HLA-DR-negative AML (M1 and M2): FLT3 mutations (ITD and D835) and cell-surface antigen expression

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【 学位論文題目 】

HLA-DR-negative AML (M1 and M2): FLT3 mutations (ITD and D835) and cell-surface antigen expression
(DR陰性AML (M1/M2) におけるFLT3遺伝子変異 (ITD及びD835) 及び形質の研究)

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INTRODUCTION

Acute myeloid leukemia (AML) cells usually express HLA-DR (DR) antigen, with the exception of M3 (French-American-British; FAB) subtype, which is characteristically DR-negative (DR^-). Although the infrequent existence of DR^- non-M3 AML cases has been known, the phenotypic spectrum and the gene abnormalities of the DR^- non-M3 AML cases have yet to be clarified. One study pointed out that there is a correlation among 3 rare characteristics found in AML: the DR^- status, the cup-like nuclear structure and the fms-like tyrosine kinase (FLT3) internal tandem duplication (ITD) (FLT3-ITD), but not a direct correlation between FLT3-ITD and DR^- status. This led us to investigate the mutations of the FLT3 gene and the expressions of cell-surface antigens (CSAs) in DR^- AML (M1/M2) cases in comparison with DR^+ cases.

MATERIALS AND METHODS

Twenty-nine DR^- M1/M2 samples were found among 259 AML samples that were submitted to the Department of Laboratory Medicine, Kobe University Hospital, for CSA analysis and additional tests. CSA analysis was performed by direct or indirect cell-surface immunofluorescence, using a total of 18 monoclonal antibodies, in 29 DR^- M1/M2 samples and 30 DR^+ M1/M2 samples. Samples were defined as positive when the indicated antigen was detected on $\geq$ 20% of cells in the leukemic gate, except for CD13, CD33, and CD34 which used $\geq$ 15%.

RT-PCR for PML-RARα was performed in the DR^- M1/M2 samples to confirm the absence of PML-RARα fusion gene. RT-PCR to detect the FLT3-ITD and D835 mutation were performed in 55 (27 DR^- and 28 DR^+) M1/M2 samples, in which the cDNAs were available. Samples, which were positive for FLT3-ITD and D835 mutations, were subsequently sequenced after ligation with a TA cloning vector (Invitrogen, Carlsbad). The obtained sequences were compared with the wild-type FLT3 mRNA reference sequence (GenBank accession no. NM_004119.1).

SPSS version 10 for windows was used to analyze the CSA data and the incidence of FLT3-ITD and D835 mutations to examine the significance of differences between DR^- and DR^+ M1/M2 AML samples. The analyses were performed by the Chi-square test (Fisher's exact test for two-sided test) and the $P<0.05$ were considered as statistically significant.

RESULTS

Cytogenetic analysis and PML-RARα fusion mRNA

The cytogenetic analysis revealed normal karyotypes in 19 available data of DR^- samples and PML-RARα fusion mRNA was not detected by RT-PCR in any of the DR^- M1/M2 AML samples.

Cell-surface Antigen (CSA)

The expression patterns of several antigens were different between the DR^- and DR^+ groups. These antigens included CD34 antigen ($P<0.0001$), CD7 antigen ($P=0.002$), CD45RA antigen ($P<0.0001$), and CD45RO antigen ($P<0.0001$). CD34 was negative in 24 of 29 DR^- samples. CD7 was not expressed in 26 of 28 DR^- samples. CD45RA/RO isoforms in the DR^- AML were tested in 25 samples (10 M1 and 15 M2). In the DR^- M1 group, 8/10 expressed RA^-/RO^+ (RO-type) and 2/10 expressed RA^+/RO^- (RA-type), and no RA^+/RO^+ (mixed-type) was observed. In the DR^- M2 group, each of the expression patterns of CD45RA/RO isoforms were found at almost equal rates. The RO-type, RA-type, and mixed-type were observed in 5/15, 4/15, and 6/15 of DR^- M2 samples, respectively. The $CD34^-$ and $CD7^-$ status was found in 12 of 13 RO-type DR^- M1/M2 samples. Almost all DR^+ M1 and M2 cases expressed the RA-type. No RO-type was observed in either M1 or M2 DR^+ samples. In contrast, The RO-type was observed only in the DR^- group, and almost all DR^- M1 samples.

Incidence of FLT3 mutations in DR^- M1/M2 AML

FLT3-ITD and D835 mutations were detected in 21 of 55 (38.2%) and 3 of 55 (5.5%) M1/M2 AML samples, respectively. Of 27 cDNAs that were available from the 29 DR^- samples, 18 samples (66.6%) had FLT3 mutations: ITD in 16 samples and D835 in 2 samples. In contrast, the incidence of FLT3 mutations in the DR^+ group was lower, found only in 6 of 28 samples (21.4 %), with ITD in 5 samples, and D835 in 1 sample. The incidence of FLT3-ITD was higher in the DR^- group than in the DR^+ group ($P=0.002$), whereas the incidence of D835 mutation showed no significant difference between the two groups ($P=0.611$).

FLT3 mutations analysis

A simple duplication was found in 11 cases, and a duplication with insertion in 7 cases. An insertion derived from intron 14 was detected in 2 cases. All mutations resulted in in-frame transcripts and ranged in size from 18 to 93 nucleotides (median 60).

Two mutation-type sequences showing deletion in exon 14 of the juxtamembrane (JM) region were newly found in a case of DR^+ M2 having t(8;21). Four new additional point mutations (PMs) and polymorphism in exon 13 nt 1740, besides the encountered ITD, were detected in 3 DR^- samples and in 2 DR^- samples respectively.

Sequencing of the D835 point mutations revealed a common mutation D835Y, in two DR^- samples. In DR^+ Sample, the sequencing revealed 2563-2564T>AinsCCTCCC, resulting in the substitution of Ile by Thr, Ser, and His. Nonetheless, the mutant sequence within the kinase domain remained in-frame after the insertions.

DISCUSSION

FLT3 mutations

The incidence of FLT3 mutations (ITD and D835) in the DR^- M1/M2 AML samples was high (66.6%). The incidence of FLT3-ITD mutations (59.3%) in the DR^- M1/M2 cases is comparable to that in the reported M3 variant cases (45%-65%). Thus, FLT3 mutation is the most common genetic alteration in DR^- M1/M2 AML cases, since there is no known common chromosomal abnormality or other gene alteration in this subset. The high incidence also indicated that DR^- M1/M2 is one of the AML subsets in which FLT3-ITD mutation is found most frequently. There was a significant association (P=0.002) between the DR^- status and the FLT3-ITD in the tested M1/M2 AML cases.

The incidence of D835 mutations in DR^- M1/M2 AML (7.4%) coincided with those found in other studies of AML (7%-10%). In contrast to FLT3-ITD, there was no significant association (P=0.611) between the D835 mutation and the DR^- status in the M1/M2 AML samples.

FLT3-ITD mutations always led to in-frame transcripts, and each of the mutant ITD sequences were unique for each sample, except in the two samples that had a duplication derived from intron 14. Interestingly, one case of DR^+ M2 AML, exhibited not only duplication and insertion but also deletion of the JM-domain coding region. This is the second report of in-frame deletions in the JM-domain coding region, and the first report in AML. This sort of small deletion (11-12 amino acids) has already been demonstrated to cause constitutive activation that might result from alteration of the length of the JM domain.

The 4 newly found point mutations (PMs) in exon 14 (S574 and E598), exon 13 (C553) and exon 12 (N520), were detected in the allele in which the ITD mutations were

found (manuscript in press). The point mutations in the JM region result in dimerization and activation of the FLT3 receptor, although the resultant activation is weaker than that which the FLT3-ITD or D835 mutations can cause.

Cell-Surface Antigens (CSA)

CD45RO expression accompanied by the $CD34^-$ and $CD7^-$ status was the commonest pattern in the DR^- M1 cases. The $CD34^-$ and $CD7^-$ status was also the commonest pattern in the DR^- M2 cases. However, the pattern of CD45RA/RO isoforms was diverse, and included the mixed-type (40%), RO-type (33.3%), and RA-type (26.7%). This diverse pattern of CD45RA/RO expression in the DR^- M2 phenotype entity can be explained by the interpretation that the RO to RA shift is just starting along with maturation.

The DR^- status is not the isolated absence of an antigen, but is a change of pattern in which the expression of multiple antigens is turned on or off in a coordinated manner. One study reported that 10% of non-M3 cases (M1/M2 AML) were DR^- and $CD34^-$. A class of lineage-marker-negative (Lin^-) human hematopoetic repopulating cells of which the distinguishing features were $CD34^-$, DR^-, and Thy-1^- was discovered previously. The phenotype of the DR^- M1/M2 cells can be explained by the aberrant over carry of the $Lin^-CD34^-CD38^-$ phenotype in the immature myeloid stage. These $Lin^-CD34^-CD38^-$ cells may possibly express CD45RO.

General Comments

The high incidence of FLT3-ITD in the DR^- M1/M2 AML has not been reported by direct analysis. We found that the DR^- status in M1/M2 AML is associated with the $CD34^-$, $CD7^-$, and $CD45RO^+$ expressions. Nonetheless, the overall phenotypic spectrum and the gene abnormalities in the DR^- AML (M1/M2) have yet to be delineated.

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論文審査の結果の要旨

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（要旨は　１０００字～２０００字程度）

一般に急性白血病細胞は、FAB分類M3を除きHLA-DR (DR)抗原を発現する。一方、DR抗原陰性の非M3白血病も稀にではあるが報告されているものの、それらの細胞形質や遺伝子異常については明らかになっていないのが現状である。近年、急性白血病細胞におけるDR抗原陰性状態（DR^-）と杯様核構造、fms-likeチロシンキナーゼ（FLT3）のinternal tandem duplication (ITD)三者が相関するが、DR^-とFLT3-ITDの間には直接の関連が見出されないことが報告されている。この様な背景に基づき、本研究ではAML (M1/M2)におけるDR抗原発現状態とFLT3遺伝子変異を比較検討するとともにCD45RA/ROアイソフォームの発現を始めとした各種表面抗原の発現状況を検索し、DR^- M1/M2 AMLの特徴付けを試みた。

研究に供された検体は1989年から2001年までに神戸大学医学部附属病院ならびに関連施設にて細胞表面抗原検索を行った259例のAML保存資料であり、就中29検体（26例）がDR^- M1/M2 AMLであった。対照には1991年から1999年の間に保存されていた30検体のDR^+ M1/M2を用いた。DR^- M1/M2 AMLに関しては、RT-PCR法により PML-RARα融合転写産物が存在しないことを確認し、さらにその内１９検体については細胞遺伝学的解析を加えた。細胞表面抗原CD2, CD5, CD7, CD11b, CD10, CD13, CD14, CD15, CD19, CD33, CD36, CD45, CD45RA, CD45RB, CD45RO, CD56, HLA-DRに対するモノクローナル抗体を用い、フローサイトメトリーにより発現を解析した。

FLT3-ITD検索にはRT-PCRにより増幅した重複配列挿入部位断片を電気泳動し、野生型PCR産物より分子量の大きなものをスクリーニング後直接塩基配列決定を行った。一方、FLT3 D835変異はRT-PCR産物の*Eco*RV制限酵素断片多型によりスクリーニングし、切断されなかった産物をTAベクターにクローニング後塩基配列を決定した。

得られた結果は以下のごとくである。まず、RT-PCR解析が可能であった５５検体のM1/M2 AMLにおいて、FLT3-ITDは２１検体（38.2%)、D835変異は３検体（5.5%）に認められ、これらの頻度は過去に報告されたものとよく一致していた。その内訳は、DR^-検体ではFLT3-ITDは59.3%、D835変異は7.4%、DR^+検体ではFLT3-ITDは17.9%、

D835 変異は 3.6%であり、DR-群において DR+群に比較して有意に FLT3-ITD が頻発していた（$P = 0.002$）。一方、D835 変異頻度に関しては両群間に有意差は認められなかった（$P = 0.661$）。次に、細胞表面マーカー解析では、CD34 は DR^-群（17.2%）、DR^+群（79.3%）；CD7 は DR^-群（7.1%）、DR^+群（40.7%）と有意に DR^-群で低頻度であり、CD45RO は DR^-群（76.0%）、DR^+群（11.1%）と DR^-群で有意に高頻度であった。以上から、FLT3-ITD が DR- M1/M2 AML における最も一般的な遺伝子変化であるとともに $CD34^-$, $CD7^-$, $CD45RO^+$が本型白血病を特徴付ける表面マーカー発現形質であることが示された。

本研究は、急性白血病 M1/M2 についてその遺伝子異常ならびに表面マーカー解析を行ったものであるが、従来殆ど行われなかった DR^- M1/M2 AML の特徴的遺伝子異常ならびに表面抗原形質について重要な知見を得たものとして、価値ある集積と認める。よって本研究者は医学（博士）の学位を得る資格があると認める。