**Stem Cell Disorders**

**Multilineage involvement in the 5q- syndrome: a fluorescent in situ hybridization study on bone marrow smears**

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**Background and Objectives.** A pluripotent progenitor cell was demonstrated to be involved in myelodysplastic syndromes (MDS) with normal karyotype or with numerical chromosome aberrations, but the pattern of lineage involvement by the 5q31 deletion in the 5q- syndrome is unknown. We performed this study in order to define the distribution pattern of the 5q- anomaly better in the non-lymphoid cell compartment.

**Design and Methods.** Bone marrow (BM) smears from 8 patients with the 5q- syndrome were studied by a modification of the fluorescent in situ hybridization (FISH) technique that allowed direct visualization of cell morphology. A commercial LSI EGR1 probe (Vysis Inc.) for the 5q31 band was used simultaneously in dual-color experiments with a chromosome-5-centromeric probe (Vysis Inc.) on BM smears from 8 patients with the 5q-syndrome. As additional internal controls a chromosome-7-centromeric probe and a 7q31 probe were used. To establish the sensitivity limit of this approach 5 normal BM smears were studied. All 8 patients had the 5q- chromosome as the sole anomaly in 45% to 75% of the interphase cells.

**Results.** For each patient 20-40 erythroblasts were analyzed: they were mostly proerythroblasts and basophilic erythroblasts. In all patients a clone carrying the 5q31 deletion was detected (35-50% of the cells, median 45%). Between 20-50 granulocyte precursors were scored; the 5q31 deletion was found in 40%-50% (median 45%) in all cases. The proportion of neutrophils carrying the 5q deletion was consistently lower than the corresponding value in promyelocytes (28.7% vs 45.6%). In the 20-25 megakaryocytes analyzable in all patients, the overall incidence of 5q31 deletion was 52-68%. Equal proportions of large multilobular megakaryocytes and hypolobular megakaryocytes characteristic of the 5q-syndrome were scored: the latter cells showed the 5q31 deletion more frequently than the former cells (93.6% vs 19.3% of the cells). In 66% to 100% of the cases (median 83%) a few cells with uncondensed nuclear chromatin pattern, and two or three prominent nucleoli with cytoplasmatic hypogranulation were seen in each sample carrying the 5q31 deletion.

**Interpretation and Conclusions.** We arrived at the following conclusions: i) the transformation in the 5q- syndrome involves an early progenitor cell retaining the ability to proceed along multiple differentiation pathways; ii) there is a preferential distribution of the 5q31 deletion within immature cells and morphologically abnormal megakaryocytes.

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**Key Words :** 5q- syndrome, MDS, FISH on BM smears

**Myelodysplastic syndromes (MDS) are a group of hematopoietic neoplasms associated with cytopenia and morphologic aberrations involving one or more cell lineages.** Studies including the analysis of G6PD isoenzyme expression, restriction fragment length polymorphism, combined immunophenotyping and in situ hybridization, provided compelling evidence for an acquired disorder occurring at a stem cell level.1,4

Despite the heterogeneity of evolution patterns, three prognostically distinct groups were recognized,5 based on the assessment of hematologic and cytogenetic variables. Among the low-risk MDS group, a distinct disease carrying a partial deletion of the 5q chromosome as the sole anomaly was first described by Van den Berghe et al. in 1974.6 Peculiar hematologic features in this cytogenetic entity include refractory macrocytic anemia, hypolobulated megakaryocytes with normal or elevated platelet counts and modest leukopenia. There is wide consensus that the association of the above mentioned cytogenetic and hematologic features identifies a distinct hematologic entity occurring more frequently in females and having a benign clinical course with a low propensity for transformation into acute leukemia.7

The application of fluorescent in situ hybridization (FISH) on bone marrow (BM) smears proved to be a reliable method of allowing the simultaneous visualization of cytogenetic anomalies and cell morphology.8-12
liciation of which cell lineages are affected by the 5q- anomaly may allow for a better understanding of the biology of this disease, especially when considering that heterogeneity of lineage involvement may occur in MDS and that macrocytic anemia is frequently the only remarkable hematologic alteration in the 5q- syndrome.

To define the distribution of the 5q- anomaly better in the non-lymphoid cell compartment, eight patients with morphologic, cytogenetic and hematologic features typical of the 5q- syndrome were analyzed using a modification of the conventional FISH technique permitting the direct visualization of hybridization signals on cytologically intact BM smears.

Design and Methods

Patients

Eight patients with morphologic, clinical and cytogenetic features consistent with the diagnosis of 5q- syndrome were included in the present study. These patients were classified according to the FAB criteria. An independent review of bone marrow (BM) morphology was carried out, with reference to the presence of dyserythropoiesis, dysgranulopoiesis and dysmegakaryocytopoiesis.

Cytogenetic studies

Chromosome studies were successfully performed in all cases as part of the diagnostic workup; in 3 cases a second investigation was performed after 1-2 years.

Bone marrow cells were cultured for 24 and 48 hours in RPMI medium to which 10% fetal calf serum had been added. The cytogenetic procedure included mitotic block by colcemid, hypotonic shock in 0.075M KCl solution and fixation in 3:1 methanol/acetic-acid solution. When possible, a minimum of 20 metaphases were analyzed for each patient; karyotype analysis was performed according to the ISCN.

FISH studies

Probes. A commercial probe (LSI probe EGR-1, cod. 32-190021, Vysis Inc., Illinois, USA) recognizing sequences at the 5q31 band (conjugated with Spectrum green fluorophore) was used simultaneously in dual-color experiments with a chromosome-5-centromeric probe (conjugated with Spectrum orange fluorophore) throughout this study. As additional controls the following probes were used in all experiments in BM smears as outlined below: a chromosome-7-centromeric probe (conjugated with Spectrum orange fluorophore) was used simultaneously in dual-color experiments with a chromosome-5-centromeric probe; the second control was cohybridization of the 5q31 probe with the chromosome-5-centromeric probe; the second control was rehybridization of the same slide with the chromosome-7-probes. The chromosome-7-probes were selected as controls because they had similar characteristics (size, direct labeling) as the chromosome-5-probes used in this study.

Diagnostic BM smears in eight patients with the 5q-syndrome were studied. The slides were stored at room temperature for 1-6 years. In patients #1, 3, 7, a second FISH analysis was performed on follow-up BM smears obtained 1-2 years after diagnosis.

Because hybridization efficiency on these cytologic preparations was sub-optimal as compared with FISH on conventional cytogenetic preparations, two types of controls in MDS samples were used. The first control was cohybridization of the 5q31 probe with the chromosome-5-centromeric probe; the second control was rehybridization of the same slide with the chromosome-7-probes. The chromosome-7-probes were selected as controls because they had similar characteristics (size, direct labeling) as the chromosome-5-probes used in this study.

Hybridization conditions. The same slides previously colored with May-Grünwald-Giemsa stain (MGG) and used for clinical observation were first incubated for 60 min with RNAase (100 ug/mL; Boehringer Mannheim, Mannheim, Germany) washed twice in 4×SSC 5 min each, subsequently dehydrated in ethanol alcohol series (70%, 80%, 90%, 100%) and air dried.

The slides were prewarmed on a hot plate and then immersed in a 70% formamide/2×SSC solution at 72°C for 5 min and dehydrated again with ice alcohol series.

Fifteen microliters of each probe were added to each slide, which was covered with a covers lip. Rubber cement was used to seal the edges and the slide was incubated overnight at 37°C, in a moist chamber.

Post-hybridization washes included baths at 45 °C in 50% formamide in 2×SSC for 15 min, in 1×SSC for 10 min, and in 0.1×SSC for 5 min, without intermittent agitation. No antifade solution was applied to the slides.

Evaluation of FISH results was performed using a Nikon fluorescence-equipped microscope with couple charged camera device and appropriate hardware and software (Cytopision System, Applied Imaging distributed by Nikon Italy, Florence, Italy); FISH images and the MGG microscopic field previously recorded were fused for the final view.

Signal screening. To prevent data misinterpretation deriving from inefficient hybridization only those areas with more than 70% cells showing two centromeric signals at a 1,200× magnification were analyzed.

Granulocytic and erythroid cell were counted as affected by 5q deletion if two centromeric signals and one 5q31 signal were present. The same criterion was applied for the detection of 7q31 deletion.

All megakaryocytes without overlapping nuclear lobes and with well delineated signals were analyzed. Fluorescent spots disclosed on different focus planes were recorded separately and fused in the same cell image.
using computed-assisted image recording. Because the ploidy of megakaryocytes is variable we first calculated the mean green-to-red signal ratio ±2 SD in 50 normal megakaryocytes and then used the lower interval limit as the cut-off point for recognizing 5q deletion in MDS megakaryocytes. In each megakaryocyte, the number of 5q31-signals (green fluorescence) and of chromosome-5-centromere signals (red fluorescence) was counted and the 5q31/5q-centromere ratio was calculated.

A total of 250 erythroblasts and granulocyte precursors and 50 megakaryocytes were scored in the five normal BM smears to set the cut-off point for recognition of 5q31 and 7q31 deletion.

Forty or more erythroblasts and granulocyte precursors and a minimum of 20 megakaryocytes with well spread nuclei were examined in each MDS slide.

**Results**

**Hematologic features**

Hematologic and cytogenetic data of our patients are summarized in Table 1. Their median age was 64.5 years, with a 1:4 male-to-female ratio.

Five patients are alive with stable disease (follow-up of 48-96 months), 1 patient developed progressive transfusion-dependent anemia after 36 months (patient #3), 1 patient died after 48 months of an MDS-unrelated condition (heart attack; patient #1); 1 patient was lost to the follow-up (patient #4). No patient developed leukemia.

Macrocytic anemia (median hemoglobin value 9.6 g/dL, range 8.5-10.2 g/dL, median MCV value) with low count was required for each individual megakaryocyte to be counted as affected by 5q31 deletion.

**Cytogenetic and FISH findings**

All 8 patients had the 5q- chromosome as the sole anomaly, with the classical 5q13-q31 breakpoints (Table 1). The percentage of metaphases with the 5q31-33 deletion ranged from 33% to 75% (median 55%); similar data were obtained by interphase FISH, showing 45%-75% nuclei with the 5q deletion (median 59%). In three patients the percentage of 5q- cells was reassessed by interphase FISH after 1-2 years: the proportion of deleted cells increased from 45%, 55% and 58% to 67%, 68% and 77%, respectively.

**FISH on BM smears**

Controls and cut-off point for the recognition of 5q deletion. The hybridization efficiency was found to vary in different areas of the slides.

Optimal results, i.e. in the presence of the expected 2-red and 2-green signals, were observed in well spread areas with preserved cell morphology, a minority of cells having clumped chromat in these areas, usually representing a minority of 1,200× microscopic fields, more than 70% of the cells had the expected signal configuration (Figure 1).

In the five controls the percentage of erythroblasts having a false 5q31 deletion was in the range of 5-10% (median value 7%); 4-10% of the cells (median 6%) were granulocyte precursors carrying a false 5q31 deletion. The ploidy of the 50 analyzed megakaryocytes ranged between 4 and 32 (modal ploidy 8). The ratio of chromosome 5q31/chromosome-5-centromere signals was in the range of 0.75-1.00 (median value 1.00). The cut-off point for recognition of 5q deletion was set at the mean values plus 2 SD, corresponding to 12.1% for erythroblasts, and 12.5% for granulocytic cells. A <0.70 ratio of chromosome 5q31/chromosome-5-centromere was required for each individual megakaryocyte to be counted as affected by 5q31 deletion.

Hybridization of the samples with chromosome-7-
probes showed a false deletion in 4-9% of erythroid cells (median value 6%) and in 6-10% of granulocytic cells (median value 7%). A 0.80-1.00 ratio of red-to-green signals was observed in megakaryocytes.

**MDS marrows**

Detailed results of FISH investigations on BM smears are presented in Table 2.

**Erythroid cells**

Forty erythroblasts were analyzed in each patient: they mostly consisted of proerythroblasts and basophilic erythroblasts; only few mature erythroid precursors having two centromeric signals could be analyzed due to inefficient hybridization in cells with clumped chromatin. In all patients, a clone carrying the 5q31 deletion was detected, accounting for 35-50% of the cells (median 45%). Globally, 43.7% of 320 erythroblasts carried the 5q- chromosome in the eight patients analyzed at diagnosis.

**Granulocytic lineage and undifferentiated cells**

The 5q31 deletion was detected in 40 to 50% (median 45%) of the granulocyte precursors. Due to chromatin clumping only a total of 80 neutrophils could be analyzed in the eight patients: 23 neutrophils (mean 28.7%) showed the 5q31 deletion, as compared with 146/320 (mean 45.6%) granulocyte precursor cells.

A total of 25 cells with uncondensed nuclear chromatin, 1 or more nucleoli and scanty cytoplasm, corresponding to blast cells, could be visualized in the eight MDS patients. Twenty of these cells (80%) showed a 5q deletion.

**Megakaryocytic lineage**

A minimum of 20 megakaryocytes were analyzed in each patient. The number of chromosome-5-centromere signals ranged from 2 to 8.

The percentage of platelet progenitors carrying a deletion was in the range of 52%-68% (median 62%). Although the expected 0.50 ratio of 5q31/chromosome-5-centromeric signals was found in the majority of megakaryocytes with deletion, a ratio in the range of 0.50-0.70 was observed in some cells. Counting errors due to difficulty in recognizing hybridization spots located on different focus planes may account for this observation and indeed similar findings were reported by van

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**Table 2. FISH studies on BM smears: no. of cells with deletion/total.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mono/hypo-</th>
<th>Other</th>
<th>Erythroblasts</th>
<th>PM, M, Metamyelocytes</th>
<th>Neutrophils</th>
<th>Blasts</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mono/hypo-</td>
<td>Other</td>
<td>Erythroblasts</td>
<td>PM, M, Metamyelocytes</td>
<td>Neutrophils</td>
<td>Blasts</td>
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<tr>
<td>1*</td>
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<td>3/11</td>
<td>18/40</td>
<td>20/40</td>
<td>3/10</td>
<td>3/3</td>
</tr>
<tr>
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<td>12/12</td>
<td>3/12</td>
<td>16/40</td>
<td>16/40</td>
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<td>2/2</td>
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<tr>
<td>3*</td>
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<td>16/83</td>
<td>140/320</td>
<td>146/320</td>
<td>23/80</td>
<td>20/25</td>
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</tbody>
</table>

*Patients studied at diagnosis and after 1-2 years. °PM: promyelocytes; M: myelocytes; MM: metamyelocytes.
Lom et al. who adopted a sensitive confocal laser microscopy method to detect numerical abnormalities in dysplastic megakaryocytes. As detailed in Table 2, mono/hypolobular megakaryocytes were found to carry the 5q31 deletion more frequently than multilobular megakaryocytes (93.6% vs 19.3% of the cells). The areas with the expected chromosome-7-signal configuration in MDS patients were essentially the same as those that had shown successful hybridization with the chromosome-5-probes. The percent of cells with false 7q31 deletion was below the sensitivity limit of this technique in all cases.

Discussion

Even though the primary transformation event in MDS occurs in an undifferentiated hematopoietic precursor, a heterogeneous pattern of lineage involvement by chromosome lesions was described, possibly reflecting selective differentiation of stem cells carrying a cytogenetic abnormality.

The approach described here, using FISH on BM smears, has the advantage over molecular genetic methods and cell culture studies, of permitting the direct visualization of cell morphology and hybridization pattern. Two factors appeared to have a positive impact on the efficiency of hybridization in this study: an open chromatin structure and the presence of areas with well-dispersed cytology and preserved morphology. Indeed, cells of all lineages at various stages of maturation, including megakaryocytes, could be recognized in this study.

Whereas the involvement of cells of the erythroid and granulocytic lineage in MDS with -7 and +8 was well documented, as reviewed by Knuuttila, scanty information is available about lineages affected by the 5q31 deletion. This issue is of particular interest, the 5q- syndrome representing a specific entity, distinct from other refractory anemias with or without multilineage dysplasia. The patients included in this report were typical examples of the 5q- syndrome, in that they showed the classical abnormalities of megakaryocyte morphology, moderate neutropenia, macrocytic anemia, and 5q deletion affecting the q13-31 bands. The relatively benign clinical course in our patients is in line with previous reports.

We found in this study that mosaicism of 5q31 deletion and cytogenetically normal cells was present at diagnosis in all the patients with the 5q- syndrome. The frequency of cells with deletion was in the range of 45-75% and concordance was observed between data obtained by karyotyping and by interphase FISH. The coexistence of cytogenetically normal and abnormal clones is a general phenomenon in MDS, possibly reflecting the persistence of residual non-clonal hematopoiesis or, alternatively, the secondary nature of chromosome anomalies in these conditions. In this study the proportion of cells with deletion increased over the time in three patients studied at different times. This finding is in line with previous reports describing a proliferative advantage of the 5q- clone, which may sometimes become detectable by cytogenetic analysis late in the course of the disease.

Even though morphologic aberrations in the 5q- syndrome were mostly confined to the erythroid and megakaryocytic lineages, the 5q31 deletion affected the three principal hematopoietic lineages in all our cases, suggesting involvement of a multipotent myeloid stem cell retaining the capacity of differentiation along multiple cell lineages; this finding reflects the data from Nilsson et al. The direct demonstration of megakaryocyte involvement in the 5q- syndrome has not been reported in the literature, whereas the presence of the 5q- anomaly in granulocytes, monocytes and erythroid cells was previously documented in a few patients by molecular genetic methods and cell culture studies.

A homogeneous pattern of involvement of erythroid cells was found in this study, with the 5q deletion occurring in 35-50% of the cells. Because erythrocytes in our patients were consistently macrocytic it is reasonable to assume that perturbed DNA synthesis leading to macrocytosis affected the majority of erythroid progenitors, independently of the presence of the 5q anomaly in each individual cell.

It is noteworthy that the involvement of the granulocytic lineage in the 5q- syndrome occurred despite only moderate neutropenia without major infections. Due to sub-optimal hybridization in neutrophils and to the low number of blast cells in this subset of MDS, it was impossible to compare, in individual patients, the frequency of the 5q- in the early precursor cells and in mature cells. Interestingly, the global frequency of deletion in the blast cells was higher than in mature granulocytes (see Table 2) suggesting that inefficient maturation along the granulocytic lineage may be a feature of the 5q- clone and that most neutrophils may derive from stem cells not affected by the 5q anomaly.

Mosaicism of the 5q- clone and normal cells was previously documented in a FISH study of peripheral blood myeloid cells. Direct demonstration that megakaryocytes were part of the malignant clone in MDS with numerical chromosome abnormalities was recently provided by Van Lom et al. who showed that -7 and +8 were present in megakaryocytes using dual color fluorescent in situ hybridization and confocal laser scanning microscopy. Recently, Godon et al. demonstrated that the 5q31 deletion was present in all megakaryocytes from 5/6 patients with MDS. In our study we were able to confirm that megakaryocytes were part of the abnormal clone in the 5q- syndrome and showed that this chromosomal anomaly was associated with mono/hypolobular megakaryocytes, the presence of which is one of the defining criteria for this condition. The loss of the cluster of hematopoiesis-related genes, including growth factors and their receptors, interleukins as well as the dihydrofolate reductase gene may affect the maturation capa-

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bility of the hematopoietic progenitors carrying the 5q-
chromosome, altering the endomitotic activity of mega-
karyocytes.

In conclusion, we have shown by FISH on morpho-
logically intact BM smears that mosaicism of cells with
the 5q- anomaly and chromosomally normal cells con-
sistently affected the 3 principal hematopoietic lineages
in the 5q- syndrome. The 5q anomaly correlated with
megakaryocyte morphology as well as with cell immu-
nerity in the granulocytic lineage, whereas no obvious
correlation with erythroblast morphology was noted.
This method may be useful in clinical practice to verify
the fluctuation of the size of the 5q- clone within each
cell lineage during the natural history of the disease, or
after treatment with hematopoietic growth factors.

Contributions and Acknowledgments

GLC, AC, RB, were responsible for the conception of the
study, its design, funding, and data handling. RB, M GR,
RM, AB, FC, GS, PA, MP performed cytogentic and FISH
analyses. RB and AC contributed to the analysis and writ-
ing of the paper. With respect to the order in which the
names appear, the authors are listed according to their
contribution to the work.

The last names are those of the principal clinician
involved and the senior author.

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Potential implications for clinical practice

FISH on intact BM smears may demonstrate the
selective involvement of the hematopoietic lineages.
This technique is particularly useful for analyzing the
molecular pattern of monolobulated megakaryocytes
which constitute a peculiar finding in 5q- syndrome.

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