High CD33 expression levels in acute myeloid leukemia cells carrying the nucleophosmin (*NPM1*) mutation

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ABSTRACT

The CD33 antigen is expressed on the blast cells of most cases of acute myeloid leukemia and represents a suitable tumor-associated target antigen for antibody-based therapies. The aim of this study was to investigate the relationship between the CD33 levels quantified by mean fluorescence intensity and antibody binding capacity, and the presence/absence of *NPM1* and *FLT3* gene mutations in 99 newly diagnosed acute myeloid leukemia cases. The CD33 intensity evaluated as mean fluorescence intensity and antibody binding capacity was significantly higher in the *NPM1*-mutated acute myeloid leukemia cases compared to the *NPM1*-unmutated cases (P=0.0001 and P=0.0088, respectively). On the contrary, *FLT3* gene mutations did not influence the levels of CD33 expression on the leukemic cells. These results establish a rational basis for the therapeutic use of anti-CD33 antibodies in *NPM1*-mutated acute myeloid leukemia patients.

Key words: acute myeloid leukemia, AML, monoclonal antibody therapy, mean fluorescence intensity, MFI, antibody binding capacity, ABC, *NPM1*.

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by the presence of acquired genetic alterations in hematopoietic progenitor cells that alter the normal mechanisms of cell growth, proliferation and differentiation.[1] One of the most frequent mutations in AML involves the nucleophosmin (*NPM1*) gene[2] encoding a nucleus-cytoplasm shuttling protein known to be involved in rearrangements in leukemia and lymphoma.[3] The abnormally mutated NPM1 protein shows an aberrant cytoplasmic localization (NPM1c+),[2,4] whereas the wild-type protein is mainly located in the nucleolus. Cytoplasmic NPM1 in AML is strongly associated with a normal karyotype and is mutually exclusive with recurrent cytogenetic abnormalities such as t(15;17), t(8;21), inv(16)/t(16;16) and 11q23 abnormalities or with complex karyotypes.[2] The *NPM1* mutation in AML is associated with a frequent CD34 negativity[2,5-6] and a number of distinct biological features, including a unique gene expression signature, microRNA profile and association with leukemic stem cells, that indicate *NPM1*-mutated AML is a distinct leukemia entity.[7,8]

Another important mutation in AML, that occurs in 25-30% of patients, involves the *FLT3* gene.[9] There are two main types of *FLT3* mutations: the internal tandem duplication (ITD) that maps to the juxtamembrane region, and point mutations that most frequently involve aspartic acid 835 (D835) of the kinase domain (KD), but have also been found less frequently in several other sites. *FLT3-ITD* mutations in AML are associated with an unfavorable prognosis both in pediatric and adult patients.[10,11] Conversely, *NPM1*-mutated AML patients without *FLT3-ITD* show a relatively good prognosis.[12]

Together with molecular analysis, immunophenotyping represents a key component of the diagnostic workup of AML. The highest diagnostic yield is achieved when information derived from a selected panel of monoclonal antibodies (MoAbs) is combined with the assessment of the expression level of a given antigen, which can be quantified by mean fluorescence intensity (MFI) and antibody binding capacity (ABC). In AML, one of the antigens usually expressed is CD33. Physiologically, CD33 expression is restricted to early multilineage hematopoietic progenitors, myelomonocytic precursors and more mature myeloid cells, being absent on normal pluripotent hematopoietic stem cells. About 85-90% of AML cases express the CD33 antigen. CD33 has consequently gained clinical importance as a suitable tumor-associated antigen and target for antibody-based AML therapies.[13] Considering the availability of an anti-CD33 MoAb for clinical use,[14,15] the aim of this study was to investigate the relationship between the qualitative and quantitative CD33

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expression and the presence of mutations of the *NPM1* and *FLT3* genes in AML cells.

Design and Methods

Patients

Ninety-nine adult AML samples (with the exclusion of M3), routinely investigated at diagnosis were selected after exclusion of the major genetic aberrations (Aml-Eto, Inv16, Dek-Can, Bcr-Abl major and minor Bcr, MLL). All samples were studied in order to identify *NPM1* mutations by direct sequencing or its subcellular localization in bone biopsy specimens using immunohistochemical methods.[4] The same samples were also analyzed for the *FLT3-ITD* and *D835* point mutations by RT-PCR. All patients were enrolled in different GIMEMA protocols, which were approved by the local ethical committee. All patients gave their informed consent for these biological studies.

Forty-eight patients were males and 51 females; median age was 50 years (range 19-83). Median white blood cell (WBC) count was 21,200×10^9/L (range 470-292,000 ×10^9/L). According to the FAB classification, 3 cases were M1, 28 were M2, 36 were M4, 10 were M5, 5 were M6 and 4 were M7. Thirteen cases were analyzed on peripheral blood and the FAB classification was not available.

Analysis of NPM1 mutations

Exon-12 *NPM1* mutations were analyzed by direct sequencing as previously described.[2] One microgram of total RNA was retrotranscribed using the MMLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). cDNA sequences were amplified with primers NPM1_25F, 5'-GGTTGTTCTCTGGAGCAGCGTTC-3' and NPM1_1112R, 5'-CCTGGACAACATTTATCAAA-CACGGTA-3' using Taq Gold DNA Polymerase (Applied Biosystems). PCR products, purified by standard methods, were sequenced directly from both strands using the same primers employed for the amplification of the region in which the mutations fall.

Immunohistochemical staining

Immunostainings were performed using the APAAP technique as previously described.[4] The NPM1 subcellular distribution (nucleus-restricted *vs.* cytoplasmic) was assessed and cases were classified as either NPM1c$^+$ (cytoplasmic-positive) or NPM1c- (cytoplasmic-negative). All cases were stained in parallel for nucleolin (C23) that, in NPM1c$^+$ cases, was required to be nucleus-restricted.[4]

ASO-PCR FLT3-ITD

A multiplex PCR approach was adopted using 2 μL of the same cDNA to amplify both exon 11 and exon 17 of the *FLT3* gene, as reported elsewhere.[16] Of the single step PCRs, 15 μL were digested with *Eco*RV (Amersham International, UK) and electrophoresed on a 3% agarose gel containing ethidium bromide. The products were visualized under UV light.

Immunophenotype on leukemic cells

Whole bone marrow (86 cases) or peripheral blood (13 cases) cells collected in EDTA were stained with various combinations of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin chlorophyll-protein (PerCP)- and allophycocyanin (APC)- labeled MoAbs (Table 1), according to the manufacturer's instructions.[17] All MoAbs were from BD Biosciences (San Jose, CA, USA), with the exception of MPO, CD3, CD79-a, CD117 (Immunotech Coulter Company, Marseille, France) and Glycophorin-A (Dako, Glostrup, Denmark).

Cells were acquired on a FACSCalibur cytometer (BD Biosciences), by collecting at least 30,000 ungated events per tube. Data analysis was performed using the PAINT-A-GATE software (BD Biosciences).

CD33 expression

The PE-conjugated anti-CD33 MoAb (BD Biosciences) was included in the diagnostic panel and was used for further evaluations. Its expression was estimated by assessing the proportion of positive leukemic cells and quantified on the basis of the MFI values using the CELLQuest software (BD Biosciences) and ABC values through the QuantiBRITE PE system (BD Biosciences), designed for PE-labeled MoAbs. QuantiBRITE PE is a set of four beads with R-PE covalently attached at four different levels that was used for standardization before acquisition of samples. Ten thousand events were acquired from each tube using a FACSCalibur flow cytometer and data were analyzed using the CELLQuest software. The QuantiCALC software program was used to convert flow cytometry data on CD33 in terms of antibodies bound per cell.[18]

The MFI and ABC values of the CD33 antigen were determined using the same antibody combinations for all cases analyzed (Table 1).

Results and Discussion

NPM1 is considered the hallmark of a distinct subtype of AML that constitutes about one third of primary adult AML cases (with the exception of the M3 subtype)[2] and is included as a provisional entity in the WHO classification of lympho-hemopoietic neoplasms.[19] In our series of non-M3 adult AML cases studied at presentation which did not carry major genetic abnormalities, we identified 43 of 99 cases that showed the presence of *NPM1* mutations by immunohistochemistry and/or mutational screening. Among the 43 mutated patients, 34 were subject to direct sequence analyses with the purpose of identifying the

Table 1. MoAb combinations used to characterize AML cells. MoAbs are ordered by conjugated fluorochromes: FITC, PE, PerCP, APC. The combination used to determine MFI and ABC values of the CD33 antigen is reported in bold.

cyMPO/cyCD79-a/cyCD3
CD34/CD117/CD4/HLA-DR
CD34/CD33/ CD13/ HLA-DR
CD15/CD11-b/CD45/CD34
CD16/CD11-b/CD45/CD34
CD7/CD56/CD33/CD34
CD2/CD14/CD45/CD34
CD41-a/CD13/CD33/CD34
CD61/CD13/CD33/CD34
CD45/Glycophorin-A

cy: cytoplasmic

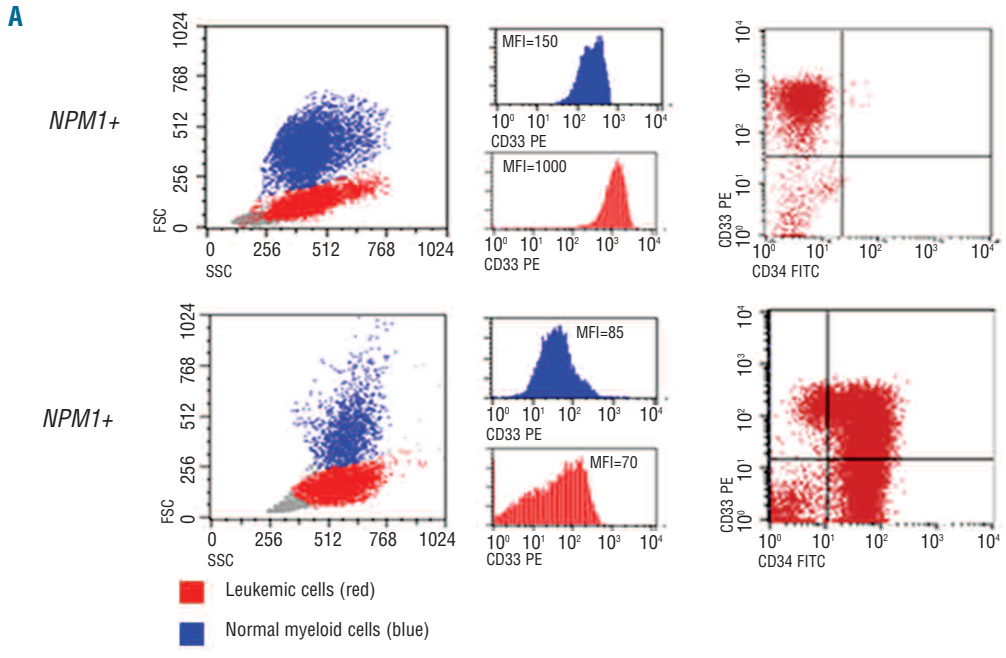

Figure 1. (A) **Example of two AML patients (*NPM1*⁺ and *NMP1*⁻) with different CD33 expression levels. The histograms show the different expression intensity (MFI) of CD33 between leukemic cells and normal myeloid cells; (B) CD34 expression in *NPM1*⁺ and *NMP1*⁻ AML cells.**

exact type of *NPM1* gene alteration. Of these, 27 showed type A, 5 type B and 2 the type D mutation. Forty-four of the 56 *NPM1* unmutated patients were analyzed by immunohistochemistry and showed the normal nuclear distribution of the NPM1 protein (absence of mutations was confirmed by sequencing in 15 of 44 cases); in 12 of 56 cases, the absence of a mutation involving the 12 *NPM1* exon was performed by sequencing analysis only.

All 99 cases expressed the CD33 antigen on a median proportion of 71% of cells (range 13-94%). Considering the *NPM1*-positive and *NPM1*-negative cases, significant differences in CD33 MFI and ABC values were found: a median MFI value of 429 (range 15-1,806) was found in the 43 *NPM1*-mutated patients, while in the 56 *NPM1*-unmutated patients the median value was 210 (range 27-990). This difference was highly significant (P=0.0001). CD33 ABC values were studied in 31 cases. This analysis demonstrated that 12 of 31 *NPM1*-mutated cases showed a median ABC value of 11,016 (range 2,690-19,264), while 19 of 31 *NPM1*-unmutated cases had a median value of 3,803 (range 288-15,941) (P=0.0088). Furthermore, in *NPM1*-mutated patients a significantly higher MFI expression of the CD33 antigen was recorded in the leukemic cells compared to the normal residual myeloid population (P=0.038) (Figure 1A). On the contrary, no difference was observed between the leukemic and normal myeloid population in *NPM1*-unmutated patients; similarly, no significant difference was observed in the levels of CD33 antigen expression in the normal residual myeloid population between *NPM1*-mutated and *NPM1*-unmutated patients.

In agreement with previous results,[2] a higher WBC count was found in the *NPM1*-mutated cases compared to the unmutated group (47,570×10⁹/L, range 1,520-228,800×10⁹/L *vs.* 8,480×10⁹/L, range 470-292,000×10⁹/L, P=0.027). Finally, as previously reported,[2,5] *NPM1*-mutated patients showed a higher proportion of CD34 negative cases (79%) compared to *NPM1*-unmutated cases (32%) (Figure 1B).

The *FLT3* mutation was detected in 22 of 99 patients (22.2%): *FLT3-ITD* in 18 of 22 patients, *FLT3-D835* in 3 of 22 patients, while one patient carried both the *FLT3-ITD* and *FLT3-D835* mutations. In keeping with previous observations,[2,7] *FLT3*-mutated cases showed a closer association with the *NPM1*⁺ than the *NPM1*⁻ group: 18 of 43 (41.8%) and 4 of 56 (7%) *FLT3*-mutated cases, respectively. Nevertheless, the alterations of the *FLT3* gene did not influence the CD33 expression levels on the leukemic cells. In fact, if we consider the *NPM1*⁺/*FLT3*⁺ and *NPM1*⁺/*FLT3*⁻ groups, no differences were observed in terms of CD33 MFI (average 435.4 *vs.* 578.4, P=0.38) and ABC (average 9,143.7 *vs.* 10,270.1, P=0.72) values. Taken together, these results demonstrate that although there is an association between *FLT3* and *NPM1* mutations within the *NPM1*⁺ and *NPM1*⁻ groups, alterations of the *FLT3* gene do not influence the CD33 expression levels on the

leukemic cells.

Determining the levels of CD33 expression on the surface of AML cells may have clinical implications. A higher expression intensity of the antigen implies a higher binding of the therapeutic antibody and, consequently, a better delivery of conjugated chemotherapy. Indeed, cells displaying a higher CD33 intensity have a greater likelihood of capturing and internalizing the anti-leukemic agents, as observed for acute promyelocytic leukemia patients.[20] Our study highlights that AMLs with *NPM1* mutations show a significantly higher degree of expression of the CD33 antigen and suggest that this observation may have implications in the clinical management of this important subgroup of AML patients.

Authorship and Disclosures

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