Growth advantage of CD34+ cells in trisomy 8 high-risk myelodysplastic syndrome despite enhanced apoptotic signals

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ABSTRACT This study examined haematopoietic stem cells of 19 high-risk cases of myelodysplastic syndrome (MDS) for apoptotic and anti-apoptotic signals and cellular proliferation and correlated these with clinical and cytogenetic subtypes, particularly trisomy 8. The aim was to identify cellular and cytogenetic markers of prognostic relevance to survival of high-risk MDS cases. High-risk MDS cases had a significantly higher percentage of apoptotic CD34+ cells and anti-apoptotic survivin+ cells than controls, particularly for trisomy 8 cases. Trisomy 8+ cells showed a significant positive correlation with apoptotic CD34+ cells and capacity for colony formation. The latter was significantly lower in trisomy-8-negative cases than normal controls, while that of trisomy 8 cases was comparable to controls. Our results suggest that although trisomy 8 cells are in a pro-apoptotic state, they are regulated by the enhanced expression of anti-apoptotic signals which provide them with their proliferative advantage.

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Introduction

Myelodysplastic syndrome (MDS) is a group of clonal stem cell disorders characterized by cytopenias, ineffective haematopoiesis, bone marrow dysplasia and a risk of progression to acute myeloid leukaemia [1]. All patients diagnosed with MDS have a reduced life expectancy compared with age- and sex-matched normal controls, but particularly those patients classified as high-risk of disease [2] using the international prognostic scoring system (IPSS) [3].

The essence of MDS is damage of colony-forming units [4], but the defect of the haematopoietic stem cells is not well characterized. Prior reports have shown that an increase in CD34+ cells, a marker denoting increased blasts in peripheral blood and bone marrow, was associated with poor survival and higher risk of leukaemia transformation in MDS [5]. The effects of cytopenias and leukaemia transformation are the major causes of death in MDS [6].

Apoptosis has a crucial role in MDS, being responsible for the ineffective haematopoiesis that is characteristic of the disease [7]. In previous studies, early MDS was associated with an elevated ratio of apoptosis to cellular proliferation, but the mechanisms of the findings were not then established [8]. In later studies, cases of low-risk MDS showed enhanced intramedullary apoptosis, while high-risk MDS was associated with cellular proliferation, giving the abnormal clone a growth advantage [9]. Survivin, a member of the inhibitor-of-apoptosis family, facilitates cell cycle progression and is found in increased levels in low-risk MDS [7]. However, survivin levels in high-risk MDS and its prognostic impact are still questionable.

Meanwhile, specific cytogenetic abnormalities strongly correlate with prognosis in MDS [10] and about half of patients with primary MDS have cytogenetic abnormalities, the most common of which are trisomy 8, monosomy 7 and 5q− [11]. In trisomy 8+ MDS, patients suffer predominantly from pancytopenia, and the diagnosis can be confounded by aplastic anaemia since some cases of aplastic anaemia can evolve into trisomy 8+ MDS [12], in which 8+ appears to confer a favourable prognosis [13]. Chromosomal defects, as grouped by IPSS, and blast cell percentages are so far the most relevant parameters for predicting overall survival and progression-free intervals in MDS [14].

In the current work we examined haematopoietic stem cells of high-risk MDS cases for apoptotic and anti-apoptotic signals, and cellular proliferation as indicated by capacity for colony formation, and correlated these with clinical and cytogenetic subtypes, particularly trisomy 8. The aim of the study was to identify cellular and cytogenetic markers of prognostic relevance to survival of high-risk MDS cases.

Methods

Sample

Bone marrow samples were obtained from 19 high-risk primary MDS patients admitted to the department of haematology, Ain Shams University hospitals, and 10 control subjects free of haematological problems. Approval for the study was obtained from the local ethical committee and participants gave their informed consent for participation.

The 19 cases of high-risk MDS were selected over the 4-year period January 2003 to January 2007 from the total pool of MDS cases (76 cases) presenting to the haematology unit during that period.

At the time of sampling all MDS cases were newly diagnosed and had received no previous therapy. The IPSS of each MDS patient was determined according to the definition of Greenberg et al. [15]. The percentage of bone marrow blast cells for estimation of the IPSS score were calculated from average counts performed by 2 experienced haematologists from Leishman stained bone marrow smears and not from flow cytometry quantification of CD34+ cells, since acquiring adequate samples for flow cytometric analysis renders the specimens more dilute than the original smear.

Treatment strategy

The patients in our study were treated according to standard guidelines for the management of MDS patients [16]. All our patients were of the high-risk group and none of them was eligible for stem cell transplantation. They were treated with intensive chemotherapy using cytarabine (100 mg/m²/24 h continuous IV infusion) on days 1 to 7 and idarubicin (12 mg/ m² IV bolus) on days 1 to 3 with supportive treatment for associated cytopenias.

Follow-up study

Follow up was carried out until January 2009 (2 years after the last patient was recruited). Follow up included clinical and laboratory evaluations including regular peripheral blood and bone marrow examinations. The response to therapy was assessed based on the report of the international working group to standardize response criteria for myelodysplastic syndromes [17]. Overall survival was calculated from the time of diagnosis until the last follow up or death, while progression-free survival was defined as the period during which the disease was stable with no hospitalization required.

Data collection

The following was performed for patients at diagnosis and for controls:

Flow cytometric quantification of apoptotic CD34+ cells

Tricolour flow cytometric analysis of erythrocyte-lysed bone marrow specimens (using ammonium chloride...
Quantification of survivin expression in bone marrow mononuclear cells using immunocytochemistry

Immunocytochemistry methods were used to determine survivin expression in bone marrow mononuclear cells. Using Ficoll–Hypaque density gradient centrifugation, bone marrow mononuclear cells were isolated, washed, their counts adjusted to 1–3 × 10⁶/ul, and used to prepare cytospin slides on the Cytospin III (Shandon Corp.). Slides were fixed in acetone 100% for 10 min. at 4 °C, prior to immunostaining for survivin using polyclonal rabbit anti-survivin antibody and the rabbit ABC staining system with the anti-rabbit Ig secondary antibody and avidin biotinylated horseradish peroxidase detection reagent (Santa Cruz Biotechnology). Internal control slides were prepared by replacing the primary antibody with non-immune sera. Slides of controls and cases were mounted, covered and examined under light microscopy. Duplicate slides were evaluated for at least 200 cells for each case, with average counts estimated and positivity established at a cut-off of 5% [19].

Cell culture for haematopoietic colony formation and immunophenotyping

Immunocytochemistry methods were used to quantify colony-forming capacity of haematopoietic progenitors. Then 2 mL of bone marrow were added to 4 mL of Dulbecco’s modification of Eagle’s medium, low glucose containing 10% (v/v) heat-inactivated fetal bovine serum and penicilllin/streptomycin (Gibco) and centrifuged to pellet the cells and remove the fat layer. Cell pellets were then resuspended and fractionated on a density gradient generated by centrifugation with 70% Percoll solution at 13 000 g for 20 min. Cells were collected, rinsed with control medium and plated for primary culture. An aliquot of 0.3 mL (1–5 × 10⁶/mL) of cells were cultured in 3 mL methyl cellulose based haematopoietic differentiation medium, vortexed and left to stand for 5 min. A aliquot of 1.1 mL of the cell suspension was put in a low-adherence 35 mm Petri dish (1–5 × 10⁵ cells per dish, 5 dishes per cultured case) then incubated at 37 °C in a humified atmosphere with 5% CO₂. The medium was changed every 3 days. After 10 days, adherent cells formed homogeneous colonies. Colonies were enumerated based on morphology recognition in situ by light microscopy and counted per culture. Cells were detached with trypsin-EDTA, washed in phosphate-buffered saline, and immediately incubated on ice with labelled mouse anti-human antibodies for determination of CD34 and CD45, then analysed by flow cytometry [20].

Statistical analysis

Data analysis was performed using SPSS software, version 10.0. Quantitative data was expressed as mean and standard deviation (SD). Inter-group comparisons were evaluated by Student t-test or Mann–Whitney test. Multiple regression and Spearman correlation were used for quantitative intra-group analysis. Statistical significance was calculated at P-value of < 0.05.

Results

Table 1 presents the cytogenetic profile and levels of cellular markers as well as survival times for this sample of high-risk MDS patients.

Apoptosis of CD34-positive cells

A histogram representing the flow cytometric analysis of apoptotic signals in our MDS cases, comparing trisomy 8+ and 8− cases is shown in Figure 1. A significantly higher percentage of CD34+/Annexin+/PI− cells was
obtained in high-risk MDS cases as compared with normal controls. Also a significantly higher percentage of CD34+/Annexin+/PI– cells was found in trisomy 8+ cases compared with cases having other cytogenetic abnormalities (P < 0.05) (Table 2 and Figure 1).

A significant positive correlation was found between the percentage of CD34+/Annexin+/PI– cells and the percentage of trisomy 8+ cells (P < 0.05) (Figure 2). There was no significant correlation between the percentage of apoptotic cells and survivin+ cells or haematopoietic colony counts (P > 0.05) [data not shown].

**Cytogenetic findings in high-risk MDS cases**

Using conventional cytogenetics 17/19 (89%) successful metaphases were obtained, showing 4/19 cases (21%) with normal karyotype, 4/19 cases (21%) with trisomy 8, 1/19 case (5%) with 20q–, 4/19 cases (21%) with monosomy 7, 2/19 cases (11%) with both monosomy 7 and 5 (complex karyotype), 1 case (5.3%) with monosomy 5 and 1 case (5%) with 5q– (Table 1). Using FISH, 10/19 cases (53%) were found to have trisomy 8. In cases with trisomy 8+ there were 3200 CFCs by FISH compared with only 1720 in trisomy 8– (Table 2).

**Survivin expression**

The mean percentage of survivin cells were significantly higher in MDS cases compared with controls (P < 0.05) and among trisomy 8+ MDS cases compared with trisomy 8– MDS cases (P < 0.05) (Table 2 and Figure 1). However, there was a significant positive correlation between colony-forming cell counts and percentage of survivin+ cells or CD34+/Annexin+/PI– cells (P > 0.05) [data not shown].

**Survival studies**

There was a longer progression-free survival among trisomy 8+ MDS cases compared with cases with other cytogenetic abnormalities; however the difference was not statistically significant.

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### Table 1 Descriptive data of the sample of patients with high-risk myelodysplastic syndrome (n = 19)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytogenetic profile (by conventional methods)</strong></td>
<td></td>
</tr>
<tr>
<td>No. of patients</td>
<td>%</td>
</tr>
<tr>
<td>Normal karyotype</td>
<td>4</td>
</tr>
<tr>
<td>Monosomy 5</td>
<td>1</td>
</tr>
<tr>
<td>Monosomy 7</td>
<td>4</td>
</tr>
<tr>
<td>Trisomy 8</td>
<td>4</td>
</tr>
<tr>
<td>20q–</td>
<td>1</td>
</tr>
<tr>
<td>5q–</td>
<td>1</td>
</tr>
<tr>
<td>Failed metaphase</td>
<td>2</td>
</tr>
<tr>
<td>Complex karyotype</td>
<td>2</td>
</tr>
<tr>
<td><strong>Cytogenetic profile (by FISH)</strong></td>
<td></td>
</tr>
<tr>
<td>Trisomy 8</td>
<td>10</td>
</tr>
<tr>
<td><strong>Cellular marker profile</strong></td>
<td></td>
</tr>
<tr>
<td>Blast cells in smears (%)</td>
<td>Mean</td>
</tr>
<tr>
<td>CD34+ cells by flow cytometry (%)</td>
<td>9.0</td>
</tr>
<tr>
<td>CD34+/Annexin+/PI– cells (%)</td>
<td>8.0</td>
</tr>
<tr>
<td>Survivin+ cells (%)</td>
<td>30.0</td>
</tr>
<tr>
<td>Colony-forming cells (no./culture)</td>
<td>10.7</td>
</tr>
<tr>
<td>2180</td>
<td>1920</td>
</tr>
<tr>
<td><strong>Patient survival times</strong></td>
<td></td>
</tr>
<tr>
<td>Overall survival (months)</td>
<td>23</td>
</tr>
<tr>
<td>Progression-free survival (months)</td>
<td>15</td>
</tr>
</tbody>
</table>

20q– = deletion of the long arm of chromosome 20, 5q– = deletion of the long arm of chromosome 5; FISH = fluorescent in situ hybridization; PI = propidium iodide; SD = standard deviation.
The rate of overall survival was also not significantly different comparing trisomy 8+ and 8− MDS cases (Table 2 and Figure 1).

**Discussion**

MDS is a pre-neoplastic condition that frequently develops into overt acute leukaemia [21]. Leukaemic transformation has been presumed to be the result of multiple tandem chromosomal lesions and genetic mutations, some affecting cellular proliferation and others conferring resistance to apoptosis [10].

The pathogenesis of MDS is thought to include involvement of the MDS clone itself in abnormal apoptosis, signalling and immune deregulation of the bone marrow microenvironment [22]. One area of active clinical research in the treatment of MDS is the role of disruption of stromal production of the inflammatory cytokines that induce premature apoptosis of bone marrow cellular elements, which in turn contributes to the ineffective haematopoiesis in MDS [23]. However, although initial studies on purified CD34 cells in some MDS cases showed increased numbers of apoptotic cell markers, other investigators have demonstrated that CD34 cells from patients with MDS are resistant to apoptosis [10]. These contradictory reports prompted us to conduct the present study on MDS patients, focusing on high-risk cases, who are more liable to disease progression and poor outcomes, mostly due to leukaemic transformation.

To ensure that our findings regarding apoptosis were not related to differences in cellular differentiation we conducted our research on CD34+ cells. These cells expressed increased numbers of apoptotic cell markers, other investigators have demonstrated that CD34 cells from patients with MDS are resistant to apoptosis [10]. These contradictory reports prompted us to conduct the present study on MDS patients, focusing on high-risk cases, who are more liable to disease progression and poor outcomes, mostly due to leukaemic transformation.

![Figure 1](image_url) **Comparison of apoptosis, proliferation and patient survival rates in cases of high-risk myelodysplastic syndrome characterized as trisomy 8+ or trisomy 8−**
amounts of annexin V in MDS patients compared with controls and among trisomy 8+ MDS cases compared with other MDS cases and with controls. This confirms previous reports of increased apoptosis among the haematopoietic progenitor cells in MDS patients and an even higher increase among trisomy 8+ cases. Bone marrow cells from MDS patients with trisomy 8+ demonstrated significantly higher cell-surface Fas expression and comprised a higher proportion of annexin V+/PI− cells and caspase 3+ cells compared with cells from other MDS patients, particularly those with monosomy 7 or having a normal karyotype [10].

The findings in previous research that trisomy 8+ cells are Fas+ and annexin V+ is consistent with a cell population under immune attack and undergoing apoptosis [12]. This was postulated to be due to an immune response directed against neoantigens on trisomy 8+ cells. In this scenario, activated T-cells in proximity to trisomy 8+ cells would release cytokines (interferon gamma and tumour necrosis factor–alpha) upregulating apoptotic signals on the surface of haematopoietic cells. This was further confirmed in other studies by the ability of trisomy 8+ cells to increase their proliferative activity in the absence of an immune response in T-cell depleted samples [10].

Despite the apparent apoptotic activity in our trisomy 8+ MDS cases, the cellular capacity for colony formation was maintained at near normal levels. The increased expression of survivin in these cases could provide the rationale for the anti-apoptotic effect, a findings that concurs with studies demonstrating increased levels of survivin in MDS patients, even those low-risk cases [7,24]. Other research has revealed several patterns of aberrantly reduced myeloid nuclear differentiation antigen, which functions to enhance apoptosis induced by tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) in intermediate and high-grade MDS [25]. Research too has shown elevated levels of the anti-apoptotic agent NF-KappaB, whose inhibition induced rapid apoptosis of bone marrow cells from high-risk MDS cases [21]. The latter data, which indicate the presence of proliferative activity within MDS clones, is also confirmed by the growth advantage observed in MDS cells particularly those from patients having trisomy 8, in our study as well as another recent study [10].

The rate of survival of the trisomy 8 cases in our study did not vary significantly from other cases of high-risk MDS, possibly since treatment was not tailored to individual cases and was mostly palliative, pending the availability of bone marrow transplantation in suitable cases.

Recent advances in understanding the pathogenesis of MDS have led to significant therapeutic progress [1]. Currently there is no standard treatment for MDS and, since the aim of therapy is to eliminate the damaged colony-forming units [4], allogenic stem-cell transplantation remains the only curative strategy [22]. The pharmacological treatment provided for patients who cannot qualify for transplantation aims to induce differentiation of haematopoietic cells and also to inhibit apoptosis [4]. New therapeutic options include thalidomide and its analogue lenalidomide, which has proven extremely effective for patients with 5q− syndrome [1]. However, based on the results of the current study and previous work, we strongly believe that in high-risk MDS cases enhanced apoptosis may have a beneficial role in ameliorating the anti-apoptotic activity in these cases and may defer the risk of leukaemic transformation. New DNA hypomethylating agents azacytidine and decitabine have shown efficacy in patients with high-risk MDS and they may delay the disease progression [1]. Novel therapeutic strategies include molecular inhibition of MDS stem cell apoptosis as well as trials to use small-interfering-RNA to knock down survivin [12].

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**Figure 2** Correlation of trisomy 8+ cells to apoptosis and capacity for haematopoietic colony formation in high-risk myelodysplastic syndrome cases (\(n = 19\))
Although trisomy 8+ MDS cases are known to be have intermediate prognosis [3], our study showed a difference in the response to therapy and survival between trisomy 8+ and 8– high-risk cases. The fact that this difference was not statistically significant may be attributed to the small size of the sample and warrants further studies of larger samples. The findings of this study, as well as the review of previous work about this topic, provides reasonable evidence that cytogenetic and flow cytometric analysis can help map the profile of different risk groups in MDS. We believe that the prognoses for patients with this disease may be predicted according to the balance of apoptotic and anti-apoptotic activity of their CD34+ bone marrow cells, in conjunction with their cytogenetic analysis, particularly for trisomy 8 cases, since this group of patients seem to stand out as a prognostically separate entity. This information, once available, can facilitate therapeutic decisions for individual patients. We also recommended evaluating the apoptotic/antiapoptotic profile of the newer drugs that are directed against survivin in trisomy 8+ high-risk MDS cases.

References