Published in final edited form as:

Cancer Genet. 2014 March ; 207(3): 98–102. doi:10.1016/j.cancergen.2014.02.005.

Copy neutral loss of heterozygosity in 20q in chronic lymphocytic leukemia/small lymphocytic lymphoma

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Abstract

Single nucleotide polymorphism (SNP)-based chromosome microarray analysis was used to uncover copy neutral loss of heterozygosity (LOH) in the long arm of chromosome 20 in blood or bone marrow specimens from three patients with chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL). All three patients presented with lymph node enlargement. While one of the patients has had a complicated clinical course, the other two have a more indolent disease. Sequence analysis of the tumor suppressor gene *ASXL1*, which is located in 20q and is commonly mutated in malignant myeloid diseases and occasionally in CLL/SLL specimens, revealed no mutations in our three patients with copy neutral LOH in 20q. The possible contribution of other imprinted microRNAs and antisense genes residing in 20q to the pathogenesis of a subset of CLL/SLL patients is discussed. These findings illustrate the value of SNP arrays for the detection of novel recurrent genomic alterations that may contribute to CLL/SLL onset or progression.

Keywords

Chronic lymphocytic leukemia; copy neutral loss of heterozygosity; chromosome 20; chromosome microarray analysis; imprinting

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Introduction

Identification of cytogenetic alterations in chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) was hindered in the past due to the characteristic low mitotic activity of malignant cells from these patients. In subsequent years, substantial improvement was achieved by stimulating CLL/SLL cells either with CD40 ligand or with a combination of IL-2 and the immunostimulatory CpG-oligonucleotide DSP30 to increase the yield of metaphase spreads for karyotypic analysis (1, 2). In most clinical cytogenetics laboratories, however, CLL/SLL samples are currently analyzed using a disease-specific DNA probe panel and fluorescence *in situ* hybridization (FISH), which permits the detection of chromosomal imbalances in interphase cells, circumventing the need for mitotic stimulation (3). FISH analysis is used to detect trisomy 12 and deletions of 13q14.2, 11q22.3, and 17p13.1, findings that have prognostic implications (4, 5). Loss of 13q14.2 is associated with down regulation of two microRNAs, *MIR15A* and *MIR16-1*, located at this site (6). Deletions of 11q22.3 typically affect the tumor suppressor gene *ATM*, whereas deletions of 17p13.1 result in loss of one copy of *TP53*, often accompanied by a mutation in the remaining allele (7).

Despite its well-accepted clinical utility and wide applicability, FISH analysis of CLL/SLL is limited to a small set of aberrations detectable by the DNA probe set. In contrast, single nucleotide polymorphism (SNP) arrays permit high-resolution analysis of DNA copy number imbalances throughout the genome, while also allowing for the detection of loss of heterozygosity (LOH). In one recent study that compared various conventional cytogenetic and array technologies in samples from patients with either CLL or myelodysplastic syndrome (MDS), the highest detection rate of genomic alterations was achieved with oligoarrays containing SNPs (8).

SNP-array studies of several large series of CLL patients have been reported (9-15). Using SNP arrays, aberrations previously shown by FISH to have prognostic importance in CLL/SLL have been identified at the expected frequency (9). Moreover, the use of such arrays has uncovered previously undocumented recurrent genomic alterations, e.g., trisomy 19 and gain of *MYCN* (16). SNP-array analysis of CLL/SLL samples has also uncovered genomic complexity and copy-neutral LOH (cnLOH) not detectable with the classical FISH panel. For example, in one CLL/SLL case, we observed chromothripsis, a phenomenon by which regions of the malignant genome are shattered and recombined to generate frequent oscillations between two DNA copy number states (17). While LOH is typically the result of genomic copy number loss in CLL, cnLOH is relatively rare in this disease; however, when it does occur, e.g., in cases with cnLOH at 17p13.1, it can have prognostic implications (18). The identification of such regions of cnLOH are significant, because the resulting LOH may unmask a mutant tumor suppressor allele or imprinted gene important in CLL/SLL disease onset or progression.

In this report, we describe clinical and genetic findings in three CLL/SLL patients who presented with lymph node enlargement and whose CLL/SLL cells were found to have cnLOH encompassing all or nearly all of 20q. The findings illustrate the value of SNP arrays for the detection of novel recurrent genomic alterations that may contribute to CLL/SLL

pathogenesis. We also discuss the possible involvement of several imprinted microRNAs and antisense genes residing in 20q.

Materials and methods

SNP array analysis

SNP array analysis was performed using Affymetrix CytoScan HD arrays containing >2.6 million copy number markers, of which 750,000 are SNPs. Total genomic DNA from each sample was digested with *NspI* restriction enzyme and ligated to adapters that recognize cohesive 4-basepair overhangs. A generic primer recognizing the adapter sequence was used to amplify adapter-ligated DNA fragments. Amplification products were purified using magnetic beads, fragmented, biotin-labeled, and hybridized to arrays according to the manufacturer's recommendations. The hybridized array was then washed and scanned with a GeneChip Scanner 3000 7G. Intensities of probe hybridization were analyzed by using Affymetrix GeneChip Command Console, and copy number and genotyping analyses were performed using Affymetrix Chromosome Analysis Suite software with default settings.

Sequence analysis

Eleven PCR products encompassing *ASXL1* exons 8, 9, 11, and 12 were amplified for sequence analysis. For amplification, QIAGEN Fast Cycling PCR (Qiagen, Valencia, CA) was used according to the manufacturer's protocol. PCR products were gel purified and Sanger sequenced using the primers M13-F (GTAAAACGACGGCCAGT) and M13-R (CAGGAAACAGCTATGAC).

Results

Clinical findings

Patient 1 is a 61-year-old male who in 2008 presented with fatigue and new onset lymphadenopathy, but with no increase in peripheral white cell count. A lymph node biopsy revealed CLL/SLL. Flow cytometry studies were significant for partial CD23 expression on clonal B-cells. FISH studies performed at that time identified trisomy 12. He was started on rituximab treatment followed by rituximab maintenance for the next 2.5 years. In early 2011, his peripheral blood counts began to fall, and a marrow biopsy showed widespread involvement of disease. He was started on therapy consisting of fludarabine, Cytosan and rituximab (FCR), and after an initial response, he relapsed again in early 2012. SNP array analysis was performed on peripheral blood in May and October of 2012, during active disease and after FCR therapy, respectively, the latter when few lymphocytes were present in his blood. He was later switched to ibrutinib therapy and is currently in a stable remission.

Patient 2 is a 56-year-old male who presented with neck stiffness in July 2011. A CT scan showed extensive lymphadenopathy above and below the diaphragm. Peripheral blood counts were normal, with the exception of absolute lymphocytosis (4,100/mm³). He underwent a lymph node biopsy diagnosed as atypical CLL/SLL, with the lymphoma cells being CD5-negative. Peripheral blood flow cytometry showed involvement by a monoclonal B-cell population with only partial expression of CD5 and CD23. SNP-array analysis of a

blood sample was performed in May of 2013. His latest scans showed a slight increase in the size of the involved nodes and an increase in the absolute lymphocyte count (9,300/mm³).

Patient 3 is a 69-year-old male who in 2004 was found to have cervical lymphadenopathy during routine physical examination and was ultimately diagnosed with CLL/SLL. He received rituximab treatment followed by intermittent rituximab maintenance. In 2007, when he first presented to our institution, his absolute lymphocyte count was 32,200/mm³. In January 2013, a marrow biopsy showed 70% involvement by lymphoma. At that time, flow cytometry analysis on a bone marrow sample revealed a clonal B-cell population with a classic CLL/SLL immunophenotype, and SNP-array analysis was performed on bone marrow. He has been treated with Revlimid and rituximab since March 2013 with a good response (current absolute lymphocyte count 2,200/mm³).

Genetic findings

SNP-array analysis of CLL/SLL cells from patients 1 and 2 each showed both cnLOH in 20q and trisomy 12. Additionally, the sample from patient 1 also exhibited gain of 17q21.31q25.3, whereas patient 2 had cnLOH at 9q13q34.3. A follow up SNP-array study on blood from patient 1, carried out after FDR therapy, revealed a normal genomic profile, consistent with the presence of mostly granulocytes when the sample was drawn, and indicating that cnLOH in 20q is a somatic change. Patient 3 had a single copy deletion in chromosome band 13q14.2-q14.3 encompassing *MIR15A/MIR16-1* as well as cnLOH in 20q. Although matched normal DNA was not analyzed independently from patients 2 and 3, the fact that the regions with cnLOH in 20q and (in patient 2) 9q were large (>30 Mb), and included terminal portions of these chromosome arms, strongly suggested that these alterations are acquired, not constitutional (19). DNA profiles of chromosome 20 for each sample are shown in Fig. 1, and a summary of all of the SNP-array findings is presented in Table 1.

The additional sex combs-like 1 gene, *ASXLI*, is one of the most commonly mutated genes in malignant myeloid diseases, including MDS, chronic myelomonocytic leukemia and acute myeloid leukemia (20, 21). In these disorders, *ASXLI* mutations are associated with disease aggressiveness and poor prognosis. Truncating mutations of *ASXLI* have also been reported in other malignancies, including 3 of 105 (2.9%) CLL/SLL specimens (22). Given that the *ASXLI* locus resides in 20q, at band 20q11.21, and that the protein encoded by *ASXLI* belongs to a polycomb repressor complex implicated in tumorigenesis, Sanger sequencing was performed on CLL/SLL cells from all three of our patients. No mutations were found in *ASXLI* exons 8, 9, 11 and 12, where pathologic mutations typically occur (21, 22).

Discussion

Copy neutral LOH is thought to be pathogenetic either through the acquisition of two identical mutant copies of an underlying recessive gene or two silenced copies of an intact (wild-type) allele (23). In some cancers, cnLOH is a frequent occurrence (24). In MDS, Gondek et al. reported cnLOH in 24 of 72 (33%) patients evaluated with SNP arrays (25). The cnLOH was found in regions such as 7q that are often affected by deletions as

demonstrated by conventional karyotyping, and all of the cnLOH lesions were somatic, as they were not found in germline DNA isolated from CD3⁺ lymphocytes. In MDS, cnLOH is thought to provide a useful marker for identifying chromosomal segments that may harbor mutated genes such as *TET2* and *TP53* important in disease pathogenesis (26, 27). Similarly, in CLL/SLL, one study identified cnLOH in 4 of 56 (7%) cases, including one site that encompassed *TP53* (10); in another CLL/SLL study, cnLOH was detected in 9 of 144 (6%) patients, with the most frequently affected sites being the clinically relevant sites 13q14.2, 17p13.1, and 11q22.3 (28).

To date, we have performed SNP array analysis on 47 CLL patients, 3 (6%) of which exhibited cnLOH in 20q. To our knowledge, cnLOH in 20q has not been reported previously as a recurrent alteration in CLL/SLL. The net effect of cnLOH is essentially the same as with a chromosomal deletion. Thus, cnLOH in 20q and deletions of 20q would have similar consequences with regard to allelic loss. Recurrent deletions of 20q have been reported in a large spectrum of hematological malignancies primarily of myeloid origin, including polycythemia vera, MDS, and acute nonlymphocytic leukemia (29, 30). Although del(20q) is usually not reported in patients with lymphoid malignancies, our earlier karyotypic studies of CLL/SLL uncovered three cases with a del(20q), one of which also showed del(6q). Furthermore, 15 CLL/SLL cases with del(20q) were found in a search of Mitelman's Database of Chromosome Aberrations and Gene Fusions in Cancer [<http://cgap.nci.nih.gov/Chromosomes/Mitelman>]. In 5/15 cases, del(20q) was the sole abnormality observed, suggesting that LOH at 20q may play a significant role in the pathogenesis of some CLL/SLL patients.

With regard to candidate genes in 20q potentially involved in CLL/SLL, we considered *ASXL1* because of its location in 20q and the fact that occasional truncating mutations of this gene have been reported in CLL/SLL specimens (22). However, we found no mutations in *ASXL1*, indicating that some a yet-to-be identified imprinted or mutated gene(s) in 20q is involved. Notably, at least four paternally imprinted genes or microRNAs are located in 20q, including *MIR296*, *MIR298*, and two genes encoding GNAS-related antisense RNA: *SANG* (*GNAS1 antisense*) and *GNASAS* (*GNAS antisense RNA*). *GNAS* is a complex imprinted locus that produces multiple transcripts through the use of alternative promoters and alternative splicing. The most well characterized *GNAS* transcript, Gs-alpha, encodes the alpha subunit of the stimulatory guanine nucleotide-binding protein, which plays essential roles in many physiologic processes. Other transcripts produced by *GNAS* are expressed exclusively from either the paternal or the maternal *GNAS* allele (31). Thus, it is possible that cnLOH in 20q results in the presence of two silenced (imprinted) copies of one or more genes/microRNAs of relevance to CLL/SLL pathogenesis, which we hope to explore in future investigations. Notably, a possible influence of imprinting in familial CLL/SLL has been proposed (32). Our findings illustrate the value of SNP array analysis for the detection of novel genomic alterations that may contribute to CLL/SLL onset or progression.

Acknowledgments

We thank Yu Cao, MS, for technical assistance with the CMA studies. This work was supported in part by National Cancer Institute Award CA-06927 and an appropriation from the Commonwealth of Pennsylvania. The Genomics Facility of Fox Chase Cancer Center was used in the course of this work. The study sponsors had no involvement in

the study design, collection, analysis and interpretation of data, in the writing of the manuscript, or in the decision to submit the manuscript for publication.

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Figure 1.

Depiction of DNA copy number and allele peaks in chromosome 20 identified in blood (patients 1 and 2) or marrow samples from three CLL/SLL patients. For each sample shown, the y axis depicts DNA copy number (*Upper*) and allele peaks (*Lower*).

Allele peak panels normally show three distinct “bands,” representing all homozygous (*Top* and *Bottom* bands) and heterozygous (*Middle* band) allele calls, with allele peak panels showing absence of middle band for most or all of 20q corresponding to cnLOH. Profiles of chromosome 20 in patient 1 are from the time of active disease (**A**) and after aggressive therapy, the latter when few CLL cells were evident (**B**). Profiles of chromosome 20 in patients 2 (**C**) and 3 (**D**) were obtained at time of active disease. Note loss of middle band (*arrows*) in samples showing cnLOH in 20q.

Table 1

Summary of SNP microarray findings in three CLL/SLL cases with cnLOH in 20q

Case no.	SNP array findings in CLL/SLL samples	% abnormal ^a
1	(12)×3,17q21.31q25.3(41,399,892-81,041,938)×3, 20q11.2q13.33(30,295,756-62,915,555)×2 hmz	70%
2	9q13q34.3(67,983,161-141,025,328)×2 hmz,(12)×3, 20q11.2q13.33(29,413,471-62,915,555)×2 hmz	50%
3	13q14.2q14.3(50,523,537-51,506,413)×1, 20q11.2q13.33(30,869,097-62,915,555)×2 hmz	80%

Abbreviation: hmz, homozygous.^aEstimated percentages are based on copy number and allele analyses of SNP arrays.