PhD Thesis

LINEAGE SPECIFIC CLONALITY ANALYSIS OF MYELODYSPLASTIC SYNDROMES AND MYELOPROLIFERATIVE NEOPLASMS

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INTRODUCTION

For better understanding the pathogenesis of neoplastic hematologic diseases, proper diagnosis as well as prognostic and therapeutic point of view, an important aspect is that the lesion at which point of the hemopoesis occurs. In other words, it is useful to determine whether the disease has pluripotent, or committed stem cell origin. The knowledge of the molecular pathogenesis is also used by WHO classification as a principle in the classification of neoplastic hematologic diseases. The determination of the starting point of a tumor may help to answer the question whether beyond symptomatic therapy the chemotherapy could serve as curative treatment or the allogeneic bone marrow transplantation may be the only solution. In case of committed stem cell disease, the chemotherapy may result complete recovery, because if the therapy is able to kill the committed cancer stem cells, the normal hemopoesis may regenerate from the pluripotent stem cells. Conversely, in case of pluripotent stem cell disease and in absence of targeted therapy, real treatment can be expected only if all the hematopoietic stem cells could be killed, however, this kind of treatment should be followed by allogeneic bone marrow transplantation to restore the hemopoesis.

The examination of the involvement of different cell lineages may help to identify the origin of the tumor. Justifying cell lineage involvement several methods can be used. First, investigation of disease specific molecular aberrations (e.g. point mutations, deletions) can be used, second the use of clonality tests may be considered.

The group of myelodysplastic syndromes includes such clonal hematologic diseases which in clinical and morphological point of view are characterized by ineffective hemopoesis. These diseases associated with a preneoplastic monoclonal proliferation phase and often progradiate into acute leukemia. In the literature the MDS is considered as hematopoietic stem cell disease. The involvement the myeloid line is clear and characterized by monoclonal proliferation. In contrast, there are conflicting data in regard how the lymphoid lineage is involved in the neoplastic process.

The myeloproliferative neoplasms (MPN) also belongs to the large group of myeloid tumors. These diseases are characterized by abnormal and increased proliferation in the bone marrow. Morphologically, in the different types, different cell lines dominate, but there is evidence that a number of bone marrow cell lineages are involved in the abnormal proliferation in a greater or lesser extent. The MPNs are also considered as stem cell diseases, but the results of the clonality tests of the individual cell lineages, like in MDS, are not unambiguous in regard of the involvement of the lymphoid lineage.

OBJECTIVES

The primary objective of our studies through the investigation of different cell lineages was to demonstrate that in MDS and MPN at which step of the hemopoesis the disease originates from. We assumed that if there is only myeloid involvement, it is likely that the disease is based on myeloid committed stem cell. If, however, lymphoid involvement occurs, there can be pluripotent stem cell origin in the background. Establishing the cell lineage involvement, sorted lymphoid and myeloid cells were analyzed by three methods. At first, a genetic marker independent clonality test the HUMARA (human androgen receptor assay) X chromosome inactivation assay was used. The second method was to examine the del(20q) and del(5q) by microsatellite-PCR (MS-PCR) technique. Besides demonstrating the involvement of cell lineages by MS-PCR tests we also looked for the answer whether the MS-PCR technique which is suitable to detect much smaller deletions than traditional metaphase

cytogenetics could detect the deletions at higher rates of incidence. Finally, we investigated the presence of the JAK2V617F point mutation in MPN patients' cell lineages using mutation specific PCR technique.

SAMPLES AND METHODS

Samples

The peripheral blood and bone marrow aspirate samples of MDS and MPN patients were used. According to the WHO classification and based on the comparison of clinical and pathological findings the cases were classified into the appropriate disease group. For the MS-PCR tests control buccal mucosa scrape samples were collected from the patients. To determine the evaluation criteria of the HUMARA, peripheral blood samples of 27 healthy women and 4 B-cell chronic lymphocytic leukemia (B-CLL) were used. For the MS-PCR and JAK2V617F mutation testing samples from male patients were also collected. We have done the HUMARA in 103 MPN and 40 MDS female patients. MS-PCR tests were done in 12 MDS and 23 MPN cases, of which 20 cases HUMARA were conducted in parallel. JAK2V617F mutation test was made in 27 cases of MPN, of which 11 were done in parallel with HUMARA. The patients' age ranged between 12 and 82 years.

Flow cytometric cell sorting

Lymphoid and myeloid cells were sorted from blood and bone marrow samples by flow cytometry. In the HES cases, eosinophils, and in one MDS-RAEB case blasts also were sorted. The sorting is based on the CD45 expression intensity and granularity of cells. To determine the sensitivity of the HUMARA method, mixtures of CD5/CD23 or CD5/CD19 dual-positive monoclonal lymphoma cells, and polyclonal myeloid cells sorted in a single step from B-CLL patients. The immunnolabeling were done by using CD5-FITC, CD23-RPE, CD19-RPE-Cy5 and CD45-RPE-Cy5 (DAKO A / S, Denmark) antibodies, the sorting was carried out on a BD FACSort flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA USA). Generally 10^5 cells were sorted per cell lineages, per patient. The sorting purity of the samples was greater than 95% according to reanalyzing the sorted samples. Sorted cells were stored in 500 µl TE8 buffer, or 200 µl PBS at -20 °C until DNA isolation.

DNA isolation

DNA isolation from the sorted cells was made by proteinase-K digestion and phenol-chloroform-extraction method or using Qiagen Blood Mini Kit (Qiagen, Hilden, Germany). After isolation by phenol-chloroform, DNA was dissolved in TE8 buffer, so the amount of the DNA corresponding to 3000 sorted cells was in one microliter volume. The DNA isolated by Qiagen Blood Mini Kit was stored 50 µl AE buffer. The DNA samples were stored at +4 °C for use and then archived at -20°C.

Human androgen receptor assay - HUMARA

X chromosome inactivation occurring in the cells of women affect the two X chromosomes randomly. During the subsequent mitotic cycles the inactivation pattern will be

stably transmitted into the daughter cells. In case of monoclonal proliferation, for example in tumors, all cells carry the inactivation pattern of the starting clone. To analyze this process, investigation of the methylation pattern of short tandem repeat polymorphisms (STRP) can be used. The most commonly used method is the HUMARA based on the human androgen receptor gene, which is highly polymorphic (close to 90%) and reliably reflects the methylation of the X-inactivation.

The test sequence contains methylation points, which depending on the methylation status can be cleaved or not by methylation-sensitive restriction enzymes HhaI, HpaII. In heterozygous females, without the methylation-sensitive cleavage two different-length androgen receptor sequence (approximately 220 bp) can be amplified by PCR, while the samples of homozygous women are not informative because the two alleles cannot be separated from each other. In case of monoclonal proliferation after methylation-sensitive enzyme treatment the PCR can amplify only one allele, therefore, only one band is obtained after the electrophoresis. The PCR reaction was done as described by Allen et. al. with some modifications. First the DNA from 10000 cells was digested with 10U HhaI enzyme in Multi-Core buffer (Promega, Madison, WI, USA), containing 0.1 mg/ml BSA in 20µl final volume. Digestion was performed at 37 °C, overnight. After digestion the DNA was precipitated in 2.5-fold volume of ethanol and after drying was dissolved in 10 µl distilled water. The total 20 µl PCR reaction volume composed 10 µl of DNA dissolved in water, 200 µM of dNTP's (GibcoBRL, Invitrogen Corporation, Carlsbad, CA, USA), 0.5 unit RedTaq DNA polymerase (Sigma, St. Louis, Missouri, USA), RedTaq buffer and 10 pmol of each primers (5 'GCT GTG AAG GTT GCT GTT CCT C 3', 5 'AGA GGC CGC GAG CGC AGC ACC TC 3'). The PCR amplification was carried out on MJR Minicycler (MJ Research, Watertown, Massachusetts, USA) and BioRad iCycler (BioRad Laboratories, Hercules, CA, USA). Cycling conditions were a single predenaturation step at 94°C for 5 minutes followed by 40 cycles of denaturation 94°C for 40 seconds, annealing at 68°C for 40 seconds, and extension at 72°C for 1 minute, and a final elongation at 72°C for 5 minutes.

After amplification, the full 20 µl of PCR mixture was loaded to 8% non-denaturing polyacrylamide gel and run at 120 V, 25 mA overnight, until the xylene cianol tracer dye reached the bottom of the 24 cm long gel. The gel was stained with SYBR Gold DNA dye (Invitrogen, Carlsbad, CA, USA) at the recommended concentration by the manufacturer, and finally photographed with Polaroid or digital camera on UV transilluminator. In case of Polaroid pictures the photos were digitized by a scanner.

Those informative cases in which the PCR amplified only one allele and only one band corresponding this allele could be seen on the photograph, were accepted as monoclonal. However, in a substantial part of the samples the decrease of the ratio of either allele was not clear, so those samples were classified polyclonal or monoclonal after densitometric analysis. The densitometric analysis was made by ImageJ 1.4 software. In the international literature the commonly used criterion is that if the ratio of the two alleles is at least 3 to1, or bigger, the sample considered as monoclonal. However, several studies have shown that in healthy women the allele ratio may differ from 1 to 1 and neither the 3 to 1 ratio is always reliable criteria for monoclonality. To have a method with acceptable specificity, we set up our own criteria for evaluation of the HUMARA results, using lymphoid and myeloid cells sorted from blood samples of healthy women. To determine the sensitivity, the HUMARA method was tested on mixtures with known ratio of sorted monoclonal lymphoma and polyclonal myeloid cells of B-CLL patients.

Microsatellite-PCR

Known microsatellite regions of the genome are used for investigation of deletion or amplification of particular loci in tumors. After the PCR amplification of a microsatellite region the quantification the PCR products can be used to detect the occurrence of the alleles. In this study del(20q), the most common genetic aberration in MPN, and del (5q) described in MDS was tested in lymphoid and myeloid cells by MS-PCR technique. The microsatellite markers were selected from the common deleted region (CDR) according to the literature data. MS-PCR tests were performed in the 20q11.2-q13.2 region, at D20S607, D20S170, D20S110, D20S96 and D20S119 loci, and in the 5q31 region, at D5S476 and D5S414 loci.

The PCR amplifications were made on MJR PTC-100 and PTC-200 thermal cyclers (MJ Research, Watertown, Massachusetts, USA) using the following program: predenaturation at 94°C for 2 min, followed by 35 cycles denaturation at 94°C for 40 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 40 seconds, and final elongation at 72°C for 5 minutes. The PCR reaction mixture contained DNA approx. of 10,000 cells, 0.5 units of Taq polymerase-T (GibcoBRL, Invitrogen Corporation, Carlsbad, CA, USA), 50mM KCl, 10 mM TRIS (pH 8.3), 1.5 mM $MgCl_2$, 200 μM dNTP, and 5-5 pmol primers. One primer was marked with Cy5 fluorescent dye (MWG-Biotech AG, Ebersberg, Germany). The following primers were used: D20S607: AAA ATG TCC CAG AGG CAA AG and CTG TTT ATT GAC AAA GGC TG; D20S170: TTC TCA GGC TCC TGG C GGG GGC TTC CAT GAG T; D20S96: CAC TCT TGC AAC AAC CTG CCT GG and GTA ATT TGC TGC TCC TG; D20S119: ACA CAG TTT CTG CAG TAT TAT CTC C TTA GGG TTT CCA GAT GTG TAT G; D20S110: TTG ACA GCC AAA TGA ATT TA and CTA GAC TTA TCT GGT TGA CAT CG; D5S414: GGC CAG TTC AGT CAA GTG TTC CAG CAT ATA TGG and CGC; D5S479: GCA ATA GCA AGA CTC TGA CC and GCC TTC CTG TGT CAG TTA TT. The primer sequences were chosen from the online database of the Whitehead Institute Center for Genome Research and The Genome Database. The data above can be checked at http://www.ensembl.org/Homo_sapiens/Info/Index. The detection and quantitation of the PCR products was performed on ALFexpress DNA Analysis System automated DNA sequencer (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) using 5% denaturing polyacrylamide gel, run at 1,500 V, 38 mA, 15 W, 55°C. For the evaluation of the densitometric curves the own software of the device was used. The evaluation was performed according to the presence and area of the peaks corresponding to the alleles, and the samples were classified into retained (R) deleted (D), and allelic imbalance (AI) categories. The categories were assigned with values from 0 to 4. Samples which showed the same pattern like the control buccal mucosa got the value 0. Value 4, or D ratings were assigned to the samples in which one allele were completely lost. The value 2 assigned to those AI samples, where the relative ratio of an allele decreased to approx. 50% compared to the peak seen in the control sample. The sample classified as AI3 if the decrease of the relative ratio of an allele was significantly higher than 50%, but there was not complete deletion. The sample classified as AI1 if the decrease of the relative ratio of an allele was significantly smaller than 50%. Because of the inaccuracy of the PCR reaction and fluorescence detection the AI1 classification was considered acceptable only if occurred in parallel at least at 2 loci. This MS-PCR evaluation system was applied according to Langbein et al..

JAK2V617F mutation testing

In 2005, several independent publications described the V617F mutation of the JAK2 gene in chronic myeloproliferative diseases. The JAK2 protein plays a key role in cell growth and in differentiation regulating JAK-STAT signal transduction processes. The mutated JAK2 protein tyrosine kinase shows constitutive activity and hypersensitivity to its activation signals. The incidence of JAK2V617F mutation in MPNs according to the literature data is 90-95% in PV, 55-60% in PMF, 50% in ET and around 50% in a rare subtype of MDS RARS-T.
We examined the presence of the JAK2V617F mutation in flow sorted lymphoid and myeloid cell cells in MPN patient samples. The tests was carried out by PCR using JAK2V617F mutation-specific primers. According to Baxter et al. the following PCR primers were used: mutant allele specific forward primer (5'AGC TGG ATT TTT TAA ATT ATG GAG TAT ATT3 '), internal control forward primer (5'ATC TAT AGT CAT GCT GAA AGT AGG AGA AAG3 '), and a common reverse primer (5'CTG AAT ACA CCT AGT TCA TTT GTG GTT TCA3'). The two forward and one reverse primer allows the simultaneous amplification of two PCR products. The internal control primer and the reverse primer produces an amplicon which is 364 base pairs in length, and provides a control for assessing the quality of the DNA template. If the mutation is not present in the test sample, only the internal control product will be obtained. If the DNA template contains the JAK2V617F mutation, the mutation-specific primer produces a second, 203 base pair long PCR product along with the 364 base pair control. The PCR reaction components in 25 µl final volume were as follows: approx. 10,000 cells' DNA in TE8 or AE buffer, 1 unit of Sigma RedTaq Genomic DNA polymerase (Sigma, St. Louis, MO, USA), 3 mM $MgCl_2$, 200µM dNTP, 12.5 pmol reverse primer, 6,25 pmol internal control forward primer, 6,25 pmol mutation-specific forward primer, and Sigma Redtaq buffer. The PCR reaction run with the following program: 95°C predenaturation for 5 minutes followed by 40 cycles of denaturation at 95°C, annealing at 53°C, 30 seconds, and extension at 72°C, 1 min, and final elongation at 72°C for 5 minutes. In some cases, where small amount of DNA remained after the completion of the HUMARA, whole genome amplification was performed using Qiagen REPLI-g Kit (Qiagen, Hilden, Germany) as recommended in the manufacturer's protocol, and 1 µl of the amplified DNA was used for the JAK2V617F PCR. The PCR products were separated on 2% agarose gel, stained with SYBR Gold DNA stain, and using UV-light illumination were documented as digital photographs. The samples were classified to positive or negative categories on the basis of the visible presence or lack of the JAK2V617F product in the photograph.

RESULTS

Determination of the evaluation criteria, sensitivity and specificity of the HUMARA

The inactivation ratio of the two androgen receptor allele is characterized by the ratio of peak areas in the densitometric curve. For the calculation of this ratio the area of each peaks was divided by the total area of both peaks. To quantitatively characterize the X chromosome inactivation (XI) the ratio of the smaller molecular weight allele expressed in percentages were used, which value called X inactivation number - XIN. The XIN value may ranges between 0% and 100%. Assuming completely random XI the XIN value should be of 50%. In case of non random XI, the XIN differs from 50%. Homogeneous sample of monoclonal cells show XIN value of 0% or 100%.

Among samples of 27 healthy women 21 were heterozygous and thus suitable for testing. A slight shift in favor of the smaller allele appeared, which may refer to non random XI. The average of XIN was 65.2% and 64.1% in the lymphoid and myeloid cell lines with a significant standard deviation (S.D.), 20.0% and 22.4%, respectively. Beyond the non random XI a technical explanation may exist for the higher than 50% XIN values, since the high number of PCR cycles (40) may bias towards the smaller allele during amplification. This may be due to the high GC content of the amplified sequence which may result in developing relatively stable secondary structures affecting the amplification of the allele with higher number of CAG repeats negatively.

To properly assess the patient samples in which the non random XI may due to the presence of monoclonal cells, we had to determine such XIN threshold values which provide acceptable false positivity in polyclonal samples of control healthy women tested, in other words they are sufficiently specific.

We examined the XIN mean ± 1 S.D. and the XIN mean ± 2 S.D. limits. The specificity of the XIN mean ± 1 S.D. criterion was found to be low (80%, 76%). The XIN mean ± 2 S.D. criterion was too strict as it exceeded the theoretical maximum value of 100%. The 3:1 allelic ratio criterion commonly used in literature had the lowest specificity, 70% in lymphoid cells and 52% in myeloid cells, respectively. In order to obtain specific criteria we determined a polyclonal XIN range with upper and lower limits for the lymphoid and myeloid cells including 90% of the control samples, consequently it has at least 90% specificity. For determination the threshold values, both the lower and upper extreme XIN values were omitted in an equal proportion from the control women XIN values measured. The polyclonal XIN range was 34-90% in the lymphoid cells and 37-86% in the myeloid cells.

Specificity of clonality criteria by different threshold values.

Lymphoid cells		Myeloid cells	
XIN range	Specificity	XIN range	Specificity
Mean ±1S.D 45,2-85,2%	80%	Mean ±1S.D 41,7-86,5%	76%
Mean ±2S.D. 25,2-105,2%	100%	Mean ±2S.D. 19,3-108,9%	95%
3:1 ratio	70%	3:1 ratio	52%
34-90%	90%	37-86%	90%

As moderately strong correlation (correlation coefficient = 0,646) were found in the lymphoid and myeloid XIN values, we examined whether they can be mutually used each other as control in those cases where only one cell lineage is monoclonal. If only one of the cell lineages is monoclonal, its XIN value might be significantly differ from XIN value of the other polyclonal cell lineage. We searched for that XIN difference value between the lymphoid and myeloid cells in the samples of healthy control women, of which greater value did not occurred in 90% of the cases. This XIN difference value was 26%. This means that bigger difference between the lymphoid and myeloid cells XIN values of the control women in less than 10% occurred, and therefore for this type of criterion at least 90% specificity has been adopted. It should be noted, however, that using only this criterion it's not possible to determine whether which of the two cell lineages is monoclonal.

To determine the sensitivity of the HUMARA, mixtures of monoclonal lymphoma cells and polyclonal myeloid cells with known proportions were examined in four B-CLL patient samples. If the test sample contained at least 75% monoclonal cells, the monoclonality

was clearly identifiable as we found 0% or 100% XIN values. In case of mixtures with less than 75% of monoclonal cells our evaluation criteria were applied. In all samples with 50-60% of monoclonal cells monoclonality was justified at least by 1 criterion. In contrast, in samples containing only 20-25% of monoclonal cells, the possibility of monoclonality arose in only one case out of four and only by one criterion.

Finally, we examined the correlation between the real monoclonal cell ratio and the ratio calculated from the measured XIN values in the mixtures containing known proportion of monoclonal cells. Considering the following algorithm the ratio of monoclonal cells in the sample (M%) can be calculated using the XIN value of the pure monoclonal samples (XIN_M), pure polyclonal samples (XIN_P), and the XIN value measured ($XIN_{measured}$) in the mixture samples.

$$\frac{M\%}{100} \times XIN_M + \frac{100 - M\%}{100} \times XIN_P = XIN_{measured}$$

In the samples containing 20-60% of monoclonal cells, strong correlation was found between the calculated and the real monoclonal cell ratios (r = 0.86), which supports the quantitative nature of the method and the suitability for testing mixture samples.

HUMARA - clonality results

In terms of the success of the HUMARA tests the following results were obtained. Out of the 143 cases 98 (69%) cases were heterozygous and informative. Forty-five cases (31%) was not informative because of the homozygosity of the two androgen receptor alleles or because of the smaller difference in size than the resolution of the gel, or weak PCR reaction, respectively.

Clonality results by HUMARA

		Clonality of cell lineages				
	Case number	L+ M+	L- M+	L+ M-	L- M-	Lnd M+
MDS-RA	6	2	4			
MDS-RAEB	3	2			B+:1	
MDS-u	17	4	6	3	2	2
MDS-AML	3	1	1		1	
MDS total	**29**	**9**	**11**	**3**	**4**	**2**
MPN-ET	34	10	13		6	5
MPN-PV	10	5	4			1
MPN-PMF	2	1		1		
MPN-u	15	5	6	1	1	2
MPN-HES	8	Eo+: 2 | Eo-: 1	Eo+:1 | Eo-: 2		Eo-: 2	
MPN total	**69**	**24**	**26**	**2**	**9**	**8**

L+: lymphoid monoclonal, L-: lymphoid polyclonal, Lnk: lymphoid not done, M+: myeloid monoclonal, M-: myeloid polyclonal, B+: blast monoclonal, Eo+: eosinophil monoclonal, Eo-: eosinophil polyclonal

Overall, in MDS and MPN similar proportions of monoclonality was found. The ratio of double monoclonal lymphoid-myeloid cases was 31% in MDS, and 35% in MPN. The ratio of cases showed only myeloid monoclonality was 38% in both disease group. Exclusive lymphoid monoclonality occurred in 10% of MDS and 3% of MPN. The ratio of polyclonal cases was 18% in MDS and 13% in MPN. Myeloid monoclonality occurred in 76% of MDS and 85% of MPN. The lymphoid monoclonality rate was 41% and 38% in MDS in MPN, respectively. Monoclonality in any cell lineage occurred in 82% of MDS and 87% of MPN.

The ratios of MDS-RA cases were somewhat different from the average rates of MDS where 8% lower proportion of lymphoid (33%) and 24% higher proportion of myeloid (100%) monoclonality was found. However, because the MDS-RA results derived from only 6 cases, these differences cannot be considered statistically reliable. The lymphoid monoclonality ratio in ET and PV cases also deviated from the average MPN. In ET it was 9% was lower (29%), in PV was 12% higher (50%) compared to the average rate in MPN. Regarding the monoclonality of the myeloid cells only PV cases showed significant difference from the average, since 100% of the myeloid cells were monoclonal. As only 2 cases of PMF were studied, its significant deviation from the mean is not considered reliable.

Microsatellite-PCR results

MS-PCR was done in thirty-five cases. In one MDS-RA out of twelve MDS cases (8%) were found a complete deletion of the D5S476 locus, only in myeloid cells. 20q deletion was found in one MDS-u (8%) at D20S96 and AI1 at D20S110 loci in myeloid cells. Twenty-three cases of MPN was tested by MS-PCR for the deletion of 5q and 20q. 20q deletion, or allelic imbalance was found in 6 MPN cases (26%). Of these, in 3 cases (13%) both the lymphoid and myeloid cell lineages, whereas in the other 3 (13%) only the lymphoid cells were involved. Out of twenty-two MPNs 3 cases (13%) had 5q involvement. The myeloid lineage had been involved in all three cases, while the lymphoid cells were involved only in one case. In one case (4%) both 5q and 20q involvement occurred in a HES patient, but del(20q) only in the lymphoid, 5q AI only in the myeloid cells was detected.

Parallel HUMARA and microsatellite-PCR results

Parallel HUMARA and MS-PCR test were carried out in twenty cases. Four out of twelve MPNs (33%), one ET, 2 MPN-u and one HES showed parallel deletion or AI by MS-PCR and monoclonality by HUMARA. The MS-PCR detected involvement only in those cases which proved to be monoclonal by HUMARA. Parallel 5q involvement by MS-PCR and monoclonality by HUMARA could be detected only in one MDS-RA out of 8 MDS cases in the myeloid cells. In the rest of the MDS cases, we could not find any deviation from the normal karyotype by MS-PCR.

Parallel HUMARA and JAK2V617F mutation results

JAK2V617F allele specific PCR was done in 28 MPN cases in cell lineages, and in 11 of these cases HUMARA was made in parallel. These cases were chosen from routine pathological diagnostic cases which carried the JAK2V617F mutation in whole blood samples. The JAK2V617F mutation was detected in all cases in the myeloid cells. In 5 out of 6 PMF (83%), 8 out of 8 PV (89%), 8 out of 12 ET(67%) and in one MPN-u the lymphoid cells also carried the mutation. The two tests were compared in both cell lineages to determine the overlap between the JAK2V617F mutation and clonality results. In 7 out of 11 cases

(64%) the test results matched at cell lineage level. In four cases (36%) the HUMARA could not demonstrate monoclonality in one lineage which carried the JAK2V617F mutation.

DISCUSSION

HUMARA optimization

New clonality criteria have been established for the HUMARA X-inactivation test. We determined a polyclonal XIN range for lymphoid and myeloid cells, from which at least 90% specificity was required. Samples were considered monoclonal if the XIN value fell outside the 34-90% and 37-86% polyclonal range in the lymphoid and myeloid cells, respectively. We set up one more monoclonality criterion. We assumed that if only one of the two cell lineages is monoclonal, their XIN value will differ significantly from each other. Accordingly, we found that if the difference in the XIN values is greater than 26% it means that one of the lineages will be monoclonal with 90% probability. It is important to note that it's not possible to tell which of the two lineages is monoclonal, so this criterion may be rather complementary.
The sensitivity of the HUMARA was also tested. To do this, mixtures with known proportions of flow sorted leukemic and myeloid cells from 4 B-CLL patients were examined. The HUMARA was capable to detect monoclonality in all samples containing at least 50-60% of monoclonal cells at least by one of our criteria.
We demonstrated that the method is quantitative in mixture samples containing monoclonal cells. It was proved by the strong correlation ($r = 0.86$) of the known monoclonal ratio and ratio calculated from the measured XIN of the mixtures.

Lineage specific HUMARA studies

The results of our HUMARA studies in MDS was in concordance with the relevant literature data, and takes place between two extreme opinions. According to the first opinion the lymphoid lineage is not affected, or only in very rare cases, suggesting committed myeloid stem cell origin. The other view presumes that the lymphoid involvement occurs in a much larger part of the MDS cases favoring pluripotent stem cell origin. In 38% of our cases only the myeloid cells were monoclonal suggesting a committed myeloid stem cell derived process. However, in additional 31% of the cases monoclonality occurred in both lineages, implying unambiguous pluripotent stem cell origin. Pluripotent stem cell origin indicated also by the fact that in 10% of MDS lymphoid monoclonality and myeloid polyclonality was detected. This latter result may be explained if the ratio of the monoclonal cells in the sorted myeloid sample was below the sensitivity (50-60%) of the method.
According to literature data, in MPN a higher rate of lymphoid involvement occurs compared to MDS. In our study, 69 MPN cases were examined by HUMARA. We found that the incidence of double lymphoid-myeloid monoclonality indicating pluripotent stem cell origin was similar to MDS. Thirty-five percent of all cases showed double lymphoid-myeloid monoclonality, while in 38% only the myeloid lineage were monoclonal. Only the PV subtype showed higher ratio (50%) of double lymphoid-myeloid monoclonality.
To sum up the results of the HUMARA clonality tests, we assume that in MDS and MPN in more than one-third of the cases the tumor derived from pluripotent hematopoietic stem cell. The reliability of our results and the method is supported by the good concordance with the pathological diagnosis, as monoclonality was demonstrated in 82% of MDS and in 87% of

MPN cases. Consequently, in those female MDS or MPN patients where other pathognomonic marker cannot be not detected, the HUMARA may be used as diagnostic tool.

Cell lineage specific MS-PCR tests

Cell lineage involvement was examined in 35 patients using MS-PCR by detecting 20q and 5q deletion in sorted lymphoid and myeloid cells.
In MDS del(5q) or del(20q) was detected only in myeloid cells, except one patient. This finding on the one hand may imply that the disease may originates from committed myeloid stem cells, on the other hand the pluripotent stem cell origin cannot be excluded, because the deletions may occur at a later point of the tumorigenezis in a committed myeloid stem cell, and may play a role only in the malignization and manifestation of the disease.
Regarding the del(5q) MS-PCR results in MPN, cell lineage involvement were similar to those of MDS, i.e. del(5q) occurred only in myeloid cells, and may also indicate the committed myeloid stem cell origin of the disease. However, the del(20q) lineage involvement was different. One half of the del(20q) carrying cases showed double lymphoid-myeloid and the other half showed only lymphoid involvement. The dual lineage involvement clearly supports the possibility of the pluripotent stem cell origin. The exclusive occurrence of the del(20q) in lymphoid cells may be explained by the low sensitivity of the MS-PCR method which is similar to HUMARA, as requires also the measurement of the quantitative changes in the relation of two alleles. It is possible that the frequency of cells carrying deletion in the myeloid lineage fell below the sensitivity of the method in these cases.

Parallel HUMARA and MS-PCR studies were made in 20 informative cases. In 1 out of 8 MDS parallel del(5q) and monoclonality was found, while additional 6 cases showed only monoclonality by HUMARA. In all 21 of the MPN cases monoclonality was justified by HUMARA, and 4 cases showed deletions with MS-PCR in parallel in the same cell lineages. The phenomenon that with similar detection sensitivity far less cases showed deletion, than monoclonal proliferation further strengthens the assumption that the tested del(5q) and del(20q) may occur in a later step of the tumor development on the basis of a monoclonal proliferation.
We have also examined whether the MS-PCR molecular technique detecting smaller deletions than the more conventional cytogenetic methods is able to detect higher rate of 20q and 5q deletions in MDS and MPN. We found that in MDS neither deletions was more frequent. However, in MPN the del(5q) occurred in 14% of the samples, although this type of deletion is not characteristic of this disease group. The del(20q) approx. showed twice the frequency (26%) than its incidence detectable by conventional metaphase cytogenetics.

Lineage specific JAK2V617F mutation studies

The test was performed in 28 MPN patients for whom the routine diagnostic procedure confirmed the mutation in the whole blood. In eleven cases the occurrence of JAK2V617F mutation was compared with HUMARA monoclonality results at cell lineage level. The JAK2V617F mutation was detected in all cases in the myeloid cells. The involvement of lymphoid cells was over 80% in PMF and PV, while in ET was also found in the two-thirds of the patients. This suggests that the JAK2V617F mutation in the large majority of cases occurs in pluripotent stem cells.
The results of the parallel HUMARA tests in 7 out of 11 MPN cases (64%) agreed with the JAK2 mutation involvement at cell lineage level. In the remaining 4 cases the HUMARA could not demonstrate monoclonality in either cell lineage where the JAK2 mutation test was

positive. This is explained by the higher sensitivity of JAK2V617F PCR (3%), as for positive HUMARA, 50-60% of monoclonal cells in the sample is necessary.

Summary

New, criteria with 90% specificity have been developed for the HUMARA X-inactivation test, and the sensitivity of the method was also determined. The HUMARA studies in concordance with the pathological diagnosis in more than four-fifths of the cases proved monoclonality, accordingly, the HUMARA based on our new criteria may be used as diagnostic test. The results of the lineage specific HUMARA X-inactivation tests suggest that in more than one-third of the patients MDS and MPN are pluripotent stem cell diseases.
Using lineage specific MS-PCR it was also demonstrated that the del(5q) occurs mainly in myeloid cells in both of diseases, whereas the del(20q) can also be found in the peripheral lymphoid cells. The presence of del(20q) in the lymphoid lineage, implies that this deletion is earlier than del(5q), and may occur at the pluripotent stem cell level. Because the frequency of monoclonality detected by HUMARA is higher than frequency of deletions detected by MS-PCR, it is assumed that del(5q) and del(20q) represent later steps in the tumor development on the ground of a preexisting monoclonal proliferation. The incidence of deletions by MS-PCR did not show difference compared to conventional cytogenetics in MDS. However in MPN, in which the del(5q) is rare, we found this aberration in 14% of the cases. In MPN we found approx. 2-fold frequency (26%) of del(20q) compared to the literature data based on conventional cytogenetics.
The lineage specific JAK2V617F mutation studies confirmed the pluripotent stem cell origin in more than 80% of PMF and PV, and in the two-thirds of ET cases, by demonstration the involvement of both cell lineages.
In conclusion, using 3 different methods or the combination of these we demonstrated that the significant proportion of MDS and MPN - depending on the type of disease in a range of 31-89% - is pluripotent stem cell disease. It is likely that in most cases the JAK2V617F mutation appears at an early stage of the disease, in a pluripotent stem cell. The del(20q), if it's present, also occurs in pluripotent stem cells in MPN. In the greater proportion of MPNs, monoclonality can be detected by HUMARA without the presence of the del(20q) and del(5q), suggesting that these deletions are secondary aberrations in the tumor development.

SUMMARY OF NEW FINDINGS

1. New HUMARA X-inactivation test evaluation criteria have been developed for lymphoid and myeloid cell lineages with 90% specificity, omitting the need of control tissue. We determined the sensitivity of the HUMARA, and showed that the method is suitable to test samples containing at least 50-60% monoclonal cells. The quantitative nature of the method was tested and approved in mixtures of monoclonal and polyclonal cells.

2. According to our HUMARA clonality results, demonstrating double lymphoid-myeloid monoclonality we proved that in more than one-third of MDS and MPN cases the tumor derived from pluripotent hematopoietic stem cell.

3. MS-PCR showed that the del(5q), in both diseases occurs mainly in myeloid cells, whereas the del(20q) can also be found in peripheral lymphoid cells. The lymphoid appearance of del(20q) implies its pluripotent stem cell origin, which consequently may be an earlier event in the tumorigenezis than del(5q). Because the frequency of monoclonality detected by

HUMARA is greater than the frequency of deletions detected by MS-PCR, it is assumed that del(5q) and del(20q) represent later steps in the tumor development and occur on the ground of a preexisting monoclonal proliferation.

4. The incidence of deletions by MS-PCR method showed no difference compared to conventional cytogenetics in MDS. However higher incidence of del(5q) (14%), and del(20q) (26%) was detected in MPN.

5. The lineage specific JAK2V617F mutation studies confirmed the pluripotent stem cell origin in more than 80% of PMF and PV, and in the two-thirds of ET cases, by demonstration the involvement of both lymphoid and myeloid lineages. According to our results the majority of MPNs carrying the JAK2V617F mutation can be considered as pluripotent stem cell diseases.

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