Multidrug resistance 1 gene expression and AgNOR in childhood acute leukemias

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Abstract The multidrug resistance 1 (MDR1) gene product, P-glycoprotein (Pgp/p170) is a membrane protein, which acts as an ATP dependant efflux pump that expels a wide variety of organic compounds including chemotherapeutic agents from the cell. Pgp over expression has been demonstrated to be linked with poor treatment outcome and poor prognosis in a number of malignant tumors. AgNORs is a simple, reliable and inexpensive method of evaluating the proliferative activity of a tumor. We have studied MDR1 expression and AgNORS in 41 cases of acute leukemia in children. In this study, AgNOR counts in patients with acute lymphoblastic leukemia (ALL) L2 subtype (FAB classification) were significantly higher as compared to the ALL L1 subtype. Similarly, mean AgNOR count in the acute

myeloid Leukemia (AML) M2 subtype was significantly higher as compared to the ALL L1 subtype. However, there was no correlation between AgNOR and treatment outcome or between AgNOR counts and MDR1 expression in any of the subtypes of acute leukemia included in this series. In AML, MDR1 gene expression was found to be related to reduced remission induction rates and hence poorer prognosis. In ALL, our study has shown no difference in remission induction between MDR1 positive and MDR1 negative cases. This would suggest that factors other than MDR1 may be of relevance in Pediatric ALL.

Keywords Acute leukemia · AgNOR · Multidrug Resistance 1 · P-glycoprotein.

Abbreviations

ALL – Acute lymphoblastic leukemia

AML – Acute myeloid leukemia

MDR1 – Multidrug resistance 1

Pgp – P-glycoprotein

AgNOR – Nucleolar organizer regions

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Introduction

The use of chemotherapeutic drugs in the treatment of leukemia has been plagued by the emergence of resistant cells, either at initial presentation or at the time of relapse [1]. The membrane transporter protein p-glycoprotein (P-gp or P 170) has been found to be a major contributory factor in many cases. P-gp is encoded by the Multidrug resistance 1 gene (MDR1) and acts as an ATP dependent efflux pump, transporting a wide variety of apparently unrelated organic

compounds out of the cell. This protein is expressed in a wide variety of normal tissues and is said to play a role in the excretion of various substrates into bile, intestinal lumen and urine. P-gp also prevents the entry of toxic metabolites into cells [2]. Notably, the MDR1 encoded protein actively expels many drugs used to treat leukemias such as anthracyclines, vinca alkaloids and epipodophyllotoxins [3], thus decreasing intracellular drug accumulation. P-glycoprotein mediated efflux can be blocked by a number of chemo sensitizing agents such as verapamil, cyclosporin and quinine

In normal hematopoietic cells, MDR1 is expressed at very low levels [4]. However, overexpression is common on neoplastic cell membranes at diagnosis and at relapse of acute leukemias. Agents capable of modulating MDR-1 have been used for treating poor-risk AML including quinine, tamoxifen, calcium channel blockers, cyclosporine A, and its analog, PSC8335 [6].

Nucleolar organizer regions (AgNORS) are loops of DNA relating to the production of precursor of ribosomal RNA – AgNOR is a simple, inexpensive and reliable method of evaluating proliferative activity of tumors [7, 8].

In the present paper, we report our studies on MDR1 gene expression using immunohistochemistry and AgNOR count using the silver staining technique in 41 cases of acute leukemia in children.

Materials and Methods

Peripheral blood/bone marrow smears from 41 cases of acute leukemia in children were used in this study. Leishman stained smears were used to study morphology. The following cytochemical stains were carried out according to standard procedures [9]: Myeloperoxidase, Sudan black B, PAS and non specific esterase. The leukemias were classified according to FAB criteria [10, 11]. AgNOR staining was carried out on one smear from each case using the silver staining method of Crocker et al [8]. AgNORS are visualized as black intranuclear dots. Five hundred cells from each smear were examined under oil immersion objective, the number of AgNOR dots counted and the mean number per nucleus determined. The enumeration of AgNORS was done by method of Crocker et al [8]. MDR1 immunostaining [12] was carried out on smears using the APAAP technique. Briefly, the smears were fixed in cold acetone for 10 minutes and then rinsed in Tris buffer, 0.05M, pH 7.6. The smears were then incubated with prediluted primary mouse monoclonal antibody to P-glycoprotein (clone JSB1, Zymed) followed by rabbit antimouse secondary antibody (Dako, Denmark). Mouse APAAP (Dako) was then added followed by substrate solution containing Naphthol AS MX

phosphate and Fast Red TR with Levamisole. The slides were rinsed in TBS buffer (2 changes, 2 minutes each) in between the incubation steps. Positivity was indicated by red color at the sites of binding of primary antibody. Normal renal tissue was used as positive control. Patient samples were considered MDR positive if $\geq 1\%$ of the blasts showed reactivity with MDR antibody as per standard criteria [13, 14]. Remission was assessed at the end of 1 month of induction therapy according to the established criteria [15]. The results were analyzed statistically using the SPSS package.

Results

Patient Characteristics

41 pediatric patients with acute leukemia were studied. The median age of the patients was 3 years (range –36 days to 12 years) and sex ratio M: F was 1:4:1 (24 and 17 patients respectively). The mean WBC count was $25.5 \times 10^9/l$ and mean blood blast percentage was 62.2%. The population studied included 36 newly diagnosed cases and 5 cases presenting at the time of relapse. The numbers of patients in different FAB categories is indicated in Fig. 1.

MDR Expression

The results of MDR immunostaining are given in Fig. 2. There was no statistically significant correlation between expression of MDR1 and known prognostic parameters-total WBC count, age and blast count at the time of presentation. Cytoplasmic positivity was observed in 12 cases and membrane positivity in 4 cases (Fig. 3). The intensity of MDR immunostaining was weak to moderate. Of the 41 cases enrolled in our study, 8 (5 ALL, 3 AML) had to be excluded from evaluation of treatment results as 2 patients died due to sepsis and 6 patients were lost to follow up. Remission induction data for ALL is indicated in Table 1.

Table 1 All – MDR1 positivity and response to treatment.

Presentation	MDR1 positive		MDR1 negative*	
	No of cases	no in CR**	No of cases	no in CR
New cases	9	8 (89%)	11	10 (91%)
Presenting at relapse	1	0 (0%)	4	3 (80%)
Total	10	8 (80%)	15	13 (87%)

* 5 MDR1 negative cases lost to follow up.

** CR= Complete remission

Fig. 1 Distribution of cases under different subtypes of acute leukemia

Fig. 2 MDR1 positivity in different subtypes of Leukemia

Fig. 3 MDR1 positivity in blasts of ALL (arrows) (APAAP-MDR1 X 630).

Fig. 4 AML – MDR1 phenotype and clinical remission acute leukemia.

In ALL, the differences in remission induction between MDR positive and negative cases were not statistically significant. Among the 5 ALL cases presenting at the time of relapse, 4 of MDR negative cases achieved remission and the only MDR positive case did not obtain remission.

In the AML group (Fig. 4), complete remission was achieved only in 33% MDR positive cases in contrast to 100 % of MDR negative cases. However, as the number of cases studied was small, the difference between MDR positive and MDR negative population in AML and in relapsed ALL groups could not be analyzed statistically.

AgNOR Count

AgNORs counted by Technique 1 of Crocker [8] in different FAB categories are given in Fig. 6. The mean AgNOR count in acute lymphoblastic leukemia was lower than the mean AgNOR count in acute myeloid leukemia. The difference in AgNORs counts between L2 and L1 and between L1 and M2 were statistically significant ($P < 0.01$). There was no statistical correlation between AgNOR count and remission induction or between AgNOR count MDR1 expression. It was observed that the appearance of AgNORs was small dots in ALL while in AML larger dots and bleb like structures in addition to smaller dots were observed (Fig. 5).

Fig. 5 AgNOR counts in subtypes of AML M2

Fig. 6 AgNOR dots and blebs are seen within blast cells of AML. (AgNOR X 630)

Discussion

The discovery of P-glycoprotein marked an important starting point since cancer cells with this membrane protein provided investigators with a prototypical system for studying the drug resistance phenomenon. It is of particular significance to the area of acute leukemia as the overexpression of p glycoprotein can lead to resistance to drugs that are important in the treatment of this group of malignancies. We have studied MDR1 expression and AgNOR counts in 41 children with acute leukemia.

MDR1 expression was seen in 33% of cases of ALL as compared to 55% of cases of AML. We did not observe any statistically significant correlation between MDR1 expression and known prognostic parameters – age, total, initial WBC count, blast percentage at presentation, Hb levels and platelet count. These results are similar to those reported by other workers [16, 17]. A significantly higher proportion of patients with the FAB L2 subtype of acute lymphoblastic leukemia expressed p-glycoprotein as compared to the L1 subtype. Similar correlation with AML subtypes could not be studied statistically in view of the small no of samples in each subgroup. Although MDR1 expression has been studied in relation to immunological subtyping of ALL by other authors, correlation with FAB subtypes has not been analyzed so far. We did not find a significant difference in remission induction rates between MDR1 positive and MDR1 negative ALL cases. This is consistent with the observation of Goasguen et al [4] in pediatric ALL. These authors found a significant difference between remission induction in the two groups in adult ALL and this led them to conclude that factors other than MDR1 may be important in children. However, in a series reported by Casale et al [17], it was found that MDR1 expression in childhood ALL was adversely related to treatment outcome and could be useful biological marker of treatment response. These workers and others [18] also reported a significant negative correlation between Pgp expression in pediatric ALL and relapse rate and the 10 year event free survival rates. It is interesting to note that the TEL/AML gene, which is associated with good prognosis in acute lymphoblastic leukemia has been shown to overcome drug resistance through transcriptional repression of multidrug resistance -1 gene expression [19].

In the AML patients included in our study, only 33% of MDR positive cases achieved complete remission, while 100 percent of the MDR1 negative cases achieved complete remission. However, this data could not be analyzed statistically because of the smaller number of cases in the MDR negative subgroup.

AgNORs have been widely studied in a variety of neoplasia. The morphology of AgNOR dots was small and dot like in ALL cases while they were larger and bleb like in AML as reported earlier [20]. We found a statistically significant increase in AgNOR counts in ALL L2 and AML M1 as compared to the ALL L1 subtype. These results are similar to those observed by certain workers [20, 21]. We found no correlation between AgNOR count and remission induction in the 41 cases of acute leukemia included in this series. Pich et al [7] have reported that patients with mean AgNOR count above 6.6 showed a correlation with clinical remission and also with longer survival. AgNOR number has been reported to show direct correlation with Ki67 reactivity of leukemic blasts [20]. We found no correlation between AgNOR counts and MDR1 expression in our study. Such a correlation has not been previously investigated, to the best of our knowledge.

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