Evaluation of 13q14 Status in Patients with Chronic Lymphocytic Leukemia Using Single Nucleotide Polymorphism-Based Techniques

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Deletions of chromosome 13q14 are common in chronic lymphocytic leukemia and other cancers, demonstrating the importance of this region in tumorigenesis. We report the use of two single-nucleotide polymorphism (SNP)-based techniques to determine 13q loss of heterozygosity (LOH) status in 15 patients with CLL: (i) digital SNP (dSNP), where analysis of heterozygous SNPs detects allelic imbalances, and (ii) DNA sequencing, where LOH is identified by comparison of allelic peak heights in normal and neoplastic cells. The SNP-based techniques were compared with established molecular techniques, fluorescence *in situ* hybridization and multiplex ligation-dependent probe amplification, to determine their utility and relative sensitivity. dSNP proved to be the most sensitive technique, identifying 13q14 LOH in 11 of 13 (85%) patients (95% CI: 55%, 98%) without the need for neoplastic cell enrichment. Three cases showed evidence of LOH by dSNP that was not apparent by other techniques. In 8 of 13 (62%) cases, partial or interstitial patterns of LOH were observed by dSNP. Our findings demonstrate that dSNP represents a useful, sensitive technique for the analysis of chromosomal aberrations that result in LOH. It may have applications for the analysis of other malignancies that are difficult to assess by conventional molecular techniques. (J Mol Diagn 2009, 11:298–305; DOI: 10.2353/jmoldx.2009.080167)

Chronic lymphocytic leukemia (CLL) is the most common leukemia among adults in the Western world, occurring predominantly in individuals over the age of 65 years.¹ It is a heterogeneous disease associated with a highly variable clinical course.² At present, the molecular etiology of CLL remains largely undetermined. However, it is widely acknowledged that the disease, like most

cancers, is associated with marked chromosomal instability.³ Fluorescence *in situ* hybridization (FISH) has identified recurrent chromosomal abnormalities in approximately 80% of CLL patients, and specific genomic aberrations now serve as prognostic indicators for disease progression.³

The most frequent chromosomal aberration in CLL is the loss of genetic material from the long arm of chromosome 13. Chromosome 13q14 deletions are reported in over 50% of CLL cases and are associated with a favorable prognosis when present as the sole genomic abnormality.³ The deletion of chromosome band 13q14 has also been reported in a variety of other malignancies,^{4–7} leading researchers to postulate that this chromosomal region harbors tumor suppressor gene(s) involved in the etiology of these diseases. Delineation of the minimal deleted region at 13q14 may facilitate the identification of such genes.

Several well-established techniques are available for the detection and characterization of chromosome 13q14 deletions. These include metaphase cytogenetics,⁸ interphase-FISH,³ array-based comparative genome hybridization,⁹ single nucleotide polymorphism (SNP) arrays¹⁰ and multiplex ligation-dependent probe amplification (MLPA).¹¹ While extremely useful, each technique has limitations and no technique is universally applicable. Conventional cytogenetic analysis has been traditionally hampered by the low mitotic activity of CLL cells. FISH analysis is limited to the genomic regions covered by the probes; therefore, deletions not covering these specific regions may remain undetected. Low density SNP arrays may fail to identify cases where the deleted region is very small,¹² whereas both array-based comparative genome hybridization and MLPA are acknowledged to have limited sensitivity for the detection of aberrations present in a low percentage of cells.^{9,11} Consequently, there is a need for a sensitive technique that allows high-resolution screening of chromosome band 13q14.

We report here the novel application of two molecular techniques, digital single nucleotide polymorphism (dSNP) and loss of heterozygosity (LOH) DNA se-

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quencing, for the characterization of LOH at chromosome 13q14 in patients with CLL. Both techniques rely on the genotypic analysis of heterozygous SNPs to determine LOH status.

Digital SNP is a sophisticated innovative technique that allows identification of LOH through the direct counting of alleles.¹³ Briefly, the technique relies on the identification of heterozygous SNPs in patient samples and subsequent separation of the heterozygous alleles through serial dilution of the patient DNA. PCR amplification of the heterozygous alleles is performed and the allelic frequencies counted. Deviation from the expected 50:50 ratio for heterozygous alleles represents LOH at that specific locus and is highly suggestive of a deletion. Sequential probability ratio testing (SPRT)¹⁴ confirms the significance of such deviations.

DNA sequencing may be used to determine LOH status through the comparison of heterozygous SNPs in neoplastic and normal cells. Briefly, paired patient DNA samples from isolated mononuclear cells and non-neoplastic buccal mucosa cells are obtained for each patient. Heterozygous SNPs present in the buccal DNA samples are compared with mononuclear cell DNA samples by PCR/sequence analysis. The quantification of heterozygous alleles is determined using Mutation Surveyor software (version 3.2, SoftGenetics, State College, PA). Tumor cells harboring LOH show reduced quantities of a heterozygous allele. The reduced allele quantity is not noted in the normal buccal mucosa cells.

The aim of this study was to assess the utility and relative sensitivity of SNP-based technology to identify and characterize 13q14 LOH status in a cohort of 15 patients with CLL. High-resolution screening of chromosome band 13q14 may allow for the delineation of the minimal deleted region and identification of candidate tumor suppressor genes for further investigation.

Materials and Methods

Patients

Fifteen patients with CLL under the care of the Hematology Unit at the Royal Devon and Exeter Hospital were enrolled in this study. Each patient had an established diagnosis of CLL based on current World Health Organization classification guidelines.¹⁵ Patients had a history of persistent lymphocytosis $>5 \times 10^9/L$ and an immunophenotypic profile typical of CLL, confirmed by a flow cytometry score of ≥ 4 in all cases.¹⁶ The majority of patients (13/15) had received treatment for their disease before involvement in this study. The study protocol had received prior approval by the local research ethics committee and all patients provided written informed consent in accordance with the Declaration of Helsinki.

Sample Preparation

DNA was extracted from whole blood using the Wizard Genomic DNA purification kit (Promega, Southampton, UK) according to the manufacturer's instructions. Mono-

nuclear cells were isolated through Lymphoprep gradient centrifugation (Axis Shield, Oslo, Norway) and processed for DNA extraction using the Wizard Genomic DNA purification kit (Promega, Southampton, UK). DNA concentrations were measured using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington). Normal genomic DNA was extracted from non-neoplastic buccal mucosa cells using standard procedures. Cells were collected by rubbing a cytology brush against the inside of the patient's cheek. The cells were lysed in 50 mmol/L NaOH and DNA was precipitated in 1M/L Tris/HCL (pH 7.5).

Digital SNP

A panel of 10 SNP markers covering the 13q14.2 to 13q34 region of chromosome 13 (Figure 1A) was chosen on the basis of high population heterozygosity (0.3 to 0.5). Probes and primers to these polymorphisms were designed by the Applied Biosystems Assays-on-Demand service (ABI, Foster City, CA) (probe and primer sequences are available in Table 1). In each case, allele-specific probes were labeled 5' with either 6-FAM or VIC and 3' with a minor groove binding (MGB) protein serving as a non-fluorescent quencher. Assays were validated using standard curves generated from seven serial (1:2) dilutions of heterozygous DNA to ensure equivalent amplification and probing efficiency, and by amplification of homozygous individuals of both genotypes to ensure that background fluorescence was within acceptable limits.

Real-time PCR amplification using the ABI 7900HT platform (ABI) was initially performed for all 15 patient samples to identify individuals who were heterozygous for each SNP. PCR was performed in 10- μ l reactions including 5 μ l universal master mix, (no AMPerase) (ABI), assay mix (0.9 μ mol/L probe, 1.8 μ mol/L each primer), and 40 ng of DNA. PCR reaction conditions were 95°C for 20 seconds, followed by 60 cycles of 95°C for 1 second and 60°C for 20 seconds. Following amplification, appropriate baseline and threshold levels were set and the genotype of each sample was determined for each SNP.

The optimal DNA concentration to yield good separation of alleles was calculated from the average molecular weight of a mole of base (342 g), the number of molecules in a mole of substance (the Avogadro constant, 6.023×10^{23}) and the number of bases in the human genome (3×10^9). However, since this figure takes no account of the presence of PCR inhibitors or DNA degradation in the sample, optimal dilution factors were further determined empirically.

Optimally diluted DNA samples were distributed to the wells of 96-well optical plates. Real-time amplification of the DNA was performed. PCR reactions were performed in a total volume of 8 μ l including 4 μ l universal master mix (no AMPerase) (ABI), assay mix (0.9 μ mol/L probe, 1.8 μ mol/L each primer), and 2 μ l appropriately diluted DNA. PCR cycles were 95°C for 20 seconds, followed by 60 cycles of 95°C for 1 second and 60°C for 20 seconds. Following amplification, each well was genotyped as allele 1, allele 2, allele 1 + 2, or no product detected (nd).

Likelihood analysis¹⁴ was applied to assess the strength of evidence for deviation from a 50:50 distribution of alleles. The specific method used was the SPRT, as described in previous studies.^{13,17} This technique allows two probabilistic hypotheses to be compared as data accumulates. A heterozygous sample is expected to have allelic balance, with the two heterozygous alleles appearing at approximately the same frequency (null hypothesis H^0) (Figure 2A). A sample

with LOH will have allelic imbalance (alternative hypothesis H^1) with over-representation of one allele (Figure 2B). The degree of imbalance will be directly proportional to the number of clonal B-lymphocytes that are present in the sample and that harbor the aberration. Threshold curves representing the two hypotheses are generated for a specific tumor load based on a predetermined likelihood ratio. Analysis is concluded by plotting the number of informative alleles tested against the major allele ratio (Figure 3A). If the point is plotted above the upper threshold curve, it is considered as evidence of allelic imbalance and hence LOH at that locus. Points plotted below the lower threshold curve are categorized as having allelic balance and therefore no LOH.

A likelihood ratio of >8 was accepted as evidence of LOH since it provides a post-test probability of LOH of 89%, if the pre-test probability of the presence of LOH at 13q is assumed to be 50%. Since SPRT allows for repeat testing, the post-test probability of LOH can be increased further by testing each sample with several allelic probes.

The proportions (P) of the major allele for a likelihood ratio of 8 for the presence of LOH in test samples with varying proportions of B-lymphocytes were calculated according to the formula $P = \log(16)/(n \cdot \log[(100-I)/100]) + \log[(100-I)/(100-I/2)]/\log[(100-I)/100]$, where n = total number of informative alleles counted and I = the percentage of lymphocytes in the peripheral blood sample. The results of the equation were tabulated with Microsoft Excel 2000 software.

B Heterozygous

Loss of Heterozygosity

C Normal

13q14 Deletion

LOH Analysis by DNA Sequencing

Primers were designed to amplify the SNPs rs3809325 and rs9535499 within *EBPL* and *DLEU7* respectively (Table 2). Paired patient DNA samples from isolated mononuclear cells and non-neoplastic buccal mucosa cells were amplified by PCR and sequenced in one direction by Big-Dye terminator chemistry using the ABI 3730 genetic analyzer (ABI). The LOH status of each sample was determined through the quantification of each allele by peak height analysis using the DNA quantification tool of Mutation Surveyor software (version 3.2, SoftGenetics, State College, PA) (Figure 1B). Coefficient of variation

Figure 1. Techniques used to investigate 13q14 status. **A:** Schematic representation of the relative positions of dSNP and DNA sequencing polymorphisms and the FISH and MLPA probes at 13q14. The polymorphisms analyzed by dSNP and DNA sequencing are indicated by the stars, while the loci of the FISH and MLPA probes are indicated by gray boxes. Numbers within the boxes denote the number of probes targeting that locus. **B:** DNA sequencing to detect LOH status at 13q. The upper sequencing trace represents the normal genomic DNA sequence obtained from buccal mucosal cells. The arrow highlights the heterozygous SNP with similar peak heights for the two heterozygous alleles. The lower sequencing trace represents the DNA sequence obtained from mononuclear cells harboring LOH at 13q14. The arrow highlights the heterozygous SNP with reduced peak height for one of the two heterozygous alleles. **C:** MLPA to detect deletions at 13q. The upper trace represents gene dosage analysis in a normal control. The lower trace represents gene dosage analysis in a patient with a deletion at chromosome 13. The two arrows highlight the genes on chromosome 13. A reduction in the peak heights is evident in the lower trace indicating reduced gene dosage and hence the presence of a deletion.

Table 1. Sequences of Probes and Primers Used for Digital SNP Analysis

Name	Sequence	Labels
RB-1 F	5'-GTTGCTGGACAGCCTATGGAT-3'	None
RB-1 R	5'-AAGGAATTATACCAAAGCAGCTAACTGAA-3'	None
RB-1 P(1)	5'-TCAGGTGACTAT C TTTGT-3'	5' VIC, 3' MGB
RB-1 P(2)	5'-CAGGTGACTAT G TTTGT-3'	5' 6-FAM, 3' MGB
EBPL F	5'-CCCACCCTTCTACTTTTCTGGATT-3'	None
EBPL R	5'-CACCAGCCGGCTTAAAGC-3'	None
EBPL P(1)	5'-AAATGGAAAGATTGT C AGTA-3'	5' VIC, 3' MGB
EBPL P(2)	5'-ATGGAAAGATTGT C GAGTA-3'	5' 6-FAM, 3' MGB
KPNA3 F	5'-CAAGTCTGTTTGCTTAATGTCCCATG-3'	None
KPNA3 R	5'-CCAGTCCCGCACTGACT-3'	None
KPNA3 P(1)	5'-CCAAAGC A AGTCACAC-3'	5' VIC, 3' MGB
KPNA3 P(2)	5'-CAAAGC G AGTCACAC-3'	5' 6-FAM, 3' MGB
C13Orf1 F	5'-CTCATTAGATCCAATCCCACTGCTT-3'	None
C13Orf1 R	5'-CCACTGTAAATGACTAAATTTGAATGCTT-3'	None
C13Orf1 P(1)	5'-AAACTCCCA A ACAAAG-3'	5' VIC, 3' MGB
C13Orf1 P(2)	5'-AACTCCCA G ACAAAG-3'	5' 6-FAM, 3' MGB
KCNRG F	5'-GATCTCTCAAAAATTGATGAGTGTGGTT-3'	None
KCNRG R	5'-TCGAGACCTTGCTGTGATTCTG-3'	None
KCNRG P(1)	5'-AAAGTCATT T GAATTC-3'	5' VIC, 3' MGB
KCNRG P(2)	5'-AAGTCATT C AATTC-3'	5' 6-FAM, 3' MGB
DLEU2 F	5'-ACTTCCCTACCTATTCTCCTGGAAAA-3'	None
DLEU2 R	5'-GGAGCTAGAGGCATTTTGTAGCAA-3'	None
DLEU2 P(1)	5'-AATCAAGAAAT G ACACTTT-3'	5' VIC, 3' MGB
DLEU2 P(2)	5'-CAATCAAGAAAT T ACACTTT-3'	5' 6-FAM, 3' MGB
DLEU1 F	5'-GCCAGTGTCTAAACTCCAAACAAC-3'	None
DLEU1 R	5'-GGCGGTTTTAAATGCACGTGTATC-3'	None
DLEU1 P(1)	5'-CAATGAGACCT A GATATG-3'	5' VIC, 3' MGB
DLEU1 P(2)	5'-CAATGAGACCT T GATATG-3'	5' 6-FAM, 3' MGB
DLEU7 F	5'-GGGTCTTGAAAGAAAAGTACAGAGCTA-3'	None
DLEU7 R	5'-CCACTCAGTTTCCCAACCTAA-3'	None
DLEU7 P(1)	5'-AAAGAAAGGCATC C CCCCAG-3'	5' VIC, 3' MGB
DLEU7 P(2)	5'-AAAGGCATC G CCCCAG-3'	5' 6-FAM, 3' MGB
SLC15A1 F	5'-GCTGCTGATTTCAGTTGGAGACA-3'	None
SLC15A1 R	5'-TTTGGATTAAATCACCTCCAGCTT-3'	None
SLC15A1 P(1)	5'-TCTAGATGCA A GATCTG-3'	5' VIC, 3' MGB
SLC15A1 P(2)	5'-TAGATGCA A TATCTG-3'	5' 6-FAM, 3' MGB
UBAC2 F	5'-GAATCCTAAGATGCTTAATTTGTAAAGTTTGCA-3'	None
UBAC2 R	5'-GGTGCATGATGAGCAGTGAAAA-3'	None
UBAC2 P(1)	5'-CTCGGATC A TATTTAG-3'	5' VIC, 3' MGB
UBAC2 P(2)	5'-CTCGGATC G TATTTAG-3'	5' 6-FAM, 3' MGB

Probes are labeled 5' with 6-FAM or VIC and 3' with a MGB to enhance probe specificity. Nucleotides representing the SNPs under investigation are highlighted in bold.

analysis was applied to establish normal variation observed for peak height quantification.

FISH

FISH analysis for the detection of chromosome 13q deletions was performed by Bristol Genetics Laboratory as a routine clinical test using LSI S13S319 (Spectrum Orange) and LSI 13q34 (Spectrum Aqua) (Vysis Inc., Abbott Molecular, Maidenhead, UK) probes mapping at 13q14 and 13q34 respectively. FISH was conducted according to the manufacturer's protocol using co-denaturation and rapid wash procedures.

MLPA

MLPA was performed according to the manufacturer's protocol (MRC-Holland, Amsterdam, Holland). Two kits, Salsa MLPA kit PO37 CLL1 and PO38 CLL2, provided a total of 12 probes that specifically target 13q14. The locations of the probes are shown in Figure

1A. Ligation products were amplified using a common 6-FAM labeled primer set. Products were analyzed on an ABI 3100 genetic analyzer (ABI) and dosage analysis was performed using Genemarker software (Version 1.70, SoftGenetics, State College, PA). The software calculates dosage quotients by comparing peak height values for each sample with those produced by normal controls (Figure 1C).

Results

Digital SNP

Assay Design and Validation

Real-time PCR assays were designed and validated for 10 SNPs spanning the 13q14.2 to 13q34 region. The median distance between the SNP probes at 13q14 was 92 kb (range, 10 kb to 1348 kb). All assays were deemed suitable for further analysis on the basis of yielding reliable and specific amplification with an

Figure 2. Schematic representation of dSNP analysis. **A:** Schematic of dSNP results for patient CLL 13 (marker DLEU2) with no allelic imbalance. Wells scoring as "allele 1" are marked in light gray, wells scoring as "allele 2" are marked in dark gray. Wells containing both alleles 1 and 2 are hatched while those with no signal are blank. The total number of informative alleles is 58 (30 allele 1 and 28 allele 2). The major allele frequency is thus 0.52. **B:** Schematic of dSNP results for patient CLL 1 (marker EBPL) showing allelic imbalance. Wells scoring as "allele 1" are marked in light gray, wells scoring as "allele 2" are marked in dark gray. Wells containing both alleles 1 and 2 are hatched while those with no signal are blank. The total number of informative alleles is 67 (54 allele 1 and 13 allele 2). The major allele frequency is thus 0.81.

efficiency difference of less than 0.5 between alleles, and minimal cross allele reactivity. All assays demonstrated a linear relationship between crossing point and DNA concentration over seven serial 1:2 dilutions (data not shown).

Assessment of SNP Genotype for Each Sample

Of the 15 patients studied, 13 were heterozygous for at least one of the 10 SNPs within the panel and were therefore suitable for dSNP analysis. The median number of heterozygous SNPs per patient was 3.0 (range, 2.0 to 5.0). In patients with more than one informative SNP at 13q14, the median distance between SNPs per patient was 169 kb (range, 42 kb to 2478 kb).

Calculation of Optimal DNA Concentration for dSNP Analysis

The average mass of one molecule of DNA was calculated as 2.6×10^{-12} g. Therefore, a DNA concentration of 2.6×10^{-12} pg/ μ l should yield one template

Figure 3. Digital SNP analysis of 13q in patients with CLL. **A:** Sequential probability ratio test (SPRT) to detect LOH at 13q. The x axis represents the number of alleles tested and the y axis represents the major allele frequency. Threshold curves are calculated for a 60% tumor load based on the likelihood ratio of 8. Analysis is conducted by plotting the number of alleles tested against the major allele frequency. Points plotted above the upper curve represent LOH, while those below the lower curve represent no LOH. **B:** Schematic representation of chromosome 13 from band 13q14.2 to band 13q32. The sample number is given at the left side of the figure and the markers tested are shown at the top. Markers colored in black represent loci with LOH determined by dSNP analysis, while those in white represent loci with no LOH.

molecule per 1 μ l. In practice, the presence of PCR inhibitors and the occurrence of variable amounts of DNA degradation in the samples meant that the optimal DNA concentrations needed to be determined em-

Table 2. Sequences of Primers Used for DLEU7 and EBPL LOH Sequencing

Name	Sequence
DLEU7 F	5'-TGTAACACGACGCCAGTGTGAGGTGGG CCAGAAATAA-3'
DLEU7 R	5'-CAGGAAACAGCTATGACCAATTACAAT AGGGTAGGCAGCA-3'
EBPL F	5'-TGTAACACGACGCCAGTGTATCGCCAAG CCAATAGGAC-3'
EBPL R	5'-CAGGAAACAGCTATGACCAAGTAGACCGG GGCAGAATTT-3'

Table 3. Summary of 13q14 Status as Determined by dSNP, LOH Sequencing, FISH, and MLPA

Identifier	CLL Load	13q14 status			
		dSNP	LOH Sequencing	FISH	MLPA
CLL 01	73%	LOH	LOH	Deleted	Deleted
CLL 02	95%	LOH	—	Deleted	Deleted
CLL 03	70%	No LOH	No LOH	Not deleted	Not deleted
CLL 04	78%	—	—	Not deleted	Not deleted
CLL 05	97%	LOH	No LOH	Deleted	Deleted
CLL 06	62%	LOH	LOH	Deleted	Deleted
CLL 07	98%	LOH	No LOH	Not deleted	Not deleted
CLL 08	26%	LOH	No LOH	Not deleted	Not deleted
CLL 09	37%	LOH	LOH	Deleted	Deleted
CLL 10	89%	LOH	LOH	Deleted	Deleted
CLL 11	41%	No LOH	No LOH	Not deleted	Not deleted
CLL 12	100%	—	—	Deleted	Deleted
CLL 13	88%	LOH	—	Not deleted	Not deleted
CLL 14	84%	LOH	—	Deleted	Deleted
CLL 15	91%	LOH	LOH	Deleted	Deleted

Discrepant results are highlighted in bold.

pirically. Optimal DNA concentrations ranged from 2 to 40 pg/μl.

Assessment of CLL Load

The CLL load was calculated for each patient as the percentage lymphocytes of the total leukocytes at the time of sample collection. In the majority of cases, the CLL load ranged from 70% to 100% (Table 3). In several cases, the CLL load was notably lower at 26% (CLL 08), 37% (CLL 09), and 41% (CLL 11).

dSNP Analysis

The LOH status of selective informative markers spanning the region 13q14.2 to 13q34 was determined using dSNP analysis for each of the 13 CLL patients with heterozygous SNPs. Allelic imbalances at 13q14 were identified in 11/13 (85%) (95% CI: 55%, 98%) CLL patients using this technique (Figure 3B). When analysis was extended to cover the 13q34 region, allelic imbalances were observed in all 13 patients. Deviations from the expected balanced allele frequency were tested for statistical significance by SPRT analysis, thus confirming the LOH status (Figure 3A). The extent of LOH varied considerably between patients and no common region of LOH was evident. Six cases (CLL 03, CLL 05, CLL 07, CLL 10, CLL 11, and CLL 13) showed evidence of LOH extending along just part of the chromosome arm. Two cases (CLL 02 and CLL 15) showed evidence complex chromosomal rearrangements, with regions of LOH disrupted by small, interstitial regions of heterozygosity. Digital SNP analysis allowed the identification of LOH in patients with low CLL loads (CLL 08, CLL 09, and CLL11) without the need for mononuclear cell enrichment techniques.

Comparison of dSNP with FISH and MLPA

FISH and MLPA allowed direct assessment of the 13q14 deletion status at specific loci in all 15 patients. FISH and

MLPA showed complete concordance, both identifying chromosome 13q14 deletions in 9/15 (60%) patients. Of the nine patients with a confirmed deletion at 13q14 by FISH and MLPA, eight showed evidence of LOH by dSNP analysis. The LOH status of the remaining patient with a confirmed 13q14 deletion could not be determined by dSNP analysis due to the lack of informative heterozygous SNPs within our panel.

Three discrepancies (CLL 07, CLL 08, and CLL 13) were observed between the dSNP, FISH, and MLPA techniques (Discrepancies highlighted in Table 3). Each discrepancy showed evidence of LOH by dSNP analysis that was not apparent by FISH or MLPA.

DNA Sequencing to Identify LOH

Of the 14 CLL patients for whom both buccal and mononuclear cell samples were available, 10 were heterozygous for either rs3809325 (*EBPL*) or rs9535499 (*DLEU7*) and were tested by DNA sequencing to identify LOH. The mean coefficient of variation for repeat measurements of allele peak height was 6.4 (95% CI: 3.7, 9.1). We therefore considered a reduction of $\geq 6.5\%$ in the allele quantification in mononuclear cell DNA, when compared with that observed for the corresponding allele in buccal mucosa DNA, to be indicative of LOH at that locus. DNA sequencing identified 13q14 LOH in 5/10 (50%) patients with CLL. There was good concordance between the DNA sequencing, FISH and MLPA techniques, with just one discordant result reported (CLL 05) (discrepancy highlighted in Table 3). This case had a 13q14 deletion confirmed by FISH and MLPA but did not show LOH by DNA sequencing analysis.

Comparison of SNP-Based Techniques

Digital SNP and DNA sequencing were both used to determine 13q14 LOH status for 10 patients. Concordant results were reported for 7/10 (70%) patients. The three discordant results (CLL 05, CLL 07, and CLL 08) showed

evidence of LOH by dSNP analysis that was missed by DNA sequencing (discrepancies highlighted in Table 3).

Discussion

We report here the novel application of two SNP-based molecular techniques, dSNP and LOH DNA sequencing, for the evaluation of 13q14 LOH status in a cohort of 15 patients with CLL. We have compared these approaches with FISH and MLPA to determine their utility and relative sensitivity. In the diagnostic setting, FISH is widely regarded as the gold standard for the identification of chromosome deletions in patients with CLL, and MLPA is emerging as an equally valid technique for this application.¹¹ The advantages and limitations of the techniques are discussed further below.

Digital SNP proved to be the most sensitive of the techniques, identifying 13q14 LOH in 11/13 (85%) (95% CI: 55%, 98%) patients in our cohort. The dSNP technique also allowed the identification of LOH in samples with heterogeneous cell populations with as few as 26% circulating lymphocytes (CLL 08), without the need for tumor cell enrichment. The incidence of 13q14 LOH in our cohort is higher than that reported in the literature, where most studies report chromosome 13q14 deletions in around 50% of CLL patients.³ A possible explanation for this discrepancy is that our small patient cohort may not be entirely representative of the CLL population and, as such, inclusion of additional cases may have resulted in an incidence of 13q14 deletions that would more accurately reflect current data in the literature.

Digital SNP analysis enabled high-resolution screening of chromosome band 13q14. This was accomplished through the application of a panel of 10 SNP probes targeting the 13q14.2-13q34 region. The median distance between the SNP probes at 13q14 was just 92kb (range, 10 kb to 1348 kb). This permitted LOH analysis to extend beyond the boundaries of the FISH and MLPA probes that are used diagnostically, thereby giving additional information about the 13q14 region. This high-resolution approach allowed the identification of complex chromosomal rearrangements such as those observed for CLL 02 and CLL 15 (Figure 3B), which were not apparent by FISH, MLPA, or DNA sequencing. In these cases, large regions of LOH were disrupted by small interstitial patterns of heterozygosity. Such rearrangements have not been previously described in patients with CLL. Identification of these complex rearrangements strengthens the evidence for the existence of recombination hot spots within the 13q14 region.¹⁰

Of the 15 CLL patients in the study cohort, 13 could be analyzed by dSNP. The two remaining patients could not be assessed by this technique due to the lack of appropriate heterozygous SNPs within our panel. This represents a significant limitation of SNP-based approaches but the inclusion of additional SNPs within the panel may help to overcome this limitation.

The requirement of informative heterozygous SNPs also limited the utility of the DNA sequencing approach for LOH analysis. Of the 14 CLL patients for whom both

buccal mucosa cell and mononuclear cell samples were available, only 10 were heterozygous for either rs3809325 (*EBPL*) or rs9535499 (*DLEU7*) and could therefore be analyzed by this technique.

DNA sequencing for the identification of LOH allows high-throughput analysis of multiple samples. However, the technique is associated with limited sensitivity. One case (CLL 05) showed heterozygosity by DNA sequencing, yet the patient had a deletion at 13q14 confirmed by FISH and MLPA, and showed LOH at that locus by dSNP. A further two cases (CLL 07 and CLL 08) showed evidence of LOH by dSNP that was not apparent by DNA sequencing, demonstrating the superior sensitivity of the dSNP approach. These results highlight the limitations of using DNA sequencing as a technique for quantification.

FISH and MLPA are universally applicable for the assessment of 13q14 deletion status in all patients. They are robust techniques that are commonly used in diagnostic laboratories. In our study, FISH and MLPA showed complete concordance, both identifying chromosome 13q14 deletions in 9/15 (60%) patients. The sensitivity and resolution of FISH and MLPA are limited by the locality of the probe(s) targeting the region of interest. In the present study, three patients (CLL 07, CLL 08, and CLL 13) showed evidence of LOH at 13q14 by dSNP that was missed by FISH and MLPA. In all three cases, dSNP identified LOH at loci occurring beyond the boundaries of the FISH probes and could therefore not be assessed accurately by this technique. The LOH observed for one case (CLL 13) also occurred beyond the boundaries of the MLPA probes. This discrepancy may therefore represent a small, interstitial deletion that would have been missed by both FISH and MLPA due to the locality of the probes.

The sensitivity of FISH and MLPA is also limited when applied to the analysis of samples with heterogeneous populations of normal and malignant cells. This is exemplified by case CLL 08, where dSNP identified LOH at a locus that showed normal gene dosage by MLPA. This discrepancy may be attributed to the sample having a low proportion of clonal B cells in the circulating lymphocyte population, which was also low (26%). MLPA is acknowledged to have limited sensitivity for the detection of genomic aberrations when the percentage of neoplastic cells is low.¹¹

FISH and MLPA lack the capacity to detect chromosomal aberrations that do not alter chromosomal copy number, such as uniparental disomy. In our cohort, one sample (CLL 07) with a high lymphocyte load (98%) showed LOH by dSNP analysis that was missed by an MLPA probe targeting that locus. This discrepancy may represent an example of uniparental disomy LOH due to mitotic recombination. LOH, without loss of genetic material, at chromosome band 13q14 has been previously described in patients with CLL¹⁰ and would not be detectable by the gene dosage techniques such as MLPA.

Other techniques, such as SNP-chips, have previously been used for the high-resolution screening of the 13q14 band.¹⁰ However, the SNP density of such chips would have to be high to achieve a resolution comparable with dSNP. The smallest detectable deletions in CLL patients

are 3 Mb and 240 kb with the 10-k and 50-k arrays respectively.¹⁰ Additionally, SNP chips only reliably detect aberrations in the presence of 25% abnormal cells.¹⁸ This may limit their application for the analysis of chromosomal aberrations in heterogeneous cell populations in other study cohorts.

Despite the high-resolution analysis afforded by the dSNP approach, no common region of LOH was apparent in the cohort of 15 CLL patients studied. This is in contrast with the results reported from recent array-based studies that have identified a minimal deleted region at chromosome band 13q14 that almost always includes *MIR15A* and *MIR16-1*.^{10,19} In our study cohort, we were not always able to assess the LOH status at this locus due to the lack of informative SNPs within our panel, and therefore, more of our samples may be deleted at this point in concordance with the current literature. However, one patient (CLL13) in our study may not demonstrate a deletion at this point since normal gene dosage was demonstrated for the *MIR15A* and *MIR16-1* locus by MLPA and heterozygosity was apparent at either side of this locus by dSNP. This patient showed evidence of LOH by dSNP at a locus centromeric to *MIR15A* and *MIR16-1*. This may be an indication that although the majority of CLL cases manifest deletions at this locus, there may be a small proportion of cases in which this region is not deleted.

We have shown that SNP-based technology represents a useful approach for the assessment of 13q14 LOH status in patients with CLL. Both dSNP and LOH DNA sequencing compare well with established molecular techniques for 13q14 LOH analysis. The dSNP approach offers the advantage of enhanced sensitivity and high-resolution screening. This is a relatively straightforward technique that requires no specialist equipment other than a real-time PCR machine. Additionally, the analyses of dSNP data are far simpler than either high-resolution SNP arrays or array comparative genome hybridization. Digital SNP may have applications for the analysis of DNA extracted from tissues, such as paraffin embedded biopsies, that are more difficult to assess by conventional molecular techniques.

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