Methods for Sample Acquisition and Processing of Serial Blood and Tumor Biopsies for Multicenter Diffuse Large B-cell Lymphoma Clinical Trials

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Abstract

Increasingly, targeted therapies are being developed to treat malignancies. To define targets, determine mechanisms of response and resistance, and develop biomarkers for the successful investigation of novel therapeutics, high-quality tumor biospecimens are critical. We have developed standard operating procedures (SOPs) to acquire and process serial blood and tumor biopsies from patients with diffuse large B-cell lymphoma enrolled in multicenter clinical trials. These SOPs allow for collection and processing of materials suitable for multiple downstream applications, including immunohistochemistry, cDNA microarrays, exome sequencing, and metabolomics. By standardizing these methods, we control preanalytic variables that ensure high reproducibility of results and facilitate the integration of datasets from such trials. This will facilitate translational research, better treatment selection, and more rapid and efficient development of new drugs.

See all the articles in this *CEBP Focus* section, "Biomarkers, Biospecimens, and New Technologies in Molecular Epidemiology."

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Introduction

Personalized medicine in oncology, designed to target specific genes or pathways altered in an individual tumor, is becoming a new paradigm. Such tailored approaches rely on the profiling of malignant tissues by high-throughput technologies, followed by the selection of appropriate treatment, targeting tumor-specific aberrations. However, challenges remain, some of which are due to inherent properties of cancer biology, study design, regulatory issues, and costs (1). One additional underestimated challenge is the isolation of suitable biospecimens for tumor characterization in a reliable and reproducible manner.

Differences in biospecimen collection, processing and storage, within and among institutions, lead to sample variability, which can affect the results of downstream assays (2, 3). In recognition of this, the field is beginning to develop and implement standard operating procedures (SOPs) and best practice guidelines (3–5). A repository of protocols relating to biospecimen collection and handling exists (5), but contains limited guidance for biopsy collection and processing, which typically differ from primary tissue acquisition due to the small size of biopsies (6).

We hypothesized that serial biospecimens could be collected safely in the context of multicenter clinical trials in patients with diffuse large B-cell lymphoma (DLBCL). Our intention was to develop protocols to standardize the acquisition and processing of peripheral blood samples and needle core biopsies (NCB) from multiple hospitals, including those with limited access to laboratory equipment or liquid nitrogen. Thus, the tissue/blood could be acquired at each site, but processed at a central laboratory (Jewish General Hospital, JGH). We now describe protocols for tissue collection and nucleic acid isolation from NCBs, which allow for serial biopsies from the same lymph nodes at multiple time points from the same patient. Protocols for the

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isolation of plasma and peripheral blood mononuclear cells (PBMC) from blood are also described. We provide details about the yield and quality of the isolated nucleic acids, as well as data describing the effect of shipment versus immediate processing. Finally, we present data on the utility of the isolated material in downstream applications.

Materials and Methods

Patient population

Correlative studies were performed in the Q-CROC-02 clinical trial in relapsed/refractory DLBCL (Clinicaltrials.gov identifier: NCT01238692), in which four Canadian sites (Jewish General and Sacré Coeur Hospitals in Montréal, Princess Margaret Hospital in Toronto, and Queen Elizabeth II Hospital in Halifax) recruited patients; the JGH also served as the central processing laboratory. Ethics board approved the project at each site and patients provided consent to conduct the biopsies in the context of this clinical trial. Peripheral blood and NCBs were collected before and after 15 days of treatment (Fig. 1).

Collection and shipping of NCBs and blood

To ensure enough material for all applications, four NCBs from an accessible lymph node were isolated (Supplementary File S1: Collection). The first core was placed immediately in 10% formalin for use in immunohistochemistry (IHC) assays. The three subsequent cores were pooled together in cell culture medium containing 10% FBS. All non-JGH biopsies were shipped at 4°C using overnight delivery (Supplementary File S2: Shipping).

Peripheral blood samples (approximately 15–18 mL) were collected in BD Vacutainer sodium heparin tubes and shipped to the central processing laboratory at ambient temperature using overnight delivery.

Sample processing and nucleic acid extraction

A single-cell suspension was created from the three biopsies, and tumor cells were isolated using a magnetic bead-negative selection technique (human B cell enrichment cocktail without CD43; Stem Cell Technologies) to enrich for tumor cells and reduce contamination by non-malignant cells, which significantly contribute to variability, as well as limit sensitivity for gene-expression profiling and detection of somatic alterations (Supplementary File S3: DNA and RNA isolation; ref. 6). Blood samples were centrifuged to separate plasma from cellular elements. Plasma was frozen, whereas PBMCs were isolated by Ficoll gradient separation (Supplementary File S4: Blood protocol). DNA and RNA were simultaneously extracted from the isolated tumor cells and PBMCs using the All-Prep RNA/DNA (Qiagen) Kit. Nucleic acid quality and yield were measured using a NanoDrop 1000 spectrophotometer.

Immunohistochemistry

IHC was performed at the Segal Cancer Centre Research Pathology Facility (JGH) using standard IHC protocols, which are detailed in Supplementary File S5: IHC. Every biopsy had a section stained with hematoxylin and eosin (H&E) and tumor content/viability was assessed. CD20 immunostaining was performed using the Discovery XT Autostainer (Ventana Medical System) and a standardized diagnostic applications protocol. Two reference tissue samples were included on each slide as a positive and negative control. Sections were analyzed by conventional light microscopy.

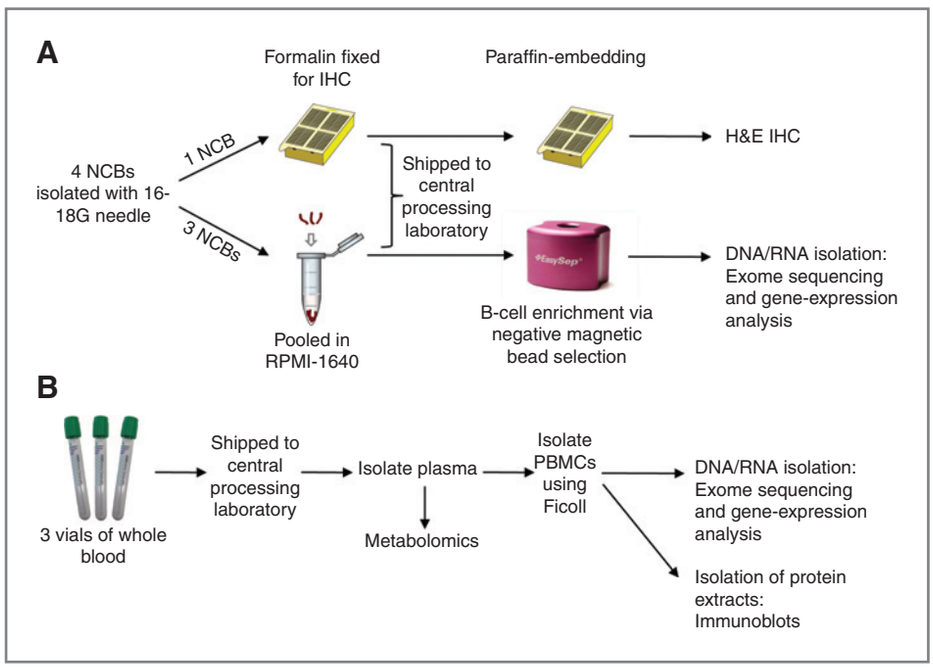

Figure 1. Schematic of Correlative Studies for Q-CROC-2. A, four NCBs were collected. The first biopsy was fixed and used for IHC, whereas the next three were combined in a tube with media. All samples were shipped to a central processing laboratory. The formalin-fixed sample was embedded in paraffin for use in IHC. The other biopsies were combined into a single-cell suspension and tumor cells were isolated by magnetic bead selection. These samples were used for DNA/RNA isolation. B, peripheral blood samples were shipped to the central processing laboratory, where plasma and PBMCs were separated. PBMCs were used for DNA/RNA isolation and if adequate sample was acquired, protein extracts were made.

Exome sequencing

Exome sequencing was performed at either the Institut de Recherche en Immunologie et Cancérologie (13 cases) at the Université de Montreal or at the Genome Québec Innovation Centre (13 cases) using the TruSeq Exome Capture Kit and the Nextera rapid capture exome kits, respectively. All samples were sequenced on HiSeq 2000 instruments with 100-nt paired reads.

cDNA microarray analyses

Microarray analyses were performed at the Genome Québec Innovation Centre, comparing Agilent (Sureprint 8 × 60 K one-color human expression array) and Affymetrix (Human Gene 2.0 ST Array) platforms. Before hybridization and scanning, RNA quality was assessed using an Agilent BioAnalyzer 2100 to determine the RNA integrity number (RIN).

Metabolomics

Metabolomic approaches measure all the metabolites present within a system simultaneously. Plasma for metabolomics analysis (100 μL) was kept at −80°C until processing by a combination of gas chromatography and ultraperformance liquid chromatography coupled with mass spectrometry with positive and negative electrospray ionization (GC-MS^{+ESI}, UPLC-MS/MS^{+ESI}, and UPLC-MS/MS^{-ESI}) at Metabolon, Inc. For technical quality control, replicate samples were created from a homogenous pool containing a small amount of all study plasma samples. Overall process variability was determined by calculating the median relative SD for (i) the internal standards that were added to each sample before the injection into the mass spectrometers (acceptable instrument variability is set at 5%), and (ii) all endogenous metabolites (i.e., noninstrument standards) present in 100% of the pooled sample, which are technical replicates of pooled samples (acceptable total process variability is set at 10%).

Results

We developed SOPs to collect and process biomaterials from patients with lymphoma enrolled in multicenter clinical trials. Then, we validated our SOPs in the context of a clinical trial in which biopsies and peripheral blood were collected pre- and posttreatment.

Biopsies

Of the 40 patients enrolled in the trial, 27 (67.5%) underwent a pretreatment biopsy, and 14 patients (35%) also underwent the day 15 biopsy. Most biopsies were performed by an interventional radiologist using ultrasound guided techniques. No serious adverse events occurred in relation to the biopsies. However, 3 patients had biopsy-related bleeding, all of which were self-limited; 2 were minor bleeds not requiring intervention, and one bleed required medical intervention (grade 1 and grade 2, respectively, by the Common Terminology Criteria for Adverse Events version 4; ref. 7).

Biopsies were all processed at the central processing laboratory (JGH), where tumor B cells were separated and nucleic acids extracted. We assessed whether the quality of biomaterial differed depending upon the research site where collection was performed and by transport to the central processing laboratory. We isolated fewer tumor cells from samples collected at non-JGH sites (see Table 1). This may be attributed to the biopsy system (8) or to shipping. The JGH used larger (16 gauge) needles for biopsy collection and recovered more cells than other participating sites (4.75 vs. 1.98 million, $P = 0.05$). The time from tissue acquisition to tissue processing was also significantly shorter for JGH biopsies compared with other sites (27 minutes vs. 23 hours). There were only four shipped biopsies in which the temperature indicator tag showed exposure above 10°C. While these tended to have lower quality by the 260/280 ratio, it was not significant (1.875 vs. 1.788; $P = 0.0889$). Despite the decreased cell number, there was no difference in the DNA quantity or quality between research sites (Table 1). In contrast, we recovered significantly less RNA when biopsies were shipped from non-JGH research sites. However, while we recovered less RNA, it was of excellent quality with a RIN average of 9. Furthermore, nucleic acids isolated from day 15 biopsies were also of excellent quality, independent of collection site. Thus, we conclude that high-quality tumor-derived nucleic acids were isolated from all enrollment sites.

Blood samples

We collected pretreatment blood samples from all enrolled patients ($N = 40$) and day 15 blood samples in all but 1 patient, who went off study due to a drug-related adverse event. The yield and quality of DNA isolated from PBMCs were comparable among sites (Table 1), but the yield of RNA was greater when the blood did not require shipping. Despite this, the RNA quality was high.

Immunohistochemistry

The first NCB was preserved in formalin immediately upon procurement from the patient, embedded, and subsequently used for immunohistochemical analyses at the central processing laboratory. In our experience, the core obtained from the first pass provides the best tissue integrity, important for histologic assessment. Representative images of H&E stains and IHC (stained with anti-CD20) are shown (Fig. 2A). We found that all biopsies, regardless of collection site, were of high quality. Thus, shipping did not affect the quality of fixed tissue.

Sequencing and microarrays

Of the 27 patients with pretreatment biopsies in this study, we submitted DNA on 26 tumor/germline pairs ($n = 52$ samples) for exome sequencing, in which 1 patient was excluded because of low DNA yield from the tumor biopsy. For the first 13 patient pairs, libraries were constructed using TruSeq in pools of 4 to 5 samples. Unfortunately, due to technical difficulties unrelated to DNA quality, this resulted in an imbalanced distribution of reads

Table 1. Comparison of nucleic acids from biopsies and blood with and without shipping

	JGH central laboratory	Non-JGH sites	*P*
Tissue collection			
Number of biopsies obtained			
At time of accrual (day 0)	11	16	
At day 15	9	5	
Needle gauge			
<18 gauge	13	3	0.029[a]
18 gauge	6	9	
Mean time from tissue collection to processing (HH:MM)	0:27 ± 0:06	23:33 ± 0:56	
Cell count from biopsy ($\times 10^6$)			
Day 0	5.27 ± 1.4 ($n = 11$)	0.79 ± 0.15 ($n = 12$)	0.004[a]
Day 15	3.86 ± 1.19 ($n = 8$)	0.43 ± 0.04 ($n = 4$)	0.074
Plasma volume collected (mL)			
Day 0	3.97 ± 0.41 ($n = 15$)	2.70 ± 0.31 ($n = 25$)	0.02[a]
Day 15	3.82 ± 0.32 ($n = 15$)	2.79 ± 0.31 n = 24)	0.03[a]
Nucleic acid yield and quality			
Tissue DNA			
Yield from day 0 (μg)	12.2 ± 2.7 ($n = 11$)	11.9 ± 3.1 ($n = 11$)	0.95
Yield from day 15 (μg)	15.6 ± 6.0 ($n = 8$)	10.3 ± 5.8 ($n = 4$)	0.59
DNA 260/280 ratio day 0	1.86 ± 0.02 ($n = 11$)	1.86 ± 0.03 ($n = 11$)	0.98
DNA 260/280 ratio day 15	1.93 ± 0.06 ($n = 8$)	1.92 ± 0.04 ($n = 4$)	0.83
Tissue RNA			
Yield from day 0 (μg)	7.81 ± 2.7 ($n = 11$)	0.99 ± 0.25 ($n = 12$)	0.02[a]
Yield from day 15 (μg)	5.78 ± 2.14 ($n = 8$)	3.38 ± 3.04 ($n = 4$)	0.53
RNA 260/280 ratio day 0	1.82 ± 0.1 ($n = 11$)	1.92 ± 0.12 ($n = 9$)	0.57
RNA 260/280 ratio day 15	1.96 ± 0.07 ($n = 8$)	2.32 ± 0.72 ($n = 4$)	0.50
RNA RIN day 0	9.06 ± 0.18 ($n = 5$)	8.88 ± 0.30 ($n = 5$)	0.62
Blood DNA			
Yield from day 0 (μg)	17.5 ± 3.2 ($n = 15$)	20.3 ± 3.3 ($n = 25$)	0.57
Yield from day 15 (μg)	23.9 ± 5.4 ($n = 15$)	20.9 ± 2.6 ($n = 24$)	0.80
DNA 260/280 ratio day 0	1.88 ± 0.02 ($n = 15$)	1.86 ± 0.02 ($n = 25$)	0.65
DNA 260/280 ratio day 15	1.87 ± 0.09 ($n = 15$)	1.89 ± 0.02 ($n = 24$)	0.80
Blood RNA			
Yield from day 0 (μg)	5.7 ± 1.0 ($n = 15$)	2.1 ± 0.4 ($n = 24$)	0.0006[a]
Yield from day 15 (μg)	5.9 ± 1.0 ($n = 15$)	5.1 + 0.8 ($n = 24$)	0.54
RNA 260/280 ratio day 0	2.1 ± 0.07 ($n = 15$)	1.98 ± 0.06 ($n = 22$)	0.18
RNA 260/280 ratio day 15	2.05 ± 0.04 ($n = 15$)	2.05 ± 0.02 ($n = 22$)	0.98

NOTE: We compared DNA and RNA isolated from biopsies or PBMCs. The *P* value was determined by the nonparametric *t* test.
[a]$P < 0.05$.

among the individual samples. The second batch of libraries was prepared using the Nextera Rapid Human Exome Kit and yielded more balanced libraries. Overall, we obtained at least 30× redundant sequence coverage across targeted regions for 41 of 52 exomes prepared from these samples. The average coverage achieved across all exomes in targeted regions was 71.5×. As a measure of quality, we assessed the number of duplicate reads in the libraries prepared using the Nextera Kit. When duplicate reads represent less than 5% of the library, this indicates high quality. Of these 26 libraries, only one had higher than 5% duplicate reads (6.7%). There was no significant difference between the libraries derived from blood and biopsy DNA (3.94% vs. 3.98%, $P = 0.8841$). Furthermore, there was no difference in the quality of libraries derived from JGH or non-JGH hospitals (4.15% vs. 3.80%, $P = 0.2131$).

For cDNA microarray analyses, 0.2 μg of RNA is required. Two pretreatment biopsies (both from non-JGH) did not yield enough RNA. We found that both the Agilent and Affymetrix microarrays yielded high-quality data. We used Agilent Feature Extraction Software V10.7.3.1 for 1 Color Gene Expression to assess the overall data quality. The GE1_QCMT_Sep09 thresholds were used for quality control criteria of the microarray data. Any arrays with values outside of the threshold ranges were further evaluated (see Supplementary Table S1).

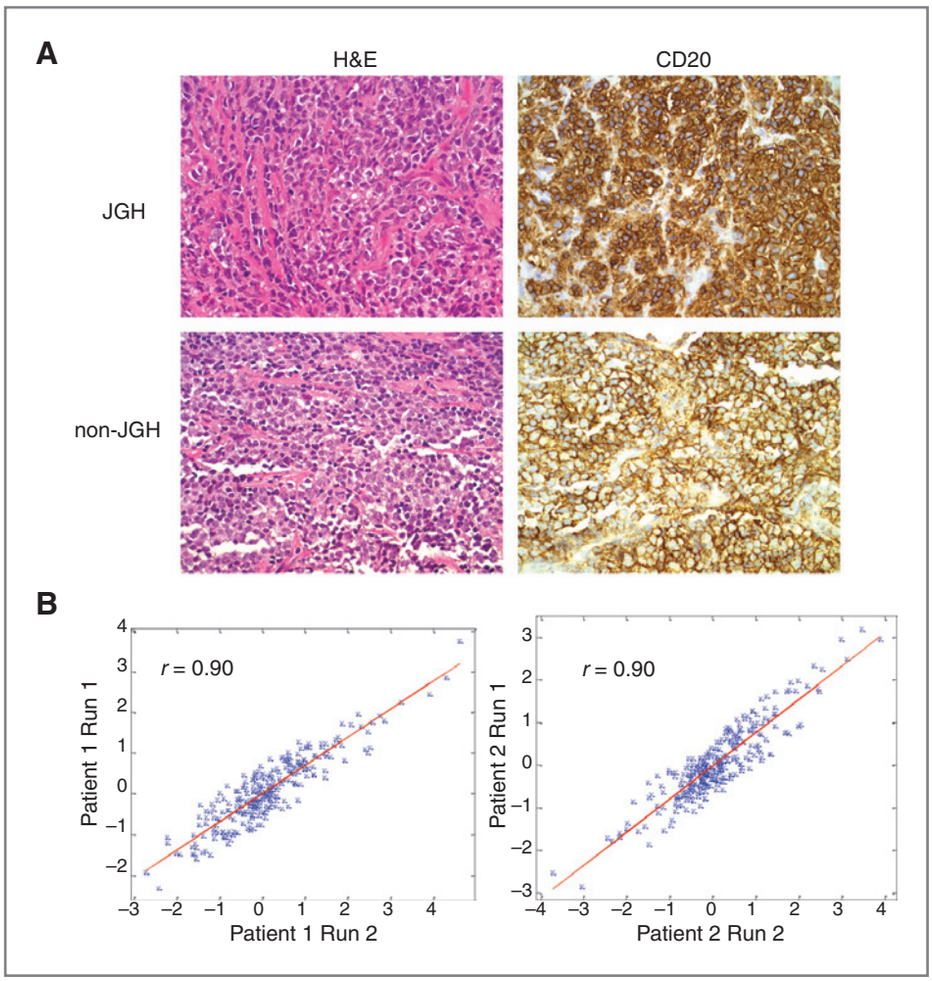

Figure 2. Examples from downstream applications. A, representative H&Es and anti-CD20 IHC are shown for one patient at the site of the central processing laboratory (JGH) and one from an outside hospital. B, metabolites from two samples were run 1 year apart and compared by Pearson coefficient.

Only one sample showed poor results, with values outside of the acceptable range for 8 of the 10 metrics reported. Microarrays using RNA extracted from biopsies collected at JGH and non-JGH sites had equal quality (see Supplementary Table S1).

Metabolomics

All samples passed the technical quality control criteria and were normalized in terms of raw area counts. Each raw data metabolite was then rescaled to set the median equal to 1 and missing values were imputed with minimum values to allow inter sample comparisons. A total of 605 metabolites were identified and quantified in all the samples (389 named and 216 unnamed metabolites). To estimate metabolite measurement variability from sample storage conditions and batch effects, we processed two sets of duplicate samples in two runs, 1-year apart, and found excellent correlation (Pearson correlation $R = 0.9$; Fig. 2B).

Discussion

We have shown that biopsy collection in the context of multicenter clinical trials is feasible, safe, and yields valuable biomaterial for several downstream applications. In DLBCL, tumor subclassification based on gene-expression profiles is predictive of response (9). As more novel therapies are tested, biomarkers of response and resistance will be critical to their development. Thus, protocols such as those defined here may become commonplace in clinical research.

Our goal was to optimize protocols by controlling important preanalytic variables. We did note that RNA yield was most affected by shipping. Several collection methods for blood are available that yield high-quality RNA (10), and should be incorporated into future trials. B cells were isolated from the biopsies after shipping, and thus, precluded the use of stabilizers. However, RNA quantity and quality were sufficient for downstream applications.

The published literature on patient willingness to undergo biopsies solely for research use varies considerably. El-Osta and colleagues (11) found that only 4.4% of patients agreed to an optional biopsy when participating in trials, whereas Gomez-Roca and colleagues (12) found that 68% of patients on optional biopsy protocols had at least one biopsy done and 44% had sequential biopsies.

The latter study is in good agreement with our data, in which we find that 67.5% and 35% of patients had a pre- and posttreatment biopsy, respectively. In our study, a biopsy was required unless deemed unsafe or tissue inaccessible by the treating physician. On the basis of this experience, there are patients in whom biopsy is still not feasible within the context of a clinical trial. For DLBCL, this would include patients with certain retroperitoneal masses or lymph nodes in the celiac axis, porta hepatis, or mediastinum. Most of our biopsies originated in peripheral lymph nodes and were obtained using ultrasound guided techniques. With these resources now available for collection of high-quality material, we hope to enhance the development of targeted agents for use in molecularly defined populations. Our experience will provide a useful starting point for other investigators to consistently and reliably sample lymphoma tissue by NCB in multicenter, biopsy-driven lymphoma trials.

Disclosure of Potential Conflicts of Interest

S.E. Assouline received speakers' bureau honoraria from Roche Canada. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.H. Nielsen, Z. Diaz, S. Qureshi, C. Rousseau, N. Benlimame, K. Klein Oros, J. Krumsiek, R.D. Morin, L. Cerchietti, N.A. Johnson, W.H. Miller Jr, S.E. Assouline, K.K. Mann

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Other (clinical research assistant): R. Christodoulopoulos

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