Clonality Assay of Hematopoietic Disorders: Significance of the Buccal Epithelium as Non-hematopoietic Control and of 95% Rejection Limit as a Novel Criterion for Monoclonality

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In clonality assays using X chromosome inactivation patterns (XCIPs), several factors such as constitutive and acquired XCIP skewing, lack of appropriate controls for hematopoietic diseases including multilineage disorders, and ambiguous criteria for monoclonality, have complicated determination of clonality. To address these issues, we studied the significance of the buccal epithelium as a non-hematopoietic control and the usefulness of the 95% rejection limit as a criterion for monoclonality. Sixty-nine females informative for human androgen receptor gene (*HUMARA*) were divided into "young," "middle-aged" and "elderly" groups. When XCIP correlation between the buccal epithelium, peripheral granulocytes, and peripheral lymphocytes was analyzed, the buccal epithelium showed a good correlation with granulocytes and lymphocytes in "young" and "middle-aged" groups, whereas the correlation was poor for the "elderly" group. For all age groups, there was an excellent correlation between granulocytes and lymphocytes. When we performed clonality assay for seven "young" and "middle-aged" patients with various leukemic phases using buccal epithelium as a non-hematopoietic control, all cases were accurately evaluated with the aid of a novel criterion, the 95% rejection limit. Our findings suggest that the buccal epithelium may constitute an effective control, especially when a non-hematopoietic control is required, and that the 95% rejection limit may serve as a statistically-defined criterion for monoclonality.

Key words: HUMARA clonality assay — Hematopoietic disorders — Buccal epithelium — 95% rejection limit

Clonality assays using X-chromosome inactivation patterns (XCIPs) have provided valuable data for establishing the current concepts of hematological disorders. Although their application is restricted to informative females, these clonality assays have several advantages over cytogenetic analysis, somatic mutation determination, or viral integration analysis in that they do not require any tumor-specific markers. Clonality is essentially a quantitative definition and does not equate with malignancy. However, recognition of clonal cells in a lesional tissue may have both biological and clinical implications.

Glucose-6-phosphate dehydrogenase isoenzyme was for many years the only suitable X-linked polymorphic marker.[1] Unfortunately, assays using this protein are limited by the low frequency of the protein polymorphisms in most racial groups. However, it is now possible to examine XCIPs in the majority of females, by using a number of X-linked foci generated with DNA induction techniques.[2] Paternal and maternal genes of informative females can be distinguished either by the presence of a restriction enzyme polymorphism, e.g. phosphoglycerate

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kinase and hypoxanthine phosphoribosyl transferase, or a variable number tandem repeat sequences, e.g. the DXS255 locus recognized by the probe M27β and the human androgen receptor gene (*HUMARA*). Because active and inactive X-chromosomes show different methylation patterns, they can be distinguished by methylation-sensitive restriction enzymes, such as *Hpa*II and *Hha*I. Currently, polymerase chain reaction (PCR)-based clonality assays for the *HUMARA* locus are widely used because the assays can be performed rapidly with only a small amount of DNA and polymorphism at this locus is frequently observed. The results of HUMARA methods correlate well with those of Southern blot-based assays for phosphoglycerate kinase, hypoxanthine phosphoribosyl transferase, or M27β.[3]

Although many hematological disorders have been investigated for clonal status by using these modern techniques,[4–9] interpretation of the XCIPs and determination of monoclonality are sometimes problematic. The first reason is that XCIPs of hematopoietic cells vary in females. Some neonates show extremely skewed XCIPs (constitutive skewing) in their hematopoietic cells. In addition, recent studies[10–13] have shown that aging signifi-

cantly influences XCIPs (acquired skewing). Secondly, when performing clonality assays, pathological samples should be compared with their normal counterparts, which should provide the original X-inactivation patterns of the tissues being examined. However, XCIP correlation between hematopoietic and non-hematopoietic tissues has not been established.[13, 14] For this reason, lymphocytes are often used as controls when myeloid disorders are investigated.[8] In some myeloid disorders, however, it is difficult to completely exclude the possibility that lymphocytes are included in the neoplastic clones[2] while the widely accepted concept of multilineage dysplasia involves further complications.[15] Therefore, non-hematopoietic tissues will be necessary as a normal control in clonality assay for hematopoietic disorders. Finally, there are currently no definite criteria for distinguishing between monoclonal and polyclonal processes even when suitable controls have been obtained. In previous studies, a lesion was considered to be monoclonal when the lesional XCIP was "considerably" skewed in comparison with the control XCIP, but the degree of this skewing varied with each study. Distinguishing criteria should therefore be statistically defined and established.

To address these issues, we analyzed the XCIP correlation between peripheral granulocytes, peripheral lymphocytes, and the buccal epithelium obtained from normal females belonging to different age groups. In addition, we introduced the 95% rejection limit as a novel criterion for monoclonality in order to interpret accurately the clonal status of patients with various leukemic phases.

MATERIALS AND METHODS

Normal females Both peripheral blood and buccal epithelial samples were obtained from 82 healthy female volunteers. Informed consent had been obtained from all participants. The healthy volunteers were hematologically normal with full blood counts and white cell differentials normal for their age. The initial screening of blood samples for establishing heterozygous status at the *HUMARA* loci revealed that 69 (84%) of the normal females were heterozygous for *HUMARA*, and all of them were included in the present study. They were divided into three age groups, 22 "young" adults (age range, 18–39; median, 25 years); 21 "middle-aged" adults (age range, 41–67; median, 50 years); and 26 "elderly" adults (age range, 70–92; median, 80 years). Peripheral blood samples (20 ml) were separated into polymorphonuclear and mononuclear cell fractions by means of standard density gradient centrifugation (Ficoll-Paque, Amersham-Pharmacia Biotech, Tokyo). The purity of the granulocytes in the polymorphonuclear fractions was more than 90% when cytologically assessed after lysis of the erythrocytes with hypotonic saline. Monocytes were removed from the mononuclear fractions by gentle incubation on plastic trays for 3 h at room temperature. The resultant fraction contained more than 90% lymphocytes.

Leukemic patients Nine female patients with *de novo* leukemias were included in the present study after their informed consent had been obtained. Seven of these patients were informative for the *HUMARA* locus (Table I); four of them had acute myeloid leukemia (AML), one acute lymphoid leukemia (ALL), and two chronic myeloid leukemia (CML). One of the AML patients (case 4) was in complete remission (CR), one CML patient was in a myeloid crisis (case 6), and the other was in the chronic phase (case 7). Peripheral blood samples were obtained from all the patients. Mononuclear fractions were retrieved from six acute leukemias and one blastic phase CML. In one of the three non-remissive AML cases, samples were col-

Table I. Profiles of Leukemic Patients and Results of Clonality Assay

Case	Age (Group)	Leukemia[a]	% tumor[b]	Lineage	Tumor %LA[c]	BE %LA	"Normal" %LA[d]	Results	Plot No.[e]
1.	27 (Young)[f]	AML (M3)	83[g]	Gran	88 (MNC)	59	36–80	Abnormal	I
2.	60 (Middle)[h]	AML (M1)	87	Gran	5 (MNC)	68	32–90	Abnormal	II
3.	68 (Middle)	AML (M2)	94	Gran	11 (MNC)	59	24–82	Abnormal	III
		AML (M2)-CR	0	Gran	48 (MNC)	59	24–82	Normal	IV
4.	66 (Middle)	AML (M2)-CR	0	Gran	45 (MNC)	42	8–66	Normal	V
5.	59 (Middle)	ALL (L2)	75	Lym	82 (MNC)	54	23–76	Abnormal	VI
6.	36 (Young)	CML-Mc	95	Gran	13 (MNC)	60	37–81	Abnormal	VII
7.	66 (Middle)	CML-Cp	1[i]	Gran	4 (PMN)	47	13–71	Abnormal	VIII

%LA, % lower allele; MNC, mononuclear cell; BE, buccal epithelium; AML, acute myeloid leukemia; CR, complete remission; ALL, acute lymphocytic leukemia; CML, chronic myeloid leukemia; Mc, myeloid crisis; Cp, chronic phase; Gran, granulocyte; Lym, lymphocyte; MNC, mononuclear cell fraction; PMN, polymorphonuclear cell fraction; *a*) tumor subtype is shown in parenthesis; *b*) % tumor is cytologically estimated; *c*) subject fraction is shown in parenthesis; *d*) "normal" %LA is calculated according to the 95% rejection limit (Table II); *e*) patients are plotted in Fig. 2 according to age and lineage; *f*) young (18–39 years old); *g*) blasts + promyelocytes; *h*) middle (41–67 years old); *i*) blastic cell count.

lected in the CR as well as the leukemic phase (case 3). Polymorphonuclear fractions were obtained from one case with chronic phase CML. Blood samples were subjected to analysis after monocytes had been removed from them. The percentage of cytologically assessed tumor cells per leukemic patient is shown in Table I.

Buccal epithelial samples The epithelial cells of the buccal mucosa were obtained from both normal and leukemic females by scraping the buccal cavity several times with a cotton swab. A Giemsa-stained cytological smear was immediately prepared to ensure the retrieval of adequate buccal epithelial cells in order to prevent sampling bias. Especially with the leukemic females, great care was taken to prevent hematopoietic cell contamination. Sample purity assessed morphologically was >95% buccal epithelial cells. The cotton swab was rinsed in phosphate-buffered saline, and buccal epithelial cells were collected after mild centrifugation.

Sample manipulation High-molecular-weight DNA was prepared by proteinase K/detergent digestion with phenol/chloroform extraction and ethanol precipitation. The X-chromosome inactivation status was established by PCR analysis of DNA methylation at the *HUMARA* loci by means of a fluorescent modification.[16, 17] DNA was incubated overnight at 37°C with 20 units of methylation-sensitive *Hpa*II in a final volume of 20 μl. In the control tube, the same amount of DNA was incubated under similar conditions except for the absence of the restriction enzyme. After digestion, DNA was purified by phenol/chloroform extraction. The PCR mixture consisted of 10 m*M* Tris (pH 8.4), 50 m*M* KCl, 0.01% gelatin, 1.5 m*M* MgCl$_2$, dNTP (0.2 m*M* each), 0.5 μ*M* each of the primers,[16] AR1 (5′-TCCAGAATCTGTTCCAGAGCGTGC-3′) and AR2 (5′-GCTGTGAAGGTTGCTGTTCCTCAT-3′), 0.5 units of *Taq* DNA polymerase, and template DNA. To perform fluorescent PCR, the AR2 primer was modified by means of incorporation of fluorescein at the 5′ end. One cycle of PCR consisted of 60 s at 95°C and 60 s at 60°C. A total of 25 cycles of amplification was performed after initial DNA denaturation at 95°C for 5 min. The PCR products were then mixed with formamide loading buffer, denatured at 95°C for 3 min, and run on a Shimadzu DNA sequencer DSQ 500S (Shimadzu Corp., Kyoto) in an 8% polyacrylamide denaturing gel containing 12% deionized formamide. After migration for about 5 h, fluorescent peaks were quantified with Shimadzu DSQ software (Shimadzu). Results were reported as the mean percentage expression of the lower allele (%LA) from at least two analyses. %LA was defined as the ratio of the lower-molecular-weight allele to the sum of both alleles in *Hpa*II-digested samples, following normalization for band intensities in the undigested samples[11, 14]: %LA = normalized lower allele/(normalized lower + higher alleles). For example in Fig. 1B, the normalized ratio of lower (left) and
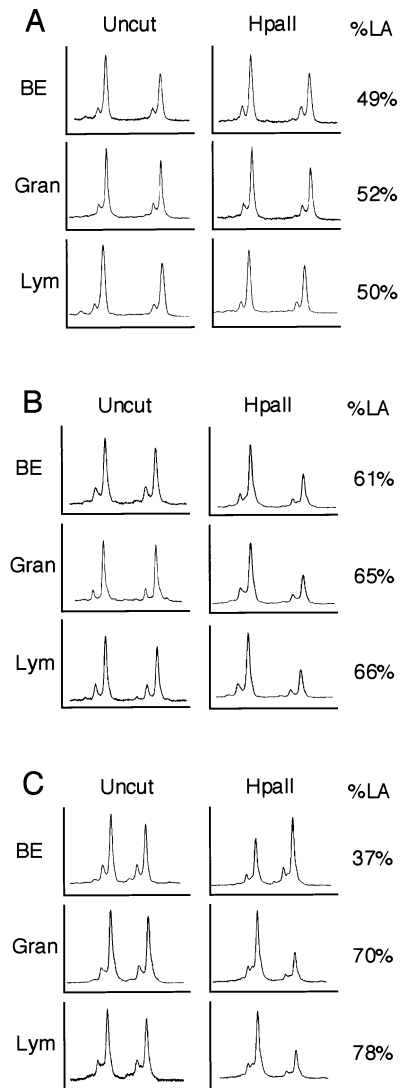

Fig. 1. Representative results of HUMARA assay for "young" (A), "middle-aged" (B), and "elderly" (C) females. The lower allele is the left of the two peaks. Uncut, no *Hpa*II treatment; *Hpa*II, treated with a methylation-sensitive restriction enzyme, *Hpa*II; %LA, percent lower allele, expression of the lower allele after normalization for band intensities with the uncut sample. Values are means of more than two experiments. BE, buccal epithelium; Gran, granulocyte; Lym, lymphocyte.

higher (right) alleles for the buccal epithelium (BE) is 61:39. %LA is therefore calculated as 61%. The median difference between the two analysis was 3% (average, 3.6%; range, 0% to 17%).

Ninety-five percent rejection limit and other statistical analyses Association between buccal %LA, granulocytic %LA, and lymphocytic %LA was analyzed for each of the three age groups. A simple regression line, a correlation

coefficient (r), and 95% rejection limit curves were calculated. The 95% rejection limit curves yielded limits of the criterion variable (Y) for an explanatory variable (X) with 95% accuracy. The area between the upper and lower 95% rejection limit curves represents the 95% rejection limit.[18]

RESULTS

XCIP correlation of buccal epithelial cells with peripheral granulocytes and lymphocytes in normal females DNA samples digested with or without *Hpa*II were amplified by fluorescent PCR for *HUMARA* loci. XCIPs were obtained by running the PCR products through 8% poly-

acrylamide denaturing gels containing 12% deionized formamide. Fig. 1 shows representative results obtained with this HUMARA assay for normal females.

XCIP correlation of the buccal epithelium with granulocytes and lymphocytes was analyzed in three different age groups, "young" ($n=22$), "middle-aged" ($n=21$), and "elderly" ($n=26$) females. Fig. 2 (A–I) shows a simple regression line, correlation coefficient (r), and 95% rejection limit for each age group. The expressions of the 95% rejection limit curves are shown in Table II. The "young" group showed an excellent correlation of buccal %LA with granulocytic %LA ($r=0.91$, Fig. 2A). The 95% rejection limit area, showing the "normal" range of granulo-
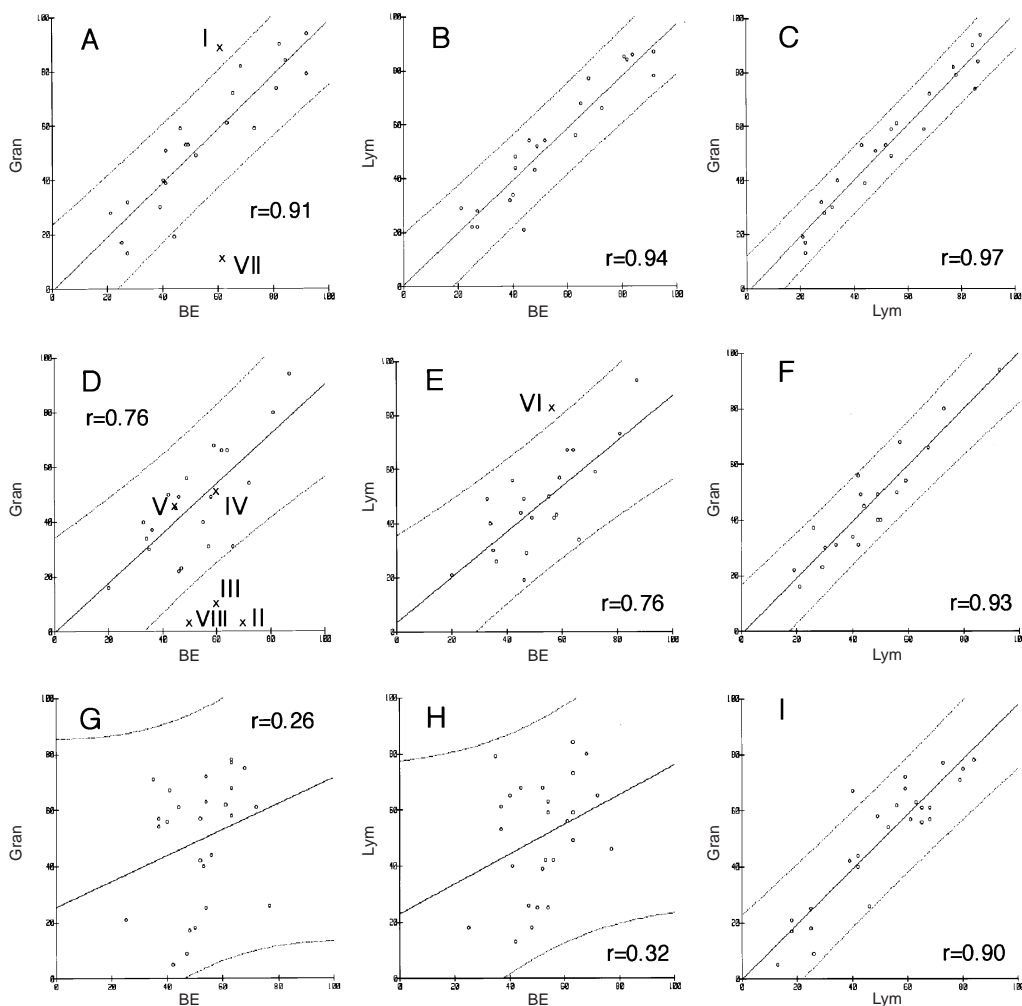

Fig. 2. Correlation in XCIP between the buccal epithelium (BE), granulocyte (Gran), and lymphocyte (Lym). Simple regression line, correlation coefficient (r), and 95% rejection limit curves are shown in each chart. Plot ($\times$) represents the leukemic cases of various phases (I–XIII, see Table I). A–C, "young" group (18–39 years old); D–F, "middle-aged" group (41–67 years old); G–I, "elderly" group (70–92 years old).

Table II. Expression of 95% Rejection Limit Curves for Young, Middle-aged and Elderly Groups

Group	X, Y	Expression
Young (18–39 years old)	BE, Gran	$Y=-1+X\pm2.1\sqrt{\{108+0.010(X-55)^2\}}$
	BE, Lym	$Y=0.97X\pm2.1\sqrt{\{66+0.0060(X-55)^2\}}$
	Lym, Gran	$Y=-2+X\pm2.1\sqrt{\{34+0.0030(X-53)^2\}}$
Middle-aged (41–67 years old)	BE, Gran	$Y=-1+0.91X\pm2.1\sqrt{\{187+0.032(X-52)^2\}}$
	BE, Lym	$Y=4+0.84X\pm2.1\sqrt{\{158+0.027(X-52)^2\}}$
	Lym, Gran	$Y=-1+X\pm2.1\sqrt{\{55+0.0078(X-47)^2\}}$
Elderly (70–92 years old)	BE, Gran	$Y=25+0.46X\pm2.1\sqrt{\{505+0.13(X-52)^2\}}$
	BE, Lym	$Y=23+0.53X\pm2.1\sqrt{\{413+0.10(X-52)^2\}}$
	Lym, Gran	$Y=0.98X\pm2.1\sqrt{\{100+0.0090(X-51)^2\}}$

BE, buccal epithelium; Gran, granulocyte; Lym, lymphocyte.

cytic %LA calculated from the buccal %LA, was narrow. There was an equally excellent correlation between buccal %LA and lymphocytic %LA ($r=0.94$, Fig. 2B), as well as lymphocytic %LA and granulocytic %LA (Fig. 2C, $r=0.97$), each with a narrow 95% rejection limit. The "middle-aged" group showed a good correlation of the buccal epithelium with granulocytes (Fig. 2D, $r=0.76$) and with lymphocytes (Fig. 2E, $r=0.76$), and the 95% rejection limit areas were slightly wider than those of the "young" group. Lymphocytes correlated excellently with granulocytes (Fig. 2F, $r=0.93$), with a narrow 95% rejection limit. In the "elderly" female group, the buccal epithelium correlated poorly with both granulocytes and lymphocytes (Fig. 2G, $r=0.26$ and Fig. 2H, 0.32, respectively). The 95% rejection limit areas were much wider than for the other two age groups. However, a good correlation between granulocytic and lymphocytic %LAs was maintained in this age group, too (Fig. 2I, $r=0.90$). There was thus a clear trend for the correlation between the three elements to be strongest for the "young" group and weakest for the "elderly" group.

Clinical significance of 95% rejection limit for leukemic patients Fig. 3 shows representative results of the HUMARA assay for leukemic patients. Leukemic samples were evaluated for clonality by using the buccal epithelium as a control and the 95% rejection limit as the criterion for monoclonality. It should be noted that the mononuclear fractions of AML patients after monocyte removal were not of single lineage derivation: they usually contained myeloid tumor cells and non-neoplastic lymphocytes.[15, 19–21] However, regression analysis of the normal "young" and "middle-aged" groups showed an excellent XCIP correlation between granulocytes and lymphocytes ($r>0.93$, Fig. 2), so that these two elements were considered to be almost identical from the statistical point of view. Therefore, the entire mononuclear fraction of AML
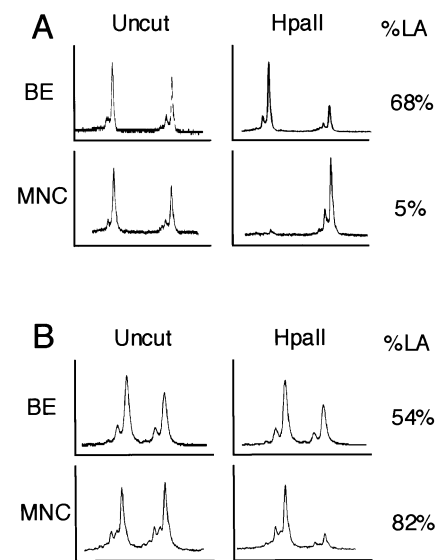

Fig. 3. The representative results of HUMARA assay for leukemic patients. A, AML patient, case 2; B, ALL patient, case 5 (Table I). The lower allele is the left of the two peaks. In both cases, X-chromosome inactivation patterns are extremely skewed in the mononuclear fraction in comparison with that of the buccal epithelium used as a control. For abbreviations, see the legend to Fig. 1.

patients was considered to consist of myeloid tumor cells and non-neoplastic granulocytes regardless of the lymphocytic involvement in the neoplastic clones.

When AML cases in the leukemic phase (cases 1, 2, and 3, Table I) were plotted on buccal epithelium-granulocyte charts according to age, all cases fell outside the 95% rejection limits, i.e., outside of the "normal" limits esti-
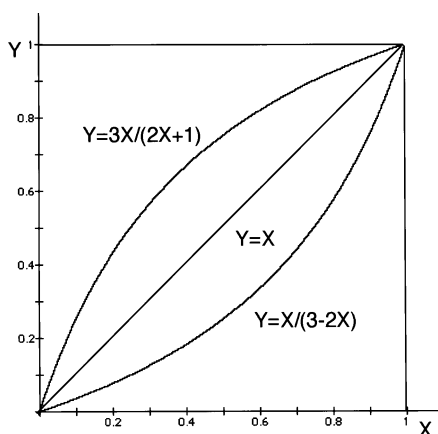
Fig. 4. Theoretical "normal" limits using a criterion of "triple skewing." The area between the curves shows the "normal" limit for X that perfectly correlates with Y using a "triple skewing" criterion for monoclonality: a lesion is considered to be monoclonal when the lesional XCIP (corresponding to Y) is "3 times" greater or less than control XCIP (corresponding to X).

mated from the buccal %LA of the patients (Fig. 2, I–III, for cases 1–3, respectively). Two AML cases in CR (cases 3 and 4, Fig. 2, IV and V, respectively) could be plotted within normal limits. One ALL (case 5, Fig. 2, VI) with 75% tumor cells in the mononuclear fraction was interpreted as "abnormal" on the basis of plotting on the buccal epithelium-lymphocyte chart. The %LA of the mononuclear sample of CML in myeloid crisis (case 6, Fig. 2, VII) fell outside "normal" limits, while CML in the chronic phase (case 7, Fig. 2, VIII) showed a skewed XCIP in the polymorphonuclear fraction resulting in this case being interpreted as "abnormal."

DISCUSSION

In hematopoietic disorders, interpretation of XCIP changes in clonality assays has been complicated by several factors, including constitutive and acquired XCIP skewing of the hematopoietic cells, lack of appropriate controls, and ambiguous criteria for clonality. Our study showed that XCIP of the buccal epithelium correlated well with those of peripheral granulocytes and lymphocytes in "young" and "middle-aged" females. In addition, the 95% rejection limit provides statistical grounds for distinguishing monoclonal from polyclonal processes. Using the buccal epithelium as a control and employing the 95% rejection limit as a distinguishing criterion, all seven leukemic cases were accurately interpreted.

Recent studies show that many neonatal blood samples have skewed XCIPs.[10, 11, 13] This constitutive skewing is partly explained by the pool size at the time of random X-

chromosome inactivation. The pool size of hematopoietic cells has been estimated as 6–16 cells.[11, 13, 22] Acquired skewing is another factor that renders XCIP interpretation difficult. Several hypotheses have been proposed for acquired skewing: a change in methylation status, selective advantage of one X-chromosome, stem cell depletion or exhaustion, emergence of a true clonal hematopoiesis, and changes in stem cell usage. However, no definitive explanation has been established to date.[13] Although the precise mechanisms of constitutive and acquired XCIP skewing have not been fully clarified, the results of clonality assays using XCIPs have led to the general acceptance of age as one of the critical factors.

When performing clonality assays, pathological samples should be compared with their normal counterparts, which yield the original XCIPs of the tissues being examined. Lymphocytes are derived from the same embryonic precursors as those of hematopoietic stem cells and are therefore supposed to show the same inactivation pattern as normal granulocytes. We showed strong concordance between granulocytic and lymphocytic XCIPs for all age groups ($r > 0.90$). This finding supports the data presented by Gale et al.[11]: they found an excellent correlation in XCIP between lymphocytes and granulocytes for normal females aged 17 to 50 years and a fairly good correlation for females aged ≥ 75 years. On the other hand, Toron et al.[13] obtained a somewhat poor correlation between lymphocytes and granulocytes for females more than 75 years of age. Overall, these data indicated that it is currently appropriate to consider that there is a good correlation between lymphocytes and granulocytes, at least in "young" and "middle-aged" females. In previous studies, lymphocytes were often used as controls when investigating myeloid disorders,[2, 8] although it is important to exclude neoplastic clone involvement in the lymphocytic lineage.

Non-hematopoietic controls are important as a reference for hematopoietic disorders. Some investigators[19–21] have suggested that the AML origin is not homogeneous. Similarly, more than one line is involved in the neoplastic clone in multilineage dysplasia.[15] However, whether non-hematopoietic tissues can serve as controls for hematopoietic disorders has been controversial.[14] Our study showed that XCIPs of the buccal epithelium correlated well with those of granulocytes and lymphocytes in the "young" and "middle-aged" groups, suggesting that the buccal epithelium is a good non-hematopoietic control in the "younger" population. The usefulness of non-hematopoietic tissues as controls for hematopoietic cells documented in our study was previously examined in a study by Gale et al.[14] They reported that the skin was not suitable as a control tissue for the interpretation of XCIPs in hematopoietic cells, showing that the granulocytes correlated poorly with cutaneous tissues. At that time, however, the importance of

age for clonality assays was not known. When we re-evaluated the raw data used by Gale *et al.* and took the subjects' age into consideration, we found that there was a good correlation in XCIPs between hematopoietic cells and non-hematopoietic tissues (skin and muscle) for normal females under 56 years in age (Table I, Gale *et al.*, reference 14). These data partly support our finding that non-hematopoietic tissues can be used as a control for hematopoietic cells for "young" and "middle-aged," but not for "elderly" females. We speculate that the buccal epithelium and hematopoietic cells have a similar XCIP in a female at birth. Acquired XCIP skewing, probably an event independent of constitutive skewing, may proceed gradually with age, and finally become prominent in "elderly" females, resulting in a poor XCIP correlation between the buccal epithelium and hematopoietic cells.

Whether hair bulbs, which are non-hematopoietic tissues, represent an effective control of hematopoietic cells has been investigated.[13] In contrast to the buccal epithelium, hair bulbs were reported not to correlate well with polymorphonuclear cells. Several factors may account for this discrepancy, such as the small amounts of DNA obtained from hair bulbs and the number of samples analyzed, in addition to the actual differences in the tissues analyzed.

One of the problems that render the interpretation of clonal assay ambiguous is that there have been no definite criteria for distinguishing monoclonal from polyclonal processes even when appropriate controls are obtained. These criteria have been arbitrarily defined in previous studies. For example, a lesion is considered to be monoclonal when the lesional XCIP is "considerably" or "3 times" greater (or less) than control XCIP. Such criteria have no statistical basis. Fig. 4 shows the theoretical "normal" limits when the criterion of "triple skewing" is applied to parameter Y (lesional %LA) which perfectly correlates ($Y=X$, $r=1$) with parameter X (control %LA). The "normal" limits for an X of 0.5 are 0.25–0.75, which are similar to those when the buccal epithelium is used as a control for the "middle-aged" group (Fig. 2, D and E). However, when X approaches 0 or 1, i.e., skewing of control XCIP, the "normal" limit area becomes narrower, indicating that the assay becomes more sensitive in cases with extremely skewed controls than in those with minimally skewed controls. This increase in sensitivity does not match our experience; many investigators have excluded cases with extremely skewed controls from clonality assays.[22–24] We concluded that the "triple skewing" criterion is not sufficient for accurate determination of monoclonality, and therefore introduced a novel criterion for monoclonality, 95% rejection limit, according to which minimally skewed controls give superior sensitivity.

The present approach for clonality assay may give proof of clonality in individual patients, and serve for a comprehensive understanding of clonality assay using XCIP. However, there are still some issues to be considered; the first is that it should be verified into how many groups normal females should be categorized in order to obtain the reference 95% rejection limits. We divided the normal females into three age groups, "young," "middle-aged" and "elderly," but a more precise grouping might be necessary. There is currently no statistical method for entering age as an independent explanatory variable into the calculation of the 95% rejection limit. The second is that when the buccal epithelium is collected in leukemic patients, white blood cells often get mixed in the sample. White blood cell contamination influences the XCIP interpretation and should be minimized. This can be achieved by filtering the buccal samples, because the buccal squamous cells are much larger in size than hematopoietic cells (unpublished data). The last issue to be considered is that the assay using the buccal epithelium as control is not highly sensitive. For example, a "middle-aged" ALL patient with 75% tumor cells in her mononuclear fraction (case 5, Table I) was barely interpreted as "abnormal" (Fig. 2E, plot No. VI). However, the sensitivity should be enhanced by using hematopoietic cells (lymphocytes as a control for myeloid disorders and *vice versa*) when the single lineage derivation of the disease is established. Cell sorting technique should also enhance the sensitivity by increasing the percentage of tumor cells in the hematopoietic fractions. This technique will allow more precise investigation; clonality assay for each lineage fraction obtained by cell sorting may provide new insights into the pathogenesis of various hematopoietic disorders.

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