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Exploration of residual disease in stem cell products from mantle cell lymphoma using next-generation sequencing

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ABSTRACT

High-dose chemotherapy followed by autologous stem cell transplantation (ASCT) has become a treatment option for fit patients with mantle cell lymphoma (MCL). However, these patients often relapse within few years, potentially caused by contaminating lymphoma cells within the reinfused stem cell product (SCP). Studies have shown that measurable residual disease, also termed minimal residual disease (MRD), following ASCT predicts shorter survival. Using next-generation sequencing, we explore whether the diagnostic MCL clonotype is present within the infused SCP. MRD was detected in 4/17 of the SCPs, ranging 4–568 clonal cells/100,000 cells. With a median survival of 17 months, 3/4 of patients with MRD+ graft succumbed from MCL relapse versus 2/13 in the MRD– fraction. Patients receiving MRD+ grafts had increased risk of mortality, and thus screening of SCPs may be important for clinical decision-making.

1. Introduction

Mantle cell lymphoma (MCL) is an aggressive subtype of non-Hodgkin's lymphoma, arising from the often rapid neoplastic lymphoproliferation of differentiated B cells. In the vast majority of cases, MCL is initiated by the t(11;14)(q13;q32) translocation that involves transposition of the cell cycle regulator, CCND1, to the immunoglobulin heavy chain (IgH) locus residing on chromosome 14, leading to constitutive overexpression of the cyclin D1 protein.

The MCL cells have a common clonal origin and the unique B cell receptor produced by the rearrangement of the IgH locus can thus be used as a molecular fingerprint for the longitudinal tracking of malignant cells for each patient. Traceable persistence of lymphoma cells during or after treatment [1] is an essential clinical concept defined as measurable residual disease, also termed minimal residual disease (MRD), and has been tied to an adverse prognosis. Generally, achieved MRD negativity after treatment is directly associated with prolonged progression-free survival and improved overall survival [2], as shown for other hematologic malignancies.

Prior to the implementation of next-generation sequencing (NGS) MRD status has been investigated by real-time quantitative PCR (qPCR), using patient-specific primers to identify any clonal IgH rearrangement [3]. However, now NGS has the advantage of greater specificity and high sensitivity, moving away from patient-specific assays and standard curves [4], and recently backed by the released guidelines for detecting B cell clonality by the EuroClonality-NGS Working Group [5]. Commercial assays have gained ground as well, with the two most prominent being ClonoSEQ (Adaptive Biotechnologies, Seattle, WA, USA), approved in 2018 by the U.S. Food and Drug Administration (FDA) for MRD testing in patients with acute lymphoblastic leukemia, multiple myeloma and MCL, and the LymphoTrack assay (Invivoscribe, San Diego, CA, USA). Likewise, the latter has also been applied for several B cell malignancies such as multiple myeloma and MCL.

Recent therapeutic advances have led to an increase in the median survival from 3 to 5 years to about 10 years [6], especially for patients eligible for autologous stem cell transplantation (ASCT). In addition, more effective tumor eradication has led to complete responses after induction therapy in most younger patients with MCL (≲65 years of

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age), with MRD negativity in blood and bone marrow achieved for many patients following ASCT. However, some patients still suffer from early post-transplant relapses, partly explained by lymphoma cells in the infused stem cell products (SCPs) [7, 8].

In the presented study, we add to the recent findings by Kumar et al. [9], where longitudinal NGS-based MRD surveillance helped to identify patients bound for relapse following autologous stem cell transplantation and extends the association between graft-MRD detected by flow cytometry and decrease in progression-free and overall survival made by Roerden et al. [10]. Although studies have proven that MRD status following treatment is of paramount prognostic importance, little is still known concerning residual disease in SCPs used for ASCT and its potential role in disease relapse. Thus, with the implementation of NGS, our aim was to demonstrate detection of residual lymphoma cells directly in SCPs from patients with MCL receiving ASCT and explore the possible association with clinical outcome in terms of disease relapse or disease-related death.

2. Materials and methods

Seventeen MCL patients with ASCT performed as part of the first-line therapy were included in the study, retrospectively. All 17 patients were diagnosed at Odense University Hospital, Denmark (OUH), between 2011 and 2016, with a median age of 61 years (50–66) at the time of diagnosis. All were classified as Ann Arbor stage IV, except for one patient, who was stage IIIA. 65% (11/17) were categorized as high-risk patients based on the Mantle Cell Lymphoma International Prognostic Index (MIPI). 41% (7/17) had blastoid or pleomorphic MCL variant, and bone marrow involvement was observed in 88% (15/17) (Table 1). All patients were treated according to the *Nordic MCL2 regimen* [11]. Induction therapy consisted of six cycles of alternating R-maxi-CHOP (rituximab, cyclophosphamide, vincristine, doxorubicin, and prednisone) and R-AraC (rituximab and cytarabine). The last cycle served as stem cell mobilization with granulocyte-colony stimulating factor and additional rituximab for in vivo purging. Following stem cell harvest of a minimum of 2 million CD34-positive cells/kg body weight, consolidation with BEAM (carmustine, etoposide, cytarabine and melphalan) was performed with subsequent stem cell rescue.

Table 1
Characteristics of 17 patients with mantle cell lymphoma treated with autologous stem cell transplantation (ASCT).

	Total
Age, years: median (range)	61 (50–66)
Male gender	12/17 (71%)
Diagnostic characteristics	
Ann Arbor stage IV	16/17 (94%)
MIPI	
Intermediate risk	6/17 (35%)
High risk	11/17 (65%)
Involved tissues	
Bone marrow	15/17 (88%)
Spleen	6/17 (35%)
Extranodal sites	6/17 (35%)
Blastoid/pleomorphic variant	7 /17(41%)
LDH elevated	10/17 (59%)
Ki-67 expression	
<29%	3/16 (19%)
30–49%	5/16 (31%)
>49%	8/16 (50%)
Treatment response	
Pre-ASCT CR[a]	13/17 (76%)
Post-ASCT CR[b]	14/16 (88%)

Mantle Cell Lymphoma International Prognostic Index (MIPI); lactate dehydrogenase (LDH); complete remission (CR).

[a] Pre-ASCT treatment response was assessed in bone marrow and peripheral blood after the 5th cycle of induction therapy before stem cell mobilizing and harvest.

[b] One patient died before post ASCT treatment response assessment.

Diagnostic material from cryopreserved lymph nodes, fixed bone marrow cells, previously cultured with methotrexate and colcemide, and peripheral mononuclear blood cells were used for the MRD investigation, performed in frozen aliquots of 0.5–1.0 ml of the apheresed autograft at the time of ASCT.

The stem cell products were cryopreserved for a mean period of 70.4 months (50.5–109.8) and contained 0.27–1.98% CD34-positive cells according to flow cytometry analysis (single platform ISHAGE gating strategy) performed following harvest. Subsequently, DNA from SCPs were sequenced to detect MRD (Table 2).

B cell IgH sequences from the diagnostic samples and SCPs were amplified using the LymphoTrack Dx IGH FR1 assay and workflow (Invivoscribe) (Fig. 1). PCR amplification of diagnostic samples was run in singlets (50–340 ng of genomic DNA) and SCPs in four replicates (2.5–4.2 µg DNA). Spike-in control DNA, equivalent to 100 cells, was added to each reaction to calculate the frequency of clonal cells. Amplicons were purified with AMPure XP beads (Beckman Coulter, Brea, CA, USA) and quantified on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using the Agilent 1000 kit (Agilent Technologies). Indexed sequencing libraries were pooled, adjusted to a concentration of 33 pM, and loaded onto an ion 530 chip using the Ion Chef System, and sequencing was performed with the Ion GeneStudio S5 Prime System (Thermo Fisher Scientific, Waltham, MA, USA).

Raw sequences from the diagnostic samples were analyzed using the LymphoTrack Dx Software (version 2.4.6, Invivoscribe). The identified clonal IgH rearrangements from diagnosis were used for MRD tracking, and sequencing reads derived from the SCPs were subsequently analyzed using the LymphoTrack MRD Software, version 1.2.0 (Invivoscribe). According to protocol, a two-mismatch difference was used as threshold for the identification of distinct clonotypes.

Median follow-up time was calculated using the reverse Kaplan-Meier method, and median survival with hazard ratio was calculated using the Kaplan-Meier method. Calculations were based on elapsing days following ASCT, terminating at relapse, death (related or unrelated to MCL), or being alive and in remission at end of study (May 28, 2021). Differences in survival between MRD positive and negative graft groups were analyzed using the log-rank test. For further information, see *Supplementary Methods*.

3. Results

A single malignant clone was identified, by sequencing, in each diagnostic sample. The median clonal read frequency was 71.1% (17.2%–83.8%; Table S1), with the lymphoma cells constituting the majority of the IgH rearranged cells in 14/17 of the diagnostic samples.

The median cell input for the PCR was 533,333 cells (424,000–704,000) per SCP (Table 2), which generated a median of 9% (5–50%) of reads mapping to the IgH locus (Table S2). In all but one sample (patient (pt.) 13), 50–80% of these reads originated from the added spike-in control (Table S2).

MRD was observed in 4/17 SCPs, each case in all four replicates (Table 2). The estimated MRD levels were 9, 4, 568, and 4 clonal cells per 100,000 cells for patients 3, 5, 13, and 16, respectively. One of these patients (pt. 5) remained in first remission almost nine years after ASCT treatment at study termination, whereas the three others succumbed following MCL relapse 30, 21, and 6 months post-ASCT (pt. 3, 13, and 16, respectively, Fig. 2 and S1). Of the 13 patients without MRD, 8 were in remission at study termination, including one remaining in second remission at study termination (pt. 6). The five other patients succumbed, of which two experienced MCL relapse at 4 and 11 months after ASCT (pt. 15 and 12, respectively) and three from causes not related to MCL, ASCT complications (pt. 4), colorectal cancer (pt. 2), and therapy-related myelodysplastic syndrome four years following transplant (pt. 7). The median follow-up of the cohort patients was 71 months, and the median survival was 77 months (MRD negative 77 months; MRD positive 17 months, Fig. 2). Patients infused with MRD positive graft were

Table 2
Summary of the MRD results from the sequencing of 17 stem cell products. The DNA from each stem cell sample was distributed in four replicates.

Patient ID	DNA input *µg*	Cell input *n*	B cell input *n*	Non-spike-in B cell input *n*	Clone detected in replicates	Clonal cells (ECE) *n*	MRD value *clonal cells per 100,000 cells*
1	3.2	549,333	525	125	ND	ND	ND
2	3.2	533,333	528	128	ND	ND	ND
3	3.2	533,333	877	477	4/4	46	8.6
4	3.2	533,333	809	409	ND	ND	ND
5	2.5	424,000	607	207	4/4	16	3.7
6	3.0	506,667	595	195	ND	ND	ND
7	3.1	508,667	754	354	ND	ND	ND
8	3.6	602,667	573	173	ND	ND	ND
9	3.2	538,667	645	245	ND	ND	ND
10	3.2	533,333	847	447	ND	ND	ND
11	3.2	533,333	863	463	ND	ND	ND
12	4.2	704,000	493	93	ND	ND	ND
13	4.2	698,667	5117	4717	4/4	3965	567.5
14	2.7	448,000	939	539	ND	ND	ND
15	4.1	688,000	530	130	ND	ND	ND
16	3.1	518,000	547	147	4/4	23	4.4
17	2.6	436,000	501	101	ND	ND	ND
Median:	3.2	533,333	595				

Cell input was calculated from the DNA input based on 6 pg DNA per cell. Estimated B cell input was calculated as the ratio between total reads and spike-in reads, multiplied by the number of spike-in cells (100). The number of spike-in B cells was subtracted from the estimated B cell input to obtain the estimated non-spike-in B cell input. MRD was defined as the number of clonal cells per 100,000 cells. Estimated cell equivalent (ECE); not detected (ND).

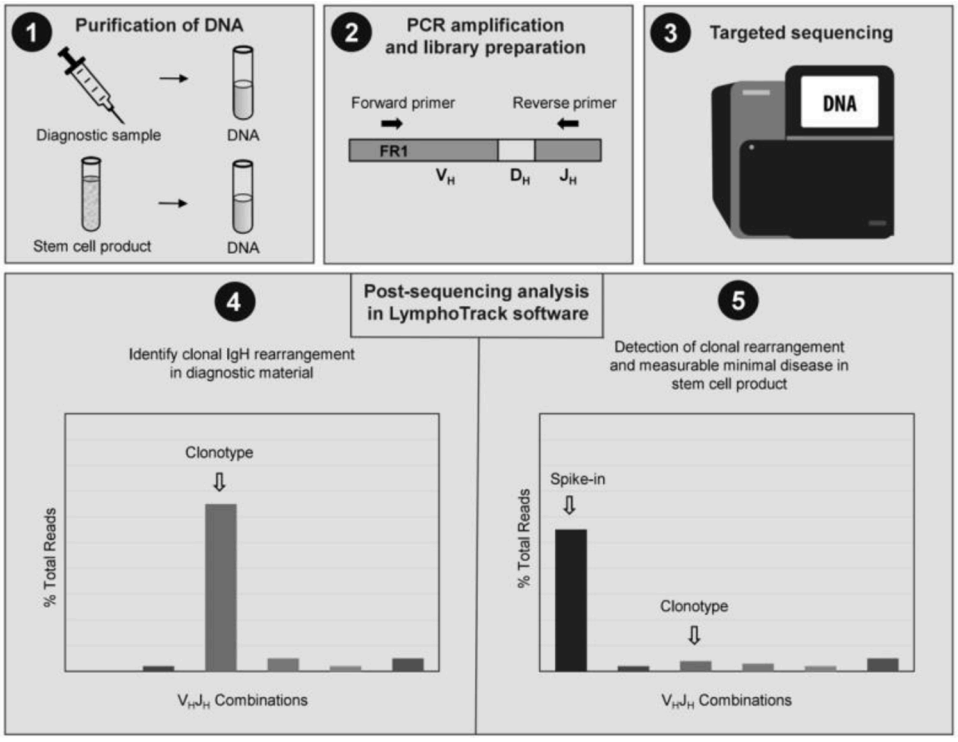

Fig. 1. Project workflow for detecting residual disease in infused stem cell products using next-generation sequencing. (1) DNA was extracted from diagnostic lymph nodes and stem cell samples. (2) DNA was PCR amplified with forward and reverse primers targeting the conserved regions of the immunoglobulin heavy-chain (IgH) rearrangement, framework region 1 (FR1) of *VH* gene segments and *JH* segment. Amplicons were purified, diluted, and pooled for library preparation. (3) The sequencing library was sequenced on the Ion Torrent platform. (4) Raw sequencing data were analyzed using the LymphoTrack Dx Software with the clonotype identified from diagnostic material. (5) This clonal IgH rearrangement was used for measuring residual disease in the stem cell products relative to spike-in DNA quantity.

found to have an increased risk of death (hazard ratio 1.79, 95% confidence interval 0.37–8.70), but no significant difference in survival ($p_{log\text{-}rank} = 0.39$).

4. Discussion

Around the turn of this century, several studies suggested [8, 12] or found a correlation [7] between the MRD burden in SCPs and relapse, and efforts were made to eliminate residual lymphoma cells from the SCPs [8, 12]. With the possibility of endogenous lymphoma cell eradication following high-dose therapy, it may again be relevant to investigate the potential of MRD-free SCPs. Our study demonstrates that autografts/SCPs cannot, *a priori*, be regarded as disease-free. In our cohort of 17 patients, MRD was identified in four grafts (24%). Of these, 3/4 patients experienced a fatal relapse, while the remaining patient had the lowest burden of clonal cells in the SCP and was still in the first remission at the time of study termination. In contrast, 2/13 (15%) of the patients with MRD negative grafts succumbed from MCL relapse within the first year after ASCT. Eight patients with MRD negative grafts (62%) were still in remission, averaging six years after ASCT, including one patient in second remission at the time of study termination. In line with this observation, Roerden et al. [10] demonstrated that 4/36 (11%)

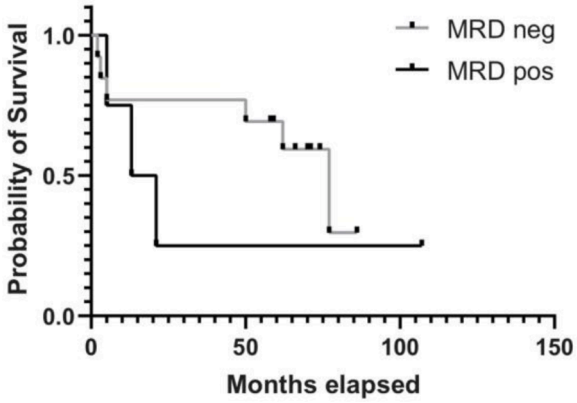

Fig. 2. Outcome following autologous stem cell transplant (ASCT) for 17 mantle cell lymphoma patients. Kaplan-Meier survival curve for patients grouped by MRD pos/neg in stem cell products. Time elapsed from ASCT and terminated when the patient relapsed, succumbed, or at the time of study termination for the patients still in remission.

patients with MRD positive grafts were identified by multi-parameter flow cytometry. Here, the progression-free and overall survival were significantly inferior for graft-positive patients. Despite limited statistical power from the small number of patients, our study corroborates that patients with MRD negative grafts may have superior survival. This tendency is also supported by a small study of 14 patients by Corradini et al. [7], who found evidence of MRD negative autografts from patients with MCL or follicular lymphoma being associated with more durable molecular and clinical remission. This association was noticeable despite the relatively short follow-up time (up to 66 months) and the small patient cohort. Also, the retrospective study in multiple myeloma by Takamatsu et al. [13] showed that after ASCT, with or without immunomodulatory drug post-ASCT, patients with MRD negative autograft tended to have improved progression-free and overall survival compared to patients with MRD positive autograft.

In contrast, patient 13 had the highest number of clonal cells in the SCP (Table 2) with an MRD value of 568 clonal cells. The presence of 1% bone marrow MCL cells prior to ASCT and 568 clonal cells per 100,000 detected from the SCP of patient 13 suggests that the relapse may have risen from residual malignant cells or infused contaminants, as discussed by Shimoni & Körbing [14]. Despite this high number of clonal cells, patient 13 experienced prolonged survival. This paradox has been approached by Pott et al. [15], who did not find a correlation between the number of reinfused lymphoma cells and outcome after ASCT in patients with MCL. MRD was detected in essentially all SCPs, so comparison of outcomes was not possible in this study.

We aimed at the theoretical sensitivity of 10^{-5} for all SCPs. Although more than 300,000 cells were analyzed, the number of reads for the patient B cells (the non-spike-in B cells) was one order of magnitude lower, and the 10^{-5} sensitivity level was thus not achieved. MRD was detected in 4 SCPs, whereas 13 samples were MRD negative. The collection of about 3 million autograft cells theoretically required for a high sensitivity of 10^{-6} [16], which is often the intended detection level of recent studies, can be challenged by the availability of patient material and sample processing. In our study, the content of rearranged IgH sequences of B cells was calculated from the number of total reads and spike-in reads generated by 100 control cells. Only a few hundred rearranged B cells were observed per SCP, most likely caused by therapy-mediated depletion of B cells and granulocytes mobilized by granulocyte-colony stimulating factor. Thus, the samples consisted mainly of leukocytes not targeted in the amplification, and the low frequency of suitable assay targets directly explains the high proportion of discarded off-target reads and two-thirds of the reads stemming from the spike-in control.

MRD could also be addressed in blood or BM before apheresis instead of addressed in SCP. This has, in a study of acute myeloid leukemia, been shown useful for outcome prediction [17]. However, the detection of MRD in SCP enables the detection of lymphoma stem cells with an enhanced sensitivity than e.g. in a blood sample from the patient.

The NGS-based methods allow for more precise tracking of clonal cells, providing information on all rearranged populations in each analysis. As example, the possible combination of the conserved immunoglobulin *leader* and framework regions enables the LymphoTrack assay to detect a clonal rearrangement in virtually all cases of MCL and nearly all other B cell malignancies. In addition, implementation of NGS is highly relevant in special cases, where the relapse clone is distinctly different from the diagnostically identified rearrangement, as it may indicate a more immature malignant capacity.

In conclusion, residual MCL cells were detected in 4 of 17 SCPs, suggesting that contamination may directly give rise to relapse. Further studies are warranted to investigate the role of MRD in SCPs in relapse and prognosis, and thus encourage a routine investigation of autografts, readjustment of planned therapy, and possibly even reconsideration of ASCT in some patients.

Author contributions

C.G.N. and J.H. conceived and designed the study. C.G.N., N.A. and L.A.L.E. raised funding for the project. C.G.N. and O.C. supervised the laboratory part of the study. J.H. and N.A. supervised the clinical part of the study. C.N. and M.B.M. contributed to the acquisition of the biologic materials. L.A.L.E. and O.C. performed the experiments. L.A.L.E., O.C. and M.H.H. analyzed the data. L.A.L.E. wrote the first draft of the manuscript. O.C., M.H.H., and C.G.N. wrote the final version of the manuscript. All authors contributed to manuscript revision, read, and approved the final version.

Ethical considerations

The project was approved and registered by the Ethical Committee of Southern Denmark (ID: S-20160069). Collection and use of biological material were approved by the Danish Data Protection Agency (file no. 16/15528).

Declaration of Competing Interest

The authors have nothing to disclose.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.lrr.2022.100341.

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