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Commentary

Role of apoptotic markers in paediatric acute lymphoblastic leukaemia

Acute leukaemia is the most common form of cancer in children, comprising approximately 30 per cent of all childhood malignancies[1]. Of the acute leukaemias, acute lymphoblastic leukaemia (ALL) occurs five times more commonly than acute myeloid leukaemia (AML). Survival rates for ALL have improved dramatically since the 1980s, with a current five-year overall survival rate estimated as greater than 85 per cent[1,2]. This improvement in survival is due to treatment of a large number of children on sequential standardized protocols, the goals of which are to improve clinical outcomes while minimizing acute toxicities and late-occurring adverse events. The current treatment protocols for ALL in children emphasize on risk-based therapy to reduce toxicity in low-risk patients while ensuring appropriate, more aggressive therapy for those with a high risk of relapse. Important risk stratification criteria for ALL in children include white blood cell count, age at the time of diagnosis, cytogenetics, immunophenotype and response to induction therapy in terms of steroid response and minimal residual disease status at the end of induction[3]. However, a significant number of patients still fail to respond to therapy despite the presence of favourable prognostic features[4,5]. In fact, management of relapsed ALL remains one of the most challenging areas of paediatric oncology.

Recent *in vitro* research studies based on chemotherapeutic resistance has renewed the focus on the role of apoptosis pathways in risk stratification and treatment of leukaemia patients. It is strongly believed that cancer chemotherapeutic agents act primarily by inducing cancer cell death through the activation of diverse apoptosis signalling pathways. In leukaemias, apoptotic processes occur both spontaneously and induced by anti-tumour therapies. At the cellular level, apoptosis is regulated by two major signalling pathways: (*i*) the receptor-mediated extrinsic pathway, and (*ii*) the mitochondria-mediated intrinsic pathway[6]. The susceptibility of cells to apoptosis depends on the relative expression of pro-apoptotic (Bax, Bcl-10, Bak, Bid, Bad, Bim, Bik and Blk) and anti-apoptotic molecules (Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, MCL-1)[7]. Through *in vitro* studies, it has been shown that functional defects in apoptosis signalling molecules or deficient activation of apoptosis pathways are responsible for chemotherapy resistance and treatment failure in acute leukaemia.

The translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the outer surface is a characteristic early change in apoptotic cells, occurring before the loss of cell membrane integrity. Annexin-V is a calcium-dependent, phospholipid-binding protein with high affinity for phospholipid phosphatidylserine[8]. Flow cytometric analysis using Annexin-V binding of translocated phosphatidylserine is a sensitive quantitative assay for detection of early apoptotic cells[9]. A potential drawback is the binding of Annexin-V to apoptotic cells even in conditions of excess necrosis. It is often used in conjunction with vital dyes such as 7-amino-actinomycin or propidium iodide (PI) which bind to nucleic acids, but can only penetrate the plasma membrane when membrane integrity is breached, as occurs in the later stages of apoptosis or necrosis. Only the cells positive for Annexin-V and PI negative are considered as true apoptotic cells. Apoptotic index (AI) can be calculated as the number of apoptotic cells divided by the total number of cells examined as a percentage during pre-treatment (AI-day 0) and post-treatment (AI-day 35) time intervals. Bax/Bcl-2 ratio is assessed by flow cytometric assay using staining for Bax and Bcl-2 apoptotic proteins and measured as a ratio of relative mean fluorescence intensity of Bax divided by that of Bcl-2. Apart from this, a few other techniques can be used to demonstrate apoptosis such as acridine orange staining or *in situ* end-labeling methods such as TUNEL[10].

Expression of apoptosis-related genes has been associated with variable clinical outcome in haematologic malignancies. High Bax levels correlate with favourable prognosis in acute myeloblastic leukaemia (AML)[11], whereas enhanced expression of Bcl-2 is a poor prognostic factor in lymphomas and chronic lymphocytic leukaemia[12]. Merchant *et al*[13] have shown that the degree of apoptosis in myelodysplastic syndromes is higher in comparison to acute leukaemias, myeloproliferative disorders and normal controls. In ALL, studies published so far have yielded conflicting results. Aref *et al*[14] have reported that Bcl-2 expression at the time of diagnosis correlates with responsiveness to induction chemotherapy, but not the patient outcome. In contrast, another study found no association between Bcl-2 levels and disease aggressiveness or resistance to therapy[15]. Prokop *et al*[16] have suggested that the Bax/Bcl-2 ratio - rather than individual Bax or Bcl-2 levels are a more reliable prognostic indicator in ALL. They demonstrated that relapse in childhood ALL was associated with a decrease of the Bax/Bcl-2 ratio and loss of spontaneous Caspase-3 processing *in vivo*. Higher Bax levels were associated with increased risk of relapse in one study[12], whereas another one[17] showed that low Bax/Bcl-2 ratio correlated with favourable prognosis. Kaparou *et al*[9] reported a significant correlation between increased Bax/Bcl-2 ratio and high-risk features such as leucocytosis, DNA index <1.16 and the del(9p) chromosomal abnormality. In their study, the ALL patients in the high-risk group had a higher Bax/Bcl-2 ratio at diagnosis than at remission, achieved after induction chemotherapy, whereas no significant change was observed in the median-risk group. It may also be speculated that alterations in the expression of apoptosis-related genes could reflect changes in the tumour-burden of the disease as a response to chemotherapeutic regimens.

Malinowska *et al*[18] found no correlation between the extent of spontaneous apoptosis and post-initial treatment apoptosis and response to therapy. However, they demonstrated that refractory/relapsed disease occurred in the group of patients with spontaneous AI below the median. Srinivas *et al*[19] observed a borderline correlation between AI and Bcl-2 expression on day 7 of induction chemotherapy with total leukocyte count at presentation, the presence of mediastinal mass and hepatosplenomegaly in paediatric ALL.

The study published in this issue by Singh *et al*[20] brings out a few interesting points regarding the significance of apoptosis as a prognostic marker in paediatric ALL cases. The authors have attempted to study the association of pre- and post-treatment AI and apoptotic protein ratio (Bax/Bcl-2) with clinico-haematologic variables and response to chemotherapy. In their study, spontaneous apoptosis index (AI-day 0) ranged from 0.9 to 16.6 per cent with a mean±standard deviation (SD) of 5.90 per cent±4.5 and a median of 4.50 per cent. The post-induction treatment apoptosis (AI-day 35) ranged from 1.4 to 62.8 per cent with a mean±SD of 19.64 per cent ±17.39 and a median of 14.0 per cent. Although the mean difference in pre- and post-treatment apoptosis index was high, but a significant increase in apoptosis between two-time intervals as defined by a cut-off of 30 per cent was noted in only 24 per cent cases. They noted a significant association between low AI at day 0 and high total leucocyte count, T-cell immunophenotype and high-risk group. AI on day 35 had no association with any of the clinical or treatment response-related parameters. In addition, no association was noted between AI day 0 and day 35 with Bax/Bcl-2 ratio. Although this study has a few limitations with a small sample size and short follow up data, it provides important clues on the clinical pattern in Indian patients with ALL.

Meng *et al*[21] indicated that the paediatric ALL patients in the drug-resistance group showed significantly higher Bcl-2 mRNA expression, compared to those in the chemotherapy sensitive group. In addition, the Bcl-2 mRNA expression in chemo-sensitive patients decreased significantly after chemotherapy, while the expression of Bcl-2 mRNA in the drug-resistant group did not alter significantly before and after chemotherapy. Chonghaile *et al*[22] have reported that the maturation state of a malignancy can determine the specific anti-apoptotic protein on which it depends for survival and that transformation of an early T-cell progenitor retains its Bcl-2 dependence, whereas the CD4+ and CD8+ positive leukaemic blasts are more dependent on Bcl-XL.

Thus, it can be concluded that the ongoing research studies on the utility of apoptotic markers in predicting the clinical characteristics or prognostic outcome of paediatric ALL patients have shown promising results. However, owing to variations in the results of different studies published worldwide probably due to differences in ethnicity, sample size, follow up data, as well as techniques used to study apoptosis, there is a need to search for newer more-specific molecular markers to study this interesting area of molecular oncology and chemotherapy resistance.

***Conflicts of Interest*:** None.

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