Distinguishing myelodysplastic syndromes (MDS) from idiopathic cytopenia of undetermined significance (ICUS): HUMARA unravels clonality in a subgroup of patients

T. Schroeder1*, L. Ruf1, A. Bernhardt1, B. Hildebrandt2, M. Aivado1,3, C. Aul4, N. Gattermann1, R. Haas1 & U. Germing1

1Department of Haematology, Oncology and Clinical Immunology; 2Institute of Human Genetics and Anthropology, Heinrich-Heine University, Düsseldorf, Germany; 3Global Clinical Development Oncology GlaxoSmithKline, Collegeville, PA, USA; 4Department of Haematology, Oncology and Clinical Immunology, St Johannes Hospital, Duisburg, Germany

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Background: Patients not fulfilling minimal criteria for myelodysplastic syndromes (MDS) but presenting with persisting cytopenia(s) not attributable to a haematological or non-haematological disease are defined as ‘idiopathic cytopenia of undetermined significance’ (ICUS).

Design and methods: We retrospectively analysed 67 of 3504 patients from our MDS Registry fulfilling the criteria for ICUS. Furthermore, we used the human androgen receptor gene-based assay (HUMARA) to look for clonality.

Results: Of all 67 patients, 66% had unilineage, 18% bilineage and 12% trilineage cytopenias. The majority of patients (67%) presented with anaemia. Median overall survival was 44 months (range: 1–199 months). In the entire group, eight patients (12%) developed acute myeloid leukaemia (AML). Of the 23 patients eligible for HUMARA, 17 had non-clonal X-chromosome inactivation patterns, while 6 patients showed clonal patterns. Two of these six patients developed AML indicating that a clonal stem cell disorder was the reason for the antecedent cytopenia, while there was no AML observed among the 17 patients with non-clonal patterns (P = 0.013).

Conclusions: Since some of the ICUS patients had a clonal bone marrow disease when presenting with cytopenia(s) and 8 of 67 patients with ICUS later developed AML, we recommend to follow these patients thoroughly. As demonstrated here, HUMARA can facilitate the discrimination between ICUS and a ‘manifest’ MDS.

Key words: clonality, HUMARA, ICUS, MDS, X-chromosome inactivation

Introduction

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of haematopoietic stem cell disorders characterised by peripheral cytopenia(s) and dysplasia in one or more cell lineages as a consequence of an ineffective haematopoiesis. Patients with MDS have also an enhanced risk to develop a secondary acute myeloid leukaemia (AML).

Representing an extended and modified proposal of the French–American–British classification system, the World Health Organisation (WHO) has recently provided a new classification of MDS. Besides morphological criteria, the WHO proposal now takes also cytogenetic as well as molecular markers into account thereby defining new subtypes [1, 2].

In the majority of the patients, the diagnosis of an MDS can be made on the basis of these WHO criteria. Still, in some patients presenting only with mild cytopenia, subtle dysplasia and without a typical karyotype aberration, a clear-cut diagnosis is not possible. On the basis of the discussions between several MDS research groups, minimal diagnostic criteria have been formulated at a Working Conference [3].

For patients who are suspected of having MDS on the basis of haematological findings and/or clinical symptoms but do not fulfill the minimal diagnostic criteria, the term ‘idiopathic cytopenia of undetermined significance’ (ICUS) has been defined. For this purpose, the following criteria have been proposed: cytopenia in one or more myeloid lineages that (I) is constant (26 months), (II) does not meet the (minimal) criteria of MDS and (III) cannot be explained by any other haematological or non-haematological disease [3, 4]. To date, the knowledge about ICUS is scarce. Wimazal et al. reported 10 patients classified as ICUS, one of whom was reclassified as MDS after careful re-examination of bone marrow cytology. In
addition, two patients developed MDS during a follow-up of 4 months and 4 years, respectively [5]. Recently, a total of 2899 patients who had undergone diagnostic marrow examination for idiopathic cytopenias, were retrospectively analysed. In this large group, the authors finally identified 10 patients to whom the diagnosis ICUS applied and who had a follow-up of at least 6 months. Of these, six patients developed overt MDS. Therefore, the authors considered ICUS a potential pre-MDS category requiring careful follow-up and additional investigations to discriminate it from a ‘manifest’ MDS [6].

A diagnostical approach, which might help to differentiate between these entities, could be the examination of clonality in haematopoietic cells. In this context, studies on X-chromosome inactivation (XCI) patterns are a widely accepted approach. For this purpose, the human androgen receptor gene-based assay (HUMARA) represents a suitable tool since the human androgen receptor gene combines a highly polymorphic CAG short tandem repeat and several cleavage sites for the methylation-sensitive restriction enzymes HpaII and HhaI within one gene locus [7]. However, since X-linked clonality analysis is on the basis of the biological phenomenon of XCI in females, the HUMARA can only be used in female patients [8].

Clonality studies using the HUMARA have frequently been applied to differentiate between clonal (i.e. malignant) and polyclonal (i.e. reactive non-malignant) states, thereby facilitating the diagnosis of a malignant disease [9]. Although the application of HUMARA has mainly been focussed on haematological neoplasms, it has also been valuable in demonstrating the transition from a precancerous lesion to a manifest cancer, e.g. in gastric carcinoma or breast cancer [10,11]. In the context of haematological malignancies, XCI pattern analyses by HUMARA have mainly been applied to myeloproliferative disorders and AML and much less frequently to lymphoid neoplasms like natural killer cell proliferations or Langerhans cell histiocytosis, due to the possibility of detecting clonality in lymphoid disorders by analysis of aberrant immunophenotypes or immunoglobulin gene or T-cell receptor gene rearrangement [9,12–15].

Regarding MDS, HUMARA has been successfully employed to determine clonality in different haematopoietic subpopulations and to investigate clonality in remission [16–18]. Clonality has also been detected in patients with aplastic anaemia who later progressed to MDS [19]. Furthermore, Mach-Pascual et al. [20] showed that a clonal XCI pattern detected by HUMARA in bone marrow cells from patients with non-Hodgkin’s lymphoma undergoing high-dose chemotherapy and autologous stem cell transplantation was a significant predictor of the development of therapy-related MDS or AML.

In the light of these data, we believe that investigating ICUS with HUMARA is a reasonable approach to find out whether the disease may still be considered benign (in case of a non-clonal pattern) or pre-malignant with a substantial risk of progression (in case of a clonal pattern).

Thus, we carried out a retrospective study using HUMARA for detection of clonality in patients with ICUS and correlated them with patients’ characteristics and their clinical courses. Furthermore, we here present clinical data on the outcome of 67 patients, which were diagnosed as ICUS.

design and methods

patients

From 1982 to 2007, 3504 patients were entered into the Duesseldorf MDS Registry. Of these, in a subgroup of 67 patients, a clear-cut diagnosis of an MDS could not be established at their first visit due to only mild cytopenia, the absence of a typical cytogenetic marker or the presence of only slight dysplasia. Therefore, at the individual time of diagnosis, these patients were informally termed ‘not yet MDS’. As soon as the ICUS definition was initially proposed in 2007, these patients fulfilling the recommended diagnostic criteria were retrospectively ‘renamed’ as ICUS at our institution [3,4]. As proposed by the definition criteria for ICUS, each of these cytopenias in one or more cell lineages (erythroid: haemoglobin <11 g/dl; neutrophilic: <1500/μl; platelet: <100 000/μl) had at least persisted since 6 months. Furthermore, a detailed case history as well as a thorough clinical investigation were carried out in each patient and did not identify any other haematological or non-haematological disease as well any other reason explaining the underlying cytopenia. Diagnostic procedures were carried out according to consensus recommendations [3]. For each patient, clinical data as well as haematological parameters were evaluated at the time of diagnosis and the course of disease was monitored. Bone marrow cytomorphology as well as peripheral blood smears were examined by the same two investigators (CA and UG) at our laboratory. Classical cytogenetic analysis was carried out in all patients, while FISH was not carried out routinely. Patients gave informed consent and were entered into the Duesseldorf MDS Registry thereby allowing a regular follow-up with a median of 48 months (range: 1–199 months).

sample preparation and DNA isolation

Blood cells were isolated from diagnostic bone marrow slides of the patients taken at the time of diagnosis. Genomic DNA was extracted using the QiAamp DNA Blood Mini Kit (Qiagen AG, Hilden, Germany) according to the manufacturer’s instructions.

HUMARA clonality assay

HUMARA was carried out as previously described [17,18]. In brief, for each patient 200 ng of DNA was incubated with 4 μl of HpaII endonuclease (Roche Diagnostics, Mannheim, Germany), 3 μl 10× buffer and 3 μl deionised water for 4 h at 37°C. Afterwards, samples were transferred into HUMARA-PCR. The following primers were used: HUMARA-specific sense primer 5’-CGGAGGAGCTTTCCA-GAATC-3’ and HUMARA-specific antisense primer 5’-TAGATGGCCTGGGAGAA-3’. PCR was carried out with a 5 s preincubation followed by 35 cycles of a 5 s denaturation at 94°C, 8 s at 62°C and 11 s at 72°C. After the last cycle, samples were incubated for 8 min at 72°C and set on hold at 4°C. Then, 10 μl of the PCR products were subjected to a labelling PCR using 2 μl of a fluorescence-labelled 5’ primer (Cy5’-TTCCAGAGCCTGCGCCGAAG-3’) per sample for six cycles with 30 s at 95°C, 30 s at 66°C and finally 30 s at 72°C. Afterwards, detection of the amplified products was carried out by automated DNA fragment analysis using ALF DNA Sequencer (Amersham Pharmacia Biotech, Freiburg, Germany). A fluorescence signal was achieved for each allele of the HUMARA gene locus and was displayed as a peak and an area under the curve by ALFwin Fragment Analyser 1.02 software (Amersham Pharmacia Biotech). Genomic DNA from healthy volunteers served as control for experimental validation.

determination of clonality

In order to determine XCI patterns, a corrected ratio was calculated according to the method described by Willman et al. [15]. In detail, the allele ratio of the digested sample was divided by the allele ratio of the non-digested sample of the same patient.

The use of this ratio corrects for preferential amplification of one allele that may occur, particularly if alleles differ markedly in the length of their repeats.
In case the calculated corrected ratio was <1, the stated corrected ratio was obtained from the inverse value of the allele ratios. Thereby, comparison between individual samples became easier. A sample was considered as clonal if the corrected ratio was ≥10.

**Statistics**

For description of the patients characteristics, medians and range of parameters were calculated and displayed in Table 1. Overall survival (OS) and AML evolution were estimated according to the Kaplan–Meier method and compared using the log-rank and \( \chi^2 \) tests.

Statistical analyses were carried out using Excel (Microsoft) and SPSS for Windows (SPSS Inc., Chicago, IL). For comparison of disease parameters between patients with clonal and non-clonal XCI patterns, the two-sided Student’s \( t \)-test was used. Only \( P \) values of <0.05 were considered as statistically significant.

**Results**

**Haematological Parameters and Clinical Data**

This is a retrospective study on 67 (49 female and 18 male) of 3504 patients included into the Duesseldorf MDS Registry, who fulfilled the recommended diagnostic criteria for ICUS [3, 4]. The median age was 69 years (range: 19–88 years).

According to the definition criteria for ICUS, at the time of diagnosis anaemia (haemoglobin <10 g/dl) was found in majority of the patients (67%) with a median haemoglobin of 10.1 g/dl (range: 4.6–14 g/dl) thereby evoking the suspicion of a reduced bone marrow function, while thrombocytopenia (platelets <100 000/\( \mu l \)) was only apparent in 36% of the patients (median: 139 000/\( \mu l \), range: 2–840 000). The median white blood cell (WBC) count of 3800/\( \mu l \) (range: 900–9280/\( \mu l \)) was almost within lower normal range, whereas neutropenia was observed in 39%. When looking for the involvement of the three haematopoietic cell lineages, 44 patients (66%) had an unilineage cytopenia. Of these, 26 patients had anaemia, 9 patients had isolated neutropenia and 9 patients had thrombocytopenia. Bilineage cytopenias were found in 12 patients (18%) with five cases of combined anaemia and neutropenia, six patients with anaemia and thrombocytopenia and only one patient with neutropenia in combination with a thrombocytopenia. Eight patients (12%) showed a cytopenia affecting all three haematopoietic cell lines. Since in three patients (4%) only information about WBC, but not absolute neutrophil count, was gathered, involvement of the haematopoietic cell lineages could not be evaluated in these patients (Figure 1).

Concentrating on further haematological parameters, the median reticulocyte count was 1.6% (0.1–6.1) and mean corpuscular volume was in median 96.8 fl (range: 78–128 fl). Serum level of erythropoietin was elevated to a median of 103 mU/ml (range: 47–1207 mU/ml). The median ferritin value was 260 \( \mu g/l \) (range: 21–3480 \( \mu g/l \)) thereby also slightly elevated. The concentrations of serum iron, vitamin B12 and folate were within the normal range in all 67 patients therefore making deficiency of one or more of these biomolecules unlikely. Serum chemistry parameters including lactate dehydrogenase were within the normal range. Due to the underlying cytopenia, 32% of the patients required transfusions at the time of diagnosis. Detailed haematological parameters of the patients are given in Table 1.

Peripheral blood smears as well as bone marrow cytomorphology, histology and immunohistochemistry

**Table 1. Haematological parameters of patients with ICUS**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ICUS (all patients)</th>
<th>ICUS (analysed by HUMARA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, n (%)</td>
<td>67 (100)</td>
<td>23 (34)</td>
</tr>
<tr>
<td>Age, years (median and range)</td>
<td>69 (19–88)</td>
<td>73 (47–84)</td>
</tr>
<tr>
<td>Leukocytes ((/l))</td>
<td>3800 (353–13 300)</td>
<td>5000 (1900–11 600)</td>
</tr>
<tr>
<td>Granulocytes ((/l))</td>
<td>2100 (360–9280)</td>
<td>3549 (1548–9280)</td>
</tr>
<tr>
<td>Haemoglobin ((g/dl))</td>
<td>10.1 (4.6–14)</td>
<td>10.3 (6.3–13.4)</td>
</tr>
<tr>
<td>MCV ((fl))</td>
<td>96.8 (78–128)</td>
<td>93 (83–112)</td>
</tr>
<tr>
<td>EPO ((mU/ml))</td>
<td>1.6 (0.1–6.1)</td>
<td>2 (0.2–3.4)</td>
</tr>
<tr>
<td>Ferritin ((\mu g/l))</td>
<td>260 (21–3480)</td>
<td>295 (92–617)</td>
</tr>
<tr>
<td>Platelets ((/l))</td>
<td>139 (2–840)</td>
<td>185 (9–541)</td>
</tr>
<tr>
<td>LDH ((U/l))</td>
<td>206 (129–1551)</td>
<td>206 (138–384)</td>
</tr>
<tr>
<td>Creatinine ((mg/dl))</td>
<td>0.9 (0.4–1.6)</td>
<td>0.9 (0.6–1.4)</td>
</tr>
<tr>
<td>TSH ((\mu U/ml))</td>
<td>0.99 (0.33–3.24)</td>
<td>0.99 (0.4–3.24)</td>
</tr>
</tbody>
</table>

Information on haematological parameters of patients with ICUS are displayed. For a better comparison, data are shown for the whole group as well as for the group analysed by HUMARA. There were no significant differences with regard to the haematological parameters between the two groups. Normal ranges for all variables are displayed in square parentheses.

ICUS, idiopathic cytopenia of undetermined significance; HUMARA, human androgen receptor gene-based assay; MCV, mean corpuscular volume; EPO, erythropoietin; LDH, lactate dehydrogenase; TSH, thyroid-stimulating hormone.

examined at the time of diagnosis and during the course did not show signs of dysplasia or an elevated blast count fulfilling the minimal criteria required for the diagnosis of an MDS. Cytogenetic analysis showed a normal karyotype in all patients without any chromosomal abnormalities.

**Outcome**

Looking at the outcome of the patients, 40 (59%) of the 67 patients included in this study are alive at the time of writing. Median OS of all patients was 44 months (range: 1–199 months) and did not differ significantly between patients with clonal and non-clonal XCI patterns.

Within the entire group, eight patients (12%) developed an AML after a median time of 27 months (range 14–186 months).

Causes of death of the 27 patients were as follows: 5 patients (19%) died due to AML, 3 patients (11%) as a consequence of fatal infection, 2 patients (7%) as a result of fatal bleeding, 1 patient (4%) due to a heart failure, but in the majority of 16 patients (59%) information of a definitive cause of death could not be ascertained.

**Analysis of Clonality by HUMARA**

Forty-nine (73%) of the patients were female and therefore eligible for assessment of clonality using the HUMARA assay.
Since blood cells were isolated from bone marrow slides of the patients obtained at the time of diagnosis and analysis was carried out retrospectively, 26 of the 49 samples were not evaluable as a consequence of too small amounts of material. From the remaining 23 patients, 17 patients (74%) had non-clonal XCI patterns, while a clonal pattern was found in 6 patients (26%) favouring the diagnosis of MDS. There was no difference with regard to age (clonal: median 72 years, range 51–81 years; non-clonal: median 75 years, range 46–84 years, \(P = 0.73\)), involvement of haematopoietic cell lineages and transfusion requirement between the two groups.

The strongest argument that a clonal stem cell disorder is the cause for the cytopenia observed in the six ICUS patients with a clonal XCI pattern is the fact that two of them developed AML, while there was no case of AML among the 17 patients with a non-clonal XCI pattern (\(P = 0.013\)). The other six patients who developed AML were within the group that was not eligible for clonality analysis as a consequence of male gender or too small amount of DNA. Of the 17 patients with non-clonal XCI pattern, 10 patients are currently alive without evidence of ‘manifest’ MDS. The other seven patients died, one due to fatal bleeding and one due to heart failure. For five patients, the cause of death could not be ascertained.

Of the four patients with clonal XCI pattern who did not develop AML, two are alive and two have died, at age 73 and 85, respectively. Their causes of death are unknown.

**Discussion**

Patients presenting with mild cytopenias and/or dysplasias, who do not fulfills the minimal diagnostic criteria of an MDS, are summarised under the term ICUS [3, 4]. Currently, there are only two publications describing clinical parameters and outcome of 20 patients with ICUS [5, 6].

Thus, on the basis of a retrospective analysis, we here present data about 67 patients with ICUS retrieved from our registry. In line with the previous study by Wimazal et al., our data confirm that cytopenia as well as dysplastic features are subtle in these patients making a discrimination between an ICUS and a ‘manifest’ MDS difficult [5]. Different from Valent et al., we observed a median serum erythropoietin level in our patients, which was three times above the upper normal limit [4].

Although cytopenia and/or signs of dysplasia were discrete in these patients, it is interesting to note that the median OS in our ICUS group was 44.3 months falling between the median OS of patients with refractory anaemia (OS 58 months) and refractory cytopenia with multilineage dysplasia (OS 36 months) [1]. Therefore, additional diagnostic tools are required to decipher whether the reduced bone marrow function in patients classified as ICUS is related to a clonal stem cell disorder.

Cytogenetic analysis or even the more sensitive FISH might fail to detect a clonal aberration since only \(~50\%\) of the patients with MDS have an abnormal karyotype at the time of disease manifestation or the karyotype abnormality might only be present in a small subset of bone marrow cells [21].

Therefore, we used the HUMARA to analyse XCI patterns. When interpreting the results, some technical considerations should be kept in mind. Firstly, on the basis of the definition proposed by Gale et al. [22] and Delforge et al. [16] that a clonal population is defined as a cell population with \(>75\%\) expression of one of both X-linked alleles, we cannot rule out that we might have missed a small monoclonal cell population representing a rising MDS clone in some of the patients included in this study [16, 22]. Secondly, a monoclonal XCI pattern can be even found in haematologically normal females as a consequence of constitutional or age-related acquired skewing [9, 23–25]. Normal tissue for comparison was not available in this retrospective study. Thus, we cannot formally exclude extreme lyonization. This issue can only be settled by a prospective investigation including collection of non-haematopoietic cells.
Nevertheless, since there was no age difference between patients with a clonal pattern and patients with a non-clonal pattern of XCI, the results of our study are unlikely to be biased by age-related skewing. Furthermore, since investigation of XCI patterns is restricted to female patients, techniques like single-nucleotide polymorphism arrays or others are required for examination of clonality in males [8]. Despite these technical considerations, HUMARA is a widely accepted method for detection of clonality and has been frequently used in the context of MDS and AML [9, 16–18]. Furthermore, it was shown that a clonal XCI pattern detected by HUMARA is associated with the progression from aplastic anaemia into MDS and that a clonal XCI pattern is a significant predictor of the development of therapy-related MDS or AML [19, 20].

We found a clonal pattern in six patients (26%), who were diagnosed as ICUS so far. The evidence of clonality observed in patients presenting with an ICUS is highly indicative for the diagnosis of MDS. There was no correlation between results of clonality analysis and survival because the number of patients eligible for clonality analysis was too small. Still, of the six patients with a clonal XCI pattern, two developed an AML during the course of time, while none of the patients with non-clonal XCI patterns suffered from AML. The retrospective character of our study did not allow us to determine the clonality status in the other six patients who developed AML. The HUMARA was not applicable, either due to male gender or due to insufficient amounts of DNA. Nevertheless, our finding is a legitimate argument to consider a clonal XCI pattern in patients with ICUS as an indicator of true MDS. This conclusion should be corroborated by prospective studies.

In summary, considering the scarcity of data available on ICUS so far [5, 6], our data add valuable new information regarding this newly defined entity. As we retrospectively shown that some of the ICUS patients actually had a clonal bone marrow disease when they first presented with cytopenia, and since 8 of the 67 patients with ICUS later developed AML, we recommend to follow these patients thoroughly once the diagnosis of ICUS has been made.

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**Disclosure**

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**References**