poor prognosis and survival.[4] We previously demonstrated that HGF may reduce the duration of the neutropenia and thus, indirectly, improve prognosis.[6]

The present data did not confirm the negative impact on hematologic recovery of age, renal failure or bone marrow appearance (Tables 1 and 2), but they confirm that: (i) neutrophil count $<0.1\times10^9$/L and the presence of bacteremia, septic shock or severe infections are associated with a poor neutrophil recovery; (ii) treatment with HGF is associated with a significantly faster neutrophil recovery (Table 2). These data are consistent with those reported in several other recent studies.[7,8] However, it should be noted that the only randomized trial in agranulocytosis induced by antithyroid drugs did not confirm the benefit of granulocyte colony-stimulating factor.[9] The present data demonstrated that a documented infection and diagnosis of antiplatelet agent-induced agranulocytosis were associated with a faster hematologic recovery (Table 2). One explanation might be that the patients treated with antiplatelet agents (e.g. ticlopidine) were closely monitored and that the drug was quickly withdrawn if neutrophil counts decreased.[10]

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Defective expression of the dihydrofolate reductase gene in patients with 5q- syndrome

We evaluated dihydrofolate-reductase (DHFR) gene expression in the marrow cells of 6 cases of 5q- syndrome and 8 patients with other myelodysplastic syndromes. DHFR mRNA was decreased in 5q- cases. Losses of transcripts were associated with low erythroblast DHFR activity, decreased progenitor growth *in vitro* and reduced erythroblast proliferative rate.

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The importance of the dihydrofolate reductase (DHFR) system is related to the key role of tetrahydrofolate in the transfer of the monocarbon unit and therefore in the synthesis of adenine and guanine bases. The human DHFR gene, 30kb long and constituted of 6 exons and 5 introns, has been mapped on the chromosome 5q11.2-q13.2.[1-3] Since abnormalities in the expression of DHFR may directly influence some phases of cell differentiation and proliferation and contribute to altering the homeostatic balance between cell growth and death, which is one of the most important physiopathologic mechanisms of myelodysplastic syndromes (MDS), we studied the expression of the DHFR gene in bone marrow cells of patients with the 5q- syndrome, to evaluate the possible role of the enzyme abnormality in the still obscure pathogenesis of the disease.[4-8]

We studied bone marrow aspirates from 6 patients with the 5q- syndrome, from 8 patients with other types of MDS, at the onset, not previously treated, and from 10 age-matched subjects without blood diseases. DHFR cytochemical reaction was performed on bone marrow smears.[9] For each erythroid cell in every specimen the optical density (OD) of the reaction product was determined by Vickers M86 scanning and integrating microdensitometry at $\lambda = 585\pm5$ nm. Total RNA was extracted by means of Trizol Reagent (Invitrogen) and evaluated and quantified by spectrophotometric analyses, using a biophotometer (Eppendorf). One microgram of total RNA for each sample was retro-transcribed using random hexamers and the High Capacity Archive kit (Applera), at 37°C for 2 hours. cDNAs were purified by Qiaquick spin columns (Qiagen). The structure and nucleotide sequence of the human DHFR gene were deduced based on the GenBank sequence AC022223. A Taq-

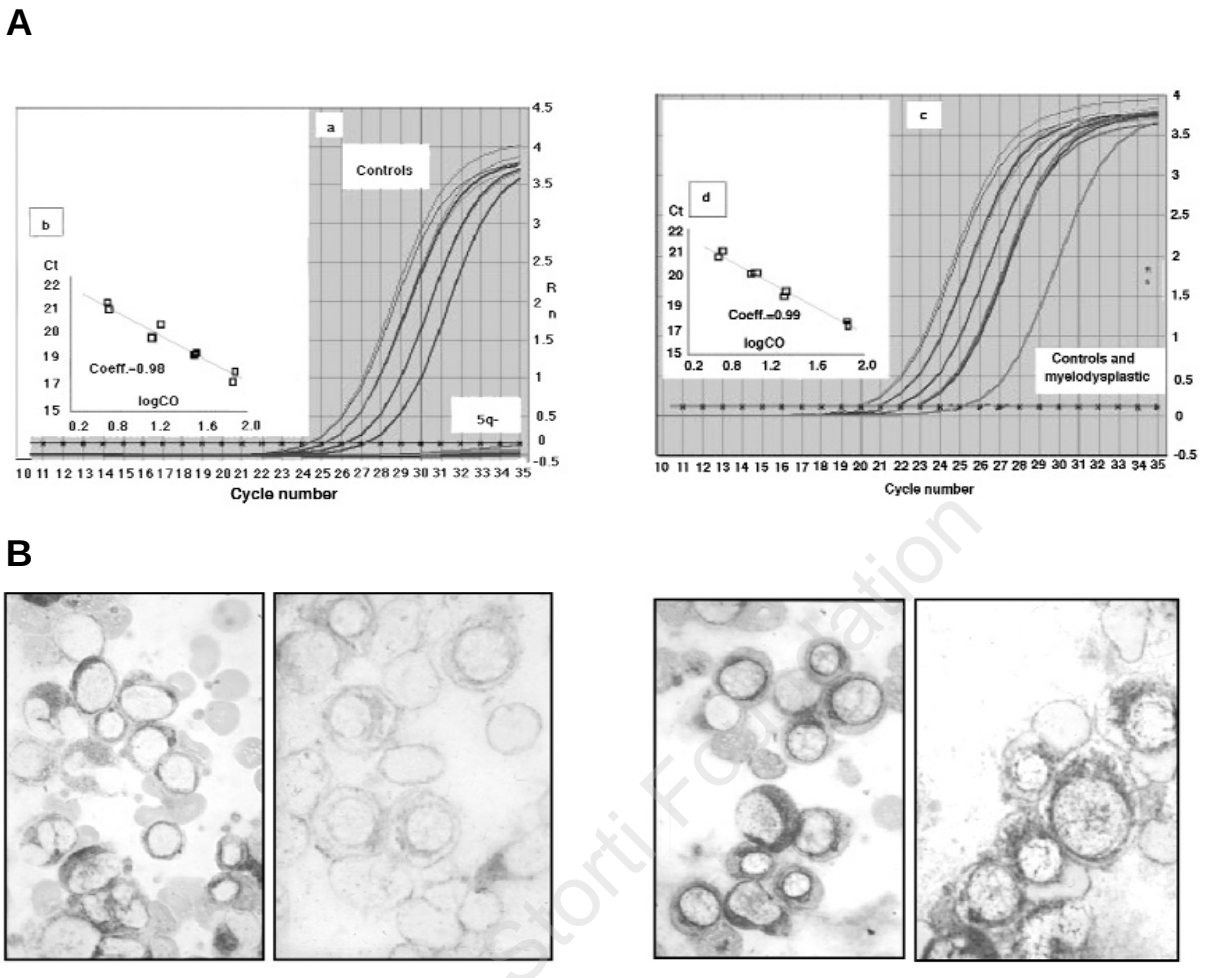

Figure 1. DHFR gene expression. A:DHFR amplification curves of controls and 5q- samples (left) and of controls and myelodysplastic samples (right). PCR was performed for 35 cycles consisting of 95°C for 10 min and 60°C for 1 min using a DHFR MGB TaqMan probe. Standard curves were generated using different amounts of RNA (1000; 500; 250; 50 ng) amplified using a GAPDH MGB TaqMan probe (inserts). Amplification plots were performed in several test tubes including samples in triplicate at known RNA concentration (1000 ng). Real-time PCR showed significantly decreased levels of DHFR mRNA in all patients with the 5q- syndrome, but not in the patients with other types of MDS. B: DHFR reaction on bone marrow smears. Left: moderate peri- or paranuclear positivity in normal erythroblasts, with intensity decreasing in relation to progressive cell maturity (left); very weak paranuclear positivity in the cytoplasm of the erythroblasts of the 5q- syndrome (right). Right: strong intensity of staining in the erythroblasts at all maturation stages of a RA case (left) and of a RARS case (right).

Man MGB probe encompassing exons 4 and 5 was designed using Primer Express V3.0 Software (Applera). Specifically, the upper and lower primers adopted were, respectively, DHFR/U 5'-CTGTTTATAAGGAAGCCATGAATCAC and DHFR/L 5'-TTGTGACAAGGATCATGCAAGAC, while the TaqMan MGB probe was 5'FAM-CAGGCCATCTTAAACTA. Real-time amplifications were performed using the 2× Master MIX, without UNG (Applera), 900 nM of the TaqMan MGB probe and 200 nM of each primer adopting a PE5700 Instrument (Applera). Commercially available expression control GAPDH Taqman probe and primers (Applera) were used as the control on the same panel of samples. The following biological parameters were also evaluated: hematopoietic progenitor cell growth *in vitro*, erythroblast apoptosis level using a TdT-mediated dUTP end labeling (TUNEL) technique[10] and erythroblast proliferative activity by an immunophosphatase alkaline technique (streptavidin-biotin complex, LSAB2 kit, Dako) using a murine monoclonal antibody to the nuclear antigen Ki-67 (MIB-1, Dako).

As reported in Figure 1, in all patients with the 5q- syndrome the real-time analysis showed significantly decreased, not quantifiable levels of DHFR mRNA in comparison with levels in normal controls. Differently, the other MDS cases showed variable gene expression profiles. In the cases of 5q- there was a significant decrease in the DHFR OD (mean 45.9±1.7) (p<0.0001), whereas in pathological erythroblasts from patients with other types of MDS the OD was significantly higher (mean 97.3±4.4) than in normal erythroblasts (mean 75.9±3) (p<0.0001) (Table 1). *In vitro* cultures of hematopoietic progenitors showed absent or severely reduced growth of BFU-E, but only slightly compromised growth of CFU-GM in all 5q- cases. The erythroblast apoptotic index was significantly higher both in 5q- cases (mean 40.9±16.0) (p=0.001) and in the other MDS patients (mean 29.5±19.2) (p=0.03) than in normal controls (mean 10.8±4.5) (Table 1). There was no relationship between erythroblast DHFR OD and apoptosis rate (p=0.5). The erythroblast proliferative rate was significantly lower in 5q- erythroblasts (mean 13.0±5.7) than in normal erythroblasts (mean 44.1±13.6) (p=0.001) and in the

Table 1. Biological features.

Case	Diagnosis	Karyotype	BFU-E*	CFU-GM*	DHFR (OD)°	AI (%)	PA (%)
1	5q- syndrome	46,XX, del(5) (q13q33)/46,XX	1	18	46.9	30	7
2	5q- syndrome	46,XY, del(5) (q13q33)/ 46,XY	0	1.5	45.1	41	9
3	5q- syndrome	46,XY, del(5) (q13q33)/ 46,XY	1.5	3	47.3	32	12
4	5q- syndrome	46,XX, del(5) (q?)/ 46,XX	2.5	4	43.6	50	30
5	5q- syndrome	46,XX, del(5) (q13q33)/ 46,XX	0	1.5	44.5	68	10
6	5q- syndrome	46,XX, del(5) (q?)/ 46,XX	1	5	48.2	24	18
7	RA	46,XY	19.5	16	89.6	42	17
8	RA	45,X,-Y/ 46,XY	4	4	95.3	41	42
9	RA	46,XY, del(3p)/ 46,XY	0	0	96.1	12	46
10	RARS	46,XX	3	5.5	97.0	32	50
11	RARS	46,XX	1.5	48	102.6	62	43
12	RARS	46,XX, t(1;22) (q12;p12)/ 46,XX	6	7.5	99.5	21	47
13	RAEB	46,XX	0.5	1.5	94.9	12	6
14	RAEB	46,XX, del(20q)/ 46,XX	3	12	103.2	1	60
15	Normal control	n.e.	50	86	76.7	2	25
16	Normal control	n.e.	44	31	79.2	16	66
17	Normal control	n.e.	56	40	71.1	14	23
18	Normal control	n.e.	28	54	80.3	14	59
19	Normal control	n.e.	37	41	75.8	6	37
20	Normal control	n.e.	35	58	77.1	14	42
21	Normal control	n.e.	66	42	76.5	9	41
22	Normal control	n.e.	36	47	78.0	7	47
23	Normal control	n.e	41	32	74.7	13	50
24	Normal control	n.e.	51	44	69.7	13	51

*OD: erythroblast optical density; AI: erythroblast apoptotic index; PA: erythroblast proliferative activity; RA: refractory anemia; RARS: refractory anemia with ring sideroblasts; RAEB: refractory anemia with excess of blasts; n.e.: not evaluated *N°/2×10⁴ MNC.*
°In each case 100 erythroblasts were evaluated and the mean for each sample was obtained.

erythroblasts of other MDS (mean 38.8±18.0) (p=0.009) (Table 1). A close correlation was observed between DHFR expression and proliferative activity (p=0.001).

In conclusion, for the first time reduced DHFR expression has been demonstrated in 5q- bone marrow. Since the DHFR gene maps outside the commonly deleted region at 5q31, as a possible explanation of our findings, we hypothesize that also deletions of distant regions of the chromosome may influence, even indirectly, transcriptional regulatory mechanisms. Chromosomal deletion mapping studies within the 5q- samples are in progress in order to identify possible DHFR gene regulatory sequences. Alternatively, changes in the levels of any of a number of cell cycle regulatory proteins may be involved. Anyway, we observed a close relationship between erythroblast enzyme levels and proliferative activity; in cases of 5q- the reduced proliferative rate, associated with increased apoptosis, could be responsible for the characteristic phenotypic feature of hyporegenerative anemia with tendentially hypocellular bone marrow.

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Membrane-bound heat shock protein 70 in acute myeloid leukemia: a tumor-specific recognition structure for the cytolytic activity of autologous natural killer cells

Patients with *de novo* acute myeloid leukemia with unfavorable and intermediate cytogenetics are frequently heat shock protein 70 (Hsp70)-membrane-positive. In contrast, no Hsp70 was detected on bone marrow of healthy donors or on CD34-enriched cells. The leukemia-selective Hsp70-membrane expression provides a recognition structure for Hsp70-peptide stimulated, patient-derived natural killer cells.

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Membrane localization of heat shock proteins has been detected selectively on tumor cell lines.[1-3] We determined Hsp70-membrane expression on leukemic blasts of patients with acute myeloid leukemia (AML) with different morphology and cytogenetics. Between March 1997 through January 2001, bone marrow and peripheral blood samples were collected from 89 patients (49 males, 40 females) with *de novo* AML, 15 (9 males, 6 females) with therapy-related, secondary AML, 10 (5 males, 5 females) with relapsed disease and 3 (all males) with persistent disease. All patients were diagnosed at the University Hospitals of Munich and Regensburg. Treatment was performed according to the German AML-Co-operative Group[4] protocol. Bone marrow aspirates of patients and healthy donors were immunophenotyped using the frequently occurring, fluorochrome-labeled, lineage-specific markers CD13, CD33, HLA-DR in combination with Hsp70-specific monoclonal antibody (C92F3B1) by multiparameter, CD45/side scatter analysis on a FACSCalibur instrument (Becton Dickinson). Chromosome abnormalities were detected using a modified Giemsa-banding technique and classified according to the International System for Cytogenetic Nomenclature (1995). In total, 59 of 89 (66%) bone marrow aspirates from patients with *de novo* AML were Hsp70-membrane-positive, as determined by the dual staining technique. Hsp70 was not common in promyelocytic AML M3: 7% (1/15), but was frequently found in other FAB types, AML M0: 100% (2/2), M1: 71% (12/17), M2: 100% (19/19), M4: 64% (14/ 22), M5: 80% (8/10), M6: 100% (1/1), and M unclassified: 67% (2/3). This is in line with the observation that the FAB M3 leukemic cell lines NB-4 and HL-60 were found to be Hsp70-negative (*data not shown*).

With respect to cytogenetics, a Hsp70-positive phenotype was frequently found in *de novo* AML with an intermediate karyotype (76%, 38/50) or unfavorable karyotype (82%, 14/17); only 32% of cases (7/22) with favorable karyotypes were Hsp70-positive (Table 1). Hsp70-membrane expression within the groups of therapy-related, secondary, relapsed and persistent AML, was comparable to that in *de novo* AML. These data are in line with clinical response rates and overall survival.

Table 1. Correlation of Hsp70-membrane expression on bone marrow aspirates of patients with *de novo*, secondary, relapsed, and persistent AML with prognostic parameters as defined by cytogenetics: *favorable*; t(15;17), t(8;21), inv/t(16); *unfavorable*; complex (≥3 abnormalities) karyotypes, del(5q), del(7q), -5, -7, inv/t(3), del/t(11q/23); *intermediate*; normal karyotype, +8, +11, 12p abn, other cytogenetic abnormalities.

AML	*Gender 65 m/52 f Hsp70⁺/n (%)*	*Favorable*	*Cytogenetics Hsp70⁺/n Intermediate*	*Unfavorable*
De novo	59/89 (66)	7/22 (32)	38/50 (76)	14/17 (82)
Secondary	12/15 (80)	0/0 (0)	8/10 (80)	4/5 (80)
Relapsed	8/10 (80)	1/2 (50)	4/5 (80)	3/3 (100)
Persistent	3/3 (100)	0/0 (0)	2/2 (100)	1/1 (100)

Hsp70-membrane expression was determined by multiparameter flowcytometry analysis together with two of the lineage-specific markers CD33, CD13, and HLA-DR in the following antibody combinations: IgG1-FITC/ IgG1-PE; CD33-FITC/ HLA DR-PerCP; CD33-FITC/ Hsp70-PE; and HLA DR-PerCP/ Hsp70-PE. The threshold of 5% for positivity was calculated with respect to the results of different normal tissues including peripheral blood mononuclear cells (PBMC, n=80), bone marrow from healthy donors (n=20), CD34-enriched cells (n=4), and bone marrow from patients in complete remission (n=10). The number of Hsp70-positive AML bone marrow cells could be correlated with the amount of immunologically defined blasts with variations of 1% - 25%. AML: acute myelogenous leukemia; Hsp70+, >5% Hsp70-positive as determined by flow cytometry; m/f: male/female; n: total number.