Oncogene expression in primary myelodysplasia: Correlation with haematological, karyotypic, and clinical progression

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Abstract

Aims: To see if the relative expressions of proto-oncogenes that are increased in acute myeloid leukaemia are raised in patients with myelodysplastic syndromes (MDS), and to see if they increase with progression to leukaemia. To note if there is a correlation between morphology, karyotype, and these proto-oncogene expressions and if any one proto-oncogene can predict prognosis.

Method: Bone marrow from 130 patients was analysed at six monthly intervals over two years for relative mRNA expression of seven oncogenes, karyotype, and morphology. The technique used slot blot hybridisation and densitometric analysis. The results were compared with 14 surgical controls and 30 people with vitamin deficiency anaemia.

Results: Six of seven oncogenes showed increased expression which progressed with time, but did not correlate with morphological or karyotypic changes. Expression of four of the seven oncogenes was increased in megaloblastic and iron deficiency anaemia. *C-mos* showed differences among the five morphological subgroups; it correlated with abnormal location ($p = 0.025$) and seemed to influence prognosis.

Conclusion: Increased proto-oncogenes reflect the overall marrow perturbation in MDS. *C-mos* may reflect persistence of monocyte pathway which confirms marrow stability.

normal cellular proliferation and differentiation. Genetic alteration in their structure or regulation results in oncogene activation, disordered control, and neoplasia. However, altered expression may arise from the disease state rather than direct genetic mutation. In AML *N-ras* and *c-myc* may be activated by point mutation, gene amplification, or translocation.³ *C-myb*, *c-mos*, *c-erb B1*, *c-fos* and *c-ets* are also increased in expression.⁴ *N-ras* oncogene point mutations occur in up to 40% of cases with MDS⁵ and loss of the mutant form may accompany clinical remission.⁶ But the role of *c-myc*, *c-myb*, and other oncogenes found in both MDS and AML is not clear.⁷⁻⁸

We therefore investigated a cohort of 130 patients over two years and studied pathogenesis at the chromosomal and molecular level to ascertain if there was any correlation with specific oncogene activation.

Methods

Primary myelodysplastic syndrome (MDS) was diagnosed on peripheral blood and bone marrow samples according to the FAB criteria⁹ in 130 consecutive patients presenting to the haematology department from 1987 to 1989. The study was approved by the local ethics committee and patients gave informed consent. Conventional Romanowsky dyes were used. Patients with vitamin B12 and folate deficiencies and those developing MDS secondary to another haematological malignancy were excluded. Bone marrow samples were submitted for karyotype and oncogene studies at six monthly intervals. No patient in the study received cytotoxic drugs.

The control group comprised 14 haematologically normal patients undergoing cold surgery. Ten patients with megaloblastic anaemia and 10 with iron deficiency anaemia were also studied.

Cytogenetic analysis was performed at diagnosis and at six monthly intervals by conventional techniques with 24 hour cultures on bone marrow using short and long term exposure to colcemid. Thirty metaphases were counted and a minimum of 10 cells studied by conventional banding.

Bone marrow (5 ml) was taken at presentation from patients and the control group, and mononuclear cells were obtained by standard Ficoll-hypaque density centrifugation. The cells were controlled rate frozen and stored in

The myelodysplastic syndromes (MDS) represent a morphologically distinct group of pre-leukaemic conditions. They are relatively common among haematological malignancies. The age specific incidence rate is 3.60/100 000 population with a relative risk of 102.5 in Leicestershire¹ (range 80 to 120).¹ Forty per cent of patients with MDS may progress over months into acute myeloid leukaemia (AML). Serial studies into the pathogenesis have been made on such patients. The syndromes are well recognised on morphological grounds, but little is known about the aetiology.

Chromosomal abnormalities are present in both MDS and AML and carry a variable prognosis.²

Proto-oncogenes are genes which control

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Table 1 Oncogene probes used for detection of expression

Oncogene	Cellular location of oncoprotein	Chromosome	Probe	Reference
<i>v-abl</i> (mouse)	Cytoplasmic	9 q34	1.9 kb <i>SacI-Hind III</i>	Reddy <i>et al.</i> , 1983 ³⁰
<i>c-erbB1</i> (human)	Plasma membrane	7 p13	2.4 kb <i>Clal</i> fragment	Xu <i>et al.</i> , 1984 ³¹
<i>c-ets1</i> (human)		1.11 q23/24	5.4 kb <i>EcoRI</i> fragment	De Taisne <i>et al.</i> , 1984 ³²
<i>c-fos</i> (mouse)	Cytoplasmic	8 q22	600 pb <i>KpnI-Hind III</i>	Oskarsson <i>et al.</i> , 1980 ³³
<i>c-mos</i> (human)	Nucleus	2 q21	1 kb <i>Sau3a</i> fragment	Trus <i>et al.</i> , 1982 ³⁴
<i>c-myc</i> (human)	Nucleus	8 q24.1	1.5 kb <i>SacI</i> fragment	Dalla <i>et al.</i> , 1983 ³⁵
<i>c-myb</i> (human)	Nucleus	6 q22	1.74 kb <i>KpnI-Hind III</i>	Klempnauer <i>et al.</i> , 1982 ³⁶

vapour phase liquid nitrogen for subsequent analysis.

Total RNA was extracted by homogenisation in guanadinium thiocyanate and purified by caesium chloride centrifugation. The integrity of the RNA was assessed by electrophoresis in a 1.0% agarose gel and stained with ethidium bromide. A 28S:18S ribosomal RNA ratio of less than 2:1 or fragmentation of either of these species was taken as evidence of degradation. Any sample showing evidence of extensive degradation was discarded because negative results can reflect degradation of the RNA rather than the absence of transcripts in the sample.

The total RNA was precipitated in 2.5 volumes of ethanol and resuspended at 20 µg/ml, denatured for 10 minutes in a solution containing 7.5% formaldehyde and 6 × SSC (saline sodium citrate), cooled, and 20 µg applied to Hybond C membrane (Amersham UK) using slot blotting manifold.¹⁰ Filters were then placed in an oven for two hours at 80°C to bake the RNA on to the membrane. Each membrane included control slots containing RNA samples from the HL60 cell line and yeast ribosomal RNA as well as vector DNA. Exon sequences from molecular cloned c-oncogene probes (table 1) were excised from the recombinant sequences by restriction endonuclease digestion and were ³²P labelled using the method outlined by Feinberg and Volgelstein.¹¹ The RNA membranes were hybridised using methods previously described by Grosso and Pitot¹² and hybridisation was detected by autoradiography at -70°C with an intensifying screen. (Dupont Cronex) using preflashed X-OMAT AR film (Kodak). The level or intensity of the slot was quantified by densitometry using a laser scanning densitometer (Pharmacia/LKB).¹³

The probes were initially evaluated using total RNA from the cell line HL60 to establish the required stringency for specific

hybridisation. The expression of each patient's RNA was determined relative to the HL60 cell line RNA sample for each probe. Samples were repeated when sufficient RNA had been extracted.

The oncogene data were submitted to a Kruskal-Wallis non-parametric one-way analysis variance. Where significant differences between groups were ascertained the groups were then tested pairwise to establish specific pairwise differences. The survival analysis involved fitting Cox's proportional hazards regression model to the survival time from date of entry to the study to assess the significance of prognostic factors.

Results

PATIENT CHARACTERISTICS (table 2)

The age of the 130 patients with MDS ranged from 55–90 years (median 77) at time of diagnosis. This compared with a range of 41–88 (median 59) for the controls. The excess of men with MDS was confirmed. Our series contained a predominance with refractory anaemia (RA) 26% of which had an abnormal karyotype. As expected, in 83% of patients with refractory anaemia with excess of blasts in transformation (RAEBT) there were chromosomal abnormalities. One of the controls had a missing Y chromosome and another a minus 12. There was no correlation between specific disease category and any one individual chromosomal gain or loss.

ONCOGENE EXPRESSION (table 3)

All proto-oncogenes in marrow samples from patients with MDS, except *c-erbB*, were significantly increased in their relative messenger RNA transcription when compared with normal control marrows. However, of all the seven oncogenes tested, only *c-mos* mRNA concentrations differentiated between the morphological MDS subgroups ($p = 0.041$) (table 4). It was lowest in sideroblastic anaemia (SA) and highest in chronic myelomonocytic leukaemia (CMML). It increased as RA evolved into RAEBT and AML, but this trend did not reach significance.

PROTO-ONCOGENE EXPRESSION IN MEGALOBLASTIC AND IRON DEFICIENCY ANAEMIAS

Table 5 shows the relative expressions of the four oncogenes which were significantly raised in this third group of patients. *C-myc*, *c-ets*, and *c-fos* were increased in both disorders; increased *c-abl* expression occurred in iron deficiency anaemia alone.

Table 2 Characteristics of patients at diagnosis and their disease subgroup and karyotype

Disease	N =	Sex M/F	Mean age	% Karyotype	
				Abnormal	Normal
RA	69	36/23	75 (55–90)	26	74
SA	21	10/11	79.9 (65–90)	19	81
CMML	14	7/7	76.1 (60–88)	9	91
RAEB	24	14/10	79 (63–86)	36	67
RAEBT	12	8/4	73 (63–93)	83	17
Total	130	75/55	77 (55–90)	30	

RA refractory anaemia; SA sideroblastic anaemia; CMML chronic myelomonocytic leukaemia; RAEB refractory anaemia with excess of blasts; RAEBT refractory anaemia with excess of blasts in transformation.

Table 3 Proto-oncogene expression in MDS and in controls with morphologically normal marrow

Oncogene	MDS expression	Number	Range	Normal expectancy	Number	Range	p Value
<i>c-mos</i>	0.7	66	0.05, 9.7	0.175	9	0.05, 1.45	0.006
<i>c-myb</i>	2.3	61	0.04, 28.8	0.162	8	0.06, 3.35	0.0006
<i>c-myc</i>	1.26	72	0.004, 126.7	0.275	9	0.012, 0.65	0.0008
<i>c-ets</i>	17.13	59	0.1, 681	0.55	9	0.075, 16.75	0.0005
<i>c-abl</i>	3.19	54	0.1, 46.1	0.23	8	0.18, 5.5	0.005
<i>c-fos</i>	1.85	59	0.07, 75.1	0.43	8	0.11, 0.95	0.001
<i>c-erbB</i>	2.09	33	0.45, 15.8	1.48	9	0.09, 3.8	0.55

Table 4 *C-mos* oncogene expression according to disease category

Disease	SA	RA	RAEB	RAEBT	CMML	p Value
N =	11	23	11	6	9	
<i>c-mos</i> oncogene expression (median)	0.38	0.55	1.19	1.3	1.57	0.041
Range	0.08, 5.2	0.05, 6.7	0.55, 9.7	0.34, 4.3	0.37, 3.9	

Table 5 Relative median expression of proto-oncogene mRNA in normal subjects and those with iron deficient and megaloblastic anaemias

Probe	Normal			Iron deficiency anaemia			Megaloblastic anaemia			p Value
	Exp	N =	Range	Exp	N =	Range	Exp	N =	Range	
<i>c-myc</i>	0.275	9	0.008, 0.5	3.11	6	0.05, 21.6	5.26	4	0.98, 46.73	0.004
<i>c-ets</i>	0.55	9	0.06, 13.4	44.2	6	3.95, 170.2	3.22	4	0.35, 68.1	0.01
<i>c-abl</i>	0.23	8	0.15, 4.4	3.20	5	1.51, 15.68	0.28	3	0.26, 8.52	0.05
<i>c-fos</i>	0.43	8	0.11, 0.95	2.27	6	0.3, 73.0	1.43	4	0.39, 68.0	0.04
<i>c-mos</i>	0.75	9	0.039, 1.16	0.68	6	0.06, 2.06	0.93	4	0.28, 256	0.09

PROTO-ONCOGENE AND KARYOTYPE (table 6)

Forty patients had an abnormal karyotype. Among these there was an association between 5q- and reduced *c-abl*; 7q- and increased *c-ets*, *c-abl*, and *c-fos*. However, only *c-mos* showed any correlation between oncogene location (on chromosome 8) and expression ($p = 0.025$). Patients with trisomy 8 had greater *c-mos* expression than those diploid for 8 ($p = 0.036$).

PROGRESSION OVER TWO YEARS

As expected, 66% of those patients with RAEBT had died by the end of the study period—all from marrow failure. In contrast, 79% of those with CMML, though having a high overall oncogene expression, were still alive two years after diagnosis. Table 7 shows that *c-mos*, *c-myb*, and *c-myc* all significantly increased in relative gene expression over time. This did not correlate with clinical, morphological, or karyotypic changes. Sixty two per cent of patients with SA had a progressive rise in the above oncogene activity but remained clinically stable with no change into a more malignant form of MDS. There was, however, a trend to an increase in their transfusion requirements with increasing erythroid hyperplasia. Unfortunately it was not possible to quantitate the degree of ineffective erythropoiesis in these patients. Those patients transforming from RA to RAEBT over the study period showed a greater difference in the increase in expression of all three oncogenes compared with patients with SA and CMML who did not progress into the more malignant subgroups of MDS.

Morphological progression occurred in 25% of the 130 patients; 7.5% progressed to AML. Presentation diagnoses were SA in two, CMML in one, RAEB in five and RAEBT in

two. In 70% of those with AML the karyotype was abnormal at presentation and in 20% it became abnormal with the change to AML. Four patients had 5q-, three 7q-, two trisomy 8 and one minus 8. In our study of the 19 patients who died with an abnormal karyotype 12 were hypodiploid and seven hyperdiploid.

Survival curves were plotted using life table survival estimates and differences between groups assessed by logrank tests. The median survival for RA, SA, and CMML had not been reached by two years. That for RAEB and RAEBT was 12 months ($p = 0.0007$). Trisomy 8 conferred a survival advantage in RA, SA, and CMML, but not in RA progressing to RAEBT ($p = 0.7$). Similarly, a *c-mos* expression of >0.6 at presentation in these three subgroups suggested a trend towards longer survival (figure).

Discussion

Unlike chronic myeloid leukaemia (CML), characterised by the Philadelphia chromosome and *bcr/abl* fusion oncogene, no one specific chromosomal abnormality or oncogene activation is pathognomonic for the myelodysplastic syndrome. In our study *c-myc*, *c-myb*, *c-mos*, *c-abl* and *c-ets* expressions were increased and increased with progression of each disorder.

Table 6 Proto-oncogene expression and karyotype in 40 patients with MDS

Chromosomal abnormality	Oncogene affected	p Value
-5	Reduced <i>c-abl</i>	0.066
-7	Increased <i>c-ets</i>	0.019
	<i>c-abl</i>	0.062
	<i>c-fos</i>	0.050
+8	Increased <i>c-mos</i>	0.025

Table 7 Change of proto-oncogene expression over time

Oncogene	N =	Median expectancy at time		Median difference	Range	p Value
		0	2 years			
RA-RAEBT:						
c-mos	7	0.44	1.19	0.43	(0, 7.43)	0.06
c-myb	7	0.62	7.34	7.19	(- 1.18, 17.20)	0.09
c-myc	6	0.47	7.34	6.49	(0.26, 94.13)	0.04
SA-SA:						
c-mos	13	0.31	2.72	2.14	(0.07, 11.77)	0.002
c-myb	9	1.01	4.40	3.23	(- 1.0, 11.36)	0.013
c-myc	9	1.00	8.99	7.85	(0, 83.73)	0.014
CMML-CMML:						
c-mos	7	1.25	3.84	1.34	(0.37, 3.97)	0.022
c-myb	7	2.60	4.53	3.05	(0, 24.29)	0.100
c-myc	7	0.38	5.76	5.44	(- 0.74, 10.29)	0.36

But *c-myc*, *c-ets*, and *c-abl* also showed increased expression in the non-neoplastic conditions of megaloblastic anaemia and iron deficiency anaemia. Nuclear *c-myc* oncoprotein is present throughout the cell cycle in normal bone marrow but most often expressed in more primitive myeloid cells and those in the G0/G1 phase of the cycle. And it is more expressed in myeloid and early erythroid cell populations.¹⁴⁻¹⁵ *C-myc* expression inversely correlates with the state of cell differentiation.¹² In both MDS and megaloblastic anaemia cells are held up in G0/G1 and there is often a myeloid maturation arrest. In iron deficiency anaemia the myeloid:erythroid ratio is reversed as additional erythroid mitotic division occurs with an emphasis on the early basophilic and late orthochromic normoblasts along with intramedullary cell death.¹⁶ All these features promote increased *c-myc* expression. In contrast, *c-myb* RNA expression was raised in MDS and not in megaloblastic anaemia and iron deficiency anaemia. This supports the recent view that *c-myb* is strictly linked to the proliferative rate of erythroid progenitors rather than to their stage of differentiation.¹⁷ *C-ets* is a proto-oncogene on chromosome 11q23 and active in erythroblastosis¹⁸ while the *abl* oncoprotein is a tyrosine kinase middle mes-

senger. Both could be increased with increased division and differentiation of erythroid cells in MDS, megaloblastic anaemia, and iron deficiency anaemia.

Only *c-mos* showed some correlation with the FAB subgroup. The *c-mos* probe used in this study was a murine genomic probe. This will hybridise to the human *c-mos* locus on 8q11¹⁹⁻²⁰ but may also hybridise to related sequences from the *mos* oncogene family.²¹ *C-mos* oncoprotein is a cytoplasmic serine-threonine protein kinase middle messenger, a protein kinase-C linking the G-proteins to the nucleus and involved in multiple signalling pathways.²² *N-ras* mutation found in one third of patients with MDS switches on the signal for division permanently²³ and *c-mos* may merely reflect this.²⁴ However, *c-mos* induces progression of meiosis and mitosis in tissue culture; at low concentrations in stem cells it increases differentiation but at a high level of expression it leads to transformation.²⁵⁻²⁷ Both observations would explain the increasing *c-mos* expression in RA transforming to RAEBT. In addition, in our study *c-mos* concentrations were highest where there was a high monocyte count or monocytic differentiation, a scenario which reflected marrow stability with a longer patient survival (trend $p = 0.079$).

Our attempts to correlate karyotype with oncogene expression were restricted by small numbers. The relation between trisomy 8 and increased *c-mos* expression is most likely to have been due to gene amplification rather than translocation during progression from MDS to AML, as Morris found no evidence for the latter.¹⁹ We confirm the findings that loss of chromosome material was accompanied by a worse prognosis.²⁸ In the small number progressing to AML 70% had abnormal karyotypes at presentation, and in 20% it became abnormal with change to AML: 80% had lost chromosome material; 20% developed trisomy 8.

MDS is a heterogeneous disorder with a mixture of clonal and non-clonal cell populations.²⁹ Further studies using a cell sorter and in situ hybridisation are needed to elucidate the precise role of the increased oncogene expressions that we have observed.

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C-mos relative expression and survival. (A) $p < 0.6$ (B) $p > 0.6$.

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