Published in final edited form as:

Br J Haematol. 2015 June ; 169(5): 689–693. doi:10.1111/bjh.13349.

Detection of classical Hodgkin lymphoma specific sequence in peripheral blood using a next-generation sequencing approach

Yasuhiro Oki¹, Sattva S. Neelapu¹, Michelle Fanale¹, Larry W. Kwak¹, Luis Fayad¹, Maria A. Rodriguez¹, Michael Wallace², Mark Klinger³, Victoria Carlton³, Katherine Kong³, Malek Faham³, and Anas Younes¹

¹Department of Lymphoma and Myeloma, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

²Department of Interventional Radiology, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

³Sequentia, Inc., South San Francisco, CA, USA

Summary

We applied a highly sensitive next-generation sequencing method to identify lymphoma-specific immunoglobulin gene segments in classical Hodgkin lymphoma (CHL) at initial diagnosis or recurrence, and assessed the ability of detecting such lymphoma-specific sequences in peripheral blood (PB). Seventeen CHL cases were tested and lymphoma-specific sequences were identified in 12 of the primary tumour biopsies. In 11 of these patients whose paired PB samples were available, tumour-specific clonotypes were detected in PB in eight patients. This data demonstrates the feasibility of detecting circulating tumour-specific sequences, creating an unprecedented opportunity to optimize the future treatment and monitoring strategies for patients with CHL.

Keywords

sequencing; minimal residual disease; Hodgkin lymphoma

Classical Hodgkin lymphoma (CHL) is considered a highly curable disease. However, up to 20–30% of high-risk patients succumb to recurrent or refractory disease (Kuruvilla, 2009; Moskowitz, 2012). Accurate determination of disease status after completion of treatment may provide new opportunities for formulating surveillance and/or management strategies. However, the scarcity of the malignant cells and the lack of protein-based tumour makers have thus far precluded the development of clinical assays to evaluate minimal residual

Correspondence: Yasuhiro Oki, MD, Department of Lymphoma and Myeloma, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd. Unit 429, Houston, TX 77030, USA. yoki@mdanderson.org.

This study was presented in part at the American Society of Hematology Annual Meeting 2013.

Author contributions

YO designed the study, provided patient care, analysed data and wrote the paper. MF, LWK, LF and MAR provided patient care and edited paper. SN and MW supported sample collection and storage and edited paper. MK, VC, KK and MF conducted laboratory work, analysed data and wrote the paper. AY provided the concept of the study and edited the paper.

Conflict of interest

MK, VC, KK and MF are employees of Sequentia Inc. All other authors reported no conflict of interest to disclose.

disease (MRD) in CHL. The current modality to assess disease status is based primarily on radiographic studies (Hoppe *et al*, 2012), which are associated with significant cost, radiation exposure and limited specificity. New methods are needed that are safe, inexpensive and predictive of clinical outcome.

The malignant Hodgkin Reed Sternberg (HRS) cells are considered of B-cell origin (Kuppers *et al*, 1994; Marafioti *et al*, 2000). A previous report suggested that clonotypic B-cells may be present in the blood of patients with CHL (Jones *et al*, 2009); however, the relationship between these circulating clonotypic B-cells and CHL is unclear. In this pilot study, we assessed the ability of a next-generation sequencing approach, the LymphoSIGHT™ method (Sequentia, Inc., South San Francisco, CA, USA) (Faham *et al*, 2012), to identify lymphoma-specific immunoglobulin gene rearrangements in primary tumour samples and detect such lymphoma-specific clonotypes in peripheral blood (PB) in patients with CHL at diagnosis or disease recurrence as well as in follow-up blood samples. This method has the sensitivity to detect one lymphoma cell per million leucocytes in PB, and has been applied to MRD detection in multiple B-cell malignancies (Faham *et al*, 2012; Gawad *et al*, 2012; Armand *et al*, 2013; Ladetto *et al*, 2014; Logan *et al*, 2013; Vij *et al*, 2014). We show that HRS cells carry clonal rearrangement of the immunoglobulin loci and thus, such loci can be used as highly specific tumour markers for detection of the disease.

Materials and methods

This study was approved by the M.D. Anderson Cancer Center Institutional Review Board. CHL cases with both primary tissue and blood samples, taken either at the time of diagnosis or at recurrence were selected for evaluation. All patients provided written consent for analysis of the biopsy specimen. First, DNA and RNA were extracted from frozen primary tumour biopsy samples and analysed for clonality at the immunoglobulin heavy chain (*IGH*) and kappa chain (*IGK*) loci using the LymphoSIGHT™ method. Briefly, rearranged immunoglobulin gene segments (*IGH*-VDJ, *IGH*-DJ and *IGK*) in the genomic DNA were amplified with locus-specific primer sets, sequenced and analysed using standardized algorithms for clonotype determination (Gawad *et al*, 2012). For D-J amplification, we used primers in the D intron to specifically amplify only incomplete (D-J) rearrangements at the immunoglobulin locus. Thus, if *IGH*-DJ clonotypes are identified, they will be different from *IGH*-VDJ clonotypes. Only rearranged immunoglobulin genes are amplified with this method, and thus, reactive cells that do not carry immunoglobulin rearrangements are purposefully disregarded in this analysis. We used an assay quantitation approach, which employs spikes of known amounts of reference sequences into each nucleic acid sample (Faham *et al*, 2012). The inclusion of spikes enabled us to quantitate the absolute number of each immunoglobulin molecule present in a sample. Therefore, lymphoma molecules, number of clone molecules and frequency of *IGH/IGK* sequences are all metrics that are calculated based on a conversion of reads to molecules according to the known spike quantities. Clonotypes with a frequency >5% in the B-cell repertoire of the tumour biopsy were considered to represent tumour clonotypes, as previously validated (Faham *et al*, 2012; Gawad *et al*, 2012). Though the frequency of the malignant cells in CHL is known to be much lower than that in B-cell non-Hodgkin lymphomas, the same threshold of 5% in the rearranged immunoglobulin sequence was applied to determine the lymphoma clonotype in

order to eliminate the false-positive rate, as this is a methodology-specific value rather than a disease-specific value. In addition, *IGH*-VDJ clonotypes with a frequency >2% in DNA were deemed to be cancer-specific if the clonotype was not present in the RNA obtained from the same specimen. Such clonotypes were deemed suitable for follow-up, and were then quantitated in serum and peripheral blood mononuclear cells (PBMC) using the same LymphoSIGHT method. The levels of the tumour clonotypes in the blood compartments were calculated relative to the total number of rearranged B-cell sequencing reads in the sample. The sensitivity to detect one lymphoma molecule in one million leucocytes has been previously validated in dilution experiments (Faham *et al*, 2012).

Results and discussion

A total of 17 CHL patients were analysed. The patient characteristics are summarized in Table I. Thirteen *de novo* cases were analysed at diagnosis, and four cases were analysed at recurrence. A high-frequency clonal rearrangement was identified for at least one receptor (*IGH*-VDJ, *IGH*-DJ and *IGK*) in 12 of 17 cases (71%), which we subsequently refer to as a 'calibrating receptor'. A single calibrating receptor was identified in four patients (33%), and two calibrating receptors were found in eight patients (67%) (Fig 1A). *IGH*-VDJ clonal rearrangements were detected in 10 patients (83%), while *IGH*-DJ clonal rearrangements were detected in six patients (50%) (Fig 1A). *IGK* clonal rearrangements were found in four patients (33%).

In 11 patients with identified lymphoma-specific clonotypes, paired serum and/or PBMC samples were also analysed for the presence of the lymphoma-specific clonotype. Lymphoma-specific clonotypes were detected in the blood samples from 8 of the 11 patients (73%). Notably, a lymphoma-specific clonotype was detected in the serum compartment in 8 of 9 cases (89%) tested (Fig 1B), while it was detected in PBMC only in 3 of 9 cases (33%) tested (Fig 1C). The patients that were positive in the PBMC compartment were also positive in the serum compartment. Follow-up samples obtained from three patients after achieving clinical remission were negative for the tumour-specific clonotype in both the serum and cellular compartments.

We conducted sequence and expression level analysis of each *IGH*-VDJ clonal rearrangement. We calculated the number of somatic mutations in each lymphoma-specific clonotype compared to the germline sequence in the interrogated region. A median of 14 somatic mutations (range 0–27) was observed in the 10 patients with *IGH*-VDJ clonal rearrangements. These findings are consistent with previous studies reporting a high load of somatic mutations in V region in HRS cells (Kanzler *et al*, 1996; Braeuninger *et al*, 1997; Marafioti *et al*, 1997). Moreover, this confirms that HRS cells correspond in their developmental stage to germinal or post-germinal centre B-cells (Marafioti *et al*, 2000). While *IGH*-VDJ clonotypes were observed frequently in DNA obtained at diagnosis, *IGH*-VDJ clonotypes were not detected in the RNA from the same sample. This confirms that the immunoglobulin heavy chain of the HL clone is not expressed.

We performed an exploratory analysis to determine the association between the presence of lymphoma-specific clonotypes in the cellular compartment at diagnosis and eventual

progression. All three untreated patients that were positive at baseline in the cellular compartment experienced relapse or progression (at 3, 11 and 17 months). In contrast, none of the five patients without detectable lymphoma-specific clonotypes in their cellular compartment at baseline experienced relapse (follow up duration 23–45 months).

This is the first clinical assay that can be used to detect MRD in CHL. HRS cells comprise only a small population of cells in tumour biopsies. Yet, we were able to identify lymphoma-specific clones in tumour biopsy specimens using the LymphoSIGHT™ sequencing method. This is largely due to the fact that the sequencing method targets only B-cells with *IGH* rearrangements, and is not affected by the presence of other reactive cells, such as T-cells, histiocytes and eosinophils in the microenvironment. Compared to a technically complicated microdissection of HRS cells or unstable flow cytometric evaluation of HRS cells, the sequencing-based approach provides a simple and sensitive method for detecting the lymphoma-specific signature. The detection rate may also depend on the quality and quantity of DNA from the tumour, and further investigation is needed to improve the successful detection rate.

More importantly, lymphoma-specific sequences were identified in the serum from 8 of 11 cases. The lymphoma-specific sequence was more frequently detected in serum than in PBMC, suggesting that the circulating lymphoma cells may lyse easily in the blood, or, more likely, DNA may be released directly from the tumour into the circulation. Our result demonstrates proof-of-principle and underscores the promise of a new methodology to measure disease burden and provide prognostic information from a blood test in patients with CHL, similar to previous work in other lymphoid malignancies (Faham *et al*, 2012; Gawad *et al*, 2012; Armand *et al*, 2013; Logan *et al*, 2013; Ladetto *et al*, 2014; Vij *et al*, 2014). Although detection of the CHL sequence in the blood was not feasible in all analysed patients, our results warrant further evaluation of this method to detect disease burden, potentially to help formulate appropriate treatment and/or follow-up plans. Additional studies will be performed to evaluate the performance of the sequencing-based method using blood specimens compared with PET scans, which will help define the clinical context in which sequencing-based MRD assessment will be applied to CHL patients.

Acknowledgments

This research project was in part supported by M.D. Anderson Cancer Center Start-up Fund to YO. The blood and tissue samples were obtained from the M.D. Anderson Lymphoma Tissue Bank supported by the Lymphoma SPOR (CA136411) to AY, LWK and SSN, the Cancer Center Support Grant CA16672, and Fredrick B. Hagemeister Research Fund. This study was supported in part by research funding from Sequentia, Inc. to MK, VC, KK and MF.

References

- Armand P, Oki Y, Neuberg DS, Faham M, Cummings C, Klinger M, Weng L, Bhattar S, Lacasce AS, Jacobsen ED, Davids MS, Jacobson C, Fisher DC, Brown JR, Fowler NH, Alma Rodriguez M, Wallace MJ, Neelapu SS, Rodig S, Younes A, Freedman AS. Detection of circulating tumour DNA in patients with aggressive B-cell non-Hodgkin lymphoma. *British Journal of Haematology*. 2013; 163:123–126. [PubMed: 23795711]
- Braeuninger A, Kuppers R, Strickler JG, Wacker HH, Rajewsky K, Hansmann ML. Hodgkin and Reed-Sternberg cells in lymphocyte predominant Hodgkin disease represent clonal populations of

- germinal center-derived tumor B cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1997; 94:9337–9342. [PubMed: 9256483]
- Faham M, Zheng J, Moorhead M, Carlton VE, Stow P, Coustan-Smith E, Pui CH, Campana D. Deep-sequencing approach for minimal residual disease detection in acute lymphoblastic leukemia. *Blood*. 2012; 120:5173–5180. [PubMed: 23074282]
- Gawad C, Pepin F, Carlton VE, Klinger M, Logan AC, Miklos DB, Faham M, Dahl G, Lacayo N. Massive evolution of the immunoglobulin heavy chain locus in children with B precursor acute lymphoblastic leukemia. *Blood*. 2012; 120:4407–4417. [PubMed: 22932801]
- Hoppe RT, Advani RH, Ai WZ, Ambinder RF, Aoun P, Bello CM, Bierman PJ, Blum KA, Chen R, Dabaja B, Duron Y, Forero A, Gordon LI, Hernandez-Ilizaliturri FJ, Hochberg EP, Maloney DG, Mansur D, Mauch PM, Metzger M, Moore JO, Morgan D, Moskowitz CH, Poppe M, Pro B, Winter JN, Yahalom J, Sundar H, National Comprehensive Cancer Network. Hodgkin lymphoma, version 2.2012 featured updates to the NCCN guidelines. *Journal of the National Comprehensive Cancer Network*. 2012; 10:589–597. [PubMed: 22570290]
- Jones RJ, Gocke CD, Kasamon YL, Miller CB, Perkins B, Barber JP, Vala MS, Gerber JM, Gellert LL, Siedner M, Lemas MV, Brennan S, Ambinder RF, Matsui W. Circulating clonotypic B cells in classic Hodgkin lymphoma. *Blood*. 2009; 113:5920–5926. [PubMed: 19188663]
- Kanzler H, Kuppers R, Hansmann ML, Rajewsky K. Hodgkin and Reed-Sternberg cells in Hodgkin's disease represent the outgrowth of a dominant tumor clone derived from (crippled) germinal center B cells. *Journal of Experimental Medicine*. 1996; 184:1495–1505. [PubMed: 8879220]
- Kuppers R, Rajewsky K, Zhao M, Simons G, Laumann R, Fischer R, Hansmann ML. Hodgkin disease: Hodgkin and Reed-Sternberg cells picked from histological sections show clonal immunoglobulin gene rearrangements and appear to be derived from B cells at various stages of development. *Proceedings of the National Academy of Sciences of the United States of America*. 1994; 91:10962–10966. [PubMed: 7971992]
- Kuruvilla J. Standard therapy of advanced Hodgkin lymphoma. *Hematology/the Education Program of the American Society of Hematology*. 2009; 1:497–506.
- Ladetto M, Bruggemann M, Monitillo L, Ferrero S, Pepin F, Drandi D, Barbero D, Palumbo A, Passera R, Boccadoro M, Ritgen M, Gokbuget N, Zheng J, Carlton V, Trautmann H, Faham M, Pott C. Next-generation sequencing and real-time quantitative PCR for minimal residual disease detection in B-cell disorders. *Leukemia*. 2014; 28:1299–1307. [PubMed: 24342950]
- Logan AC, Zhang B, Narasimhan B, Carlton V, Zheng J, Moorhead M, Krampf MR, Jones CD, Waqar AN, Faham M, Zehnder JL, Miklos DB. Minimal residual disease quantification using consensus primers and high-throughput IGH sequencing predicts post-transplant relapse in chronic lymphocytic leukemia. *Leukemia*. 2013; 27:1659–1665. [PubMed: 23419792]
- Marafioti T, Hummel M, Anagnostopoulos I, Foss HD, Falini B, Delsol G, Isaacson PG, Pileri S, Stein H. Origin of nodular lymphocyte-predominant Hodgkin's disease from a clonal expansion of highly mutated germinal-center B cells. *New England Journal of Medicine*. 1997; 337:453–458. [PubMed: 9250847]
- Marafioti T, Hummel M, Foss HD, Laumen H, Korbjuhn P, Anagnostopoulos I, Lammert H, Demel G, Theil J, Wirth T, Stein H. Hodgkin and reed-sternberg cells represent an expansion of a single clone originating from a germinal center B-cell with functional immunoglobulin gene rearrangements but defective immunoglobulin transcription. *Blood*. 2000; 95:1443–1450. [PubMed: 10666223]
- Moskowitz AJ. Novel agents in Hodgkin lymphoma. *Current Oncology Reports*. 2012; 14:419–423. [PubMed: 22706966]
- Vij R, Mazumder A, Klinger M, O'Dea D, Paasch J, Martin T, Weng L, Park J, Fiala M, Faham M, Wolf J. Deep sequencing reveals myeloma cells in peripheral blood in majority of multiple myeloma patients. *Clinical Lymphoma, Myeloma & Leukemia*. 2014; 14:131–139.e131.

Fig 1.

(A) Detection of lymphoma specific clonotypes in tumour biopsy samples. The identification of lymphoma clone (calibration) in the three tested assays (*IGH*-VDJ, *IGH*-DJ and *IGK*) in the 17 patients is shown. A black box means at least one lymphoma clone has been identified in that sample whereas a white box indicates that none were detected. (B) Detection of lymphoma-specific clonotypes in cell-free compartment from peripheral blood. The level of the different lymphoma clones in the cell free serum sample (lymphoma molecules/ml serum) at the time of diagnosis is shown for the indicated patients. Patients 4 and 7 had no serum sample and their levels are left blank. Patient 5 had no blood sample at all and is not shown. (C) Detection of lymphoma-specific clonotypes in peripheral blood mononuclear cells. The level of the different lymphoma clones in the peripheral blood mononuclear cells (PBMC) sample (lymphoma molecules/ 10^6 leucocytes) at the time of diagnosis is shown for the indicated patients. Patients 10 and 15 had no PBMC samples and their levels are left blank. Patient 5 had no blood sample at all and is not shown.

Table 1
Detection of circulating tumour material in CHL patients at diagnosis or recurrence.

Clinical characteristics				EBV		PBMC component		Serum component		Clinical outcome			
ID	Histology	Disease status [†]	Largest mass diameter (cm)	IPS	EBER or LMP1	PBMC component		Serum component		Radiation [‡]	PET2	PFS	Status
						Clone molecules (n)	Frequency among IGH/IGK sequences (%)	Clone molecules (n)	Frequency among IGH/IGK sequences (%)				
Clonotype analyses at initial diagnosis													
Clonotype identified in the primary tissue													
1*	CHL, NS	Stage III	6.0	3	Negative	2	0.08	12	50.98	No	Pos	11	Progression
2	CHL, NOS	Stage II unfavourable	6.0	1	Negative	0	0	33	60.29	Yes	NA	45	Censor
3	CHL, NS	Stage II unfavourable	1.5	2	Negative	0	0	12	19.81	No	Neg	28	Censor
4	CHL, NS	Stage IV	5.4	2	Negative	0	0	NA	NA	No	Pos	35	Censor
5	CHL, NS	Stage II unfavourable	8.0	2	Equivocal	NA	NA	NA	NA	Yes	Neg	34	Censor
6	CHL, NOS	Stage IV	4.0	5	NA	14	0.01	456	97.02	No	Neg	17	Progression
7	CHL, NS	Stage III	11.0	3	Negative	0	0	NA	NA	No	Neg	34	Censor
8	CHL, NS	Stage II unfavourable	13.0	1	Negative	0	0	61	45.35	Yes	Neg	23	Censor
9	CHL, NS	Stage II unfavourable	10.5	1	Negative	457	7.39	0.6	22.55	Yes	Pos	3	Progression
10	CHL, NS	Stage III	16.0	2	Negative	NA	NA	0	0	No	Neg	25	Censor
Clonotype not identified													
11	CHL, NOS	Stage II unfavourable	5.8	0	Negative	NA	NA	NA	NA	No	NA	30	Censor
12	CHL, NOS	Stage II unfavourable	2.9	2	Negative	NA	NA	NA	NA	Yes	Neg	32	Censor
13	CHL, LR	Stage I favourable	2.6	2	Negative	NA	NA	NA	NA	Yes	Neg	26	Censor
Analysis at recurrence													
Clonotype identified													
14	CHL, NS	Stage II at recurrence	1	–	Negative	0	0	1	4.29	–	–	–	Recurrence
15	CHL, NS	Stage II at recurrence	8	–	NA	NA	NA	1	8.7	–	–	–	Recurrence
Clonotype not identified													
16	CHL, MC	Stage I at recurrence	1.5	–	NA	NA	NA	NA	NA	–	–	–	Recurrence
17	CHL, MC	Stage III at recurrence	3.2	–	NA	NA	NA	NA	NA	–	–	–	Recurrence

ID, patient identification number; IPS, International Prognostic Score; EBV, Epstein-Barr virus; EBER, EBV-encoded RNA; CHL, classical Hodgkin lymphoma; MC, mixed cellularity; NOS, not otherwise specified; NS, nodular sclerosis; NA, not available; PBMC, peripheral blood mononuclear cells; PET2, positron emission tomography scan after two cycles of therapy; PFS, progression free survival in months; pos, positive; neg, negative.

* Paired blood sample was obtained at time of initial diagnosis, while biopsy was obtained at the time of recurrence.

Unfavourable disease is defined by a presence of any of the following: Disease involvement in more than two sites, erythrocyte sedimentation rate 50 mm/h or higher, extranodal involvement and the presence of mediastinal mass with maximum transverse diameter >33% of internal thoracic diameter. Absence of such features is considered favourable disease.

[‡] Radiation after ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine). (All patients received ABVD chemotherapy.)