Article is available online at http://www.webio.hu/por/2007/13/2/0114

ARTICLE

Lineage-Specific Clonality Analysis of Chronic Myeloproliferative Disorders and Myelodysplastic Syndrome by Human Androgen Receptor Assay

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In myelodysplastic syndrome (MDS) as well as chronic myeloproliferative disorders (CMPD) others than chronic myeloid leukemia the frequency of pathognomonic genetic aberrations is very low and, therefore, X chromosome inactivation (XI) assays may help in assessing the clonality. To establish specific clonality criteria on XI, human androgen receptor assay (HUMARA) was performed on sorted myeloid and lymphoid peripheral blood cells of 21 healthy females. Clonality criteria 1 and 2 conferring at least 90% specificity were set based on the ranges and differences of XI number (XIN) describing the ratio of representation of the two alleles in as well as in between reactive myeloid and lymphoid compartments. Spiking experiments indicated that the test identifies clonality reliably when no more than 40-50% reactive cells are admixed. In the CMPD and MDS cases peripheral myeloid cells were monoclonal by one of the two criteria in 71-100%, whereas lymphoid cells in 28-75%. The results of HUMARA, available in 73% of the female patients, supported the clinicopathological data in 84% as well as proved pluripotent stem cell origin in 31-75% and 21% of CMPDs and MDS, respectively. (Pathology Oncology Research Vol 13, No 2, 114–122)

Key words: chronic myeloproliferative disease, myelodysplasia, HUMARA

Introduction

Although the basis of pathological diagnostics of tumors is pathomorphology, this methodology needs to be combined with other, especially molecular investigations in many circumstances. One major approach among these is clonality analysis, like the antigen receptor gene rearrangement tests, which might differentiate between reactive versus neoplastic lymphoproliferation of T- and B-cell origin. On the other hand, increasing number of tumor-specific genetic aberrations becomes known whose identification is needed for the exact subclassification of the neoplastic lesions. Furthermore, in certain diseases, like in chronic myeloproliferative disorders (CMPD) and myelodysplastic syndromes (MDS) making up a considerable fraction of the clonal, neoplastic hematological diseases, not only the clonality analyses, but the determination of cell lineage involvement, defining the uncommitted versus committed stem cell origin of the process, would be beneficial. However, the known genetic aberrations in tumors arising from different cellular compartments vary in a broad range. For example, in MDS as well as in CMPDs others than chronic myeloid leukemia (CML) the frequency of pathognomonic genetic aberrations is very low.[3,7,9,11,13] One exception is the recently described JAK2 (V617F) mutation.[8,10,14,15,18] The most common genetic alteration in these CMPDs is the del(20q) occurring in 10% in polycythemia vera (PV) [17], where-

Received: Nov 16, 2006; *accepted:* May 10, 2007

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Abbreviations: chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), chronic myeloproliferative disorder (CMPD), essential thrombocytemia (ET), hypereosinophilic syndrome (HES), myelodysplastic syndrome (MDS), non-random X-inactivation (NRXI), polymorphic microsatellite region (PMR), polycythemia vera (PV), X-inactivation (XI), X-inactivation number (XIN)

as deletion of 5q as well as numerical aberrations of chromosome 7 and 8 occur approximately with the same frequency in MDS.[7] Because of the low number of specific genetic changes and the absence of cell lineage specific clonality markers, X chromosome inactivation assays remains the method of choice for clonality analysis in these disorders. These assays are suitable for clonality studies in females and, as they are not cell lineage-specific, they can be used by analyzing separate cell lineages for determination of the extent of cell lineage involvement of the disease. Currently the most commonly used method is the DNA-based human androgen receptor assay (HUMARA). The assay is based on the PCR amplification of a polymorphic microsatellite region (PMR) located in the human androgen receptor gene on the inactive X chromosome, identified by methylation-sensitive restriction endonuclease digestion. The PMRs from the two alleles are distinguishable by molecular weight, and the amount of PCR product is proportional with the allele representation. Due to the absence of full consensus in the literature, further studies are needed in various aspects of HUMARA, which are as follows: 1. the criteria to be used for determination of clonality, 2. the specificity and 3. the sensitivity of the assay. To shed light on these questions, flow-sorted myeloid and lymphoid blood cells of 27 healthy adult females as well as various mixtures of flow-sorted monoclonal leukemic and polyclonal myeloid cells from blood samples of four chronic lymphocytic leukemia patients were analyzed by HUMARA.

Based on the obtained data, criteria for clonality were set and applied to investigate the clonality of flow-sorted myeloid and lymphoid cells in blood samples of CMPD as well as MDS patients. Applying one of the two criteria, the HUMARA assay could confirm the clinicopathological diagnosis in more than three quarters of patients. Experimental conditions indicated that at least one of the clonality criteria was fulfilled in all cell mixtures with a prerequisite of 50 to 60% ratio of monoclonal cells in the samples.

Materials and Methods

Patients

Peripheral blood samples of 27 healthy women (age range: 20-65 years, mean: 42.7) were collected for flow cytometric sorting. Altogether 80 patients' peripheral blood and bone marrow samples have been investigated. Out of these only 57 could be evaluated for HUMARA X chromosome inactivation PCR test. Twenty-three patients fell into the MDS (age range: 12-81, mean: 62.4), 22 into the essential thrombocytemia (ET) (age range: 22-80, mean: 54.7), 7 into the hypereosinophilic syndrome (HES) (age range: 29-81, mean: 56.7) and 5 into the PV (age range: 32-79, mean: 58.4) disease categories based on the clinicopathological findings classified according to the WHO principles.[12] For determining sensitivity, various mixtures of polyclonal and monoclonal peripheral blood cells were used from four patients with chronic lymphocytic leukemia (CLL) diagnosed according to the WHO guidelines.

Flow cytometric cell sorting

From the blood and bone marrow samples lymphoid, myeloid and eosinophilic cells (the latter ones only in HES patients) were sorted by means of FACS. The sorting gates were set by CD45 expression and side scatter (SSC). Using these two parameters on a two-dimensional diagram, the lymphocytes (high CD45 expression and low granularity) and myeloid cells (medium CD45 expression and high granularity) could be separated. In HES patients eosinophils were gated by their CD45 expression and very high granularity combined in a logical gate with high autofluorescence in the green and orange channels. In case of CLL patients, by combining the CD5/CD23 or CD5/CD19 double positive lymphocyte and granulocyte gates distinct mixtures of mono- and polyclonal cells were sorted directly (100% CLL, 75-80% CLL + 20-25% granulocytes, 50% CLL + 50% granulocytes, 20-25% CLL + 75-80% granulocytes and 100% granulocytes). The immunolabeling was carried out using CD45-RPE-CY5, CD5-FITC, CD19-RPE and CD23-RPE monoclonal antibodies (DakoCytomation, Glostrup, Denmark), according to the instructions of the manufacturer. The $5x10^4$-10^5 cells from each lineage were sorted by means of a FACSort flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA). Based on flow cytometric analysis of the sorted samples the purity of the sorting was >95%. The sorted cells were stored in 500 µl TE8 buffer at –20°C until DNA isolation.

DNA isolation

After thawing, 10 mg/ml proteinase-K and 1% SDS were added to the sorted cells and incubated at 56°C overnight. DNA was purified by phenol and chloroform extraction followed by ethanol precipitation, finally solved in TE8 buffer and stored at 4°C.

Human androgen receptor assay – HUMARA

HUMARA was done according to the protocol described by Allen et al[1] with some modifications. DNA from $3x10^3$-10^4 cells was digested at 37°C overnight with HhaI restriction endonuclease (Promega, Madison, WI, USA) in 20 µl buffer containing 0.1 mg/ml BSA. After digestion DNA was precipitated with ethanol and dissolved in 5.5 µl distilled water. The PCR mixture contained 200 µM of dNTP

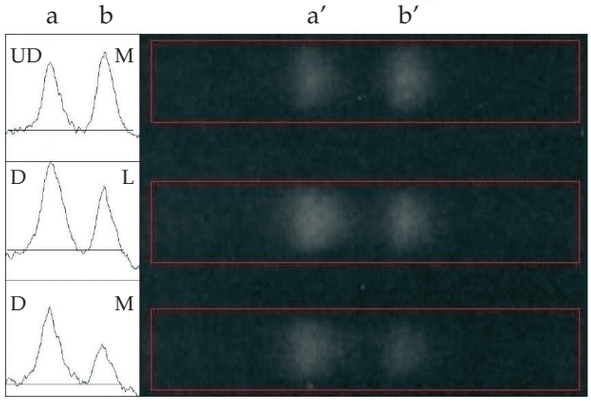

Figure 1. *Gel electrophoretic pictures* ***(a′, b′)*** *as well as densitometric curves* ***(a, b)*** *of the amplified microsatellite regions obtained by HUMARA from peripheral blood cells of a healthy, heterozygous female. The first line marked by UD (undigested) shows the PCR product obtained by HUMARA without predigestion by methylation-sensitive restriction enzyme and serves only for the detection of heterozygosity. a, a′ and b, b′ correspond to the smaller and the larger allele, respectively. The lines marked by D (digested) show the PCR products from peripheral lymphoid (L) and myeloid (M) cells following predigestion by methylation-sensitive enzyme.*

(GibcoBRL, Invitrogen Corporation, Carlsbad, CA, USA), 0.5 U RedTaq DNA polymerase, RedTaq buffer (Sigma, St. Louis, MO, USA) and 3 pmol of each primer (5' GCT GTG AAG GTT GCT GTT CCT C 3' and 5' AGA GGC CGC GAG CGC AGC ACC TC 3') in a total volume of 10 µl. The amplification was done by MJR Minicycler (MJ Research, Watertown, MA, USA) or Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA, USA) thermocyclers using the following protocol: predenaturation: 94°C 5 min; 40 cycles denaturation: 94°C, 40 sec; annealing: 68°C, 40 sec; extension: 72°C, 1 min and final elongation: 72°C, 5 min. After amplification the whole PCR reaction mixture was transferred to non-denaturing 8% polyacrylamide gel and run at 120V, 25 mA overnight until the tracer xylene-cyanol reached the margin of the 24-cm-long gel. The amplification products were visualized with SYBR Gold DNA stain (Molecular Probes, Eugene, OR, USA) by UV transillumination and finally photographed by a Polaroid camera. The photographs were scanned and densitometrically analyzed by NIH Image 1.62 or Image J 1.4 software.

Results

Fig. 1 shows the gel electrophoretic images as well as the densitometric curves of the amplified products representing the microsatellite regions on the two alleles. The individual densitometric curves are not proportional to the allele representation when HUMARA is performed without predigestion by methylation sensitive restriction endonuclease. Using predigestion, however, the density curves of the two alleles represent their activity, thus any imbalance indicates shift in the allele representation, i.e., the X-inactivation (XI) ratio. The allele representation was characterized by the area outlined by the individual curves and expressed as percentage of the total area marked by the two curves. To characterize the allele specific XI, the X-inactivation number (XIN) has been introduced which is an index referring always to the allele of lower molecular weight. The value of XIN may range from 0% to 100%; in case of random XI it is 50%, whereas non-random XI (NRXI) will result in values different from 50%. Homogeneous monoclonal cell populations are characterized by a XIN of either 0% or 100%.

Among samples of the 27 healthy control females amplifiable products of two different molecular weights were obtained in 21 cases. The ambiguous cases where the two alleles were too close to obtain reliable densitometric analysis were omitted. The XIN values obtained by HUMARA on peripheral blood lymphocytes and myeloid cells of heterozygous patients are summarized in *Table 1*. The data indicate slight preponderance for the inactivation of the smaller allele in both cell types of the healthy females investigated. The XIN corresponded to 65.2% and 64.1% for the lymphoid and myeloid cells, respectively,

Table 1. **XIN values of lymphocytes as well as myeloid cells of 21 healthy females, obtained by HUMARA**

Case number	*Age (years)*	*Lymphoid cells (XIN)*	*Myeloid cells (XIN)*
1	37	90	78
2	40	60	61
3	50	83	83
4	57	51	39
5	40	34	55
6	62	63	82
7	60	70	56
8	37	30	49
9	22	85	86
10	50	67	73
11	55	39	38
12	32	73	75
13	39	–	5
14	56	80	100
15	32	100	75
16	51	71	59
17	63	71	55
18	30	59	37
19	50	59	73
20	23	87	85
21	48	56	82
mean±SD	**44.5±12.5**	**65.2±20.0**	**64.1±22.4**

Table 2. **Relationship of various cut-off values of XIN and specificity of HUMARA in lymphoid and myeloid cells of 21 healthy females**

Lymphoid cells		*Myeloid cells*	
XIN range	*specificity*	*XIN range*	*specificity*
Mean ± 1 SD 45.2–85.2	80%	mean ± 1 SD 41,7–86,5	76%
Mean ± 2 SD 25.2–105.2	100%	mean ± 2 SD 19.3–108.9	95%
34–90	90%	37–86	90%
30–100	100%	5–100	100%

with considerable standard deviation (20.0% and 22.4%, respectively). This may be due to NRXI or the high number of the PCR cycles used which may cause the preferential amplification of the smaller allele.

For the proper evaluation of any shift in XIN due to the presence of monoclonal cells in a sample, we had to determine the cut-off values of XIN indices which still gave acceptable false positivity, in other words specificity, for the control, polyclonal cells. Specificity for the control lymphoid and myeloid cells was calculated when mean ± 1 SD or mean ± 2 SDs of XIN as cut-off values were used (*Table 2*). As we required at least 90% specificity (less than 10% false positivity) for the test to accomplish, ranges of XIN indices for the lymphoid and the myeloid cells providing 90% specificity or more were also calculated.

The correlation between XIN values of the lymphoid and myeloid cells was investigated. Because a moderately strong correlation (correlation coefficient=0.646) between the XIN values of control lymphoid and myeloid cells could be detected, we have investigated whether they could mutually serve as control for each other if one undergoes malignant transformation (monoclonal growth). To this end the difference in XIN values of lymphoid vs. myeloid cells was used as the mean difference of these values and the SD represented a narrow range (12.5 ± 9.1%). We have calculated the specificity using various ranges of difference in XIN values defining polyclonal populations, and the value of difference in XIN indices yielding at least 90% specificity proved to be 26 (*Table 3*).

The sensitivity, in term of ratio of clonal cells in a mixed cell population allowing prediction of monoclonality, was determined by HUMARA performed on DNA obtained from various mixtures of leukemic lymphoid cells and polyclonal granulocytes of four CLL patients. The cell mixtures were obtained by sorting as described in the Materials and Methods. Data shown in *Table 4* and *Fig. 2* indicate that monoclonality can be determined without any special calculation, provided that a given cell population contains at least 75% monoclonal cells, because the value of the XIN in these cases was 100 or 0. With smaller amount of leukemic cells, however, the following accomplishments of clonality criteria could be observed. First, in case 1 when 20-25% clonal cells were admixed to the polyclonal cells the XIN value proved to be 19% which fell outside of the polyclonal range (34-90%) of the lymphoid cells. However, this mixture could not fulfill our second clonality criteria because there was only 11% difference between the XIN of the mixture and that of the pure polyclonal population. In the other three cases neither of the two criteria could show monoclonality in the mixtures containing 20-25% clonal cells. In contrast, in two of the four 50-60% mixtures (case 1 and 2) one of the two criteria, whereas in the remaining two mixtures (case 3 and 4) both criteria could define monoclonality. It means that when at least 50 to 60% clonal cells were present, clonality could be predicted by at least one of the criteria, even when the NRXI of polyclonal cells was extreme (case 1).

We have investigated the correlation of the ratio of monoclonal cells calculated according to the algorithm as below and exemplified in *Fig. 3* with the true percentage of monoclonal cells (M%) in a given cell mixture (*Table 5*). The data show that in the investigated range of

Table 3. **Relationship of the various cut-off values using the mean difference in XIN of the myeloid and lymphoid cells and the specificity of HUMARA in 21 healthy females**

XIN difference	*Specificity*
Mean ± 1 SD = 21.6	80%
Mean ± 2 SD = 30.7	100%
26	90%
27	100%

Table 4. **XIN values obtained from various mixtures of monoclonal lymphoid leukemic cells and polyclonal granulocytes in four CLL patients**

	XIN values in cell mixtures containing defined proportion of monoclonal CLL cells				
Case number	*100% CLL*	*75-80% CLL*	*50-60% CLL*	*25-20% CLL*	*0% CLL*
1	100	100	53	19	8
2	–	0	30	41	52
3	0	0	23	63	82
4	0	0	27	47	70

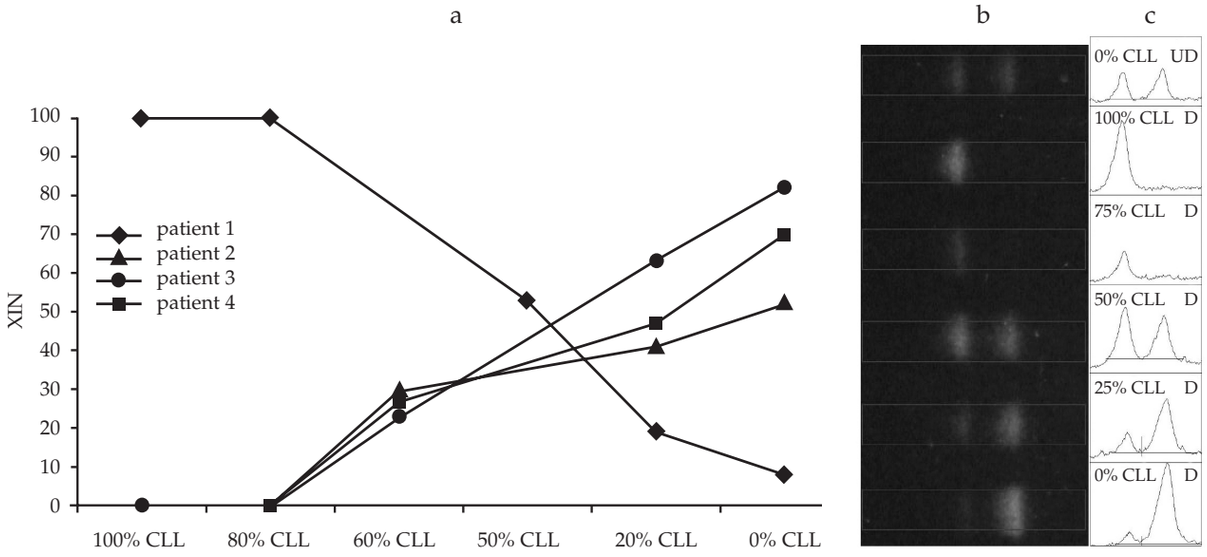

Figure 2. (a) *XIN values obtained from mixtures of monoclonal leukemic cells and polyclonal granulocytes plotted as function of the percentage of monoclonal CLL cells.* ***(b,c)*** *Electrophoretic picture (b) and densitometric curves* ***(c)*** *of the amplified PCR product as function of the various amount of monoclonal leukemic cells in the cell mixture from patient 1. UD: undigested, D: digested by methylation-sensitive restriction endonuclease.*

60% to 20% monoclonal cell ratio, the correlation between the true as well as the calculated ratio of monoclonal cells proved to be strong (r=0.86) using the following algorithm:

$$\frac{M\%}{100} \times XIN_M + \frac{100–M\%}{100} \times XIN_p = XIN_{measured}$$

where XIN_M is XIN value of the pure monoclonal sample (100% CLL cells), XIN_P is XIN value of the pure polyclonal sample (100% granulocytes), and $XIN_{measured}$ is XIN value in a given cell mixture (CLL cells + granulocytes).

One of the two clonality criteria, each providing at least 90% specificity, was applied for the HUMARA data of altogether 57 MDS, ET, PV and HES patients from which in 49 patients successful amplification of the microsatellite region was obtained in both the lymphoid as well as the myeloid cells (*Table 6*). Based on XIN values, monoclonality of the sorted myeloid cells could be claimed in the great majority of cases, ranging from 68% to 100% in the various disease categories. Beside myeloid monoclonality the lymphoid cells proved to be monoclonal in approximately 1/4 to 1/3 of the cases except in PV with relatively low number of patients where this figure reached 75%. In a surprisingly large fraction of MDS patients only the lymphoid cells proved to be monoclonal, while this figure was low (5.3%) in ET patients and zero in PV and HES patients. Among the HES patients monoclonal eosinophils could be detected in somewhat less than half of the cases. The monoclonality of the eosinophils was always accompanied with that of the myeloid cells, but in two cases polyclonal eosinophils were found beside monoclonal myeloid cells.

The value of HUMARA analysis was especially emphasized in those cases when the bone marrow histology and/or cytology were supportive for the clinically suspected neoplastic myeloproliferations, but in themselves were not pathognomonic to prove the disease. This might be frequently encountered in CMPDs and MDS as exemplified by the cases shown in *Fig. 4*. Sometimes in MDS of refractory anemia type, signs of dyserythropoiesis with various nuclear abnormalities can only rarely be observed and the massive megaloblastic transformation, which might also occur in reactive, polyclonal conditions, dominates the morphology (*Fig. 4a,b*). In a fraction of ET cases, in addition to the slightly hypercellular or normocellular parenchyma, no significant megakaryocytosis is present (*Fig. 4c*). HES especially represents a challenge for morphologists as differentiation of reactive eosinophilias from neoplastic ones can rarely be done on the basis of definitive cytological atypia (*Fig. 4d,e*). All cases of the ET as well as HES patients where no monoclonality could be identified fell into these categories.

Discussion

X-inactivation clonality tests might be useful to detect monoclonal proliferation in all circumstances when clonal genetic markers of tumorous growth are missing. One prime example for this is represented by the group of neoplastic chronic myeloproliferations. According to the liter-

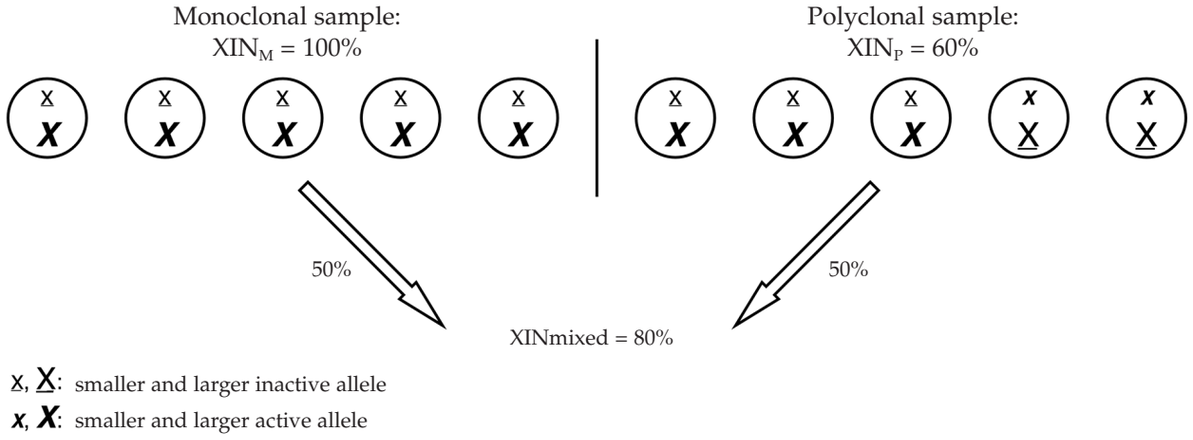

Figure 3. *Influence of the ratio of monoclonal versus polyclonal cells and the XIN value of polyclonal population on the measured XIN value of a mixed cell population*

ature, the HUMARA based X-inactivation analysis is the most informative, but widely accepted consensus is missing in several aspects with special emphasis on the matter of the reliable clonality criteria, specificity and sensitivity. In contrast to theory, the non-random X-inactivation (NRXI) is a well-known phenomenon in practice. We have found only a slight NRXI on average, but the XIN among the healthy individuals ranged in both populations on a broad scale. We cannot exclude the effect of the high PCR cycle number which may cause the preferential amplification of the smaller allele.

Determination of a rather arbitrary cut-off value in the allelic imbalance delineating the monoclonal vs. polyclonal range is widely used. In several publications, this imbalance was defined as 3 to 1 shift in the allelic representation.[5,6] However, when we have applied this cut-off value, 25% and 38% false positivity was obtained in the reactive lymphoid and myeloid cells, respectively. This represents low specificity even for a biological assay. As 100% specificity (0% false positivity) is usually not feasible and could not be obtained in our study as healthy individuals (#14, 15) with a complete silencing of one allele were included, we intended to adjust the cut-off value to give 90% specificity. This could not be achieved by using the mean XIN ± 1 SD cut-off value as it gave 80% and 76% specificity in the lymphoid and myeloid populations, respectively. The mean XIN ± 2 SDs cut-off value resulted in mathematically and biologically nonsense figure (more than 100% XIN). Thus, we have narrowed the XIN range from the two extremes and experimentally obtained the polyclonal range of 34-90% for the lymphoid and that of 37-86% for the myeloid cells fulfilling the 90% specificity. Any XIN value beyond these limits was considered to represent the presence of a monoclonal population in the sample. We have set a second clonality criteria based on the moderately strong correlation between XIN values of control lymphoid and myeloid cells. Any difference ≥26% between the XIN values of these two cell populations indicates the presence of a monoclonal population in either of these cell compartments with 90% specificity. The opposite is not against monoclonality as the presence of monoclonal population with inactivation of the same allele in both compartments will obviously decrease the difference in XIN values.

Table 5. **Correlation of the true ratio of monoclonal cell with the calculated ones according to the measured XIN values in various cell mixtures of mono- as well as polyclonal cells in four CLL patients**

Patients	*True ratio of monoclonal cells*	*Calculated ratio of monoclonal cells*
1	25	12
2	20	32.8
3	20	21.2
4	20	23.2
1	50	48.9
2	60	61
3	60	42.3
4	60	72
Correlation	**r=0.86**	

As in case of chronic neoplastic myeloproliferations the target bone marrow or peripheral blood cell population always represents various mixtures of non-neoplastic and neoplastic cells, it is a key issue at what proportion of neoplastic cells in the sample is this test capable of predicting monoclonality. This is an important issue because this type of neoplastic cells usually cannot be distinguished, consequently selected from normal counterparts even by using various cell separation techniques, thus heterogeneous samples are usually to be analyzed. The results of analyz-

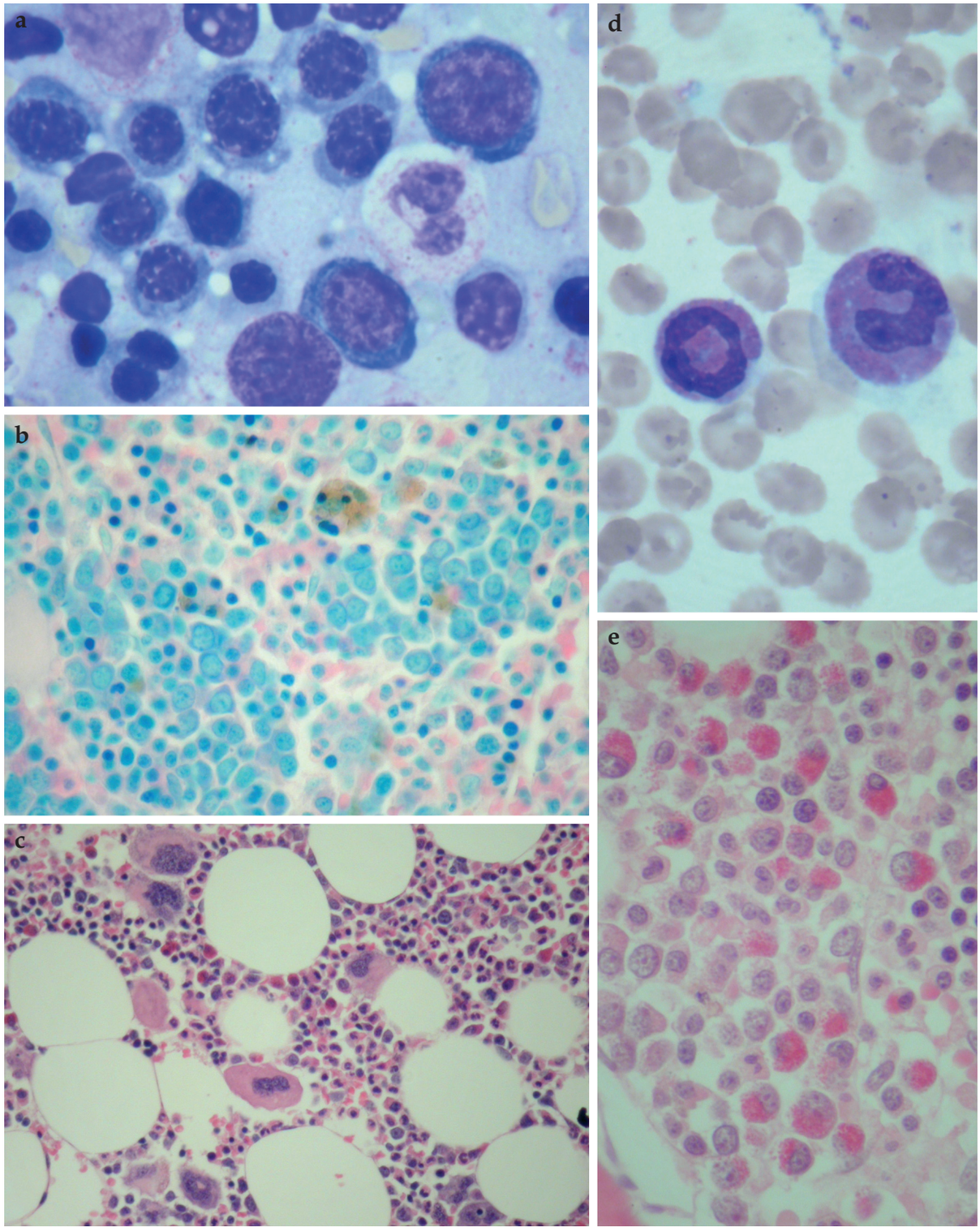

Figure 4. *Histology of clinically suspected neoplastic myeloproliferations.* ***(a,b)*** *dyserythropoiesis with slight nuclear abnormalities as well as massive megaloblastic transformation in MDS;* ***(c)*** *only slight megakaryocytosis and normocellular parenchyma, a histological change which in itself not pathognomonic for ET; (d,e) definite cytological atypia including ring-shaped eosinophilic granulocyte; cytological changes justifying the diagnosis of neoplastic proliferation of eosinophils are only rarely seen in HES.*

Table 6. **Clonality data of the myeloid (M) and lymphoid (L) cells as well as eosinophils (Eo) in MDS, ET, PV and HES patients**

	MDS		*ET*		*HES*		*PV*	
	monocl./ all	*ratio*	*monocl./ all*	*ratio*	*monocl./ all*	*ratio*	*monocl./ all*	*ratio*
No monoclonality	1/19	5.3%	5/19	26.3%	2/7	28.6%	0/4	0%
M monoclonal	14/19	73.7%	13/19	68.4%	5/7	71.4%	4/4	100%
L+M monoclonal	4/19	21.1%	6/19	31.6%	2/7	28.6%	3/4	75%
Only L monoclonal	4/19	21.1%	1/19	5.3%	0/7	0%	0/4	0%
Only Eo monoclonal	–	–	–	–	0/7	0%	–	–
M+Eo monoclonal	–	–	–	–	3/7	42.9%	–	–
L+M+Eo monoclonal	–	–	–	–	1/7	14.3%	–	–
Any clonal population	18/19	94.8%	14/19	73.7%	5/7	71.4%	4/4	100%

ing various mixtures of monoclonal CLL cells and polyclonal granulocytes indicated that 25% of polyclonal cells did not cause any shift in the XIN and monoclonality could be claimed without applying any special clonality criteria. As in spiking experiments investigation of the two clonality criteria in the four cell mixtures containing only 20-25% clonal cells indicated monoclonality in only 1 times out of 8 (4 cases, 2 criteria), thus fulfilling of one of the two clonality criteria cannot be taken as a reliable indicator of monoclonality at such a low level of clonal population. Case 1 demonstrates that at extreme NRXI of polyclonal cells even 50% of monoclonal population cannot be defined by both, only by the second criterion. However, the fact that the two clonality criteria scored positive altogether six times out of the eight in the four cell mixtures of 50 to 60% clonal cells proves that clonality can be reliable claimed by at least one of the criteria at this ratio of clonal cells. The reliability of predicting monoclonality and also the ratio of tumor cells on the basis of XIN values is demonstrated by the strong correlation between the calculated and the real proportion of monoclonal cells in mixed cell samples. Nevertheless, it can be suggested for any laboratory that for setting up cut-off XIN values for defining monoclonality a reasonable number of healthy individuals should be investigated.

By detecting monoclonality in any of the cell lineages the HUMARA X-inactivation test using strict clonality criteria supported the clinicopathological diagnosis in 100% (PV), 95% (MDS), 74% (ET) and 71% (HES), respectively. These results indicate a strong correlation between the clinical, pathomorphological and molecular data with 90% specificity for the latter one. The fact that beside the myeloid series the lymphoid ones proved to be monoclonal in MDS, ET, HES and PV at a ratio of 21%, 32%, 29% and 75%, respectively, indicates that not only the CMPD type of disorders are of pluripotent stem cell origin, but a significant proportion of MDS is of multilineage disease as suggested by some studies.[1,2,4] The exact figure cannot be obtained as this is the function of the actual ratio of monoclonal vs. reactive cells within the same cell lineage and the sensitivity of the method used. This might also explain the relatively high fraction of cases with only lymphoid monoclonality in the disease category of MDS.

In conclusion, the PCR-based HUMARA X-inactivation clonality test with the new clonality criteria each representing 90% specificity was capable of proving the clinicopathologically defined MDS and CMPD type of diseases with 84% sensitivity by detecting monoclonality according to one of the two criteria in sorted cell lineages of peripheral blood. Therefore, this assay represents a valuable asset in the diagnostic procedure of these diseases.

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