Clinical significance of p53 and Bcl-2 in acute myeloid leukemia patients of Eastern India

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Abstract
The frequency of p53 and Bcl-2 protein expression in 100 newly diagnosed and 10 relapsed acute myeloid leukemia (AML) patients was analyzed by immunocytochemistry (ICC). The Kaplan-Meier method was used for univariate and multivariate statistical analysis to assess the relationship between p53, Bcl-2 and clinico-hematologic feature with respect to overall survival (OS) using SPSS statistical software. No statistical significance was found in univariate analysis (P=0.60). However, when the subgroups of patients (+1, +2, +3 and +4) were compared, expression of p53 and Bcl-2 protein (1-10%, 11-30%, 31-50% and >50%) was statistically significant (P=0.05). However, in multivariate analysis, p53, immunopositivity was independently associated with a shorter overall survival (OS) (P=0.038) while Bcl-2 immunopositivity was associated with longer overall survival (OS) (P=0.002). Our finding shows that p53 and Bcl-2 protein overexpression is a strong indicator of response to chemotherapy and overall survival. This study reports for the first time AML in patients from Eastern India.

Introduction
Mutation of the p53 gene has been found in a wide variety of neoplastic diseases and functions as a specific transcriptional gene activator3 controlling the G1 check point of the cell cycle, DNA repair, synthesis and apoptosis.2,5 Mutations of the p53 gene are the most common genetic alteration in human cancer.7,6 The wild-type p53 protein has a short half-life and cannot be detected by immunocytochemistry in cells. Mutated p53 on the other hand, has a prolonged half-life and can be detectable by immunocytochemistry. Mutation of the p53 gene has been reported in only 5-10% of patients with acute myeloid leukemia but when present is associated with a low complete remission rate, early relapse and poor survival.7,10 Accumulation of altered p53 protein has been found to be an indicator of poor prognosis.2,5 The Bcl-2 gene, the first member of a rapidly expanding family of genes that inhibit apoptosis, was initially isolated from the t (14; 18) chromosomal translocation found in human B-cell lymphoma.11 Relatively high levels of Bcl-2 protein have been expressed in 20% of patients with AML.12 Bcl-2 is the best characterized of the proteins and its role in the pathogenesis and prognosis of AML has been studied with conflicting results.13,15 These studies have found that patients with higher levels of Bcl-2 have a lower remission rate, an inferior survival or both11,14 and, in contrast, the patients who do not express Bcl-2 had low remission rates and higher relapse rates than those with intermediate levels of Bcl-2.15,16

Several studies indicate that the protein expression levels of Bcl-2 determine the in vitro sensitivity of leukemic cells to chemotherapeutic drugs. A high level of Bcl-2 protein confers a survival advantage on B cells by inhibiting apoptosis and may block a common cell death pathway induced by chemotherapy. High levels of Bcl-2 expression confer resistance to apoptosis induced by chemotherapeutic drugs and association of Bcl-2 with poor prognosis in AML patients and other types of cancer.12,17,20 Bcl-2 protein expression has been shown to predict poor outcome in acute myeloid leukemia but conflicting results have been reported for ALL.21,22 In the present study, we examined specifically the protein expression of p53 and Bcl-2 in de novo acute myeloid leukemia patients before treatment and correlated their clinical and hematologic parameters to overall survival (OS).

Materials and Methods
Patients
This study includes 100 de novo and 10 relapsed de novo AML patients diagnosed in the Department of Clinical Hematology, S.C.B Medical College, Cuttack, India. Diagnosis and classification of AML was made using the French-American-British (PAB) classification after conventional cytochemical stains and surface marker analysis. Peripheral blood monocyte isolation
Using heparin as an anti-coagulant, 3 ml of blood were collected from AML patients. The heparinized blood was diluted in 3 ml of sterile PBS (pH 7.4). The PBS mixed blood was placed on a Ficoll Hypaque layer (density 1.077 gm/ml) in a 15 mL centrifuge tube; the proportion between the Ficoll Hypaque layer and blood should be 1:3 and 2:3, respectively. After centrifugation for 1,500 rpm for 30 min, the middle layer, which contains lymphocytes and blast cells, was collected without touching the Ficoll Hypaque layer. The lymphocytes were diluted with 1 ml PBS, and then washed twice in 1ml PBS.

Immunocytochemistry
Both p53 and Bcl-2 immunostained samples were taken from 5 positive controls and 5 negative controls. Lymphocyte fixations were performed in methanol. These slides were incubated for 10 min in 1.5% hydrogen peroxide. After rinsing in distilled water and TBS (pH=7.6), the slides were incubated with 1% blocking buffer (BSA) in TBS (pH=7.6) for 20 min to suppress the non-specific binding of immunoglobulin. Specific immunostaining was evaluated by means of overnight incubation at 4°C with the appropriate primary antibody (PAB 240, Calbiochem, USA) which can recognize both wild- and mutant type p53 protein in 1:100 dilutions. Mouse monoclonal antibody (Bcl2) was used to detect Bcl-2 proteins. The mouse monoclonal antibody (IgG1) Bcl-2, clone 124, is raised against an epitope between amino acid 41 and 54 of the Bcl-2 protein (1:100; Dako Corporation, Denmark). After washing in TBS, a secondary biotinylated goat anti-mouse/rabbit antibody (Dako Corporation, Denmark) was used to detect Bcl-2 proteins. Both p53 and Bcl-2 protein expression were evaluated by means of overnight incubation at 4°C with the appropriate primary antibody (PAB 240, Calbiochem, USA) which can recognize both wild- and mutant type p53 protein in 1:100 dilutions. Mouse monoclonal antibody (Bcl2) was used to detect Bcl-2 proteins. The mouse monoclonal antibody (IgG1) Bcl-2, clone 124, is raised against an epitope between amino acid 41 and 54 of the Bcl-2 protein (1:100; Dako Corporation, Denmark). After washing in TBS, a secondary biotinylated goat anti-mouse/rabbit antibody (Dako Corporation, Denmark) was used for 30 min.Slides were rinsed with TBS and incubated with streptavidin for 30 min. The peroxidase reaction was performed by incubating for 20 min in a developing solution containing diaminobenzidine (Sigma, USA). Finally, the slides were rinsed in TBS and then counter-stained with hematoxylin. The blast cells and lymphocytes have been differentiated accord-
ing to their morphological appearance; blast cells are larger in size with a more prominent nucleus than the smaller size lymphocytes. At least 1,000 cells were counted and the results were expressed as the percentage of positive cells. Slides were scored as immunonegative if no leukemic cells stained. Slides were scored as +1 (1-10%), +2 (11-30%), +3 (31-50%) and +4 (>50% positive cells).

### Statistical analysis

Univariate analysis was performed using the Kaplan-Meier method. The difference in survival between groups was evaluated by the log rank test. Multivariate analysis was performed using Cox's regression method with stepwise forward selection of independent variables based on the likelihood ratio. P<0.05 was considered statistically significant.

### Results

Immunostaining for p53 and Bcl-2 was successful in 110 AML patients. Patients' clinical characteristics are listed in Table 1.

Expression of p53 and Bcl-2 is heterogeneous in all-FAB types

A broad range of p53 and Bcl-2 protein expression was seen in FAB types (M1-M5) and in none of the patients with M6 and M7 FAB types, as shown in Table 1. There was a statistical difference in dispersion of p53 and Bcl-2 protein expression. Ten of the 110 patients had died at the time of analysis. Statistical analysis strongly indicated that Bcl-2 had a higher threshold effect on survival than p53.

#### p53 expression and survival

Among the 110 patients, 100 (91%) patients were p53 immunopositive while 10 (9%) patients were p53 immunonegative. Of the p53 immunopositive cases, 6 (5%) contained 1-10% of p53-positive cells, 22 (20%) had 11-30%, 16 (14%) had 31-50% and 56 (51%) had highly expressed, i.e. more than 50%, p53 immunopositive leukemic cells. The relationship between p53 and Bcl-2 protein expression for the cut off used for scoring is shown in Table 2. We compared the p53-immunostaining results with the clinical outcome of patients. The number of patients with p53 immunonegative (n=10) had a longer survival rate. However, a comparison of the p53-positive vs negative subgroup (Figure 1A) failed to reach statistical significance (P=0.60, log rank test). Kaplan-Meier curves showed that overall survival was shorter in subgroups +3 and +4 than in subgroups +1 and +2. There was a statistically significant difference in clinical outcome between the patient subgroups (+3 vs +4).

#### Table 1. Patients’ characteristics according to p53 and BCL-2 immunostaining (n=110).

| Parameter          | Category | N. patients | p53 +ve | p53 -ve | P value | Bcl 2 +ve | Bcl 2 -ve | P value |
|--------------------|----------|-------------|---------|---------|---------|-----------|-----------|---------|
| Sex                | Female   | 42          | 38 (90.4) | 4 (9.5) | NS      | 34 (90.9) | 8 (19.04) | NS      |
|                    | Male     | 68          | 62 (91.17) | 6 (91.17) | NS      | 62 (91.17) | 6 (8.82) | NS      |
| Age                | >2<10 year | 10 (9.09) | 10 (100) | NIL     | NS      | 10 (100)  | 0         | NS      |
|                    | ≥40<60   | 100         | 90 (90.90) | 10 (10) | NS      | 86 (86)   | 14 (14)   |         |
| WBC at diagnosis   | <50x10⁹/L | 72          | 62 (86.11) | 10 (13.88) | NS      | 64 (88.88) | 8 (11.11) | NS      |
|                    | ≥50x10⁹/L | 38          | 38 (100) | NIL     | NS      | 30 (78.94) | 14 (21.05)|         |
| TPC at diagnosis   | <50x10⁹/L | 72          | 64 (88.88) | 8 (11.11) | NS      | 66 (91.66) | 6 (8.33) | NS      |
|                    | ≥50x10⁹/L | 38          | 36 (94.73) | 2 (5.26) |         | 30 (78.94) | 14 (21.05)|         |
| Lymphodeno-pathy   | No       | 74          | 66 (89.18) | 8 (10.81) | NS      | 66 (89.18) | 8 (10.81) | NS      |
|                    | Yes      | 36 (32.72) | 34 (94.44) | 2 (5.55) |         | 30 (83.33) | 6 (16.66) |         |
| Splenomegaly       | No       | 68          | 64 (94.11) | 4 (5.88) | NS      | 66 (97.05) | 3 (2.94)  | NS      |
|                    | Yes      | 42 (38.18) | 36 (85.71) | 6 (14.28) |         | 30 (71.42) | 12 (28.57)|         |
| Hepatomegaly       | No       | 76          | 66 (86.84) | 10 (13.15) | NS      | 72 (94.73) | 6 (5.26)  | NS      |
|                    | Yes      | 34 (30.90) | 34 (100) | NIL     | NS      | 24 (70.58) | 10 (29.41)|         |
| Mediastinal Mass    | No       | 98          | 90 (91.83) | 8 (8.16) | NS      | 88 (89.79) | 4 (33.33) | NS      |
|                    | Yes      | 12 (10.90) | 10 (83.33) | 2 (16.66) |         | 8 (66.66)  | 10 (33.33)|         |
| Serum LDH          | Normal   | 32          | 28 (87.5)  | 4 (12.5) | NS      | 26 (81.25) | 6 (18.75) | NS      |
|                    | Elevated | 78          | 72 (92.30) | 6 (7.70) |         | 70 (90.74) | 8 (9.26)  |         |
| FAB subtypes       | M1       | 32          | 32 (100)   | 0        | NS      | 28 (87.5)  | 4 (12.5)  | NS      |
|                    | M2       | 32          | 28 (87.5)  | 4 (12.5) |         | 28 (87.5)  | 4 (12.5)  |         |
|                    | M3       | 24          | 18 (75)    | 6 (25)   |         | 18 (75)    | 6 (25)   |         |
|                    | M4       | 18          | 18 (100)   | 0        |         | 18 (100)   | 0        |         |
|                    | M5       | 4           | 4 (100)    | 0        |         | 4 (100)    | 0        |         |
|                    | M6       | 0           | 0          | 0        |         | 0         | 0        |         |
|                    | M7       | 0           | 0          | 0        |         | 0         | 0        |         |

*P value denotes the significance of difference between p53 immunopositive vs p53 immunonegative, and Bcl-2 immunopositive vs Bcl-2 immunonegative. TPC, total platelet count; LDH, lactate dehydrogenase; FAB-French-American-British, NS, not significant.
Among 100 p53 immunopositive patients, 9 patients died within 1-20 months (median 11) and 91 were alive at a median follow up of 22 months (range 1-24). On the other hand, among p53 immunonegative patients, one patient died at 15 months and 9 patients were alive at a median follow up of 22 months (range 1-24). The difference between these two groups did not reach statistical significance (P=0.60, log rank test).

**Bcl-2 protein expression and survival**

Among the 110 patients, 96 (87%) were Bcl-2 immunopositive while 14 (13%) patients were Bcl-2 immunonegative. Of the Bcl-2 immunopositive cases, 10 (9.09%) included only a small percentage (1-10%) of Bcl-2 positive cells, 24 (21.81%) had low intermediate (11-30%), 20 (14%) had high intermediate (31-50%) and 42 (51%) had highly expressed (>50%) Bcl-2 immunopositive leukemic cells.

A comparison of Bcl-2 positive vs negative subgroups did not reach statistical significance (P=0.24, log rank test, Figure 1B). Kaplan-Meier curves showed that overall survival was shorter in subgroups +1 and -4. The log rank test showed a statistically significant difference in clinical outcome between subgroups: +1 vs +2, P=0.0558; +2 vs +4, P=0.0246; +3 vs +4; P=0.0021. However, there was no statistically significant difference between subgroup +1 vs +3 (P=0.1729) or subgroup +2 vs +3 (P=0.3799). Multivariate analysis showed that Bcl-2 immunopositivity (>10%) was associated with a longer overall survival (P=0.002).

Among 96 Bcl-2 immunopositive patients who were also p53 immunopositive, 5 died within 1-20 months (median 11) and 91 were alive at a median follow up of 22 months (range 1-24). On the other hand, among Bcl-2 negative patients, one patient died at 12 months and 13 patients were alive at a median follow up of 18 months (range 1-24). There was no statistically significant difference between the two groups (P=0.24, log rank test).

**Correlation of Bcl-2 with prognostic parameter**

We examined the possible association of Bcl-2 protein expression in primary leukemic cells of AML patients and their clinical features. Both age and WBC, which were predictive of poor outcome, were associated with a high level of Bcl-2 expression. Leukemic cells from patients with a WBC of 50×10^9/L or more showed significantly higher Bcl-2 levels than leukemic cells from patients with a WBC of less than 50×10^9/L. Age between 40 and 60 years was associated with high Bcl-2 expression. Leukemic cells of patients with splenomegaly or hepatomegaly expressed a high level of Bcl-2.

**Discussion**

p53 and Bcl-2 immunostaining data of AML patients were evaluated to establish a possible correlation with overall survival outcome (Tables 1 and 2). For example, patients whose leukemic cells were p53 immunonegative and Bcl-2 immunonegative were associated with a longer overall survival. Steven et al. reported the correlation between Bcl-2 immunopositivity with clinical outcome and showed longer overall survival in Bcl-2 immunopositive cases. The reason for this is that Bcl-2 and wild-type p53 are commonly expressed in normal B cells.

Conflicting data were obtained by different authors regarding p53 and Bcl-2 expression with respect to survival analysis. We observed that p53 immunopositive leukemic cells (>10%) were associated with improved survival but Karkas et al., reported that the patients in their study with p53 immunopositive had a higher percentage of blast cells, leukocytes and platelets counts, but that these had no influence on the complete remission rate. Similar to other studies, Campos et al. reported overexpression of Bcl-2 was correlated with poor treatment outcome in AML patients. Our results showed statistically significant correlations in Bcl-2 immunopositive cases (P=0.0038) in univariate analysis, while in multivariate analysis prognostic factors such as age at diagnosis, gender, WBC count, platelet count, splenomegaly, hepatomegaly and serum LDH were associated with longer overall survival (P=0.002). To assess the importance of Bcl-2 expression in pediatric AML, we found a correlation between clinical outcome and level of Bcl-2 protein expression. The correlation of this with patient response to chemotherapy or long-term outcome did not reach statistical significance, which was similar to the findings of other workers. High levels of Bcl-2 would allow a cell to escape or suppress apoptotic signals, including those induced by chemotherapists. Bancker et al. reported a higher percentage of patients expressing high levels of Bcl-2 at relapse and this difference was not always statistically significant, as reported by Bense et al. We observed that a low level of Bcl-2 was associated with lower response rates, shorter remission duration and inferior survival. A lower level of Bcl-2 was an independent prognostic factor for both survival and remission duration. Karkas et al. reported that patients with high Bcl-2 level had a better complete remission rate (63 vs 51%). Lepelley et al. reported Bcl-2 levels in MDS patients and observed that high Bcl-2 was a favorable prognostic finding. In the study by Maung et al., none of the patients with secondary leukemia and lower levels of Bcl-2 expression achieved CR. However, this cannot be compared with other studies.
the present study due to the small sample size and low remission rate. From our study, it is clear that the Bcl-2 protein expression is significantly higher than in normal controls, which is consistent with other reports. This indicates that the alteration of Bcl-2 is an important mechanism in the pathogenesis, progress and the development of drug resistance in AML. Bcl-2 might have a different role in disease pathogenesis and leukemic progression, thus giving different prognostic implications for future therapies designed to induce apoptosis or block anti-apoptotic proteins. A recent clinical trial studied antisense deoxyligonucleotides for Bcl-2. AML patients may, therefore, show a mechanism of protein stabilization and this has been observed in interactions with viral or other cellular proteins. Alternatively, in these patients our observations may represent a high level of constitutive expression, which has been reported previously.

In summary, statistical analysis showed a significant correlation between p53 and Bcl-2 protein. Therefore, these two apoptotic-regulating proteins may be considered prognostic marker for acute myeloid leukemia.

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