Cytogenetic follow-up by karyotyping and fluorescence *in situ* hybridization: implications for monitoring patients with myelodysplastic syndrome and deletion 5q treated with lenalidomide

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ABSTRACT

In patients with low and intermediate risk myelodysplastic syndrome and deletion 5q (del(5q)) treated with lenalidomide, monitoring of cytogenetic response is mandatory, since patients without cytogenetic response have a significantly increased risk of progression. Therefore, we have reviewed cytogenetic data of 302 patients.
Patients were analyzed by karyotyping and fluorescence *in situ* hybridization.
In 85 patients, del(5q) was only detected by karyotyping. In 8 patients undergoing karyotypic evolution, the del(5q) and additional chromosomal aberrations were only detected by karyotyping. In 3 patients, del(5q) was only detected by fluorescence *in situ* hybridization, but not by karyotyping due to a low number of metaphases.
Karyotyping was significantly more sensitive than fluorescence *in situ* hybridization in detecting the del(5q) clone. In conclusion, to optimize therapy control of myelodysplastic syndrome patients with del(5q) treated with lenalidomide and to identify cytogenetic non-response or progression as early as possible, fluorescence *in situ* hybridization alone is inadequate for evaluation. Karyotyping must be performed to optimally evaluate response. *(clinicaltrials.gov identifier: NCT01099267 and NCT00179621)*

Key words: karyotyping, FISH, del(5q), banding analysis, cytogenetics, therapy control, follow-up.

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Introduction

Myelodysplastic syndrome associated with isolated del(5q) is a distinct entity with a risk of evolution into acute myeloid leukemia of approximately 10%.[1-2] It is characterized by anemia with or without other cytopenias and/or thrombocytosis. Myeloblasts comprise less than 5% of bone marrow and less than 1% of peripheral blood.[1] Lenalidomide is particularly active in myelodysplastic syndrome patients with del(5q), with over two-thirds of low and intermediate risk myelodysplastic syndrome patients with 5q deletions achieving transfusion independence.[3] In this cohort with an *a priori* increased risk, about one-third of the patients underwent clonal evolution and acute myeloid leukemia progression.[4-5] Patients without hematologic or cytogenetic response had a significantly increased risk of progression.[5] Therefore, careful monitoring of the cytogenetic response may add important prognostic information in myelodysplastic syndrome patients treated with lenalidomide.

The gold standard for the detection of aberrations is karyotyping.[6-8] Aberrations are detected in about half of myelodysplastic syndrome patients.[9] Additional fluorescence *in situ* hybridization (FISH) analyses are recommended to detect deletions 5q, 7q, 17p or 20q, monosomy 7 and trisomy 8.[6] Recently, it was suggested that FISH should be performed in cases of a suspected '5q- syndrome' and/or if karyotyping shows no metaphases or an aberrant karyotype involving chromosome 5.[10]

In order to optimize therapeutic control of myelodysplastic syndrome patients treated with lenalidomide, we evaluated the cytogenetic data of 302 del(5q) patients treated with lenalidomide and compared the results obtained by karyotyping and FISH.

Design and Methods

Patients

Our cohort included 302 patients with transfusion-dependent anemia due to low- or intermediate-1-risk myelodysplastic syndrome [according to the International Prognostic Scoring System (IPSS)] associated with a 5q deletion with or without additional cytogenetic abnormalities. This cohort includes all

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European patients enrolled in the CC-5013-MDS-003 (ClinicalTrials.gov Identifier: NCT01099267) and all patients enrolled in the CC-5013-MDS-004 study (ClinicalTrials.gov Identifier: NCT00179621) for myelodysplastic syndrome patients with del(5q) treated with lenalidomide. Cytological and morphological diagnoses were made centrally at St. Johannes Hospital Duisburg and Hannover Medical School. Patients were enrolled in the studies MDS-003 (n=42) or MDS-004 (n=260) and treated with lenalidomide (starting dose 10mg daily) according to the study protocols.[3] Written informed consent was provided according to the Declaration of Helsinki and approval from the Ethics Committee of Hannover Medical School was obtained.

Lenalidomide therapy is still ongoing in responding patients. Dose adjustments were performed in the majority of patients due to side effects such as neutropenia.

Regular follow-up investigations were performed every six months of treatment or more frequently, depending upon clinical features. As many as 11 cytogenetic analyses were performed for each patient. In total, we reviewed 1,075 cytogenetic analyses.

Standardized cytogenetic investigations: karyotyping and FISH

Bone marrow was received from European study centers for central cytogenetic review. Karyotyping was performed according to standard procedures.[11] Whenever possible, 25 metaphases were analyzed. Description of the karyotype, the chromosomal aberrations, followed the recommendations of the International System for Cytogenetic Nomenclature.[12] In all patients, an interstitial del(5q) containing the commonly deleted region 5q31 was present (Figure 1A-C). FISH for del(5q) was included in each investigation (probes supplied by Abbott, Wiesbaden, Germany). FISH was performed on fixed cells from the cytogenetic culture according to standard procedures (Figure 1D-F).[11] For each FISH analysis, at least two hundred interphase nuclei were analyzed. In agreement with the literature, mean cutoff levels were determined to be 8% (+/- 3SD) by analysis of at least 1,000 cells from 10 healthy donors.[10]

Cytogenetic response

Cytogenetic response was applied to karyotyping only. Complete cytogenetic response (CCyR) was defined as disappearance of the 5q deletion or any other chromosomal aberration. Partial cytogenetic response (PCyR) was defined as a reduction of aberrant metaphases of more than 50% compared to the previous karyotyping.[3] Cytogenetic relapse was defined as reappearance of a metaphase with a deletion in 5q after reaching a complete cytogenetic response.

Statistical analyses

Analysis of del(5q) detection rates in metaphases and interphases was carried out by Wilcoxon's test.[13] Values of $P<0.05$ were considered statistically significant.

Results and Discussion

We compare the sensitivity of detecting the del(5q) clone by karyotyping and FISH, respectively, in a large cohort of myelodysplastic syndrome patients treated with lenalidomide (n=302). A complete cytogenetic investigation based on at least 25 metaphases and FISH based on the analyses of at least 200 interphase nuclei was performed in 835 (78%) of the investigations. Here, we focus on discrepant results of karyotyping and FISH that would have led to either a different evaluation of whether the patient is eligible for treatment with lenalidomide or to a different diagnosis of cytogenetic remission, relapse or progression during follow-up.

Detection of del(5q) at study entry before treatment with lenalidomide

Twelve of 302 patients (4%) (patients 1-10, 23 and 86) showed discrepant karyotyping and FISH results already in the first cytogenetic investigation at study entry (*Online Supplementary Table S1*). In patients 1-10, del(5q) was not detected by FISH but only by karyotyping. In 5 of these 10 patients, less than 10% of the metaphases showed a deletion in 5q at study entry. In contrast, a deletion in 5q was detected in 72% and 100% of the metaphases in 2 of those

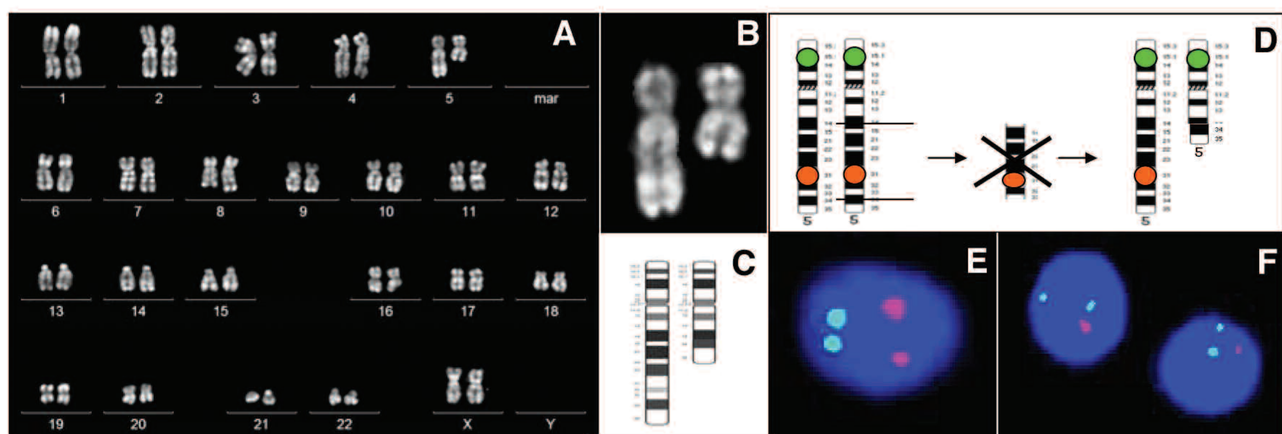

Figure 1. (A) Karyogram of a patient with myelodysplastic syndrome and a deletion in the long arm of chromosome 5 (fluorescence R-banding). (B) More detailed analysis identified the deletion as an interstitial deletion. (C) Comparison with the ideogram of chromosome 5 shows that the breakpoints are located in 5q14 and 5q34. (D) Scheme of fluorescence *in situ* hybridization to detect a deletion in 5q. The probe localized to the short arm of chromosome 5 (5p21) generates a green control signal. The probe binding to the commonly deleted region 5q31 gives an orange signal. (E) Interphase nucleus with a normal signal constellation, i.e. two green and two orange signals. (F) Interphase nuclei with a signal constellation indicative of a deletion in 5q, i.e. two green signals and one orange signal.

patients (patients 6 and 10) (*Online Supplementary Table S1*). In patients 23 and 86, del(5q) was detected by FISH only. The del(5q) was detected in 21% and 32% of the interphase nuclei, respectively. However, in both investigations, less than 10 metaphases could be analyzed. Seventeen patients showed one additional aberration and 12 patients an independent clone, e.g. +8 or t(2;11).

Follow-up after six months of treatment

Six months after treatment with lenalidomide, in 27 of 232 patients (12%) del(5q) was detected only by karyotyping and not by FISH. In 2 of these patients, a clonal evolution occurred. Neither the clonal evolution nor the del(5q) clone would have been detected if only FISH had been used. At this time point, in no case was del(5q) detected by FISH only (*Online Supplementary Table S1*).

Follow-up after 12 months of treatment

After 12 months of treatment, in 39 of 169 patients (23%) del(5q) was detected only by karyotyping. In one patient, del(5q) was detected only by FISH. In 3 patients, a clonal evolution occurred. In 6 patients, a cytogenetic relapse occurred at this time point. This relapse would not have been detected if only FISH had been used (*Online Supplementary Table S1*).

Third and later follow-up investigations after 18 months and later of treatment

In 85 of 267 patients (32%), a discrepancy was detected after at least 18 months of treatment. In only one patient was del(5q) detected by FISH only. In the other cases, del(5q) was detected by karyotyping only and not by FISH. In 31 of these patients, a cytogenetic relapse occurred and would not have been detected if only FISH had been used. In 5 of them, progression with a clonal evolution was identified by karyotyping. Neither the clonal evolution nor the del(5q) clone would have been detected if only FISH had been used (*Online Supplementary Table S2*). There was no difference regarding the detection of the del(5q) comparing patients with a discrepancy at the first time point of analysis and others.

Detection of a complete cytogenetic response

Twenty-four of 163 patients (15%) with a discrepancy between karyotyping and FISH obtained a complete cytogenetic response. Eleven patients obtained a complete cytogenetic response after six months of treatment with lenalidomide. Three patients obtained a complete cytogenetic response after 12 months, 9 patients after 18, and one patient after 24 months (median 15 months). Nine of these 24 patients (37%) reached a partial response and 15 patients (63%) did not reach a partial response beforehand. The partial response occurred once after six months, 7 times after 12 months and once after 18 months of treatment. Patient 35, for example, showed a complete cytogenetic response after 12 months without reaching a partial remission beforehand (*Online Supplementary Table S2*). On the other hand, patient 15 obtained a partial remission after six and a complete cytogenetic response after 12 months of treatment (*Online Supplementary Table S1*).

In 19 of 302 patients (6%), independent clones were detected by karyotyping. The independent clones contained trisomy 8 in 12 patients, loss of the Y chromosome in 4 patients, monosomy 7 in 3 patients, del(20q) in 2 patients, and del(11q), trisomy 14 and der(1;7) in one patient each. In 4 of these patients, two or three independent clones were found. In 8 of 16 patients undergoing a karyotypic evolution, at 17 different time points, neither the del(5q) nor the additional chromosomal aberrations were detected by FISH, in contrast to karyotyping. In patients 34, 35 and 36, karyotypic evolution occurred after they had obtained a complete cytogenetic response.

Clinical consequences

This study has three major implications for monitoring myelodysplastic syndrome patients with del(5q) treated by lenalidomide. Firstly, the del(5q) clone was detected with significantly greater sensitivity by karyotyping than by FISH, thus making the diagnosis of non-remission and cytogenetic relapse possible only by karyotyping in a significant number of patients. Secondly, without prior knowledge of the additional chromosome aberrations, clonal evolution can only be identified by karyotyping. At the time point of clonal evolution, even del(5q) was detected only by karyotyping in a significant number of patients. Thirdly, detection of the del(5q) clone was sometimes only possible by FISH, making the diagnosis of non-remission and cytogenetic relapse in these patients only possible by FISH analysis. Thus, all cytogenetic follow-up investigations must include karyotyping. At least one additional FISH analysis should be performed if karyotyping does not give sufficient results, e.g. due to poor chromosome morphology or due to a low number of metaphases (< 25 metaphases).

Karyotyping was significantly more sensitive than FISH for detection of del(5q) (P<2.2e-16). This was true for the time point of study entry as well as during follow-up. In contrast, a deletion in 5q was only detected by FISH in 3 patients. In these cases, less than 10 metaphases were available for analysis. As recommended by the European LeukemiaNet, at least 25 metaphases are necessary for a reliable karyotyping.[6] In the literature, several studies described discrepant results of karyotype and FISH analyses.[10,14-17] In most instances, the cases with a discrepant result (positive by FISH only) had an insufficient number of metaphases or the chromosome morphology was poor. Pitchford *et al*. recommended FISH in myelodysplastic syndrome patients only if an adequate karyotyping with at least 20 metaphases was not possible, since they were unable to demonstrate any advantage of FISH in the setting of an optimal banding analysis.[7] FISH is a valuable method to quantify the clone size with cytogenetic aberrations known from diagnosis. Whether it is sufficient only to perform FISH during follow-up analyses is a subject for discussion. FISH may be used to monitor for the presence of a specific chromosomal abnormality that was present before treatment.[18] Obviously, this approach has positive aspects. For example, it is cost- and time-effective as no culturing is needed since only interphase nuclei are analyzed. However, our findings question the proposal that FISH analysis alone is sufficient for follow-up analysis. Using this approach in our study, cytogenetic non-response or relapse, and even clonal evolution, would not have been detected in a significant number of patients.

One reason for the higher sensitivity of karyotyping in detecting the del(5q) clone might be that a small clone with proliferative advantage over normal cells may be more easily identified by karyotyping than by FISH. FISH is a quantitative method counting interphase nuclei with a normal signal constellation and with a signal constellation indicative of a del(5q). A small clone with a del(5q) might be

missed if it is below the cut-off level for the detection of a deletion, which is typically between 3% and 10%, and was 8% in our study.[7,9,19] In contrast, karyotyping is based on metaphases, i.e. cells undergoing spontaneous cell division *in vitro*. Therefore, the number of metaphases with a del(5q) identified by karyotyping does not necessarily represent the proportion of these cells within the bone marrow. To monitor cytogenetic response in chronic myeloid leukemia, Fugazza *et al*. did not find an advantage in using karyotyping or FISH. As in our study, one or the other method was superior in some patients.[20] In contrast, the GIMENA group recently showed that FISH is more sensitive than banding analysis in chronic myeloid leukemia and suggested this should be used for monitoring.[21] However, due to the FISH probe designed to detect the Philadelphia translocation, the cut-off level was below 1%.

A very important advantage of karyotyping is the possibility of identifying additional aberrations. Only previously selected loci can be analyzed by FISH. Moreover, an additional multicolor FISH can be applied to detect cryptic aberrations not seen by karyotyping. In our study, clonal evolution was identified in 8 patients. In most patients, clonal evolution was associated with leukemic progression as described previously.[5,22,23] In these patients, chromosomal instability leading to clonal evolution seems to be the driving force behind the expansion of tumor cell populations.[24]

In conclusion, to optimize monitoring of myelodysplastic syndrome patients with del(5q) treated with lenalidomide and to detect cytogenetic non-response, cytogenetic relapse or clonal evolution as early as possible, FISH alone is not adequate for evaluation. Karyotyping must be performed to optimally evaluate response.

Authorship and Disclosures

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