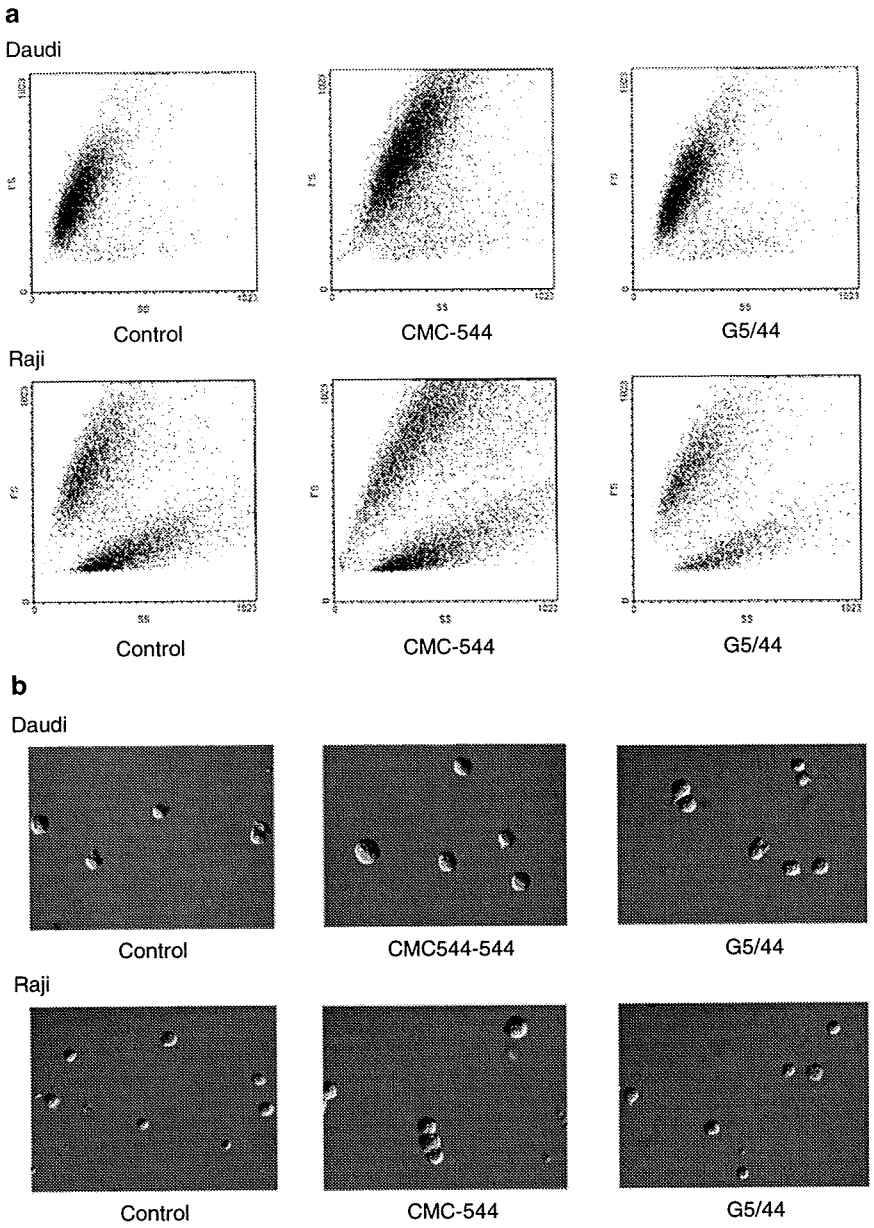

Figure 3 (**a**) Forward scatter (FS) and side scatter analyzed by flow cytometry 24 h after exposure of Daudi and Raji cells to medium with or without CMC-544. Fluorescence intensity of FS increased after exposure to CMC-544 compared with controls. (**b**) Morphological changes of Daudi and Raji cells 24 h after exposure to CMC-544 or G5/44. Cells were enlarged 12–24 h after incubation with CMC-544.

Combination effect of CMC-544 on ADDC caused by rituximab

We counted viable target cells, that is, $PKH67^+PI^-$ cells, and compared them before and after incubation with rituximab or CMC-544 in Daudi and Raji cells. Forty-nine percent of $PKH67^+PI^-$ cells changed to $PKH67^+PI^+$ after a 4 h incubation with rituximab, whereas 9 and 2% changed after incubation with CMC-544 or G5/44, respectively. The change was not significant for simultaneous incubation with rituximab and CMC-544 (53%) or G5/44 (51%) ($P=0.11$ and $P=0.21$, respectively) nor was it increased by sequential incubation with rituximab and CMC-544. Similar results were obtained from the patients' samples (data not shown).

Discussion

Rituximab has provided many encouraging clinical outcomes in the treatment of BCM.[23] CMC-544, recently introduced, is also a promising agent for BCM. These mAbs target different antigens and have different antitumor mechanisms. We investigated the effects of these agents from the viewpoint of alteration of the target molecules, that is, CD20, CD22, CD55 and CD59, which are essential for their action, and attempted to clarify the rationale for the advantage in combination of rituximab and CMC-544.

CD20 is a good target for BCM because it is expressed at high levels and is not downregulated after antibody binding.[2] Although malignant B-cells are heterogeneous and multi-step

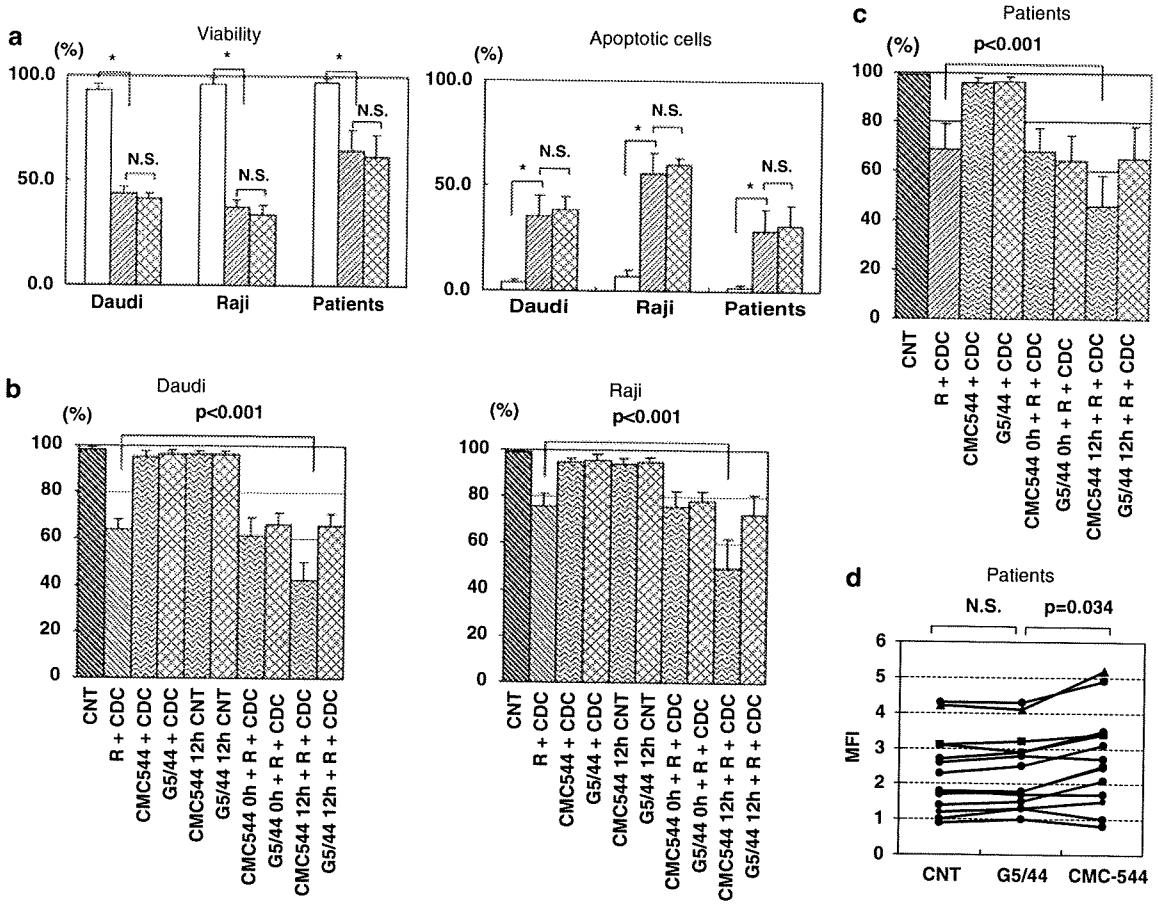

Figure 4 (**a**) Antiproliferative and apoptotic effects were determined by viable cell counts and cell cycles, respectively. These were analyzed after incubation with either rituximab (blank column) or CMC-544 (shaded column) or with both agents (mesh column) and then compared before the incubation. $*P<0.001$, NS: not significant. (**b**) The CDC effect of rituximab in Daudi and Raji cells cultured with or without CMC-544. The viable cell counts after CDC assay were compared with before, and are represented as the rate of percent (%). The viable cell count significantly decreased after rituximab treatment as a result of CDC. It was significantly enhanced by incubation in a CMC-544-free medium for 12 h after exposure to CMC-544. (**c**) The CDC effect of rituximab in cells from 12 patients with BCM cultured with or without CMC-544. The result was similar to that obtained in cell lines. (**d**) After the first 30 min of the CDC assay, cells were placed on ice to stop the reaction and analyzed the compliment deposition on the cells. This was determined by flow cytometry after staining with FITC-anti-human C3c rabbit antibody. MFIs were compared among the groups. Statistical significance is shown in the figure. Black circle, square and triangle show cells from patients, Daudi and Raji cells, respectively.

events occur before and after binding to CD20, the level of CD20 expression is presumed to be an important factor in treatments with rituximab,[3,5] and upregulation of CD20 has been attempted by some investigators.[24] In this study, the level of CD20 was constant or increased 12–24 h following exposure to CMC-544 as analyzed by flow cytometry, and constant by real-time RT-PCR. The preservation of CD20 supports the efficacy of rituximab after treatment with CMC-544.

The levels of CD20 began to decrease over time after 24 h. This might be explained by the direct antiproliferative and apoptotic effects of CMC-544, which started 24 h after incubation with CMC-544. A similar observation was reported in our study of GO.[15,19,20]

Although the level of CD20 mRNA did not change as determined by RT-PCR, the levels of CD20 surface antigen increased as determined by flow cytometry in some samples. This discrepancy might be explained by the increase in cell volume associated with cell cycle events such as calicheamicin-induced transient G2/M arrest.[15] The increase was confirmed by microscopic inspection and scatter-grams of flow cytometry.

The levels of CD22 are also important in the action mechanism of CMC-544 because CD22 directly reacts with mAb moiety of CMC-544. In our study, CD22 decreased 12 and 24 h after CMC-544 exposure as measured by RT-PCR and flow cytometry, respectively. The level of CD22 did not decrease after G5/44 or GO exposure (data not shown). Therefore, the downregulation of CD22 could be an effect of CMC-544, probably induced by a calicheamicin detached from CMC-544.

CD55 and CD59 are important regulators of CDC in BCM.[6,7] The increase of these antigens is reportedly related to their resistance to rituximab, and the decrease to their susceptibility to rituximab.[21,25] However, the underlying mechanism for the increase of these antigens with respect to the resistance to rituximab has not been well elucidated. Decreasing or inactivating CD55 is an effective idea to restore the therapeutic effect of rituximab.[8] In our present analyses, the level of CD55 significantly decreased 12–24 h after CMC-544 exposure. It is consistent with the observation that CDC from rituximab increased 12 h after incubation with CMC-544. These results may also explain the rationale for the advantage in combination of rituximab and CMC-544. The increase of compliment

deposition on CMC-544-treated cells also supports this advantage. Although, in this assay, some cases did not show an increase of compliment deposition, their viable cell counts had already decreased to 87–71% after the first 30 min of CDC assay. The cells that trapped more compliment deposition and were susceptible to CDC might have been damaged in the early phase of CDC assay. In this study, we could not show the combination efficacy of rituximab and CMC-544 on ADCC as CMC-544 might be quickly internalized after binding to CD22.

We showed here the rationale for the advantage of combined use of CMC-544 and rituximab in BCM. CMC-544 preserved the level of CD20 and decreased the level of CD55, both of which are closely related to resistance to rituximab.[6–8] Sequential combination of these agents, that is, CMC-544 followed by rituximab, may be a relevant way forward. Such combination approaches may enable more promising therapeutic approaches for the treatment of BCM especially in relapse or refractory to conventional treatments with rituximab.

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ORIGINAL ARTICLE

Comprehensive analysis of cooperative gene mutations between class I and class II in *de novo* acute myeloid leukemia

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Abstract

Acute myeloid leukemia (AML) has been thought to be the consequence of two broad complementation classes of mutations: class I and class II. However, overlap-mutations between them or within the same class and the position of *TP53* mutation are not fully analyzed. We comprehensively analyzed the *FLT3, cKIT, N-RAS, C/EBPA, AML1, MLL, NPM1,* and *TP53* mutations in 144 newly diagnosed *de novo* AML. We found 103 of 165 identified mutations were overlapped with other mutations, and most overlap-mutations consisted of class I and class II mutations. Although overlap-mutations within the same class were found in seven patients, five of them additionally had the other class mutation. These results suggest that most overlap-mutations within the same class might be the consequence of acquiring an additional mutation after the completion both of class I and class II mutations. However, mutated genes overlapped with the same class were limited in *N-RAS, TP53, MLL*-PTD, and *NPM1*, suggesting the possibility that these irregular overlap-mutations might cooperatively participate in the development of AML. Notably, *TP53* mutation was overlapped with both class I and class II mutations, and associated with morphologic multilineage dysplasia and complex karyotype. The genotype consisting of complex karyotype and *TP53* mutation was an unfavorable prognostic factor in entire AML patients, indicating this genotype generates a disease entity in *de novo* AML. These results collectively suggest that *TP53* mutation might be a functionally distinguishable class of mutation.

Key words acute myeloid leukemia; overlap mutations; TP53; multilineage dysplasia; prognosis

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Acute myeloid leukemia (AML) is a genetically and phenotypically heterogeneous disease (1). In 1999, the third edition of the World Health Organization (WHO) classification of the myeloid neoplasms classified AML into four major categories: AML with recurrent genetic abnormalities (AML-RGA), AML with multilineage dysplasia (AML-MLD), AML, therapy-related, and AML not otherwise categorized (2). The first category included AML with t(8;21)(q22;q22), (*AML1/ETO*), inv(16)(p13q22) or t(16;16)(p13;q22), (*CBFB/MYH11*), t(15;17)(q22;q12), (*PML/RARA*) and 11q23 (*MLL*) abnormalities, which create fusion genes associated with leukemogenesis. Each balanced translocation corresponded to characteristic cytogenetical and clinical

features. These translocations can be detected by reverse transcriptase-mediated PCR (RT-PCR), which becomes a sensitive, rapid and objective method for diagnosis (3). However, the classification of other categories is based on morphology of bone marrow (BM) cells and on the history of patients, although a number of genetic alterations, which are involved in the pathogenesis of AML and associated with the prognosis of patients, have been documented (4). Recently, it has been demonstrated that mutations of *FLT3*, *NPM1*, and *C/EBPA* genes are preferentially found in AML with normal cytogenetics and are highly implicated in the prognosis (5). The fourth edition of the WHO classification have included *NPM1* and *C/EBPA* mutations as provisional entities in AML-RGA, but not *FLT3* mutation because it is associated with a number of other entities (6). Furthermore, the AML-MLD category has been renamed as AML with myelodysplasia-related changes, in which myelodysplastic syndrome (MDS)-related cytogenetic abnormality, as well as previous history of MDS and MLD, has been included as a criteria for the diagnosis. However, it was suggested that AML is the consequence of two broad complementation classes of mutations: those that confer a proliferative and/or survival advantage to hematopoietic progenitors (class I mutation) and those that impair hematopoietic differentiation and confer properties of self-renewal (class II mutation) (7). In addition, clinical significance of genetic alterations in the setting of morphologic MLD remains unclear. Therefore, it is necessary to analyze genetic alterations comprehensively, taking them into account all together rather than individually to elucidate the genetic background and prognostic impact in AML (8).

It has been generally considered that *FLT3*, *cKIT*, and *N-RAS* mutations are class I mutations, and *C/EBPA* and *AML1* mutations, and *AML1/ETO*, *CBFB/MYH11*, *PML/RARA*, and *MLL* abnormalities are class II mutations, while overlap mutations of these mutations between class I and class II or within the same class in a clinical sample are not fully characterized, and the positions of *NPM1* mutation and the partial tandem duplication of the *MLL* gene (*MLL*-PTD) remain unclear. *TP53* mutations are reportedly infrequent but are associated with a poor prognosis in *de novo* AML (9–11). In addition, an association between *TP53* mutations and complex karyotype in therapy-related MDS and AML has been reported (12, 13). However, the position of *TP53* mutation remains unclear.

In this study, we comprehensively analyzed mutations of *FLT3*, *cKIT*, *N-RAS*, *C/EBPA*, *AML1*, *MLL*, *NPM1*, and *TP53* genes as well as cytogenetics in newly diagnosed *de novo* AML to disclose the feature of their overlap mutations. Furthermore, we examined the association of cooperative mutations with clinical characteristics and morphologic MLD of *de novo* AML.

Patients and methods

Patients and samples

The diagnosis of AML was based on the WHO classification. All BM smears from patients were evaluated by the authors according to the WHO criteria and morphological diagnosis was confirmed. The study population included 144 newly diagnosed *de novo* AML patients from January 1990 who were received the remission induction therapy in our institutes. We unselectively included all patients into the present study if their samples were available. The median age and WBC count at the diagnosis of the analyzed patients were 52 yr (range, 15–85 yr) and 10.3×10^9/L (range, 0.6–351 $\times 10^9$/L), respectively. Twenty-one patients were of age 65 yr or older. Cytogenetic analysis revealed that a normal karyotype was found in 54 patients and an abnormal karyotype was in 90 patients including 19 t(8;21)(q22;q22), 3 inv(16)(p13q22), 14 t(15;17)(q22;q12) and 2 11q23 abnormalities. AML-MLD was identified in 34 patients, who did not have a history of MDS. BM samples from patients with AML were subjected to Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) density gradient centrifugation. Informed consent was obtained from all patients to use their samples for banking and molecular analysis, and approval was obtained from the ethics committee of Nagoya university school of medicine.

Therapy

Among the AML patients analyzed, patients younger than 65 yr old were treated with the AML protocols of the Japan Adult Leukemia Study Group or their modifications (14, 15). Briefly, the induction therapy consisted of cytarabine (Ara-C) and idarubicin (IDR) or Ara-C and daunorubicin (DNR). Patients who achieved complete remission (CR) subsequently received three courses of consolidation therapy consisted of high-dose Ara-C or four courses of consolidation consisted of Ara-C and mitoxantrone, Ara-C and DNR, Ara-C and aclarubicin, and Ara-C, etoposide, vincristine and vindesine. Patients aged 65 yr or older received the dose-reduced induction therapy consisted of Ara-C and IDR or Ara-C and DNR. For the consolidation therapy, four courses of the dose-reduced regimen were administered.

Cytogenetics analysis

The cytogenetic G-banding analysis was performed with standard methods. A complex karyotype was defined as

at least three unrelated chromosomal aberrations. Chimeric transcripts, *BCR/ABL*, *AML1/ETO*, *CBFB/MYH11*, *PML/RARA*, and *MLL/AF9*, were examined by real-time PCR as previously described (3).

Screening for mutations of *FLT3, cKIT, N-RAS, AML1, C/EBPA, TP53, MLL,* and *NPM1* genes

High-molecular-weight DNA and total RNA were extracted from the samples using standard methods. *FLT3* gene mutations of the internal tandem duplication in the juxtamembrane domain (FLT3/ITD) and deletion and point mutation in the kinase domain (FLT3/KDM), *NPM1* gene mutation of exon 12, *N-RAS* gene mutations of codons 12, 13, and 61 and *TP53* gene mutations of exons 5–8 were examined as reported and confirmed by the sequencing procedure (11, 16–19). The partial tandem duplication of the *MLL* gene (*MLL*-PTD) was examined by RT-PCR as described previously (17). Mutations of *AML1*, *C/EBPA* and exon 8, 10–11, and 17 of *cKIT* were screened by denaturing high performance liquid chromatography (DHPLC) analysis using the WAVE Maker System (Transgenomic Inc., San Jose, CA, USA) as reported (20–22). DHPLC gradients and temperatures were determined using WAVE Maker System software. When heterozygous profiles were identified by visual inspection of the chromatograms, mutations were confirmed by cloning and sequencing procedures as reported (20).

Statistical analysis

Differences in continuous variables were analyzed with the Mann–Whitney *U*-test for distribution among two groups or the Kruskal–Wallis test for distribution among more than two groups. Frequencies were analyzed using Fisher's exact test for 2×2 tables or Pearson's chi-squared test for larger tables. Multivariate analysis to identify risk factors for achieving CR was performed using the logistic-regression model. Survival probabilities were estimated by the Kaplan–Meier method, and differences in survival distributions were evaluated using the log-rank test. The prognostic significance of the clinical variables was assessed using the Cox proportional hazards model. These statistical analyses were performed with StatView-J 5.0 (Abacus Concepts Inc., Berkeley, CA, USA). For all analyses, the *P*-values were two-tailed, and a $P < 0.05$ was considered statistically significant.

Results

Mutations of *de novo* AML

Genetic alterations of AML patients according to cytogenetics are summarized in Table 1. At least one mutation in the *FLT3*, *cKIT*, *N-RAS*, *AML1*, *C/EBPA*, *MLL*, *NPM1*, *and TP53* genes was identified in 84 of the 144 AML patients (58.3%). *FLT3* mutation was the most frequently identified in entire AML patients (35/144, 24.3%), followed by *NPM1* (29/144, 20.1%) and *C/EBPA* (17/144, 11.8%) mutations. In *FLT3* mutation, FLT3/ITD and FLT3/KDM were identified in 28 (19.4%) and seven (4.9%) patients, respectively. No overlap mutation of FLT3/ITD and FLT3/KDM was observed. In cytogenetically normal AML, *NPM1* mutation was the most frequently identified (19/54, 35.2%), followed by FLT3/ITD (15/54, 27.8%) and *C/EBPA* (13/54, 24.1%) mutations. *FLT3* mutation was also frequently identified in AML with *PML/RARA*, *AML1/ETO*, or *CBFB/MYH11*, although *NPM1* and *C/EBPA* mutations were not. In contrast, *cKIT* mutation was not identified in cytogenetically normal AML, while it was frequently identified in AML with *AML1/ETO* (3/19, 15.8%) or *CBFB/MYH11* (1/3, 33.3%). When comparing cytogenetically normal and abnormal patients, *NPM1* and *C/EBPA* mutations were

Table 1 Genetic alterations of 144 *de novo* AML patients according to cytogenetics

	PML/RARA	AML1/ETO	CBFB/MYH11	MLL abnormalities	Normal	Other abnormalities	Complex	Total	*P*-value
Number	14	19	3	2	54	38	14	144	
Mutation (%)									
FLT3/ITD	3 (21.4)	0	1 (33.3)	0	15 (27.8)	8 (21.1)	1 (7.1)	28 (19.4)	NS
FLT3/KDM	0	2 (10.5)	0	0	3 (5.6)	2 (5.3)	0	7 (4.9)	NS
cKIT	0	3 (15.8)	1 (33.3)	0	0	4 (10.5)	0	8 (5.6)	0.0208
N-RAS	1 (7.1)	0	0	0	4 (7.4)	6 (15.8)	0	11 (7.6)	NS
TP53	0	1 (5.3)	0	0	1 (1.9)	1 (2.6)	8 (57.1)	11 (7.6)	<0.0001
NPM1	0	0	0	0	19 (35.2)	8 (21.1)	2 (14.3)	29 (20.1)	0.0076
MLL-PTD	0	1 (5.3)	1 (33.3)	1 (50)	5 (9.3)	3 (7.9)	2 (14.3)	13 (9.0)	NS
C/EBPA	0	0	0	0	13 (24.1)	2 (5.3)	2 (14.3)	17 (11.8)	0.0242
AML1	0	0	0	0	1 (1.9)	2 (5.3)	0	3 (2.1)	NS

Distributions of *cKIT, TP53, NPM1,* and *C/EBPA* mutations according to cytogenetics were significantly different.
AML, acute myeloid leukemia; NS, no significance.

frequently identified in cytogenetically normal patients (33.9% vs. 11.4%, $P = 0.0014$ and 23.2% vs. 4.5%, $P = 0.0011$, respectively). *TP53* mutation was identified in 11 AML patients (7.6%), and eight of them showed complex karyotype. As *AML1* mutation was identified in only three patients, further analysis is required to confirm the statistical significance of its distribution.

Overlap mutations

In the present study, we identified a total of 165 mutations in entire AML patients. Interestingly, 103 of the 165 mutations (62.4%) were overlapped with another mutations. *AML1* (3/3, 100%), FLT3/KDM (7/7, 100%), FLT3/ITD (24/28, 85.7%), *N-RAS* (9/11, 81.8%), and *NPM1* (21/29, 72.4%) mutations were frequently overlapped with another mutations. In contrast, overlap mutations of *PML/RARA* (3/14, 21.4%) and *AML1/ETO* (6/19, 31.6%) were relatively infrequent (Fig. 1A). The most frequent overlap mutation was *FLT3* and *NPM1*, which was observed in 16 of the 51 patients with overlap mutations, followed by *FLT3* and *MLL*-PTD (five patients), *FLT3* and *C/EBPA* (four patients), *FLT3* and *PML/RARA* (three patients), *cKIT* and *AML1/ETO* (three patients), *N-RAS* and *NPM1* (three patients) and *TP53* and *MLL*-PTD (three patients). Importantly, overlap pattern of mutations was not random as shown in Fig. 1(B). The overlap mutations of *FLT3* and *cKIT* were not identified. In addition, *PML/RARA*, *AML1/ETO*, *CBFB/MYH11*, and *MLL* gene abnormality and *AML1* and *C/EBPA* mutations were not overlapped each other. These results were consistent with the genetic model of class I and class II mutations. However, overlap patterns of *N-RAS*, *TP53*, *MLL*-PTD, and *NPM1* mutations seemed different from above mutations. *N-RAS* mutation was identified in nine patients, while each one of them were overlapped with FLT3/ITD and *cKIT* mutations, respectively. Likewise, overlap mutations of *TP53* and FLT3/ITD, *TP53* and *cKIT*, *MLL*-PTD and *AML1/ETO*, *MLL*-PTD and *CBFB/MYH11*, *MLL*-PTD and *MLL/ENL*, and *NPM1* and *C/EBPA* were found in each one patient. According to the general consideration that *N-RAS* and *TP53* mutations were the class I mutation, and *MLL*-PTD and *NPM1* mutations were the class II mutation, these overlap mutations seemed to irregularly occur within the same class. Taken together, overlap mutations in the same class were observed in seven patients, while five of them showed mutations in three different genes. In this study, 51 of the 144 patients (35.4%) revealed overlap mutations, and overlap mutations consisted of two or three genes were found in 46 and five patients, respectively. However, both class I and class II mutations were always included in all patients with three mutations (Table 2). Therefore, only two patients showed two mutations within the same class: one consisted of *N-RAS* and *cKIT* mutations and the other consisted of *MLL*-PTD and *MLL/ENL* (Table 2).

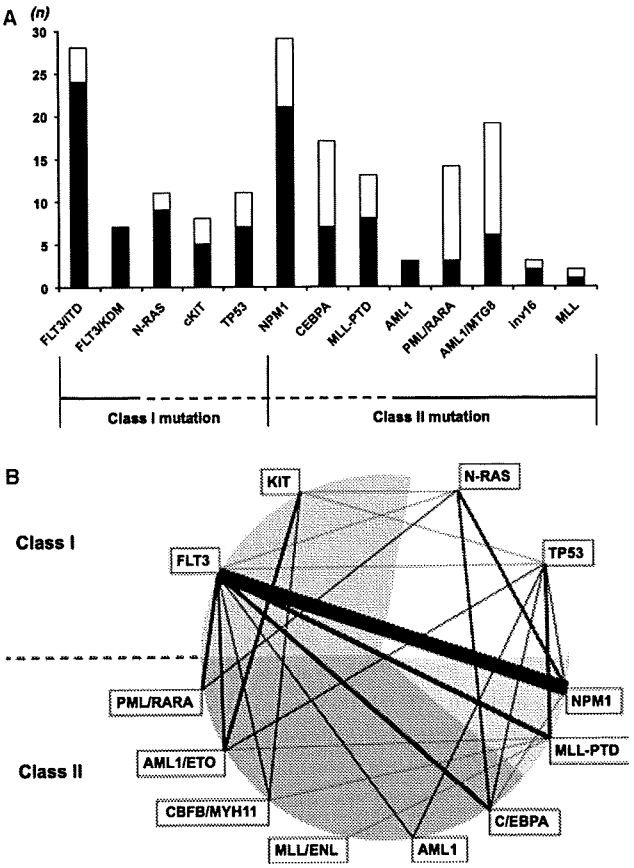

Figure 1 Prevalence and overlap pattern of class I and class II mutations in *de novo* AML. (A) We identified a total of 165 class I or class II mutations, 103 of which (62.4%) were overlapped with other mutations. Black and white bars indicate mutations with or without additional overlapped mutations, respectively. (B) Overlap pattern of each mutation. Red lines indicate mutations within the same class. Each line thickness represents the prevalence of overlap mutation. All FLT3/KDM overlapped with class II mutations, while two FLT3/ITD overlapped with class I mutations.

TP53 mutation was overlapped with a variety of gene mutations. Seven of the 11 *TP53* mutated cases showed overlap mutations: two was overlapped with *MLL*-PTD and each one with *NPM1*, *C/EBPA*, *AML1*, *cKIT*, and FLT3/ITD mutations. Cytogenetic analysis revealed that 10 *TP53* mutated cases had abnormal karyotypes: eight were complex karyotype, five of which had a deleted chromosome 17, and each one was 46XY, del(5q) and 46XY, t(8;21), del(9). Importantly, one case with *TP53* mutation and the karyotype 46XY, del(5q) harbored

Table 2 Characteristics of AML patients with overlap mutations within the same class

Age (yr)	Sex	WHO category	FAB	WBC ($\times 10^9$/L)	Mutation
Two mutations					
64	F	Not otherwise specified	M2	18.8	*cKIT, N-RAS*
46	F	Recurrent genetic abnormalities	M4	1.4	*MLL-PTD, MLL/ENL*
Three mutations					
76	M	Not otherwise specified	M4	47.5	*FLT3/ITD, TP53, MLL-PTD*
18	M	Myelodysplasia-related changes	M4	124.9	*FLT3/ITD, NPM1, C/EBPA*
50	F	Recurrent genetic abnormalities	M3	12.0	*FLT3/ITD, N-RAS, PML/RARA*
17	M	Recurrent genetic abnormalities	M2	11.0	*cKIT, TP53, AML1/ETO*
29	F	Recurrent genetic abnormalities	M4Eo	113.3	*FLT3/ITD, MLL-PTD, CBFB/MYH11*

AML, acute myeloid leukemia; WHO, World Health Organization.

FLT3/ITD, and one case with *TP53* mutation and the karyotype 46XY, t(8;21), del(9) harbored *cKIT* mutation. Although one case showed a normal karyotype, it was overlapped with the *AML1* mutation. Taken together, all *TP53* mutated cases had another genetic alterations (Fig. 2). On the other hand, a complex karyotype was found in 14 patients. Ten patients showed MLD, and eight of them had *TP53* mutation. The remaining four patients without MLD did not harbor *TP53* mutation, although three of them showed del(17) or del(17p). A genotype consisting of complex karyotype and *TP53* mutation was therefore specifically found in AML-MLD.

Prognostic implications of the mutational status

We analyzed prognostic implications of the mutational status in 130 AML patients except for acute promyelocytic leukemia. We divided AML patients into four groups: AML-MLD with or without complex karyotype and *TP53* mutation, AML with *AML1/ETO* or *CBFB/MYH11* (CBF-AML) and other type AML. Clinical characteristics of each group are shown in Table 3. Median age of the patients in CBF-AML was significantly younger than the other groups ($P = 0.012$), while there was no significant difference in median WBC count among 4 groups. The CR rate in AML-MLD with complex karyotype and *TP53* mutation (25%) was significantly lower than the other groups ($P = 0.0012$). Multivariate logistic-regression analysis revealed that WBC count (over 100×10^9/L) [odds ratio, 12.910 (95% CI: 3.101–53.742); $P = 0.0004$], wild-type *NPM1* [odds ratio, 10.640 (95% CI: 2.185–51.810); $P = 0.0034$), the genotype consisting of complex karyotype and *TP53* mutation [odds ratio, 8.755 (95% CI: 1.166–12.987); $P = 0.0271$], wild-type *C/EBPA* [odds ratio, 7.534 (95% CI: 1.111–51.103); $P = 0.0387$] and the presence of MLD [odds ratio, 3.891 (95% CI: 1.166–12.987); $P = 0.0271$] were independent unfavorable factors for achieving CR (Table 4). Furthermore, we analyzed prognostic implications of genetic alterations in addition to age, WBC count and existence of MLD. Multivariate Cox regression analysis with stepwise selection showed that the genotype consisting of complex karyotype and *TP53* mutation [odds ratio, 5.988 (95% CI: 2.681–13.333); $P < 0.0001$], not CBF-AML [odds ratio, 2.602 (95% CI: 1.107–6.116); $P = 0.0283$], FLT3/ITD [odds ratio, 1.843 (95% CI: 1.063–3.196); $P = 0.0294$] and age

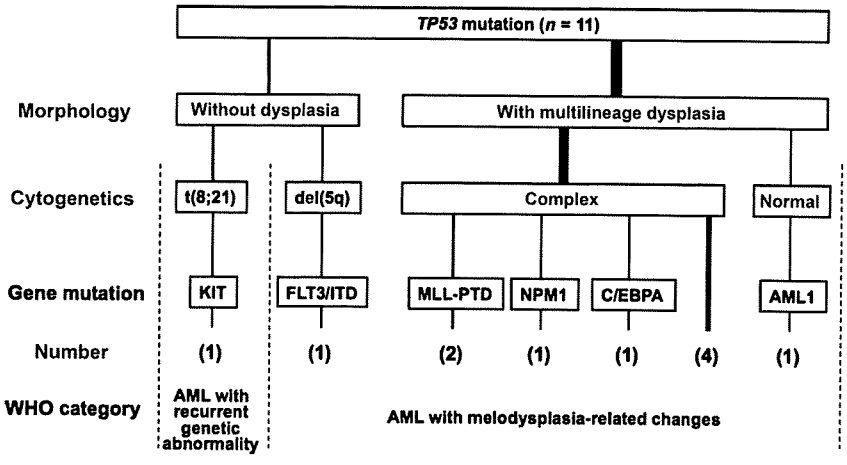

Figure 2 Association of *TP53* mutations with morphology, cytogenetics, and other mutations. *TP53* mutations were overlapped with a variety of mutations. Eight of the 11 *TP53* mutated cases showed complex karyotype, and all of them revealed morphologic MLD.

Table 3 Clinical characteristics of 130 AML patients except APL

	AML with MLD				
	With complex/ TP53 Mt.	Without complex/ TP53 Mt.	CBF-AML	Other AML	*P*-value
Number	8	26	22	74	
Age (yr)					
Median	60.5	55.5	**33.0**	53	0.012
Range	20–81	18–85	16–72	15–77	
WBC ($\times 10^9$/L)					
Median	6.6	16.9	10.3	22.9	NS
Range	1.2–22.4	0.8–139.5	3.1–113.3	0.9–351.0	
CR	**2**	19	20	60	0.0012
(%)	(25.0)	(73.1)	(90.9)	(81.1)	
Cytogenetics risk (%)					
High	8 (100)	3 (11.5)	–	10 (13.5)	
Complex	8 (100)	2 (7.7)	–	4 (5.4)	
Intermediate	–	23 (88.5)	–	64 (86.5)	
Normal	–	18 (69.2)	–	36 (48.6)	

Median age of the patients in CBF-AML was significantly younger than the other groups. The CR rate in AML-MLD with complex karyotype and *TP53* mutation was significantly lower than the other groups. Bold letters indicate significantly different groups.
AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; MLD, multilineage dysplasia; CR, complete remission.

Table 4 Unfavorable risk factors for achieving CR in *de novo* AML except for APL

Factors	OR	95% CI	*P*-value
WBC count (>100 × 10^9/L)	12.910	3.101–53.742	0.0004
Wild-type *NPM1*	10.640	2.185–51.810	0.0034
Complex karyotype/TP53 mutation	8.755	1.098–69.821	0.0405
Wild-type *C/EBPA*	7.534	1.111–51.103	0.0387
Presence of MLD	3.891	1.166–12.987	0.0271

AML, acute myeloid leukemia; CR, complete remission; MLD, multilineage dysplasia.

Table 5 Unfavorable prognostic factors for overall survival in *de novo* AML except for APL

Factors	OR	95% CI	*P*-value
Complex karyotype/TP53 mutation	5.988	2.681–13.333	<0.0001
Not CBF AML	2.602	1.107–6.116	0.0283
FLT3/ITD	1.843	1.063–3.196	0.0294
Age (>60 yr)	1.764	1.086–2.865	0.0218

AML, acute myeloid leukemia.

(over 60 yr) [odds ratio, 1.764 (95% CI: 1.086–2.865); $P = 0.0218$] were poor prognostic factors for overall survival (Table 5). For disease-free survival, not CBF-AML [odds ratio, 2.475 (95% CI: 1.176–5.208); $P = 0.0169$] and *AML1* mutation [odds ratio, 8.134 (95% CI: 1.039–63.642); $P = 0.0453$] were identified to be poor prognostic factors.

Discussion

In this study, we comprehensively analyzed mutations in the *FLT3*, *cKIT*, *N-RAS*, *AML1*, *C/EBPA*, *MLL*, *NPM1*, *and TP53* genes as well as cytogenetics in 144 newly diagnosed *de novo* AML patients. We identified a total of 165 class I or class II mutations, 103 of which (62.4%) were overlapped with other analyzed mutations, supporting that multiple genetic alterations have been accumulated at the diagnosis of AML. However, overlap mutations of *PML/RARA* (3/14, 21.4%) and *AML1/ETO* (6/19, 31.6%) were infrequent. Furthermore, overlap mutations of *C/EBPA* mutations, which are frequently found in cytogenetically normal AML, were relatively infrequent (7/17, 41.2%). It is well known that *PML/RARA*, *AML1/ETO*, and *C/EBPA* mutations are associated with favorable prognosis, while recent reports revealed that additional class I mutations, such as *FLT3* and *cKIT* mutations, reduced their favorable prognosis. Our results further indicate that it is necessary to clarify whether there are unknown class I mutations overlapped with them, which influence their prognostic impacts.

We demonstrate here that most overlap mutations found in AML consisted of class I and class II mutations. Although overlap mutations within the same class were found in seven patients, five of them additionally had the other class mutation. These results suggest that most overlap mutations within the same class might be the consequence of acquiring an additional mutation after the completion both of class I and class II mutations. Therefore, it is possible that an unknown

mutation of the opposite class was acquired in two patients, whose mutations consisted of two mutations within the same class. On the other hand, it has been reported that mutations in genes functioning in different pathways can occur in the same cancer, while genes functioning in the same pathway are rarely mutated in the same sample (23). However, it was also observed that certain overlap mutational patterns violate these rules and demonstrate tissue-specific variations (23). In addition, we found that mutated genes, which overlapped with the same class mutations, were limited in *N-RAS*, *TP53*, *MLL*-PTD, and *NPM1*. As these mutations are sometimes acquired at the relapse, it is necessary to analyze whether these irregular overlap mutations cooperatively participate in the development of AML, and whether these mutations are acquired during the disease progression (18, 24).

We further demonstrate the characteristic cooperative features of *TP53* mutation in *de novo* AML. Ten of the 11 patients with *TP53* mutation showed cytogenetic abnormality, and six of them showed an additional mutation. Furthermore, additional mutations involved both class I and class II mutations. Because of the evidence that p53 mediates quiescence of normal hematopoietic stem cells, Pedersen-Bjergaard *et al.* (25) proposed that *TP53* mutation functionally represent a new class III mutation. On the other hand *TP53* has been known as a tumor suppressor gene of importance for genomic stability, and it has been suggested that leukemic progenitor accumulates additional chromosomal alterations after inactivation of *TP53* in the development of AML (26). Although it is necessary to clarify how *TP53* mutation biologically involves the accumulation of genetic alterations during the development of AML and how their interactions are associated with the leukemogenesis, the previous and our results collectively suggest that *TP53* mutation might be a distinguishable class of mutation from class I and class II mutations.

Haferlach *et al.* (27) reported that *TP53* mutations were strongly associated with the complex karyotype in AML, but the association with dysplastic morphology was not evaluated; however, our results indicate that this genotype is closely associated with dysplastic morphology regardless of the history of MDS. It has been reported that *TP53* mutation is associated with loss of chromosome band 17p13, where the *TP53* gene is located (9, 28). Deletion of 17p, which occurred by unbalanced translocation between chromosome 17p and another chromosome, monosomy 17 or i(17), is reportedly observed in 3–4% of AML and MDS and 10–15% of therapy-related MDS or AML (29–33). Furthermore, it has been reported that 17p deletion is closely associated with dysgranulopoiesis, *TP53* mutation and additional complex cytogenetic findings in AML and MDS (34, 35). In our study, deletion of 17p was identified in eight of entire 144 AML patients (5.6%), and all showed a complex karyotype. Of note is that five of eight patients with complex karyotype and *TP53* mutation showed 17p deletion, while the chromosome 17 was intact in the remaining three patients. Prognostic implication of the genotype consisting of a complex karyotype and *TP53* mutation is particularly notable as to be an independent unfavorable prognostic factor both for achieving CR and overall survival in entire *de novo* AML patients. These results collectively indicate that this genotype generates a disease entity in *de novo* AML.

Although several genetic alterations have been shown to be associated with MDS-related AML, morphologic evidence is recommended as the most available marker for the diagnosis of AML-MLD (36); however, diagnosis of dysplasia is difficult to qualify and/or quantify. Wandt *et al.* (37) demonstrated that MLD was associated with an unfavorable cytogenetic profile and that the presence of MLD was an unfavorable risk factor for achieving CR in univariate analysis but not a poor prognostic factor for either event-free or overall survival by analyzing a large number of AML patients both with and without a history of MDS. Consistently, the presence of MLD was identified to be an independent unfavorable risk factor for achieving CR by multivariate analysis, but not for long-term survival in our analysis of all AML patients. We could not identify the AML-MLD-specific genotype other than a complex karyotype and *TP53* mutation and there were some AML-MLD without any genetic alterations, indicating that AML-MLD is still genetically heterogeneous.

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