The Human Androgen Receptor X-Chromosome Inactivation Assay for Clonality Diagnostics of Natural Killer Cell Proliferations

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Clonality is a frequently exploited characteristic of lymphoid malignancies. However, in the natural killer (NK) cell subset of large granular lymphocyte proliferations, clonality is difficult to prove because of the lack of specific genetic markers, such as immunoglobulin or T-cell receptor gene rearrangements. The human androgen receptor (HUMARA) assay, a polymerase chain reaction-based X-chromosome inactivation assay, is a potential diagnostic tool in these disorders. Although there is much experience with X-chromosome inactivation assays in myeloid proliferations, these assays have found only very limited application in clonality assessment of NK cell proliferations. We applied the HUMARA assay in laboratory diagnostics for detection of clonality in NK cell proliferations. We describe its test performance and report three cases in which clonality of NK cell populations was investigated by use of this assay. Our results demonstrate the usefulness of the HUMARA assay in the diagnostic workup of NK cell proliferations. (*J Mol Diagn* 2007, 9:337–344; DOI: 10.2353/jmoldx.2007.060155)

Natural killer (NK) cells are a lymphoid subset that normally accounts for 10 to 15% of mononuclear cells (MNCs) in peripheral blood (absolute counts: 0.1 to 0.4 × 10⁹/L). They constitute the majority of the morphologically recognizable large granular lymphocytes (LGLs) in the blood, a minority being of T-cell origin. Proliferations of LGLs form a rare and heterogeneous disorder. In contrast to normal LGLs, only 15% of these proliferations are derived from NK cells, whereas 85% are of T-cell origin. LGL disorders encompass a broad spectrum ranging from polyclonal reactive proliferation to aggressive leukemia.^{1–3} It is important to differentiate leukemia from the other proliferations. For this purpose, clonality assessment is an essential requirement. In LGL proliferations derived from T cells, clonality can be detected by use of

T-cell receptor (TCR) gene rearrangement studies. However, in LGL disorders of NK cell lineage, detection of clonality is problematic because of the absence of specific molecular markers.^{3,4}

X-chromosome inactivation assays allow the evaluation of clonality in female patients. These assays are based on the process of random inactivation of a single X chromosome in each female cell early during embryogenesis and of its subsequent stable propagation on daughter cells. This process results in the theoretically expected 1:1 inactivation ratio between the paternally and maternally derived X chromosomes in a cell population under study.⁵ If there is a significant deviation from this ratio, nonrandom X-chromosome inactivation or skewing is said to have occurred. This finding can be the consequence of clonal derivation of the cell population.⁶ The major advantage of X-chromosome inactivation-based clonality assays is that they can be performed in the absence of any tumor-specific (cyto)genetic marker. Several loci have been used to assess skewed X-chromosome inactivation, eg, the phosphoglycerate kinase (PGK) gene, the hypoxanthine phosphoribosyltransferase (HPRT) gene, and the DXS255 locus.^{7,8} Today, most published X-chromosome inactivation studies make use of the human androgen receptor (HUMARA) gene.⁵ A first reason for its popularity relates to the presence of a hypervariable CAG short tandem repeat ($n = 9$ to 36) in the first exon of the HUMARA gene.^{9,10} Distinction between the paternally and maternally derived X chromosome is only possible in females who are heterozygous for this repeat. However, because this CAG repeat is polymorphic in 90% of females, the assay is informative in most females.¹¹ A second reason for its popularity is the presence of several cleavage sites for methylation-sensitive restriction enzymes like *HpaII* in close proximity of the CAG repeat.^{9,10} Because X-chromosome inactivation is correlated with hypermethylation, these enzymes digest only the active, ie, unmethylated, alleles.⁵ In this way it is possible to distinguish the active from the inactive alleles in a subsequent polymerase chain reaction (PCR).

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Only a few studies have been published in which X-chromosome inactivation assays were performed in female patients with LGL proliferations of NK cell origin.^{12–15} They mostly used the PGK assay, which suffers from a lack of informativity. In addition, only a limited number of patients have been studied, and clonality has been demonstrated in a minority of these patients. Use of the HUMARA assay in hematology is virtually limited to myeloid disorders.⁵ In this study, we present the HUMARA X-chromosome inactivation assay as a practical diagnostic tool for the investigation of clonality in female patients with LGL proliferations of NK cell lineage.

Case Reports

Case 1

An 11-year-old girl was admitted to the hospital because of malaise, weight loss, and recurrent episodes of fever for 6 months. Her physical examination was unremarkable, except for paleness. No hepatosplenomegaly or lymphadenopathy was present. Blood analysis revealed the following: hemoglobin, 9.5 g/dl; hematocrit, 29%; platelet count, $163 \times 10^9/L$; and leukocytes, $8.9 \times 10^9/L$ with 96% lymphocytes. A majority of lymphocytes showed atypical morphology on microscopic examination. On immunophenotyping, >95% of lymphocytes were CD2-, CD7-, and CD16-positive, partially CD57-positive, and negative for CD3, CD4, CD8, and CD56, which is compatible with a NK cell phenotype. The absolute count of NK cells was $\sim 5.9 \times 10^9/L$. Bone marrow aspiration showed invasion of the marrow by these lymphocytes (50% NK cells). NK cell function tested normally *in vitro*. An extensive immunological and microbiological workup was performed. All investigations, including for autoimmune parameters such as rheumatoid factors, were negative. The patient was monitored for 2 years, during which period the NK cell count in her blood remained fairly stable. Anemia and neutropenia persisted. She had recurrent episodes of skin infections and she developed splenomegaly. Additional studies to detect clonality of the NK cell population were performed. No cytogenetic abnormalities were detected on examination of bone marrow aspiration. Detection of Epstein-Barr virus (EBV) episomes by Southern blotting tested negative. Neither TCR β (*TCRB*), γ (*TCRG*), and δ (*TCRD*) gene rearrangements nor immunoglobulin (Ig) heavy chain (*IGH*) and κ (*IGK*) gene rearrangements could be detected by Southern blotting. Finally, X-chromosome inactivation assay at the DXS255 locus was performed by Southern blotting with the M27 β probe. Later on, the PCR-based HUMARA assay was also performed (see below). Both X-chromosome inactivation assays showed that the population of NK cells had the same X-chromosome inactivated, whereas the patient's granulocytes showed random inactivation of both X-chromosomes. Thus, the X-chromosome inactivation studies demonstrated clonality of the NK cell-derived LGL proliferation. A diagnosis of LGL leukemia, NK cell

subtype was made. Treatment with cyclosporin and granulocyte colony-stimulating factor (G-CSF) was initiated, with favorable response.

Case 2

A 51-year-old female presented with malaise and subfebrile temperature for 10 months. Physical examination and medical history were unremarkable. Surprisingly, blood analysis revealed anemia and absolute lymphocytosis with hemoglobin, 14.8 g/dl; hematocrit, 43%; platelet count, $222 \times 10^9/L$; and leukocytes, $17.2 \times 10^9/L$ with 56% lymphocytes. Trephine bone marrow biopsy did show lymphocytic infiltration. Phenotypic analysis demonstrated the NK cell origin of the lymphocytes with positivity for CD2, CD7, CD56, and CD16 and negativity for CD3, CD4, CD8, and CD57. The absolute count of NK cells in blood was $\sim 8.9 \times 10^9/L$. Molecular analyses of *TCRB*, *TCRG*, and *TCRD* gene rearrangements revealed germline configurations. Cytogenetic analysis demonstrated a normal karyotype. Detection of EBV episomes by Southern blotting tested negative. The M27 β and HUMARA X-chromosome inactivation assays finally demonstrated the clonal origin of the NK cell population (see below). During the 2 years of follow-up, the clinical picture and lymphocytosis remained stable.

Case 3

An 86-year-old female was followed for type 2 diabetes mellitus for 13 years when control examination of blood revealed absolute lymphocytosis of $7.6 \times 10^9/L$. Microscopically 70% of the lymphocytes had the LGL appearance, but azurophilic granules were frequently absent. The patient did not have neutropenia, anemia, or thrombocytopenia. Trephine biopsy did not reveal bone marrow involvement. Immunophenotyping showed NK cell origin of the lymphocytic population. The absolute count of NK cells was $\sim 6.4 \times 10^9/L$. Molecular analyses for TCR and Ig gene rearrangements revealed germline configurations. Detection of EBV episomes by Southern blotting tested negative. By use of the HUMARA X-chromosome inactivation assay, no clear clonality of the NK cell population could be demonstrated (see below). In contrast, the M27 β assay did show clonality of the same NK cell population. The patient remained asymptomatic, despite persistent presence of the NK cell population during 2 years of follow-up.

Materials and Methods

We adopted the method developed by Allen and colleagues,⁹ with some modifications. DNA was extracted using the GenElute mammalian genomic DNA kit (Sigma-Aldrich, St. Louis, MO). DNA concentrations and purity were measured using the GeneQuant pro RNA/DNA calculator (Amersham Biosciences, Uppsala, Sweden). Density gradient centrifugation and cell sorting to obtain purified cell populations were performed as de-

scribed previously.¹⁶ Purity of sorted cell populations was also checked immunophenotypically as described previously.¹⁶

DNA Digestion

Samples of 0.5 to 1 μ g DNA were incubated overnight at 37°C while gently shaking in 50- μ l reactions containing 25 U of the methylation-sensitive restriction enzyme *Hpa*II. The mix also contained 5 U of *Dde*I, a restriction enzyme that is not sensitive to methylation, to improve digestion by *Hpa*II. Secondary structures can render restriction sites inaccessible. By generating DNA fragments of smaller size, *Dde*I makes the DNA more accessible to *Hpa*II.^{10,17} Simultaneously, samples were subjected to mock digestion in buffer medium without the restriction enzymes. *Dde*I was not added to these mock-digested samples because the addition of *Dde*I did not significantly influence the allelic amplification ratios of both normal and skewed cell populations. To check the efficacy of *Hpa*II digestion, 1 μ g of plasmid HP33 control DNA was systematically added to a separate aliquot of 10 μ l of each sample mixture, incubated overnight, and run on a 1% agarose gel. In addition, a control sample of known clonality was included in each run to check for complete enzymatic digestion.

Amplification at HUMARA Locus

The digested and mock-digested DNA samples were purified using QIAquick spin columns (Qiagen, Valencia, CA) before the PCR reaction. Samples were amplified in the Applied Biosystems 9800 Fast Thermal Cycler (Applied Biosystems, Foster City, CA). All PCR samples were run in duplicate. The PCR mixture consisted of 10 μ l of GeneAmp Fast PCR master mix (Applied Biosystems), 1 μ l of forward primer (10 pmol/L), 1 μ l of FAM-labeled reverse primer (10 pmol/L), 5 μ l of sample DNA, and 3 μ l of H₂O in a total reaction volume of 20 μ l. Primer sequences used were as described by Allen and colleagues.⁹ PCR conditions were 30 seconds at 95°C, followed by 35 cycles composed of 5 seconds at 95°C and 25 seconds at 62°C, followed by a single step of 10 seconds at 72°C.

Fragment Analysis

The PCR products were diluted 1:10 with distilled water. One μ l of the diluted PCR product was mixed with 10 μ l of an internal size standard in deionized formamide. The GeneScan 500 ROX size standard (Applied Biosystems) was used as size marker. The PCR products were analyzed on an automated sequencer (3100 ABI genetic analyzer; Applied Biosystems). GeneScan software was used for quantification and interpretation of raw data output. Peak heights of fluorescence intensity were used to calculate ratios.

Data Interpretation

Results were interpreted after checking digestion efficacy of the plasmid control DNA and the clonal control sample. For each sample, the peak heights of the two alleles were measured and the peak height of the shorter allele (A1) was divided by the peak height of the longer allele (A2). Assessment was based on the major peak generated from each allele only, ignoring any stutter peaks. Patients were considered heterozygous when PCR of undigested DNA samples showed two major peaks. Patients with PCR products of undigested samples showing a single major peak were considered to be nonheterozygous for the HUMARA gene. These patient samples were excluded from further analysis because in these patients the HUMARA assay is not informative. Amplification bias of the shorter allele was compensated for by calculating a corrected ratio (CR_{ampl}). CR_{ampl} was determined by dividing A1 over A2 of the digested patient target sample by A1 over A2 of the mock-digested patient target sample. The use of CR_{ampl} corrects for the preferential amplification of the shorter allele that might occur if the two alleles differ markedly in length. We also compensated for possible skewing by dividing CR_{ampl} by CR_{skew} (CR_{tot} = CR_{ampl}/CR_{skew}) of the granulocyte control sample of the same patient. CR_{skew} was calculated by dividing A1 over A2 of the digested control sample by the A1 over A2 of the mock-digested control sample. This final CR_{tot} corrects for the potential skewing of the original X-chromosome inactivation in normal tissue. The CR_{tot} ratio was inverted if necessary to obtain a value of at least 1. A polyclonal cell population would be expected to show ratios equal or close to 1. A CR_{tot} value of ≥ 3.0 was considered to represent a significant degree of skewing and was considered proof of clonality of the purified cell population under study.⁷ Such CR_{tot} value indicates that one of the parental alleles is represented in excess of 75% or more in comparison to the other allele.

Results

Performance Testing

Before analyzing diagnostic patient samples, we evaluated the performance of the HUMARA assay. For this purpose, we selected samples from females showing clear heterozygosity for the HUMARA locus and did not show skewing of their control cell population. Firstly, we assessed reproducibility of the HUMARA assay. We performed the entire assay in triplicate on DNA samples from five healthy females, 16 to 26 years of age. We used DNA from MNCs as the target cell population and DNA from granulocytes as the control population. This resulted in a coefficient of variation (CV) for CR_{tot} of 13%. We performed similar analyses on DNA from five females with B-cell chronic lymphocytic leukemia (B-CLL). Mathematically, it was impossible to calculate one value for CV to describe reproducibility because some analyses resulted in an infinite high value of CR_{tot}. This was a consequence of total digestion of the active allele in MNC samples, so

Table 1. Results of HUMARA Assay Performed on Suspected NK Cell Population and Control Cell Population from Our Three Cases

	Case 1		Case 2		Case 3	
	NK fraction	Control fraction	NK fraction	Control fraction	NK fraction	Control fraction
% of NK cells	85	57/<1	60	5	98	<1
Undigested sample						
Peak height ratio of shorter over longer allele	1.42	1.39/1.43	0.64	0.87	2.54	1.93
Digested sample						
Peak height ratio of shorter over longer allele	9.41	4.83/1.31	14.59	2.53	0.69	1.11
CR _{ampl} [*]	6.62		22.87		0.27	
CR _{skew} [†]		3.47/0.92		2.92		0.58
Final result: CR _{tot} [‡]		1.91/7.22		7.83		2.12

*CR_{ampl} = peak height ratio shorter to longer allele of digested sample divided by peak height ratio shorter to longer allele of undigested sample from the NK cell population.

†CR_{skew} = peak height ratio shorter to longer allele of digested sample divided by peak height ratio shorter to longer allele of undigested sample from the control cell population.

‡CR_{tot} = CR_{ampl} divided by CR_{skew}; in case of values less than one, the ratio is inverted to obtain a value of one or higher.

that the peak height measured 0. Our impression was that imprecision was higher in these patients, without being able to precisely quantify it. Nevertheless, this seemed of less importance because the values of CR_{tot} were always much higher than the predefined cutoff value for clonality.

Then, to determine the sensitivity of the HUMARA assay, we performed cell-mixing experiments. We mixed an increasing amount of 100% digested clonal DNA from malignant B cells with mock-digested DNA from the same cell population. We performed two independent cell-mixing experiments, both in duplicate. One experiment was performed on a sample from a B-CLL patient in which the peak of the shorter allele of the clonal cells disappeared after digestion and another experiment in another patient in which the peak of the longer allele disappeared. These experiments resulted in a detection limit of 40 to 60% clonal cells in the former and of 20 to 40% in the latter. Consequently, a test performance might show considerable individual variation in relation to sample characteristics, eg, which of the two alleles is digested.

Finally, specificity of the HUMARA assay was determined on DNA samples from the five healthy females. All analyses resulted in a CR_{tot} of less than 3 (mean, 1.16; maximum, 1.85). We also analyzed samples from three patients with suspected T-cell proliferations, in which TCR gene rearrangement studies had been negative (two cases) or positive (one case). In the two patients with polyclonal T-cell proliferations, we found a CR_{tot} of 1.20 and 1.34, respectively. In the one patient with a clonal T-cell proliferation of ~59% of MNCs, we found a CR_{tot} of 4.82 with DNA from the MNC fraction, which is compatible with the presence of the clonal population.

HUMARA Assay in Cases with NK Cell Proliferations

The results of the HUMARA assay for our three cases are presented in Table 1 and are illustrated for case 2 in Figure 1. In case 1, we were at first not able to demonstrate a clonal NK cell population. Although the NK cell

population showed skewing with a CR_{ampl} of 6.62, a control cell population of granulocytes also showed skewing, albeit to a minor degree (CR_{skew}, 3.47). We believed the latter result was attributable to contamination of the control population with NK cells. We therefore repeated the analysis with another fraction of granulocytes that contained no detectable NK cells, and found a CR_{skew} of 0.92. This indicated that normal granulocytes did show random X-chromosome inactivation and that the skewing observed in the NK cell population was

Figure 1. Result of the HUMARA assay for patient in case 2. Results are representative for the other cases. Calculations of the ratios are presented in Table 1.

indeed attributable to clonal derivation (CR_{tot} 7.22). The results from this case illustrate the importance of the use of sufficiently pure cell populations, both for the suspected NK cell population and the control cell population. We note that in this patient, the parental alleles differed by only one CAG repeat, but we were still able to analyze the results reliably because we experienced only minor interference by stutter peaks. In case 2, the parental alleles differed by four CAG repeats. The NK cell populations showed a very high degree of skewing (CR_{ampl} 22.87). The control granulocytes showed some deviation from the theoretical 1:1 X-chromosome inactivation ratio, although there was no significant skewing present (CR_{skew} 2.92). After correction for the X-chromosome inactivation pattern of the control population, we still found significant skewing of the NK cells, compatible with clonality (CR_{tot} 7.83). In case 3, analysis of the NK cell population as such showed a slight degree of skewing (CR_{ampl} 0.27). However, after comparison to the control cell population (CR_{skew} 0.58), we could not demonstrate a significant degree of skewing (CR_{tot} 2.12). Therefore, we were not able to prove the clonal origin of the NK cell population in this case. Potential pitfalls in performance and interpretation of the HUMARA assay could be excluded as cause (see below). We controlled for preferential amplification bias and improper digestion. We used highly purified cell populations, and stutter peaks did not interfere with interpretation. However, several other explanations for this result are possible. First, the NK cell population in this case could be polyclonal in nature. This has been described previously for some other patients using other X-chromosome inactivation assays.^{12–15} Second, case 3 involves an elderly woman (>80 years of age). Because correlation regarding the degree of skewing of different cell populations decreases with age, it is possible that the control cell population used did not constitute a proper control for the physiological degree of skewing of normal NK cells in this patient.^{18,19} Third, although the cutoff for clonality as used in our assay is widely accepted in the literature, it has been arbitrarily chosen and has been clinically validated in only one study.²⁰ It showed a sensitivity of 82.5% in that study, which means that the result in our third case could be false negative. Finally, it is possible that *de novo* hypermethylation occurred at the HUMARA target of the NK cell proliferation, as described in cases of non-Hodgkin lymphomas.¹⁷ This would also result in a falsely polyclonal pattern. Because clonality was demonstrated by use of another target for an X-chromosome inactivation assay, ie, the DXS255 locus, we believe that the NK cell population in case three was indeed clonal in nature. It has to be noted also that at the DXS255 locus hypermethylation has been described in lymphoid leukemias and lymphomas.²¹ However, the demonstration of clonality by use of the M27 β probe makes this very improbable in this case. This case is an illustration of the fact that a cautious interpretation of results is important, especially in elderly patients.

Discussion

In the spectrum of LGL proliferations, at least four distinct types can be distinguished: reactive disorder, chronic lymphoproliferative disorder, leukemia, and aggressive leukemia.^{1,2} Although the demonstration of clonality is fundamental in the diagnosis of leukemia, clonality is difficult to prove in case of NK cell-derived LGL proliferations because of the lack of a specific genetic marker. The distinction especially between benign chronic lymphoproliferation and chronic phase of leukemia is hard to make.^{1–4} In contrast to their T-cell counterpart, NK LGL proliferations generally show germline TCR and Ig genes. In addition, cytogenetic studies are also of limited use.^{2,4} They are of most use in cases of aggressive NK cell leukemia, which seems geographically restricted to Asia.^{22,23} Because this form is often associated with EBV infection, detection of clonal EBV episomes is an alternative diagnostic tool in these patients.²³ However, Caucasian patients more often suffer from another form of NK cell-derived LGL proliferation, which is characterized by an indolent course with neither cytogenetic abnormalities nor EBV infection.^{24,25} A promising diagnostic tool in these patients are phenotypic studies using monoclonal antibodies directed against killer immunoglobulin-like receptors (KIRs) expressed on the surface of NK cells. In healthy patients, KIRs on NK cells constitute a broad repertoire. Recent studies in patients with NK cell-derived LGL proliferations have demonstrated restricted use of KIRs on NK cells.^{26,27} However, it is not clear whether KIR repertoire studies are really useful in effectively determining clonality because comparative studies are missing. Furthermore, routine use in the clinical laboratory is hampered by the limited commercial availability of these monoclonal antibodies to cover the broad repertoire of KIRs. Recently, a microarray-based study was published on NK-derived LGL proliferations.²⁸ Although microarray analyses are beyond the capability of most clinical laboratories, the authors identified several genes of which analysis of expression level might distinguish patients from controls. However, these results need further confirmation by clonality studies before they can be used as a diagnostic tool.

Until now, X-chromosome inactivation assays have been mostly used as a tool in experimental studies. When used in a diagnostic setting, these assays have mainly been applied in myeloid disorders.⁵ In NK cell-derived LGL proliferations, only a few studies have reported on the use of X-chromosome inactivation assays, with variable results.^{12–15} Nash and colleagues¹² tested 14 female patients with NK cell LGL lymphoproliferative disorder at the PGK or DXS255 locus. Only seven patients were heterozygous. In none of them could clonality of purified NK cells be demonstrated. Kelly and colleagues¹³ analyzed the X-chromosome inactivation pattern at the PGK locus in 17 female patients with NK cell LGL disorder. Six of them were heterozygous, and two of them showed clonality of the NK cell population. Results from both studies illustrate the lack of informativity of the PGK assay. Tefferi and colleagues¹⁴ targeted the hypoxanthine phosphoribosyltransferase gene region in one

Table 2. Pitfalls of HUMARA Assay with Consequences and Possible Solutions^{5,6}

Pitfall	Consequence	Possible solution*
Skewing in normal cell populations	Erroneous interpretation of the observed skewing as proof of clonality, in case of a polyclonal population	Use an embryologically related normal cell population, eg, granulocytes ^{18,19,33,34} Cautious interpretation in females older than 60 years of age ^{18,19,33}
Indirect measure of the <i>HUMARA</i> gene inactivation status	Loss of correlation with X-inactivation status in case of acquired loss of methylation or <i>de novo</i> methylation, resulting in loss of the property to provide information on the clonal origin of cell populations	Use a second methylation-sensitive restriction enzyme ^{18,20} Use a second genetic target (eg, DXS255 locus) ^{9,10,17}
Contamination with normal cells of suspected cell fraction under study, or vice versa	Lower detection limit of clonality	Use purified cell populations, both for suspected cell population under study and for the control cell population ^{30–32}
Incomplete digestion	X-inactivation pattern and ratio mimic those of a normal polyclonal cell population, in case of a clonal population	Use digestion control: 1) Spike sample with viral phage DNA or plasmid DNA (eg, HP33) ⁵ 2) Perform PCR of inactivation-escaping gene (eg, XE169) ²⁹ 3) Use a male DNA or well-characterized clonal female sample ⁵ Choose a methylation-sensitive restriction enzyme ^{9,32} Use a second methylation-sensitive restriction enzyme ^{18,20} Use a second, nonmethylation-sensitive, restriction enzyme (eg <i>Ddel</i> or <i>RsaI</i>) ^{10,17} Use bisulphite treatment and methylation-specific PCR ³⁵
Preferential amplification bias of shorter allele	Inaccurate X-inactivation pattern and ratio, inability to perform proper analysis	Avoid use of low-quality or low amounts of DNA, eg, from paraffin-embedded tissue ³⁶ Calculate corrected ratios by use of a mock-digested sample ⁵ Choose a methylation-sensitive restriction enzyme ^{9,32} Use a second, nonmethylation-sensitive, restriction enzyme (eg, <i>Ddel</i> or <i>RsaI</i>) ^{10,34} Modify PCR conditions, eg, decrease in number of PCR cycles or use 7-deaza-deoxy-guanidine triphosphate instead of dGTP ^{36,37}
Slippage phenomenon	Inability to identify correct allelic peaks and inability to perform accurate quantification of allelic peak heights	Exclude patients with alleles that differ less than two or three CAG repeats ^{10,31} Modify PCR conditions, eg, addition of BSA (10 to 15 μ g) ³⁸ Use mathematical models for correcting stutter artifacts ¹⁸

*Contradictory results of some solutions have been reported in the literature; because we cannot exclude that these differences are related to individual characteristics of a protocol, these solutions have to be evaluated on a protocol-specific base.

female patient with NK cell lymphocytosis. They demonstrated a clonal pattern of X-chromosome inactivation in the NK cell population, whereas a polyclonal pattern was obtained in control skin tissue.¹⁴ Recently, Lima and colleagues¹⁵ evaluated 26 patients with chronic NK cell lymphocytosis on clinical, phenotypical, and molecular features. They mentioned that in four females the HUMARA assay was used and that in all of them clonality of NK cells was found. However, no further details concerning the HUMARA assay were given.

We believe that in female patients the PCR-based HUMARA X-chromosome inactivation assay is a useful diagnostic tool to evaluate clonality in NK cell proliferations. However, one has to be aware of its limitations and pit-

falls. It is clear from the literature that the HUMARA assay suffers from a lack of standardization. This might restrict its clinical use as a diagnostic test. However, the impact of the differences in methodology is not clear because comparative data are missing. Nevertheless, the performance characteristics of our HUMARA assay are in line with those reported in previous studies.^{9,10,20,29–32} In addition, many pitfalls exist in the performance and interpretation of the HUMARA assay. One has to account for all these pitfalls in the clinical laboratory, as discussed in our three cases. They can result in faulty conclusions regarding clonality of the cell population under study.^{5,6} These pitfalls, their consequences, and possible solutions are presented in Table 2. First, detection of skewing

does not always imply the presence of a clonal cell population. A small number of healthy females shows skewing, and this number increases with age.^{19,30,39} Moreover, this skewing can be tissue-specific.^{18,33} Granulocytes constitute a proper control in case of lymphoid pathology, but caution is mandatory, especially in the elderly.^{18,19,33} Second, because the HUMARA assay is an indirect measure of X-chromosome inactivation, secondary alterations in methylation might result in a loss of correlation with the true status. This is suggested by differences in the status assessed at different loci.^{9,10,30,40} In addition, *de novo* methylation has been described in lymphomas, resulting in incomplete digestion and erroneous polyclonal patterns.^{17,41} Third, falsely polyclonal results can occur because of contamination of the suspected cells with normal cells. The use of purified cell populations helps to avoid this pitfall.^{30–32} Fourth, erroneous polyclonal patterns can also arise from problems with the digestion by the methylation-sensitive restriction enzyme. The use of appropriate controls is warranted.²⁹ Fifth, one should optimize one's assay to minimize bias attributable to preferential amplification of one of the alleles.^{10,34,36} In addition, normalizing all results to a mock digest sample can help to control this. Finally, slippage of the DNA polymerase on the CAG repeat should be restricted because it results in stutter peaks that might cause confusion in identification of the correct peaks and preclude accurate quantitative analysis.^{9,10,31,32}

In conclusion, we present a protocol for the HUMARA assay, an X-chromosome inactivation clonality assay. We demonstrated that the HUMARA assay performs well and that the assay is capable of detecting clonality in female patients with NK cell-derived LGL proliferations. We believe this assay to be a practical and useful diagnostic tool in the workup of patients with NK cell proliferations. However, one should bear in mind that appropriate controls should be used at all levels of analysis and that potential pitfalls should be accounted for during performance and interpretation.

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