What does apoptosis have to do with clinical features in myelodysplastic syndrome?

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Background and Objectives. Apoptosis is thought to play a key pathogenic role in myelodysplastic syndrome. The aim of our study was to determine whether apoptotic index, p53 and bcl-2 levels correlate with the clinical consequences of ineffective hematopoiesis; namely, the development of cytopenia and the shortened survival of patients with myelodysplastic syndrome.

Design and Methods. The apoptotic index, determined by TdT-mediated dUTP nick-end labeling, as well as bcl-2 and p53 protein levels, evaluated by immunohistochemistry, were studied in bone marrow trephine biopsy archival samples from 25 *healthy* subjects aged over fifty (control group), 61 consecutive patients with myelodysplastic syndrome and 17 patients with *de novo* acute myeloid leukemia according to FAB criteria. The correlation between relevant clinical parameters was analyzed with Spearman's correlation test. Factors influencing survival were studied by Cox proportional hazards regression analysis.

Results. The apoptotic index did not correlate with blood counts at diagnosis and had no prognostic influence on the overall survival of patients with myelodysplastic syndrome. The p53 score correlated with both the leukocyte count (r=-0.274, p=0.034, Spearman's) and hemoglobin concentration (r=-0.316; p=0.014) in the myelodysplastic syndrome patients and showed an independent and significant prognostic influence on their overall survival (p=0.045, Cox's) while the bcl-2 score was not correlated with their blood counts or prognosis.

Interpretation and Conclusions. Apoptotic index and bcl-2 do not correlate with key clinical data in patients with myelodysplastic syndrome, while p53 levels show a good correlation with these data and may be a useful parameter to add to current prognostic schemes in this entity.

Key words: myelodysplastic syndrome, prognosis, apoptosis, p53 protein, bcl-2 protein.

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Myelodysplastic syndromes are clonal diseases of the bone marrow and are characterized by ineffective hematopoiesis that gives rise to peripheral blood cytopenia. Some patients with myelodysplastic syndrome may develop acute myeloid leukemia, a clinical entity that can also appear *de novo* without any antecedent hematologic disorder.

Apoptosis is a highly regulated physiologic process involved in the elimination of unnecessary, damaged or senescent cells;[1-5] it is also thought to play a key pathogenic role in myelodysplastic syndrome.[6-9] Apoptosis features an activation phase, an execution phase mediated by caspases, and a complex control mechanism in which both bcl-2 and p53 play prominent roles.[1-3] The execution phase proceeds in the nucleus in a very precise and ordered fashion:[10-12] DNA fragmentation into 300 Kbp rosettes (hexameric) or 50 Kbp loops (monomeric) is followed by characteristic morphologic changes (crescent formation) and DNA fragmentation into 180-200 bp oligonucleosomes.

Different techniques have been developed to detect the morphologic and molecular changes that occur along the programmed death process.[13] Techniques that detect late stages are fairly specific but have a very low sensitivity, while those detecting early apoptotic changes are more sensitive but, as is common in diagnostic tool technology, they lose some specificity, which complicates the choice of the best technique for assessing apoptosis.

Apoptosis in bone marrow may be studied in bone marrow aspirates or biopsy specimens. Bone marrow aspiration is straightforward and less invasive for the patient, but the use of bone marrow biopsy samples allows: i) examination of routine stored samples, ii) detection of the possible tendency of apoptotic cells to form aggregates, iii) detection of the possible participation of stromal cells and, finally, iv) help in the control of the problem of non-representative sampling associated with bone marrow aspiration.[14]

The increase in apoptosis reported in myelodysplastic syndromes[6,14,15-23] occurs in *clusters* (in the

same way as the *hot spots* commonly described in angiogenesis research) instead of randomly throughout the tissue, involves both parenchymal hematopoietic cells and bone marrow stromal cells, and appears in bone marrow samples from donors of different ages, many of whom suffered from neoplastic diseases outside the bone marrow. The percentages of apoptotic cells in bone marrow reported in the literature vary considerably, probably as a consequence of the use of different techniques or cellular populations, and certain patients with otherwise typical myelodysplastic syndrome do not show increases in apoptotic parameters. On studying $CD34^+$ subpopulations, the proportion of apoptotic cells is usually higher in early myelodysplastic syndrome than in later stages.

Among the proteins involved in cell cycle regulation, p53 and bcl-2 seem to be suitable targets for monitoring the apoptotic process. p53 protein is a key element in DNA repair and apoptosis.[24] p53 mutations have been detected in 5-10% cases of myelodysplastic syndrome[25,26] and occur mainly in blast cells, which probably explains their predominance in advanced myelodysplastic syndrome.[27] bcl-2 protein is a negative regulator of apoptosis[28] and is expressed in some normal lymphoid cells (follicle mantle cells, medullary thymocytes) as well as in the more immature myeloid cells.[29] This latter fact may explain why bcl-2 levels are increased in advanced myelodysplastic syndrome.[30] Here we report the findings of a study performed at the Hospital de León, Spain, from October 1998 to August 2000. Our aim was to determine whether apopotic index, p53 and bcl-2 levels correlated with the clinical consequences of ineffective hematopoiesis; namely, the development of cytopenia and the shortened survival of patients with myelodysplastic syndrome. We report for the first time that p53 correlates with ineffective hematopoiesis while this correlation is not statistically significant for either apoptotic index or bcl-2 parameters.

Design and Methods

Patients. Bone marrow trephine biopsy archive samples were obtained from 103 patients (62 men and 41 women) aged 23 to 89 (median 69, interquartile range 62-77) divided into three different groups: i) twenty-five consecutive patients (12 men and 13 women), aged 52 to 85 (median 64, interquartile range 61-72) without peripheral blood cytopenia who were admitted for elective surgery and whose characteristics have already been reported;[31] these were considered as the control group, ii) sixty-one consecutive patients with myelodysplastic syndrome (40 men and 21 women) aged 33 to 89 (median 74, interquartile range 65.5-82) diagnosed and classified at our center according to the standard French-American-British criteria[32] (33 had *early* myelodysplastic syndrome - [21 refractory anemia and 12 refractory anemia with ringed sideroblasts (RARS)] – and 28 had *advanced* myelodysplastic syndrome – [7 refractory anemia with excess of blasts (RAEB), 9 refractory anemia with excess of blasts in transformation (RAEBt) and 12 chronic myelomonocytic leukemia (CMML)]-, and iii) seventeen patients with acute myeloid leukemia (10 men and 7 women) aged 23 to 84 (median 62, interquartile range 59-68) diagnosed and classified at our center according to the French-American British classification group criteria, for whom a bone marrow biopsy was thought convenient for diagnosis. When patients were reclassified according to the WHO criteria,[33] we recoded the 9 RAEBt cases (six cases were upgraded to AML, while the three additional cases presenting with Auer rods were downgraded to RAEB, according to the proportion of bone marrow blast cells). All cases of RARS and RA might be renamed according to the WHO classification as *refractory cytopenia with multilineage dysplasia* because of the presence of dysplastic features in two or more cell lines, except one RA case that presented with the 5q- syndrome. Moreover, 12 CMML patients might be considered as belonging to a separate myeloproliferative/myelodysplastic category. In each case, all the commonly available clinico-biological parameters used in daily practice, including a bone marrow aspirate, were considered. The sample storage time in paraffin ranged from 40 to 3572 days (median 1280, interquartile range 797-1537). Myelodysplastic syndrome patients were assigned to risk groups according to the Spanish Scoring System (see below).[34]

The study was performed in two phases:

- phase A: a cross-sectional study, i) comparing apoptotic data among the three groups, and ii) correlating these data with several clinico-biological parameters in myelodysplastic syndrome patients (age, sex, peripheral blood counts, bone marrow blast cell percentage, bone marrow fibrosis, number of dyshematopoietic cell lines and stage – early versus advanced).
- phase B: a risk-factor study for overall survival in myelodysplastic syndrome, taking into account as a whole those variables incorporated in the Spanish Scoring System (age, peripheral blood platelet count and bone marrow blast cell proportion), together with the apoptotic index, bcl-2 and p53

levels, extent of bone marrow fibrosis, number of dyshematopoietic cell lines, CMML diagnosis (as opposed to other MDS types according to the FAB classification) and curative intent (intensive chemotherapy).

Quantification of apoptosis. Bone marrow cylinders were fixed in B5 for 90 min, decalcified in EDTA for 90 minutes using the Mielodec Kit (Bio-Optica, Milan, Italy), and paraffin-embedded. Sections of 2.5 μm thickness were deparaffinized (overnight at 37°C) and rehydrated according to standard techniques. Mercurial remains were eliminated by treating the sections with 0.5% potassium iodide and 5% sodium thiosulphate for 5 min each. Sections were then exposed to 20 μg/mL *Tritirachium album* proteinase K (Sigma Chemical Co., St. Louis, MO, USA) in 10mM Tris-HCl in a wet chamber for 15 minutes at 37°C and then for 10 min at room temperature. DNA fragmentation was detected through TdT-mediated dUTP nick-end labeling (TUNEL),[13] using the *In Situ* Cell Detection Kit (Boehringer Mannheim GmbH, Mannheim, Germany), which incorporates TdT and a mixture of nucleotides, including fluorescein-conjugated dUTP. Sections were exposed in a dark and wet chamber to the above mentioned mixture for 60 min at 37°C to standardize the reaction to end-point. Coverslips were mounted with Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA) and sections were observed with a fluorescence microscope at 1000× magnification. Type II positive cells (those showing nuclear fluorescence forming more intense aggregates close to the nuclear membrane)[35] were quantified by unit surface area (excluding both fat and trabecular bone) with the aid of the KS300 v3.0 image system (Carl Zeiss Vision GmbH, Germany). All visible cells, regardless of whether they were parenchymal or stromal, were counted. Negative controls were generated by eliminating TdT enzyme from the mixture, and positive controls were generated by exposing the sections to 160 Kunitz units/mL bovine pancreas deoxyribonuclease I (Sigma Chemical Co., ST. Louis, MO, USA) in 0.15 M NaCl for 10 min at room temperature before exposing them to the TdT-mediated dUTP nick-end labeling mix.

Bone marrow biopsy sections were stained with hematoxylin-eosin to estimate overall cellularity per unit surface area. The degree of reticulin fibrosis was estimated using Wilder's stain after Hernández-Nieto *et al.*[36] We quantified the number of hematopoietic cells per unit surface area (excluding fat and trabecular bone) by observing the sections at 1000× magnification with the above described image analyzer. The surface analyzed ranged from 0.02 to 0.04 mm^2 (median 0.031, interquartile range 0.026-0.036). The intra-assay coefficient of variation in the measurement of tissue surface area was <1%. The number of cells ranged from 247 to 813 (median 464, interquartile range 383-544). We also searched for the eventual participation in apoptosis of morphologically recognizable stromal cells (reticular cells, fat cells and fibroblasts) in the observed fields.

Apopotic index is the proportion, expressed as a percentage, between the number of type II-TUNEL-positive cells (nuclear staining, as opposed to the cytoplasmic staining observed in type I, necrotic, cells) per unit surface area and those observed in the same sample by light microscopy. Since positive cells had a heterogeneous distribution in the tissue, forming clusters, we decided to evaluate apoptotic index in such clusters. All sections were evaluated by the same observer, who was unaware of the precise diagnosis of a given sample because each sample had been previously designated a numerical code.

p53 and bcl2 score quantification. After deparaffinization and rehydration, bone marrow sections were subjected to high-temperature antigen unmasking for 5 minutes in 10 mM citrate buffer, pH 6.0. Endogenous peroxidase activity was then neutralized with 3% hydrogen peroxide in methanol for 4 minutes and the sections were exposed to monoclonal antibodies (anti-bcl-2 Concepta, clone 124, Biosystems S.A., Barcelona, Spain and anti-p53 Novocastra, clone DO-7, Novocastra Laboratories Ltd., Newcastle-upon-Tyne, UK), previously diluted 1:50 for 30 minutes at 37°C. The antibody against p53 used in this study reacts with both wild type and mutant protein. Primary antibodies were detected using an amplification system that included i) a rabbit anti-mouse-IgG and a mouse anti-rabbit-IgG that detect and amplify the primary antibody signal ii) a biotin-conjugated secondary antibody able to bind both mouse and rabbit immunoglobulins, and iii) streptavidin-conjugated peroxidase (Ventana Amplification Kit and Ventana Enhanced DAB Detection Kit, Ventana Medical Systems, Tucson, Arizona, USA). Slides were counterstained using Mayer's hematoxylin. The positivity (nuclear stain for p53, and cytoplasmic stain for bcl-2) of 500 cells was quantified by observing the sections at 1000× magnification under a light microscope, including all visible cells, regardless of whether they were parenchymal or stromal, in the count. The reactivity of individual

cells was classified as negative (0 points), weak positive (1 point) or strong positive (2 points). A global score for each antibody was calculated by adding up the values found for the 500 cells (minimum score, 0 points; maximum score, 1000 points). The positive controls were a bone marrow sample from a patient with a large-cell lymphoma that overexpressed p53, and a bone marrow core-biopsy section from a patient with a follicular t(14;18)-positive lymphoma overexpressing bcl-2. All sections were assessed by the same observer who was unaware of the precise diagnosis of a given sample because each sample had been coded numerically.

Statistical methods. The qualitative variables are described as the relative frequency of each feature considered, while percentiles are used to describe the quantitative variables. The association between dichotomic variables was studied using the chi-squared test or Fisher's exact test when appropriate. The ranks of the quantitative variables were compared using the Mann-Whitney (when dealing with two groups) or Kruskal-Wallis tests (for three groups), and the correlations among them with Spearman's correlation test. All *p*-values are two-tailed. Multivariate analysis was used to neutralize any imbalance among group means of potentially relevant variables (e.g., age) as well as to isolate (stepwise-forward multiple regression, probability of F-to-enter 0.05, tolerance 0.01) those variables predicting apoptotic index. Factors influencing survival were studied by Cox proportional hazards regression analysis. Data were analyzed with SPSS v8.0 software (SPSS Inc., Chicago, IL, USA).

Results

Patients' characteristics. The clinico-biological features of the control group subjects and the patients with myelodysplastic syndrome/acute myeloid leukemia are shown, respectively, in Tables 1 and 2. The distribution between men and women was similar in the three groups, while the median age was significantly different between them ($p<0.001$; Kruskal-Wallis); this was taken into account using multivariate techniques. Our acute myeloid leukemia patients had lower platelet counts ($p=0.002$), lower leukocyte counts ($p=0.015$) and showed a trend towards lower hemoglobin levels ($p=0.064$) as compared to the myelodysplastic syndrome patients (Table 3). The extent of bone marrow fibrosis was similar ($p=0.183$) in the patients with myelodysplastic syndrome and those

Table 1. Clinico-biological features of individual subjects (control group).

UPN	Age	Gender	Wbc	HB	Plt.	BL(BM)	APO.	P53	BCL-2	DYS.	FIB.
018	62	M	7.2	159	274	0.7	41.2	0	0	1	0
021	62	F	6.5	149	232	0.6	22.7	0	4	0	0
022	62	F	5.8	139	251	0.5	0.7	0	2	0	0
026	80	F	5.9	150	195	0.4	35.7	0	1	1	0
027	85	F	7.9	120	193	0.3	52.5	0	13	0	0
028	71	F	7.8	127	164	0.3	46.8	0	28	0	0
029	65	F	4.6	130	280	0.9	41.1	0	11	0	0
030	69	M	8.5	155	227	0.3	34.7	0	0	0	0
032	60	M	8.3	160	237	0.4	28.1	0	0	0	0
033	56	F	5.7	126	281	0.3	55.5	0	0	0	I
034	73	F	4.8	145	199	0.7	61.1	0	6	0	0
035	64	M	6.3	150	202	1.1	23.3	0	4	1	0
037	64	M	7.2	160	221	0.3	50.9	0	0	0	0
041	64	M	6.1	169	188	0.4	46.7	0	20	0	0
045	73	M	7.4	142	279	0.2	8.3	0	11	0	I
047	52	F	7.9	140	231	0.8	22.1	0	0	1	0
050	63	M	8.0	157	177	0.3	59.6	0	0	1	0
054	66	M	9.4	161	147	0.5	37.0	0	2	0	0
058	57	M	10.0	162	165	0.4	0.2	0	11	1	0
060	59	M	5.5	150	171	0.9	54.8	0	1	0	0
061	67	F	6.3	135	206	0.8	88.0	0	3	0	0
062	76	M	7.9	138	262	0.5	61.5	0	13	1	0
063	76	F	5.5	120	199	0.5	20.6	0	13	1	0
066	66	F	5.5	144	258	1.1	46.5	0	10	0	0
069	52	F	7.6	143	245	1.3	27.5	0	8	0	0

UPN= unique patient number. Gender= subject's gender (M=male, F=female). BL (BM)= proportion of blast cells (undifferentiated elements) in the bone marrow over all nucleated cells. APO.= percentage of type-II apopototic cells in bone marrow apoptotic clusters as detected by TdT-mediated dUTP nick-end labeling. P53= p53 score. BCL-2= bcl-2 score. DYS = number of dyshematopoietic cell lines (above P90 of that cell line). FIB = myelofibrosis (reticulin) grades 0-III.

Table 2. Clinico-biological features of individual patients (MDL/AML).

UPN	Age	Gender	FAB	WHO	WBC	HB	PLT	BL(PB)	BL(BM)	APO.	P53	BCL-2	Dys.	Fib.
001	68	F	AML-M4	AML-MLD	16.6	119	51	36	30.0	84	13	8	3	0
002	62	M	AML-M3	AML	1.0	108	18	65	85.0	75	7	295	0	0
003	84	F	AML-M5a	AML-MLD	2.3	65	21	32	50.0	63	297	272	3	0
004	84	M	RA	RCMD	2.5	98	128	0	2.8	60	0	20	3	0
005	75	M	AML-M0	AML	1.4	59	106	9	87.0	33	5	16	1	0
006	55	F	CMML	–	30.6	149	154	2	2.0	71	0	40	2	I
007	59	M	AML-M2	AML-MLD	0.9	67	6	6	56.6	70	1	66	3	I
008	84	M	RAEB	RAEB	4.6	92	26	0	10.4	67	26	156	2	I
009	38	F	AML-M3	AML	2.7	103	178	29	89.0	56	19	322	0	0
010	70	M	RA	RCMD	2.5	144	352	1	3.0	66	4	48	3	II
011	84	M	RARS	RCMD	3.0	81	115	0	0.4	32	5	12	3	I
012	73	M	RAEB	RAEB	3.1	98	83	0	10.0	63	7	100	3	I
013	80	M	RA	RCMD	5.4	107	50	0	2.0	32	0	2	3	II
014	62	F	AML-M5a	AML	2.1	108	67	20	92.0	82	0	44	0	0
015	65	M	RA	RCMD	4.6	103	110	1	4.3	64	21	5	3	III
016	85	F	RARS	RCMD	2.9	101	25	0	1.1	44	225	12	3	II
017	83	M	CMML	–	6.7	119	40	0	0.6	66	1	21	2	0
019	65	M	RAEBt	RAEB	7.1	53	41	0	6.0	50	0	3	3	I
020	73	F	RAEBt	RAEB	4.4	68	90	1	11.5	82	9	31	3	I
023	70	F	AML-M5b	AML	14.8	129	81	7	56.6	59	0	3	1	0
024	69	M	RAEBt	AML-MLD	2.5	88	110	6	23.2	23	0	7	3	0
025	69	F	AML-M2	AML-MLD	3.3	85	203	16	49.0	59	0	128	3	II
031	72	F	RARS	RCMD	4.6	97	247	0	0.4	43	10	8	3	0
036	82	F	RA	RCMD	2.9	88	288	0	0.8	58	0	17	3	0
038	66	M	RARS	RCMD	2.8	72	98	0	0.6	54	0	2	3	II
039	47	M	AML-M6	AML-MLD	3.9	78	61	12	40.0	43	3	0	3	I
040	74	M	RARS	RCMD	9.5	100	5	0	0.4	45	0	0	3	0
042	56	F	RAEB	RAEB	2.8	75	82	2	6.6	28	75	1	3	0
043	68	M	RAEBt	AML-MLD	2.4	103	67	0	26.2	58	0	0	3	0
044	84	M	RA	RCMD	6.0	112	563	0	1.2	34	1	10	3	I
046	45	M	AML-M0	AML-MLD	4.0	78	55	7	34.0	50	11	76	3	0
048	75	M	RARS	RCMD	3.2	107	66	0	1.1	38	0	1	2	0
049	23	M	AML-M3	AML	17.4	77	34	87	79.3	69	22	310	0	0
051	89	M	CMML	–	7.4	83	23	1	2.5	30	0	14	3	0
052	60	F	CMML	–	32.3	142	226	0	1.0	69	0	2	3	I
053	37	F	RA	RCMD	2.1	84	131	0	4.8	53	10	4	3	0
055	54	M	RA	RCMD	3.3	130	245	0	4.3	38	0	52	2	0
056	81	M	RAEB	RAEB	5.1	62	100	4	11.6	85	14	4	2	0
057	33	M	RAEB	RAEB	4.6	71	171	1	8.2	61	1	9	2	I
059	80	M	RA	RCMD	5.5	140	85	0	1.8	36	10	4	3	0
064	80	F	RARS	RCMD	6.8	103	250	0	1.1	73	2	29	3	0
065	72	M	RA	RCMD	4.7	116	109	0	1.9	36	0	11	3	0
067	69	M	RA	5q-	6.4	70	560	0	1.7	65	115	4	3	I
068	85	M	RARS	RCMD	5.6	106	94	0	1.3	18	0	4	3	0
070	85	M	CMML	–	4.2	108	108	0	1.7	51	0	7	3	0
071	67	M	AML-M4E	AML	2.1	68	74	18	80.2	79	6	562	1	0
072	63	F	AML-M6	AML	1.7	48	23	17	48.0	76	104	161	1	0
073	59	M	AML-M3	AML	1.8	146	98	0	71.5	62	2	190	0	0
074	65	M	AML-M0	AML	6.6	86	46	16	92.4	62	4	262	0	0
075	67	F	RA	RCMD	1.8	123	83	0	1.0	00	0	158	2	0
076	76	F	CMML	–	9.6	116	100	0	2.2	25	1	8	3	NA
077	84	F	RA	RCMD	1.5	97	82	0	3.2	61	104	2	3	0
078	79	M	RA	RCMD	3.7	115	167	0	0.6	41	0	16	3	0
079	62	M	AML-M0	AML-MLD	1.8	71	77	13	61.0	43	1	17	3	I
080	71	M	CMML	–	2.8	110	24	0	0.6	68	1	34	2	NA
081	86	M	CMML	–	9.1	122	66	0	0.6	43	0	46	2	I
082	76	M	CMML	–	4.8	139	79	0	0.2	50	0	50	2	0
083	72	F	RARS	RCMD	7.1	87	930	0	0.2	39	2	34	2	0
084	75	F	RA	RCMD	6.1	117	189	0	1.2	68	0	0	2	I
085	56	M	RAEBt	AML-MLD	1.8	80	208	0	20.6	45	0	36	2	0
086	66	M	RAEBt	RAEB	2.5	111	36	12	20.0	44	0	9	3	I
087	86	F	RA	RCMD	3.9	76	230	0	1.3	43	17	39	3	0
088	56	F	RARS	RCMD	8.2	123	417	0	0.4	52	0	9	2	I
089	35	M	RAEB	RAEB	11.5	45	23	4	6.8	61	0	2	3	I
090	74	F	RAEBt	AML-MLD	7.9	76	211	15	22.0	37	0	3	3	II
091	76	M	RA	RCMD	2.9	92	55	0	1.2	48	0	11	2	0
092	72	M	RA	RCMD	5.5	103	199	0	0.6	33	4	64	2	I
093	63	M	CMML	–	29.7	135	50	0	0.2	46	0	1	2	I
094	77	M	CMML	–	46.7	140	42	0	0.6	34	0	8	3	0
095	72	F	RA	RCMD	4.1	101	243	0	0.6	56	0	7	3	0
096	64	M	RAEBt	AML-MLD	0.9	48	111	0	24.2	80	4	392	3	I
097	77	M	RA	RCMD	2.2	123	316	0	0.2	NA	1	2	2	0
098	84	M	CMML	–	46.7	103	316	0	0.2	54	0	15	2	I
099	84	F	RARS	RCMD	9.4	108	961	0	0.8	73	0	3	3	0
100	82	M	RARS	RARS	9.7	117	638	0	0.2	27	0	14	3	0
101	56	M	RA	RCMD	5.9	81	125	0	1.0	41	2	10	2	0
102	76	F	RAEBt	AML-MLD	4.1	77	304	8	26.8	44	0	20	3	0
103	64	F	RAEB	RAEB	2.2	103	197	1	13.2	38	9	48	3	0

UPN = unique patient number. Gender = patients's gender (M=male, F=female). BL(BM) = proportion of blast cells in the bone marrow over all nucleated cells (except in the M6 subtype, where this proportion was calculated over non-erythroid nucleated cells). BL(PB) = proportion of blast cells in the peripheral blood. APO = percentage of type-II apopototic cells in bone marrow apoptotic clusters as detected by TdT-mediated dUTP nick-end labeling. P53= p53 score. BCL-2= bcl-2 score. DYS.= number of dyshematopoietic cell lines (above P90 of control group values). FIB.= myelofibrosis (reticulin) grades 0-III. RA= refractory anemia. RARS= refractory anemia with ringed sideroblasts. RCMD = refractory cytopenia with multilineage dysplasia. 5q-= 5q- syndrome. CMML= chronic myelomonocytic leukemia. RAEB= refractory anemia with excess of blasts. RAEBt = refractory anemia with excess of blasts in transformation. AML= "de novo" acute myeloid leukemia. AML-MLD = acute myeloid leukemia with multilineage dysplasia without prior myelodysplastic syndrome. NA = not available.

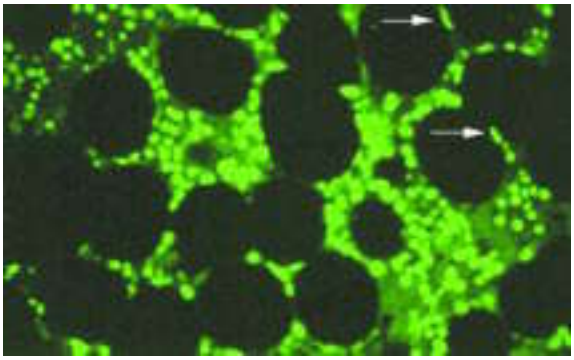

Figure 1A. Apoptotic cells in the bone marrow of an MDS patient (UPN 043) as detected by TdT-mediated dUTP nick-end labeling (T.U.N.E.L., ×400): apoptosis takes place in aggregates (*clusters*) and involves both parenchymal and fat cells (arrows).

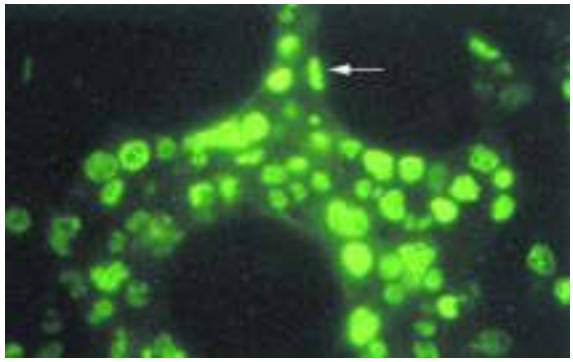

Figure 1B. Apoptotic cells in the bone marrow (T.U.N.E.L., ×1000) of an MDS patient (UPN 064): many type II positive cells showing nuclear staining are shown, including a fat cell (arrow).

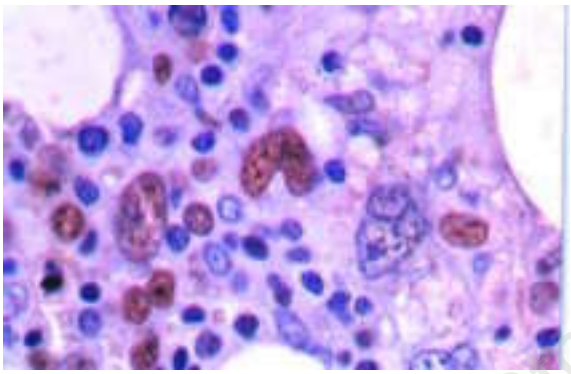

Figure 1C. p53 positive cells (nuclear staining) in the bone marrow (peroxidase, ×1000) of an MDS patient (UPN 077) as detected by monoclonal antibody reactive with both wild type and mutant protein (clone DO-7), using an amplified method.

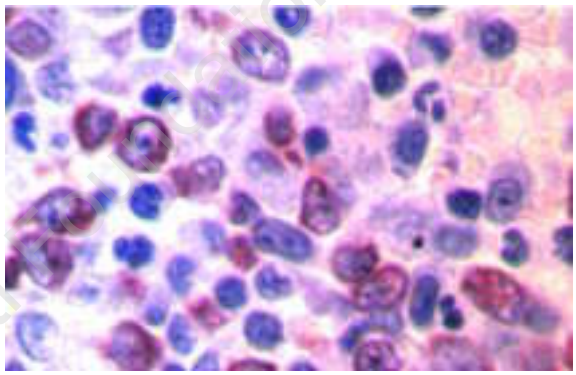

Figure 1D. bcl-2 positive cells (cytoplasmic staining) in the bone marrow (peroxidase, ×1000) of an MDS patient (UPN 008) as detected by monoclonal antibody against bcl-2 protein (clone 124).

Table 3. Peripheral blood counts and percentages of bone marrow blast cells.

Group	*Leukocytes (×10⁹/L)*	*Hemoglobin (g/L)*	*Platelets (×10⁹/L)*	*BM blast cells (%)*
Control	7.20 (5.7-7.9) (4.6-12.4)	145 (136-158) (120-169)	221 (190-255) (147-281)	0.5 (0.3-0.8) (0.2-1.3)
MDS	4.60 (0.8-7.1) (0.9-46.7)	103 (82-117) (45-149)	110 (66-240) (5-961)	1.3 (0.6-6.1) (0.1-26.8)
AML	2.30 (1.7-5.3) (0.9-17.4)	78 (68-108) (48-146)	61 (28-90) (6-203)	61.0 (48.5-86.0) (30.0-92.4)

Data shown as P50 (25-75) (range). MDS= myelodysplastic syndrome. AML= acute myeloid leukemia. BM= bone marrow.

with acute myeloid leukemia.

Apoptotic index by TUNEL Apoptosis occurred in aggregates (*clusters*), and both parenchymal (blast cells and myeloid progenitors) and stromal cells (adipocytes) were involved (Figures 1a and 1b). Regarding the apoptotic index, significant differences were found between the diagnostic groups ($p<0.001$, Kruskal-Wallis, Table 4); the apoptotic index was higher in the myelodysplastic syndrome patients than in the control group ($p=0.029$), and was also higher in the subjects with acute myeloid leukemia than in those with myelodysplastic syndrome ($p=0.005$).

The myelodysplastic syndrome patients showed a trend ($p=0.097$) towards a higher apoptotic index in advanced stages than in early stages (median 60.9; %; interquartile range 42.8%-70.8%; vs. median 43.6%; interquartile range 36.0%-59.8%). More-

Table 4. Apoptotic index, bcl-2 and p53 scores.

	Control	*MDS*	*AML*
Apoptotic	41.1	46.9	62.5
Index	(23.0-53.6)	(37.0-62.9)	(53.2-75.5)
	(0.16-88.0)	(0.1-85.0)	(33.3-84.0)
bcl-2 score	4	10	128
	(0-11)	(3.5-32.5)	(16.5-283.5)
	(0-28)	(0-392)	(0-562)
p53 score	0	0	5
	(0-0)	(0-4.5)	(1-16)
	(0-0)	(0-225)	(0-293)

Data shown as P50 (25-75) (range). MDS= myelodysplastic syndrome according to FAB criteria. AML= acute myeloid leukemia. Apoptotic Index= percentage of type-II apopototic cells in bone marrow apoptotic clusters as detected by TdT-mediated dUTP nick-end labeling.

Table 5. Risk factors for overall survival in myelodysplastic syndrome.

Variable	*Bivariate p-value*	*Multivariate p-value*
SSS	0.004	0.013
bcl-2 Score	0.463	0.805
Apoptotic Index	0.059	0.135
p53 Score	0.002	0.045
Dyshematopoiesis	0.046	0.109
Fibrosis	0.091	0.483
CMML diagnosis	0.786	0.407
Curative Intent	0.929	0.845

SSS= Spanish scoring system. Apoptotic Index= percentage of type-II apopototic cells in bone marrow apoptotic clusters as detected by TdT-mediated dUTP nick-end labeling. Dyshematopoiesis= number of dyshematopoietic cell lines. Fibrosis= extent of bone marrow fibrosis. CMML=chronic myelomonocyic leukemia. Curative Intent = patients treated with intensive chemotherapy.

over, a positive correlation between apoptotic index and extent of bone marrow reticulin fibrosis was detected (r=+0.261; p=0.043). Conversely, no statistically significant association of apoptotic index with sex, age, storage time, number of dyshematopoietic cell lines or peripheral blood counts in myelodysplastic syndrome patients was observed.

Multiple regression analysis revealed that the only parameter predictive of apoptotic index in the whole series was the *diagnostic group* variable (control vs. early myelodysplastic syndrome vs. advanced myelodysplastic syndrome vs. acute myeloid leukemia, according to FAB criteria; r= +0.353, slope = +5.78, p<0.001), confirming the trend towards a higher apoptotic index in advanced myelodysplastic syndrome seen with bivariate analysis. When the *diagnostic group* variable was excluded from the model, the only variable predicting apoptotic index was found to be the bone marrow blast cell proportion (r=+0.338, slope +0.255, p=0.001). After stratification by diagnostic group, none of the variables was predictive of apoptotic index, but when the regression was performed on the subset of patients classified as having myelodysplastic syndrome according to WHO criteria (n=43), the only determinant of apoptotic index in these patients was, again, found to be the bone marrow blast cell proportion (r=+0.308, slope +1.2, p=0.047).

p53 and bcl-2 levels. Both p53 and bcl-2 scores were significantly different among the diagnostic groups (p<0.001, Kruskal-Wallis, in both cases). The p53 score was higher (Table 4) in patients with acute myeloid leukemia than in those with myelodysplastic syndrome (p=0.005), and higher in myelodysplastic syndrome patients than in the control group (p<0.001). A statistically significant negative correlation between the p53 score and both the peripheral blood leukocyte count (r=-0.274; p=0.034) and hemoglobin concentration (r=-0.316; p=0.014), but not with the platelet count, extent of bone marrow fibrosis, number of dyshematopoietic cell lines, storage time or other clinically relevant variables, was observed in myelodysplastic syndrome patients. In acute myeloid leukemia patients, a negative correlation was seen between the p53 score and the extent of bone marrow fibrosis (r=-0.524, p=0.031).

The bcl-2 score was higher (Table 4) in patients with acute myeloid leukemia than in those with myelodysplastic syndrome (p<0.001), and higher in myelodysplastic syndrome patients than in the control group (p=0.003). No significant correlation was observed between the bcl-2 score and peripheral blood counts, extent of bone marrow fibrosis, storage time or other clinically relevant variables in myelodysplastic syndrome patients. The same lack of correlation was observed in acute myeloid leukemia patients. Finally, a statistically significant positive ordinal correlation was found between bcl-2 and age (r=+0.473, p=0.017, Spearman's) in the control group.

Correlations among bone marrow blast cell proportions, p53 and bcl-2 scores, and the apoptotic index. When we analyzed the subset of patients classified as having myelodysplastic syndrome according to the WHO classification (those selected with the most restrictive criteria), a statistically significant positive correlation between the proportion of bone marrow blast cells and apoptotic index (r=+0.319, p=0.040), and also between the proportion of bone marrow blast cells and p53 score

(r=+0.312, p=0.041), was observed. In acute myeloid leukemia patients, a positive ordinal correlation between bcl-2 and p53 scores (r=+0.504, p=0.039) was detected.

Survival analysis in myelodysplastic syndrome. Risk stratification according to the Spanish Scoring System, the p53 score and the number of dyshematopoietic cell lines predicted overall survival in bivariate analysis in this series (p=0.004, p=0.002 and p=0.046, respectively). However, the apoptotic index, bcl-2 score, extent of marrow fibrosis, diagnosis of CMML and curative intent did not show any prognostic influence on survival. The unfavorable impact on overall survival of a higher score in the Spanish Scoring System or higher p53 levels was confirmed by multivariate analysis (p=0.013 and p=0.045, respectively), but not that of the number of dyshematopoietic cell lines at diagnosis (Table 5).

Discussion

The percentage of apoptotic cells reported here is higher than that described in other reports using bone marrow aspirates,[18,19,22,23] in which most apoptotic cells are discarded.[14] The estimation of apoptotic index from apoptotic clusters also accounts for the figures observed in our series. A high apoptotic index due to B5 fixative-mediated DNA-fragmentation was ruled out since apoptotic cells did not appear in a number of our samples, in agreement with the results of a previous report using this fixative.[21]

TdT-mediated dUTP nick-end labeling is a very sensitive technique for the detection of apoptotic cells[37] and has excellent specificity when only type II positive cells are taken into account.[34] We chose this technique because it has a very high signal-to-noise ratio, its enzyme kinetics reaches the endpoint in 30 minutes,[38] and it is able to detect both 5'-recessed and 3'-recessed DNA fragments whereas *in situ* end-labeling of DNA[39] detects only 3'-recessed fragments.[40]

Our results show that control subjects aged fifty and over have apoptotic bone marrow cells in the absence of peripheral blood cytopenia. In this sense, bone marrow apoptotic cells have also been described in patients with reactive bone marrow without peripheral blood cytopenia.[41] The origin of this phenomenon is unclear but might be due to physiologic cell renewal in a very rapidly dividing tissue. The positive correlation detected in our series between the bcl-2 score and age in the control group is in agreement with the previously reported notion that senescent cells would be unable to down-regulate bcl-2 expression in response to apoptosis-inducing signals.[42]

Our data confirm the previously described increase in apoptotic changes in myelodysplastic syndrome patients as compared to in controls, stromal participation (apoptotic fat cells were a common finding), and cluster aggregation.[6,15-22] The cause of stromal apoptosis is unclear, but this phenomenon might be due to local cytokine release in the context of an immune response against dysplastic hematopoietic progenitors.

As is the case of most findings described in the literature,[15-21] we failed to detect a statistically significant ordinal correlation between apoptotic index and peripheral blood counts, or apoptotic index and dyshematopoietic features. This casts doubt on the proposed pathogenic role of increased apoptosis in the ineffective hematopoiesis of myelodysplastic syndrome, which is not supported by either the above-described correlation between the apoptotic index and peripheral blood counts, or a correlation between the apoptotic index and long-term cell growth in culture systems.[43]

Using multivariate analysis, Tsoplou *et al.*[44] also failed to detect any significant association between apoptotic parameters and transfusion requirements, leukemic conversion, or survival. In a recent report, Shimazaki *et al.*[45] reported that those patients showing a higher apoptotic rate had a poorer prognosis regardless of the French-American-British-related classification subgroup, but they did not take into account either p53 or bcl-2 levels in their analysis. Our findings do not support an independent prognostic role for apoptotic index as regards overall survival in myelodysplastic syndrome, in contrast to p53 protein levels (*see below*).

Our data show that the apopotic index is higher in patients with advanced myelodysplastic syndrome than in those with early stage disease, in agreement with Raza *et al.*,[15,16,20] but in contrast with Rajapaksa *et al.*[18] and Parker *et al.*[22,23] We believe that this discrepancy may be due to the different cell populations analyzed. In this sense, Raza *et al.* and our team focused on whole marrow cells, while Rajapaksa *et al.* and Parker *et al.*[22] used $CD34^+$ bone marrow subpopulations, whose proportions are increased in advanced myelodysplastic syndrome.[31,46] In this subset, the well-known increase in the proportion of $CD34^+$ cells could account for the increase in the global apopotic index, despite the decrease in the proportion of $CD34^+$ cells in apoptosis.

The presence of p53 reactivity in many patients with myelodysplastic syndrome as compared with

the lack of reactivity in the *control group* was one of the most striking observations found in our study. Since we did not detect false positive results, we can rule out the possibility that the high frequency of p53 positivity in our myelodysplastic syndrome series might have been due to the use of an amplification procedure. p53 positivity can be a consequence of p53 gene mutations, but the accumulation of the protein does not necessarily imply an underlying gene mutation, found in about one third of cases of myelodysplastic syndrome.[26] Since p53 mutations are not common in myelodysplastic syndrome, our results could be interpreted in terms of the occurrence of post-transcriptional modifications or interactions of p53 with other regulatory proteins. In any case, the p53 score in our series had a prognostic power equivalent to that of consolidated prognostic schemes. Its prognostic influence might be related to the observed link between p53 mutations and poor-prognosis cytogenetic abnormalities[47,48] and we, therefore, believe that the p53 score is a useful and straightforward marker, readily available for prognostic purposes in myelodysplastic syndrome patients. Furthermore, p53 score quantification obviates current uncertainty regarding the prognostic significance of several cytogenetic abnormalities belonging to the intermediate-risk subgroup.[49]

bcl-2 overexpression is considered a poor prognostic feature in acute myeloid leukemia.[50,51] According to our data, this does not seem to be the case in myelodysplastic syndrome.

Regarding the apopotic index in patients with acute myeloid leukemia, the manner in which our patients were selected should be taken into account. In this respect, patients presenting with low peripheral blood leukocyte counts or prominent dysplastic changes in the peripheral blood were deemed candidates for bone marrow biopsy, while this invasive procedure was not considered justifiable for those in whom these features were not present at diagnosis. The apopotic index is commonly considered to be decreased in *de novo* acute myeloid leukemia patients but the findings reported are contradictory[15,18,52-54] and we found an increased apopotic index in our cases of *de novo* acute myeloid leukemia. The parallel increase in apopotic index and bone marrow blast cells counts seen in the three main groups considered (control→MDS→AML) suggests a link between these parameters, probably mediated by increased cell turnover. Finally, our data revealed a positive correlation between the increase in bcl-2 and p53 scores in acute myeloid leukemia (n=17). This is surprising in view of the opposite role of p53 and bcl-2 in apoptosis.

In conclusion, the p53 score is inversely correlated with peripheral blood cytopenia and has a significant adverse prognostic influence on overall survival, which might improve the prognostic performance of current schemes. Conversely, we did not find a statistically significant correlation between the apopotic index and peripheral blood cytopenia at diagnosis in myelodysplastic syndrome patients, nor did the apoptotic index have any independent prognostic influence on their overall survival, casting doubt on the proposed pathogenic role of increased apoptosis in myelodysplastic syndromes.

Contributions and Acknowledgments

FR was responsible for the patients and data management, performed apoptotic index quantification and statistical analyses and co-ordinated the entire project. MFN performed the literature review, contributed to patient care and data management, implemented laboratory assays and performed p53 protein quantification. DSV was responsible for tissue specimens and laboratory techniques, reviewed pathology records and performed bcl-2 quantification. AFL supervised the image-analysis procedure and acted as a cell biology consultant. All authors contributed to the conception, design, data interpretation and article drafting, critically revised the manuscript and gave their approval to the final version. The order of authorship tries to take into account the time, work and scientific contribution of each author. We thank Dr. Elihu Estey (Houston, TX, USA), Dr. Suneel Mundle (Chicago, IL, USA) and Professor Francesco Lo Coco (Rome, Italy) for critical comments on a previous version of the manuscript. We would also like to thank E. Santín and R. Turiel for technical assistance.

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Disclosures

Conflict of interest: none.

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Peer Review Outcomes

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What is already known on this topic

Apoptosis seems to play a pivotal role in the pathogenesis of myelodysplastic syndromes, and p53 and bcl-2 are involved in the complex control mechanism of apoptosis. However, the possible relationship between apoptosis, its regulators, and outcome in myelodysplastic syndromes is unclear.

What this study adds

This study shows that p53, but not bcl-2, score determined by immunohistochemistry correlates with blood counts and is independently associated with survival. The paper also confirms that apoptotic index determined in marrow biopsy specimens by TUNEL technique is higher in patients with myelodysplastic syndromes than in controls and does not clearly influence survival.

Potential implications for clinical practice

The p53 score could add prognostic information to current prognostic scoring systems for myelodysplastic syndromes. However, this point must be confirmed in series of patients with cytogenetics available.

Guillermo Sanz, Associate Editor