Molecular Ontogeny of Donor-Derived Follicular Lymphomas Occurring after Hematopoietic Cell Transplantation

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INTRODUCTION

Follicular lymphoma is the most common subtype of non-Hodgkin lymphoma in North America (1) and usually follows an indolent clinical course, with 10-year survival rates ranging from 36% to 71% (2). The molecular hallmark of follicular lymphoma is rearrangement of chromosome 18q21, which results in overexpression of the antiapoptotic protein BCL2, most commonly through juxtaposition to the immunoglobulin heavy chain (IGH) locus (3). Up to 25% of healthy patients have circulating BCL2/IGH-harboring cells (4, 5), yet follicular lymphoma is diagnosed in only 1 of 24,000 persons in the United States annually (6). This suggests that additional genetic alterations are required for lymphomagenesis. Recurrent mutations have been identified in follicular lymphoma (7–10), but their timing during lymphomagenesis remains unknown.

Donor-derived malignancies after hematopoietic cell transplantation are rare (11), but they provide unique models for the assessment of clonal evolution of human tumors in vivo. Previous reports of donor-derived malignancies have lacked detailed molecular analysis. We performed...

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ultrasensitive mutation detection to define in vivo clonal diversification in follicular lymphomas from a donor–recipient sibling pair who presented more than 7 years after hematopoietic cell transplantation and subsequent donor lymphocyte infusions.

**RESULTS**

A 41-year-old woman was found to have asymptomatic leukocytosis and was diagnosed with chronic-phase chronic myeloid leukemia (CML) in 1999. In June 2000, she received myeloablative conditioning followed by transplantation of unmanipulated bone marrow from her 40-year-old human leukocyte antigen-matched sister (Fig. 1A). She received methotrexate and tacrolimus as GVHD prophylaxis and achieved a complete hematologic remission, but a BCR/ABL1 transcript remained detectable by PCR. Peripheral blood leukocytes were collected from her donor in March 2002, and donor leukocyte infusions were prepared by Ficoll separation. She received 3 donor lymphocyte infusions for a total of 1.45 × 10^8 cells/kg, with the last in June 2002. She tested positive for BCR/ABL1 by PCR with no evidence of GVHD and began treatment with IFN-α-2a in 2004, which resulted in a complete molecular remission for 2 years. In 2006, she tested positive by PCR and was switched to imatinib, which again resulted in a complete molecular remission (Fig. 1A).

In November 2009, the donor presented with palpable cervical lymphadenopathy and right leg edema. Imaging demonstrated widespread lymphadenopathy and soft-tissue masses. Biopsy of a right inguinal lymph node and bone marrow revealed grade 2 follicular lymphoma with focal areas of grade 3A (Fig. 1B). Six months later, the recipient developed shortness of breath. Imaging demonstrated pleural and peritoneal effusions, as well as extensive lymphadenopathy. Biopsy of a left inguinal lymph node and bone marrow also revealed grade 2 follicular lymphoma with focal areas of grade 3A and no evidence of CML (Fig. 1B). Morphologic and immunohistochemical findings in both follicular lymphomas were highly similar, with both cases infiltrated by tumor cells expressing CD20, CD10, BCL2, and BCL6 (Fig. 1B).

The morphologic similarity between the lymphomas and the clinical course suggested that a common follicular lymphoma ancestor was passed from the donor to the recipient. PCR with primers overlapping the major breakpoint region of BCL2 and a VH3 consensus sequence amplified identical BCL2/IGH rearrangements from the donor’s follicular lymphoma and the recipient’s follicular lymphoma (Fig. 2A and B). Thus, the 2 lymphomas were derived from a common ancestral population that harbored BCL2/IGH. PCRs with the same primers identified an identical product from the donor lymphocyte infusion, the most recently passed specimen from donor to recipient (Fig. 2A and B). This indicates that transplantation of BCL2/IGH-harboring cells occurred at least 7 years before the presentation of follicular lymphoma.

To determine the frequency of BCL2/IGH-positive cells in the donor lymphocyte infusion, we assayed BCL2/IGH by quantitative PCR. The fraction of donor lymphocyte

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**Figure 1.** Presentation of follicular lymphoma in the donor and recipient. A, time course of chronic-phase CML (cP CML) in the recipient followed by bone marrow transplantation (BMT), administration of 3 donor lymphocyte infusions (DLI), and treatment for detectable BCR/ABL1 transcript by PCR. Follicular lymphoma (FL) was diagnosed in both the donor and the recipient within a 6-month period. B, hematoxylin and eosin (H&E) stains and immunohistochemistry with antibodies against the indicated markers.
Molecular Ontogeny of Donor-Derived Follicular Lymphomas

To identify single nucleotide variants and small insertions/deletions consistent with somatic hypermutation, we compared the clonal IGH rearrangements from both lymphomas to the donor's germline VH3-66 segment that was involved in the IGH rearrangement. There were multiple identical somatic alterations within the amplified region present in both lymphomas (Fig. 2D), indicating that the common ancestor had initiated somatic hypermutation. Both lymphoma-derived IGH rearrangements also harbored distinct somatic alterations, which is consistent with ongoing somatic hypermutation during clonal divergence (Fig. 2D).

The availability of both the donor's and recipient's follicular lymphomas as well as a common ancestor population within the donor lymphocyte infusion offered a unique opportunity to delineate human lymphoma ontogeny. To identify shared and divergent genetic events, we performed exome sequencing of formalin-fixed, paraffin-embedded samples of both lymphomas and a frozen aliquot of donor lymphocyte infusion. Annotated sequence variants were intersected, filtered, sorted, and selected as outlined in Supplementary Figures S2 and S3 and Supplementary Tables S1 and S2. All mutations were verified to be somatic.
by PCR and Sanger sequencing from both follicular lymphomas and appropriate germline specimens (Supplementary Table S3). Bioinformatic predictions of the functional impact for each somatic missense mutation are included in Supplementary Table S4.

Overall, we recovered 12 non-synonymous single nucleotide variants and 2 coding insertions/deletions that were present in both lymphomas, 3 non-synonymous nucleotide variants that were unique to the donor’s follicular lymphoma, and 4 non-synonymous nucleotide variants that were unique to the recipient’s follicular lymphoma (Table 1). None of the mutations was detected within the donor lymphocyte infusion by Sanger sequencing. In addition, for the 4 variants unique to the recipient’s follicular lymphoma, we excluded a germline polymorphism by comparison with the recipient buccal swab.

Among the identical single nucleotide variants in both follicular lymphomas were two in BCL2, which is known to be a target of aberrant somatic hypermutation in follicular lymphoma (Fig. 3A; ref. 14). On the basis of sequencing of cDNA from the recipient’s follicular lymphoma, both single nucleotide variants were present in 100% of BCL2 transcript (Fig. 3A), indicating that the mutations are present in cis on the translocated allele. An in-frame deletion in EP300, an acetyltransferase that is recurrently mutated in follicular lymphoma and diffuse large B-cell lymphoma (7), was present in both follicular lymphomas (Fig. 3B). In addition, we identified an in-frame insertion in KHL6 (Fig. 3B), which was recently found to be mutated in chronic lymphocytic leukemia (15), diffuse large B-cell lymphoma, and follicular lymphoma (10).

Among the single nucleotide variants unique to the recipient’s follicular lymphoma was an R1276 premature stop mutation in ARID1A (adenine-thymine-rich interactive domain-containing protein 1A; Fig. 3C) that was previously reported in pancreatic cancer (16). The ARID1A locus is within a region of chromosome 1p that is deleted in approximately 20% of follicular lymphoma cases, and deletion is linked to inferior prognosis (17). Although the premature stop mutation was present only in the recipient’s follicular lymphoma, both lymphomas had reduced BAF250 (the protein product of ARID1A) expression by immunohistochemical analysis (Figs. 3D and Supplementary Fig. S4B). To determine whether ARID1A expression could be affected by copy number loss in the donor’s follicular lymphoma, we performed quantitative PCR on genomic DNA from both follicular lymphomas, as well as control samples. As outlined in Figure 3E, we identified copy number loss (copy number, 1.84; P = 0.022 compared with donor germline) at this locus in the donor’s follicular lymphoma but not in the recipient’s follicular lymphoma (Fig. 3D).

Exome sequencing technology currently lacks adequate sensitivity to identify very rare events, such as those occurring in 1 in 2,000 cells. To determine whether the somatic mutations that we identified are present at a low frequency within the donor lymphocyte infusion, we amplified regions flanking each mutation site from the donor lymphocyte infusions by PCR and subjected the products to ultrasensitive deep sequencing (average read coverage at mutation site, 361,723; range, 16,684–1,169,555). To correct for background frequencies of non-germline calls, we amplified and deep sequenced the same positions from the donor buccal swab by PCR (average read coverage at the mutation site, 418,499; range, 20,711–1,070,734). Mutations categorized as unique to one follicular lymphoma were also amplified by PCR from the other follicular lymphoma and deep sequenced in parallel.

Eleven of the 12 single nucleotide variants and the 2 insertions/deletions that were identified in both follicular lymphomas were “enriched” in the donor lymphocyte infusion, that is, recovered at frequencies significantly above background (Table 1). Thus, those mutations were acquired more than 7 years before presentation of either follicular lymphoma. All 4 single nucleotide variants unique to the recipient’s follicular lymphoma and the RFTN1 V254M mutation identified only in the donor’s follicular lymphoma were not enriched in the donor lymphocyte infusion or the other follicular lymphoma, which is consistent with subsequent acquisition during clonal divergence. RFTN1 (raftlin linking protein 1) is required for the integrity of lipid rafts in B cells and regulates B-cell receptor signaling (18). Recurrent mutations in close proximity to RFTN1 V254 were recently reported in patients with diffuse large B-cell lymphoma (10).

Of the final 2 mutations, ATP6V1B2 R400Q (Fig. 3F) was detected only in the donor’s follicular lymphoma but was enriched in the donor lymphocyte infusions. CTSS M185V (Fig. 3F) was initially detected only in the donor’s follicular lymphoma, but deep sequencing recovered the mutation in 4.7% of reads from the recipient’s follicular lymphoma and demonstrated enrichment in the donor lymphocyte infusion (Table 1).

DISCUSSION

The presentation of virtually synchronous follicular lymphomas in a donor–recipient pair many years after hematopoietic cell transplantation provided the opportunity to interrogate clonal evolution in separate hosts. We demonstrate that the same BCL2/IGH rearrangement in both follicular lymphomas was also present within the donor lymphocyte infusions passed from donor to recipient 7 years before the presentation of follicular lymphoma. The BCL2/IGH-harboring cells within the donor lymphocyte infusion were derived from a common ancestor, have undergone the germinal center reaction, and were capable of initiating follicular lymphoma with a surprisingly similar latency.

BCL2/IGH-harboring cells were present at a frequency of 1 in 2,000 within the donor lymphocyte infusions. This high frequency suggests that the BCL2/IGH-harboring cells had a survival advantage within the donor more than 7 years before the presentation of lymphoma. On the basis of a frequency of 1 in 2,000 cells, more than 4 million potential follicular lymphoma precursors were transferred within the donor lymphocyte infusions from the donor to the recipient. The true number of transplanted follicular lymphoma precursors may have been greater for 2 reasons.
**Figure 3.** Mutations in the paired follicular lymphomas. A, two single nucleotide variants were present in BCL2 in both follicular lymphomas (FL) but not detectable by Sanger sequencing in the donor lymphocyte infusion (DLI). Only the mutant allele is present in cDNA from the recipient’s follicular lymphoma. B, an in-frame deletion in EP300 and an in-frame insertion in KLHL6 were detectable by Sanger sequencing in both lymphomas. In contrast with BCL2, both the wild-type and mutated alleles of KLHL6 were expressed in the recipient’s follicular lymphoma. C, exome sequencing reads spanning ARID1A R1276 in the recipient’s follicular lymphoma are shown. The single nucleotide variant was only detectable in the recipient’s follicular lymphoma. GL, germline. D, immunohistochemistry for ARID1A/BAF250 (Santa Cruz) is shown in both lymphomas and a tonsil from a healthy individual. Supplementary Figure S4B shows similar findings using a different anti-BAF250 antibody and normal control. E, quantitative PCR copy number assay at the ARID1A locus. The statistically significant decrease was confirmed in repeated iterations of this experiment. F, single nucleotide variants in CTSS and ATP6V1B were detectable by Sanger sequencing only in the donor’s follicular lymphoma. Both were enriched in the donor lymphocyte infusion on the basis of ultra-deep sequencing and CTSS M185SV was also present in 4.7% of reads from the recipient’s follicular lymphoma.
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**Note:** The Bonferroni-adjusted 2-sided P value of 0.00238 was the level determining statistical significance.

**Abbreviations:** SNV/Indel, single nucleotide variation/insertion/deletion; DLI, donor lymphocyte infusion; FL, follicular lymphoma; NA, not applicable.

*Donor buccal swab served as germline.

*Mutation initially not identified in filtered exome sequencing dataset (<2 variant reads or Phred score <30) but subsequently recovered by visualization of reads using IGV (Integrated Genome Viewer v2.0).

*Mutations also not detectable in recipient buccal swab.
First, BCL2/IGH-harboring cells could have been present in the original bone marrow transplant, which was not available for analysis. Second, it is possible that somatic hypermutation affected the primer sites we used for quantitative PCR quantification of BCL2/IGH in some or all of the BCL2/IGH-rearranged cells.

To identify markers of convergent and divergent clonal evolution within the 2 follicular lymphomas, we performed exome sequencing. Previous efforts at exome sequencing have primarily used high-quality DNA from fresh or fresh-frozen tissues and cell suspensions with high tumor cell content (10, 15). In contrast, the DNA available for our study was isolated from formalin-fixed, paraffin-embedded tissue with only moderate lymphoma cell infiltration (~40%–45% and 60%–65% for the donor’s and recipient’s follicular lymphoma, respectively (Supplementary Fig. S4A)]. The number of coding single nucleotide variants in a recent study of non-Hodgkin lymphomas ranged from 20 to 135 per genome (10). Because we identified fewer than 20 coding single nucleotide variants per follicular lymphoma, it is likely that additional mutations were present but not recovered. The recovery and validation rates in our study, although within previously reported ranges (19, 20), were likely reduced compared with recent comprehensive analyses of B-cell lymphoma (7, 10), because of the use of formalin-fixed, paraffin-embedded-derived specimens with limited tumor cell infiltration. Nonetheless, the cohort of mutations we identified was sufficient to track the acquisition of somatic single nucleotide variants and insertions/deletions within follicular lymphoma ancestors over several years before clinical presentation.

Among the mutations present in both follicular lymphomas and the donor lymphocyte infusions was a novel in-frame insertion in the Kelch-1 domain of KLHL6, downstream of the single nucleotide variants recently described in chronic lymphocytic leukemia (15) and non-Hodgkin lymphoma (10). KLHL6 is a lymphoid-tissue specific BTB-kelch protein that is highly expressed in germinal center B-cells and involved in B-cell receptor signaling (21). We also recovered a novel in-frame deletion in the bromodomain of EP300, an acetyltransferase involved in transcriptional activation of p53 and inactivation of BCL6 (7). Disruption of EP300 or its partners CREBBP and MEF2B occurs in more than 50% of follicular lymphoma cases (7, 22). Our data indicate that the EP300 mutation was acquired several years before the presentation of follicular lymphoma, raising the possibility that acetyltransferase alterations can be early oncogenic events.

Loss-of-function mutations in ARID1A/BAF250, a putative tumor suppressor, are present in approximately 60% of ovarian clear cell carcinomas, 30% of endometroid carcinomas (23, 24), and other epithelial tumors but have not yet been reported in hematologic malignancies. Of note, the ARID1A locus is within a commonly deleted region on chromosome 1p within close proximity to TNFRSF14, which is recurrently mutated in follicular lymphoma (9). We demonstrate that the recipient’s lymphoma acquired a premature stop codon mutation in ARID1A that could not be retrieved from the donor’s lymphoma or the donor lymphocyte infusion, suggesting a later event in lymphomagenesis. Interestingly, both lymphomas showed reduced ARID1A/BAF250 protein staining, suggesting alternative mechanisms of ARID1A inactivation. Based on the frequency of variant reads at single nucleotide variant sites from exome sequencing (Table 1), the donor’s follicular lymphoma specimen contained approximately 45% tumor cells. Staining of the tumor for CD10-positive cells was consistent with this estimate (Supplementary Fig. S4A). Thus, we would expect single allele loss to result in a copy number of approximately 1.55. The difference between 1.55 and the measured allele frequency in the donor’s follicular lymphoma (1.84) could indicate that ARID1A allele loss was limited to a subpopulation of the donor’s follicular lymphoma or it could simply reflect the limit of precision for this assay.

The ATP6V1B2 R400Q mutation was enriched in the donor lymphocyte infusion but only present in the donor’s follicular lymphoma. Similarly, CTSS M185V was present in the donor lymphocyte infusion, the donor’s follicular lymphoma, but only a small subset of recipient follicular lymphoma cells. These findings are consistent with at least 2 scenarios. First, the recipient’s follicular lymphoma could be derived from a clonally diversified population of ancestor cells transferred from the donor. A second possibility is that the mutant allele was lost during clonal evolution in some (CTSS M185V) or all (ATP6V1B2 R400Q) of the follicular lymphoma cells within the recipient. Loss of a mutant allele would suggest that the allele is a passenger rather than a driver of lymphomagenesis.

The donor’s and recipient’s lymphomas presented after a long but surprisingly similar latency despite marked differences in immune competence between the host and recipient. Previously, Christian and colleagues (25) reported donor-derived mantle cell lymphomas diagnosed virtually simultaneously in donor and recipient 12 years after transplantation. Similar latency in donor and recipient lymphomas could indicate that host immunity plays a limited role in restraining the expansion of some follicular lymphomas and mantle cell lymphomas. Previous studies have demonstrated that prognosis among patients with follicular lymphoma correlates with transcriptional signatures derived from infiltrating, nonmalignant T cells, macrophages, and dendritic cells (26). Therefore, the pace of disease expansion may to some extent be separable from prognosis if immune competence affects the latter but not the former.

In conclusion, we used ultrasensitive mutation detection to elucidate the molecular ontogeny of follicular lymphoma during clonal evolution in separate hosts. The same approach has broad applicability for identifying genetic variants within pooled tumor populations that confer subsequent phenotypes, including therapeutic resistance and metastatic potential.

**METHODS**

The study was approved by the Dana-Farber Cancer Institute Institutional Review Board, and both participants gave written informed consent. Additional description of methods is included in the Supplementary Material.
Nucleic Acids

Nucleic acids were isolated from formalin-fixed, paraffin-embedded lymph node biopsies of both lymphomas and residual aliquots of the fresh-frozen donor lymphocyte infusion. Germline DNA of both patients was isolated from buccal swabs (QIAGEN).

Immunohistochemistry

Immunohistochemistry was performed on 5-μm formalin-fixed, paraffin-embedded tissue sections with the following primary antibodies: anti-CD20 (RTU, clone L26; DAKO); anti-CD10 (1:80 dilution, clone 56C6; Novocastra/Leica Microsystems), anti-BCL6 (1:100 dilution, catalog number sc-858; Santa Cruz Biotechnology), anti-BCL2 (1:1000 dilution, catalog number M0887; DAKO), and anti-ARID1A/BAF250A (1:50 dilution, catalog number sc-32761, Santa Cruz Biotechnology, Fig. 3E; 1:150 dilution, catalog number HPA005456, Sigma-Aldrich, Supplementary Fig. S4B).

Real-Time Quantitative PCR

Real-time quantitative PCR (TaqMan; Applied Biosystems) for BCL2/IGH and GAPDH was performed in triplicate and repeated on separate dates. A translocation-specific probe was utilized that hybridizes to the junction region. Standard curves were generated using serial dilutions of cloned BCL2/IGH and GAPDH PCR products and analyzed according to published guidelines (27). Refer to the Supplementary Methods for details.

Flow Cytometry Sorting

The donor lymphocyte infusion sample was stained for CD19 (PE-Cy7 anti-CD19, clone HIB19; BD Pharmingen) and sorted (BD ARIA II SORP Sorter, BD Biosciences). Postsorting analysis demonstrated 98.1% (CD19 positive), and 99.8% (CD19 negative) pure populations (Supplementary Fig. S1), respectively, and genomic DNA was extracted using DNA Blood Mini kit (QIAGEN).

Duplex Real-Time PCR Copy Number Assay

A duplex real-time PCR copy number assay (TaqMan; Applied Biosystems) was performed with the use of genomic DNA from both follicular lymphomas and germline samples with FAM-labeled ARID1A copy number assay (HS02336512_cn; Applied Biosystems) and VIC/TAMRA-labeled RNaseP Copy Number Reference Assay (Applied Biosystems) according to the manufacturer’s recommendations (7500 Real-Time PCR System; Applied Biosystems) and analyzed with CopyCaller Software (version 1.0; Applied Biosystems).

Exome Sequencing

Genomic DNA from both follicular lymphomas and the donor lymphocyte infusion as it represents the most recently passed specimen was equal to 1 for each comparison. We analyzed the donor lymphocyte infusion, both follicular lymphomas, and the donor’s buccal swab. PCR products overlapping the same region were divided into separate pools. Library construction from the PCR products was performed with the Amplicon Concatenation Protocol for the Applied Biosystems SOLiD system (Life Technologies). A library was constructed from each of 2 aliquots from each sample and barcoded for multiplexing. In brief, 500 ng of pooled PCR products were concatenated with T4 ligase, sheared to approximately 150 base pairs, ligated to adapters, and amplified by PCR. Barcoded libraries were then mixed and the mixture sequenced on each of 2 slides. Use of 2 aliquots of each PCR pool served as a technical replicate for library construction; sequencing the mixtures on 2 slides served as a technical replicate for the sequencing. Approximately 50 bases from one end of each fragment were sequenced with the SOLiD 4 instrument and reagents.

Resulting data were mapped to a single, custom reference generated to include all fragment sequences using either mapreads or BFAST, for single nucleotide variant and insertions/deletions detection, respectively. For both analyses, each observed substitution or insertions/deletions was tallied by position and strand across the sequence using Samtools v0.1.12a. To correct for erroneous mis-incorporations by sequencing and positional bias, the lesser of the stranded tallies were taken as the coverage.

Statistical Analysis

Statistical analysis of targeted deep sequencing was performed by calculating the relative mutation frequency (f) for each candidate gene, assuming that the number of mutations follows a Poisson distribution. We tested the null hypotheses that the mutation rate ratio between donor lymphocyte infusion and germline (fDLI/fgermline) was equal to 1 for each comparison. We analyzed the donor lymphocyte infusion as it represents the most recently passed specimen from donor to recipient. The Bonferroni-adjusted 2-sided P value of 0.00238 (= 0.05/21) was the level determining statistical significance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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