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Original Article

Applicability of next-generation sequencing to decalcified formalin-fixed and paraffin-embedded chronic myelomonocytic leukaemia samples

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Abstract: Decalcified formalin-fixed and paraffin-embedded (dFFPE) bone marrow trephines remain the primary source of gDNA in hematopathological diagnostics. Here, we investigated the applicability of next-generation sequencing (NGS) to dFFPE samples. Chronic myelomonocytic leukaemia (CMML) is a haematopoietic stem cell malignancy delineated by genetic heterogeneity. Recently characteristic mutations have been identified for this entity in a distinct group of genes (*TET2*, *CBL*, *KRAS*). We comparatively investigated DNA extracted from fresh mononuclear cells as well as dFFPE samples from four CMML patients employing a commercially available primer set covering the above mentioned and well characterized mutational hotspots in CMML followed by an amplicon based next-generation deep-sequencing (NGS) approach. As we observed high quality run data as well as complete concordance between both sample types in all cases, we further validated the potential of NGS in hematopathology on a larger cohort of CMML patients (n=39), detecting sequence variations in 84.6% of patients. Sequence analysis revealed 92 variants, including five known polymorphisms, ten silent mutations, 36 missense mutations, 14 nonsense mutations, 24 frame shift mutations and three potential splice site mutations. Our findings ultimately demonstrate the applicability of NGS to dFFPE biopsy specimen in CMML and thus allowing the pathologist to evaluate prognostically relevant mutations at a high resolution and further contribute to risk stratification for the individual patient.

Keywords: Next-generation sequencing, decalcified, formalin-fixed, paraffin-embedded samples, chronic myelomonocytic leukaemia

Introduction

Although, modern high throughput sequencing approaches have greatly advanced our understanding of various hematological malignancies, they were primarily applied to fresh mononuclear cell samples [1, 2]. However, diagnostics in hematopathology mainly rely on the use of decalcified, formalin-fixed, paraffin-embedded (dFFPE) bone marrow trephine biopsy specimen in order to elucidate immunoreactivity and morphological aspects in the topographical context of the hematopoietic system.

Chronic myelomonocytic leukaemia (CMML), for instance, is a clonal haematopoietic stem cell malignancy characterized by the presence of both myeloproliferative and myelodysplastic features as defined by the WHO Classification of myeloid neoplasms [3]. Peripheral and bone marrow blast counts are employed to divide CMML into two subtypes (CMML I <10% bone marrow and 5% peripheral blasts, CMML II up to 19% peripheral and/or bone marrow blasts) [4-6].

Clinical and haematological features regularly include monocytosis, cytopenia and/or hypercatabolic state. The clinical course however appears to be highly variable and no standardized treatment protocol could be demonstrated to positively affect overall or event-free survival [7, 8]. Moreover blastic transformation into secondary AML with dismal prognosis is frequently observed [7-9].

No single pathognomonic morphological, immunohistochemical or cytogenetical hallmark has

been established and few factors indicating prognosis and clinical course of disease have so far been reported, necessitating the search and validation of new markers for risk stratification [9, 10].

Recently a group of genes (*TET2*, *CBL*, *KRAS*), carrying mutations of prognostic relevance, in CMML was characterized by Kohlmann *et al*. by means of next-generation deep-sequencing [11]. Their initial findings were subsequently validated on a large cohort in a multi-center study and a commercial primer set (Roche, Mannheim, Germany) covering the above mentioned well characterized mutational hotspots in CMML was developed [12].

TET2 has been shown to operate as a tumor suppressor maintaining haematopoietic cell homeostasis with mutations, particularly affecting its two highly conserved regions, thus compromising protein function, inducing myeloproliferative and/or myelodysplastic malignancies [13]. CBL shows ubiquitin ligase activity and inactivating mutations have recurrently been associated with pathogenetic abnormality in clinically aggressive MPNs (Myeloproliferative neoplasms) [14]. Mutations in RAS protein isoforms resulting in a constitutively activated RAS/RAF/MAP pathway are known to play an important role in a variety of malignancies, including colorectal, lung, bladder and thyroid cancer [15]. Further, *KRAS* mutations were recently depicted to play an important role in myeloid leukaemia [16].

In previous studies, investigating *TET2, CBL* and *KRAS* mutation status in myeloid neoplasia, native peripheral blood and bone marrow mononuclear cells were analyzed based on massively parallel pyrosequencing in picotiter-sized wells on the 454 platform (Roche) [11, 12].

In order to assess the potential and applicability of massively parallel pyrosequencing to dFFPE samples on the 454 platform (454 Life Sciences, Branford, CT, USA) we comparatively investigated DNA extracted from fresh mononuclear cells as well as bone marrow trephine biopsy specimen from four CMML I patients for mutations in *TET2*, *CBL* and *KRAS* and further validated our findings on an extended cohort (n=39).

Materials and methods

Patient samples

For comparative evaluation of mutation status four paired CMML I samples of fresh mononuclear cells and dFFPE bone marrow trephine biopsies were retrieved from the registry of the Reference Center for Lymph Node Pathology and Hematopathology, University Hospital of Schleswig-Holstein, Campus Luebeck.

In order to subsequently validate our initial findings on a larger cohort additional dFFPE bone marrow trephine biopsies from 26 patients with CMML I and 13 patients with CMML II were recruited.

All samples were collected as part of standard clinical care and all studies were approved by the Ethics Committee at the University of Luebeck and are in accordance with the Declaration of Helsinki. All cases were reassessed for independent pathology review by two experienced Hematopathologists (HM & ACF) without knowledge of mutation status. Diagnosis was confirmed according to the World Health Organization classification criteria, integrating clinical, morphological and immunohistochemical findings. Immunohistochemical studies were performed on formalin-fixed paraffin-embedded (FFPE) sections according to a standard, three-step immunoperoxidase technique using the automated TechMate system (DAKO, Glostrup, Denmark) and the BrightVision Kit (ImmunoLogic, Duiven, Netherlands). Clinical and haematological features of the study group are briefly summarized in **Table 1**.

Next-generation sequencing

Genomic DNA was obtained from fresh mononuclear cells and dFFPE specimen using QiaAmp mini kit 250 according to the manufacturer's instructions as described [17]. Quality and quantity of extracted DNA samples was assessed using a Nano Drop 1000 system (Thermo Scientific, Wilmington, DE, USA). DNA fragmentation was comparatively evaluated between fresh and dFFPE samples employing a multiplex PCR approach.

Next we applied amplicon-based next-generation deep-sequencing using the GS GType

Table 1. Clinical characteristics of the patient cohort (n=39)

Gender	male	25 (64.1%)
	female	14 (35.9%)
Age [y]	Median	76.0
	Range	54.7-91.1
Diagnosis	CMML I (WHO)	25 (64.1%)
	CMML II (WHO)	14 (35.9%)
	CMML MDS (FAB)	12 (32.4%)
	CMML MPD (FAB)	25 (67.6%)
	no data available	2
Leukocyte count [Gpt/l]	Median	18.730
	Range	3.700-100.320
	no data available	2
Erythocyte count [Tpt/l]	Median	3.600
	Range	2.400-7.720
	no data available	4
Haemoglobin level [g/dl]	Median	10.15
	Range	7.1-16.6
	no data available	3
PB monocyte count [%]	Median	26.0
	Range	6.0-67.0
	no data available	4
PB monocyte count [Gpt/l]	Median	3.600
	Range	1.080-26.300
	no data available	4

Based on peripheral leukocyte counts, the FAB group proposed to distinguish between two subtypes of CMML: CMML MDS (WBC $\leq 13x10^9$/l) and CMML MPD (WBC $>13x10^9$/l) [31].

TET2/CBL/KRAS primer sets (Roche, Mannheim, Germany), designed for the investigation of three samples per 96 well plate, on a GS Junior platform (454 Life Sciences, Branford, CT, USA) with slight modifications as described [12]. Briefly, 31 PCR products covering the coding regions of *TET2*, exons 8 and 9 of *CBL* as well as exons 2 and 3 of *KRAS* were amplified. PCR reactions were performed using the FastStart High Fidelity PCR System Kit (Roche) and PCR products were pooled and purified using the Agencourt AMPure XP beads (Beckmann Coulter, Krefeld, Germany). The concentration of the amplicon pool was determined with the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies GmbH, Darmstadt, Germany). Products were prepared for emulsion PCR by diluting amplicon pools to a concentration of $2x10^6$ molecules/µl. The following emulsion PCR was performed using the Lib-A emPCR kit (Roche) with 0.6 copies per bead inserting 5,000,000 beads per emulsion oil tube in accordance with the manufacturer's instructions. Amplicon-based sequencing was then performed using the workflow as recommended by the manufacturer.

Sequencing data analysis

Sequencing runs were performed using the GS Junior Sequencer Software Version 2.7 (GType Leukemia 2.0) and sequencing data analysis was carried out with the GS Amplicon Variant Analyzer Version 2.7.

Variants detected with a frequency of 3% or higher on both strands were considered present. Regarding the patient cohort n=39, variants located outside the two evolutionarily conserved regions of the *TET2* gene, as well as silent mutations or known single-nucleotide polymorphisms were excluded from further analysis [18]. Missense variations in exon 8 and 9 of *CBL* as well as in exon 2 and 3 of *KRAS* were considered to be of significance without regard for their specific location as these regions have been shown to be essential for protein function.

Comparative sanger sequencing

Comparative conventional Sanger sequencing was performed for all samples in order to confirm data obtained by next-generation sequencing. Information on allele burden of sequence variations as measured by NGS was collected to establish differential sensitivity of both methods. The cut-off for detecting low-level variants employing the classical chain-termination method was at an average allele frequency of 20%.

Statistical analysis

Dichotomous variables were compared between different groups using Fisher's exact test and continuous variables were analyzed by the nonparametric Mann-Whitney U test. Wilcoxon signed rank test was applied for comparison of mutational allele burden of fresh mononuclear cells and dFFPE samples. All analyses were two-sided and the statistical significance level was set to 5% ($p<0.05$). All statistical data anal-

Table 2. Massively parallel pyrosequencing of TET2, CBL and KRAS in fresh mononuclear cell samples and decalcified, formalin-fixed, paraffin-embedded bone marrow trephine biopsies from four CMML patients

		Fresh mononuclear cell sample			dFFPE bone marrow trephine biopsy		
	Sequence variation	Allele frequency (%)	Reads	Sanger	Allele frequency (%)	Reads	Sanger
Case 1	*TET2* p.Q916X	42.92	890	pos.	45.9	1011	pos.
	TET2 p.I1762V	50.84	2026	pos.	49.13	2019	pos.
	TET2 p.I1873T	42.97	1145	pos.	43.48	1334	pos.
	TET2 p.I1873MfsX14	6.2	1145	neg.	6.6	1334	neg.
Case 2	*CBL* p.L380P	30.5	2279	pos.	31.6	883	pos.
	TET2 p.Y1255LfsX13	49.1	1564	pos.	37.23	462	pos.
	TET2 p.L1816X	46.5	1688	pos.	41.9	432	pos.
Case 3	*CBL* p.L380P	51.91	836	pos.	45.71	862	pos.
	TET2 p.G355D	50.68	876	pos.	50.52	1348	pos.
	TET2 p.D527EfsX6	12.78	1221	neg.	11.37	1381	neg.
	TET2 p.D527EfsX6	27.17	530	pos.	30.8	685	pos.
	TET2 p.H1380Y	50.59	597	pos.	42.23	753	pos.
	TET2 p.I1762V	48.84	1116	pos.	43.86	1058	pos.
Case 4	*TET2* p.N752KfsX59	34.59	743	pos.	38.51	1688	pos.
	TET2 p.D1704EfsX9	5.19	752	neg.	4.19	1741	neg.
	TET2 p.I1762V	48.94	1561	pos.	51.3	3302	pos.

yses were performed using GraphPad Pri sm 5.

Results

Comparative evaluation of fresh and dFFPE samples

Quantity and purity of extracted DNA were consistent between fresh and dFFPE samples. As expected, DNA from dFFPE samples revealed significantly elevated fragmentation as determined by a multiplex PCR approach.

Sequence analysis comparing fresh mononuclear cells and dFFPE bone marrow samples from four CMML patients revealed complete conformance in terms of sensitivity in the detection of sequence variants regardless of allele frequency. In all cases mutations and sequence variations found in the fresh material were confirmed in the corresponding dFFPE sample (see **Table 2**). Moreover, no significant difference in mutational allele burden between fresh and dFFPE samples was detectable employing the Wilcoxon signed rank test ($p=0.3133$).

Run quality data in dFFPE specimen

Following these observations, we sought to validate NGS applicability to dFFPE samples on a larger cohort of CMML samples (n=39).

Seeking to assess performance of amplicon-based next-generation deep-sequencing using dFFPE specimen, we comparatively evaluated our run quality data with previously published results, obtained using fresh mononuclear cell samples [11, 12].

Following DNA extraction from dFFPE bone marrow trephine biopsies, emulsion PCR and sequencing preparation procedures, NGS was performed on a GS Junior platform. Here we generated a median of 247,764 sequencing beads per run (82,588 per patient), resulting in a median of 137,097 high-quality sequencing reads per run (45,699 per patient).

A median of 49.69 Mb was sequenced per run, which is 16.57 Mb per patient.

In order to investigate the abovementioned genes a total of 31 amplicons per patient was prepared, followed by NGS. Merely, in one patient the amplicon covering *TET2* exon 6 could not be amplified and subsequent sequencing analysis failed. This equals a drop out probability of 0.0827% per amplicon.

We obtained a median sequencing coverage of 2025 reads per amplicon (range 2550; 940-3490). The highest coverage was achieved for *TET2* with a median of 2202 combined reads (for: 1149; rev: 1057), followed by *CBL* exons 8

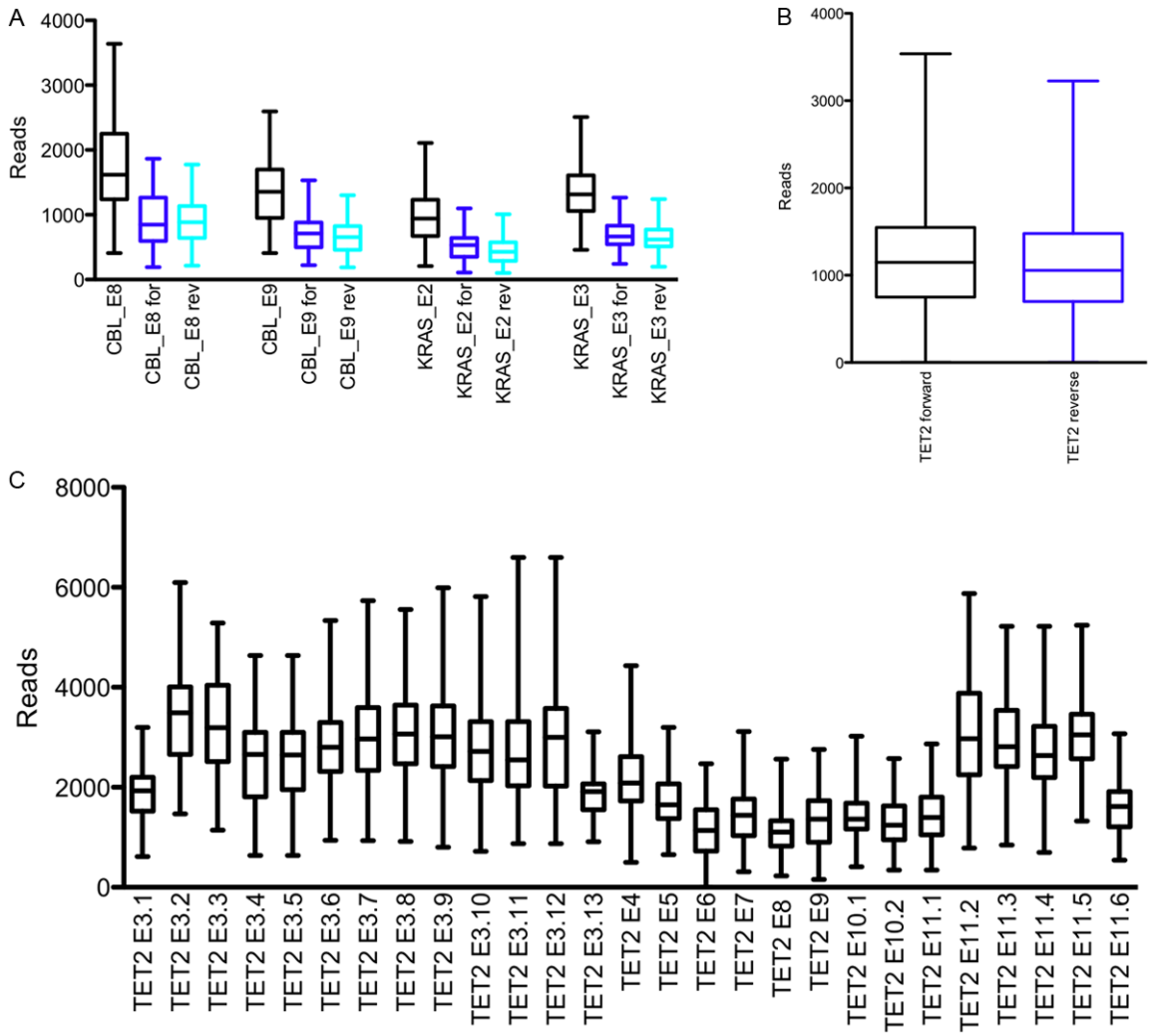

Figure 1. Combined coverage data of *TET2*, *CBL* and *KRAS* next-generation sequencing on dFFPE samples. Combined, forward and reverse reads for CBL and KRAS (A), overall forward and reverse *TET2* reads per amplicon (B) and combined *TET2* reads for all amplicons (C). Data reveal a consistent as well as high resolution coverage and summarized forward/reverse strand sequencing of *TET2* illustrate a slight predominance of the A-sequencing-bead prepared forward strand.

and 9 with 1511 reads (for: 790; rev: 742,5) and *KRAS* exons 2 and 3 with 1121 reads (for: 586,5; rev: 527,5).

Both forward and reverse strands were successfully sequenced in nearly all cases. Nevertheless, we were able to detect a significant difference between forward and reverse reads favouring the A-sequencing-bead prepared forward strand when combining all sequenced amplicons (p=0.0027) or *TET2* alone (p=0.0041). A similar trend was evaluated for both CBL exons 8 and 9 as well as *KRAS* exons 2 and 3 separately, but failed, however, to reach statistical significance. Coverage data is briefly summarized in **Figure 1**.

TET2, CBL and KRAS mutations in a validation cohort of 39 CMML samples

Amplicon-based next-generation deep-sequencing of the *TET2* gene coding regions as well as exon 8 and 9 of the *CBL* gene and exon 2 and 3 of the *KRAS* gene revealed sequence variations in 84.6% of patients. Five known single-nucleotide polymorphisms, ten silent mutations and ten previously unreported missense mutations located outside of evolutionarily conserved regions were excluded (Supplementary Table 1). In summary, sequence analysis revealed 67 putative protein damaging variances, including 26 missense mutations located within conserved regions, 14 nonsense mutations, 24

Table 3. TET2, CBL and KRAS mutations in CMML

Sample	Diagnosis	TET2	CBL	KRAS
1	CMML I	WT	p.C419S	WT
2	CMML I	c.3409+1 G>T p.V1864E	p.R420Q	WT
3	CMML I	p.P1419LfsX29 p.G1869E	p.R420Q	WT
4	CMML I		WT	
5	CMML II	c.3954+1 G>A p.R1366L	p.P417S	WT
6	CMML I	p.G1869E	WT	p.G13D
7	CMML I	p.P413HfsX14	WT	WT
8	CMML II	c.4183-16_-6del11 p.G1519DfsX52 c.3954+1 G>A p.R1366L	p.P417S p.D460del	WT
9	CMML I	p.L1340R p.R1516X	WT	p.G12D
10	CMML II	p.Q1834X	p.K392N	WT
11	CMML I	WT	p.C401S	WT
12	CMML II	p.L655NfsX45 p.Q705X	WT	WT
13	CMML I	WT	p.Y371S	WT
14	CMML I	WT	p.C384Y p.L370F	p.E37K
15	CMML I	p.L199FfsX3 p.E1178KfsX48	WT	WT
16	CMML I	p.L1081X	WT	p.D33E
17	CMML I	p.C1221VfsX5 p.M804WfsX9	p.I383M	WT
18	CMML I	p.P1741HfxX4 p.P399TfsX44	WT	WT
19	CMML I	WT	p.Q367P	WT
20	CMML II		WT	
21	CMML II	p.Q635X p.R1261H	WT	WT
22	CMML I	p.Q403X p.Q635X	p.C381Y	WT
23	CMML II	p.H1386AfsX15 p.L615SfsX24	WT	WT
24	CMML I	p.Q417X	WT	WT
25	CMML I	p.E294X p.I873DfsX28	WT	WT
26	CMML II	p.H1881N	WT	WT
27	CMML I	p.Q769X	WT	WT
28	CMML I	p.K1299fsX1 p.E576NfsX4	p.C401Y	WT

frameshift mutations and three potential splice site mutations (**Table 3**).

In total, various mutations were detected in 33 of 39 patients (84.6%) at an average of 1.92 mutations per patient. Of these mutations 19 were previously described in malignant samples. In agreement with previous studies (Kohlmann 2010) *TET2* was the most frequently mutated gene as we were able to detect nine missense mutations, 14 nonsense mutations, 23 frameshift mutations and three potential splice site mutations in 27 of 39 patients (69.2%) at an average of 1.38 mutations per patient (**Figure 2**). Four of these mutations could be detected independently in multiple patients (p.G1869E, p.Q635X, p.R1366L, c.3954+1G>A). As described, amplification of *TET2* exon 6 failed in a single patient. However, conventional Sanger sequencing was successful for this amplicon and we found no sequence variation.

In the *CBL* gene we found 13 distinct missense mutations in 14 (35.9%) of 39 patients at an average of 0.41 mutations per patient. The previously described missense mutation p.R420Q was detected in two patients.

Further we observed five missense mutations in *KRAS* exons 2 and 3 in five patients (12.8%) resulting in an average of 0.13 mutations per patient.

There was no statistically significant difference in mutation status between CMML I and CMML II (p=0.927).

Discussion

The introduction of consumer platforms, capable of massively parallel pyrosequencing has substantially enhanced the spectrum in which genomic sequencing is applicable in the routine diagnostic workup for clinical patients at affordable costs.

The molecular pathogenesis of CMML remains widely elusive, as do predictors of clinical course, outcome and response

		p.Q324X		
29	CMML II	WT		
30	CMML I	p.L1120X	WT	WT
		p.C1298MfsX2		
31	CMML II	p.T1251KfsX15	WT	WT
		p.C1271WfsX29		
		p.K306fsX1		
		p.D551AfsX15		
32	CMML II	WT		
33	CMML I	p.S393LfsX34	WT	p.E91K
		p.G1754IfsX7		
		p.T1280I		
34	CMML I	p.Q904X	WT	WT
		p.Y1245C		
35	CMML II	p.M1164K	WT	WT
		p.E1279X		
		p.P409FfsX33		
		p.R1366L		
36	CMML II	WT		
37	CMML I	p.Y1569X	WT	WT
38	CMML I	WT	p.P417S	WT
39	CMML I	WT		

CMML – Chronic myelomonocytic leukemia, WT – wildtype.

to therapy. To date, the diagnostic follow up for CMML patients focuses mainly on haematological and clinical parameters, such as anaemia, splenomegaly, or leukocytosis [5, 7, 9]. Despite ongoing discussions concerning prognostic implications, the recent identification of *TET2, CBL* and *KRAS* mutations as a highly recurrent event in MDS/MPS, particularly CMML, has greatly expanded the molecular understanding of these diseases and holds promise for upcoming targeted therapy approaches [11, 18-23].

Here, we applied an amplicon based deep-sequencing approach on a GS Junior platform (454 Life Sciences, Branford, CT, USA) to comparatively analyze the mutation status of *TET2*, *CBL* and *KRAS* in four paired samples of fresh and dFFPE specimen. Next, we validated NGS applicability on an extended cohort of dFFPE bone marrow trephine biopsy samples from 39 CMML patients. The above mentioned genes were selected due to their well-defined role as mutational hot spots in MDS/MPS. Moreover, multiple studies were recently conducted employing the same commercially available primer set and reagents (Roche, Mannheim, Germany).

Further, *TET2* mutations were recently identified to play an important role in numerous other proliferative and/or dysplastic malignancies of the myeloid linage, including MDS, polycythemia vera, essential thrombocytosis, primary myelofibrosis and systemic mastocytosis [18, 24, 25]. Moreover, *TET2* mutations appear to be of prognostic and pathogenetic significance in a subset of acute myeloid leukaemia patients [26].

Comparative evaluation of fresh and dFFPE samples revealed no difference in run quality data or diagnostic sensitivity. Next, in order to further underline NGS applicability to dFFPE specimen, we extended our investigations on a larger cohort of 39 CMML patients. In this comprehensive approach, 85 pathogenic sequence variations were detected by NGS and subsequently evaluated by Sanger sequencing. Low-level variations (allele burden <20%), however, could not be reproducibly confirmed by Sanger sequencing. We were able to detect 44 missense mutations, 14 nonsense mutations leading to a truncated translation of the protein, 24 frame shift mutations and three potential splice site mutations in 33 of 39 patients.

Of the abovementioned 67 *TET2* mutations, 19 mutations have previously been described in cancer samples adding to our understanding of potential mutation hot-spot regions of *TET2*. In accordance with previously published results, mutations were distributed throughout the entire coding regions of *TET2*, with missense mutations being predominantly found in evolutionarily conserved regions of the gene [22]. As proposed by Weissmann *et al*., we excluded ten novel single nucleotide variations located outside the two evolutionary conserved regions from further analysis [26], however, this does not exclude the possibility that these mutations might be of functional relevance in an as of yet unknown modality. The effect of substitutions leading to altered amino acid sequences outside of the conserved regions, proposed to be essential for protein function, still needs to be clarified. Moreover, little is known about TET2

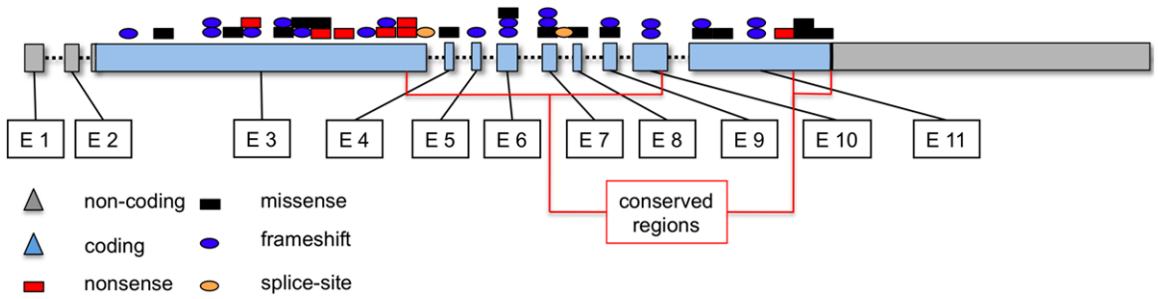

Figure 2. *TET2* Mutation types and distribution in CMML samples. Nine missense mutations within the two evolutionarily conserved regions (black boxes), 14 nonsense mutations (red boxes), 23 frameshift mutations (blue ovals) and three potential splice site mutations (orange ovals) in 27 of 39 patients (69.2%) at an average of 1.38 mutations per patient. Four of these mutations could be detected independently in multiple patients.

protein function and about the functional implications of most of these mutations [27, 28].

As dFFPE samples remain the most widely used material in both routine histopathological diagnostics as well as retrospective clinical studies, we and others have successfully focused on transferring novel investigative molecular methods from their initial application on fresh material to these more common, yet more demanding (in terms of nucleic acid quality and quantity) specimen [29].

Massively parallel pyrosequencing has been shown to be a robust, fast and highly reproducible method delivering excellent coverage and sensible detection of genetic aberrations. So far, however, no data exist about its utility using DNA extracted from FFPE samples. DNA from these samples is known, to be highly fragmented and its quality is often compromised but since hematopathological diagnostics mainly rely on these samples, their applicability is crucial to the implementation of any novel diagnostic method in the field of pathology [30]. In many instances bone marrow smears or aspirate specimen are not at hand and in addition bone marrow aspirate samples are often unavailable from patients who suffer from extensive marrow fibrosis (*punctio sicca*). Thus, any molecular method, which may be used with FFPE or even dFFPE, is of high value for the diagnostic process.

Overall sequencing coverage surpassed our expectations. The read quality data obtained in the present study on dFFPE samples were comparable to those of previously published data, applying similar approaches to fresh mononuclear cell samples from blood and/or bone marrow [11, 12]. With reference to genomic coverage as well as to average read length our results partly even exceeded published data.

Up until now the classical chain-termination sequencing was the only method applicable to dFFPE samples in routine hematopathological practice. Here we demonstrate, in a systematical comparative analysis, that a significantly improved sensitivity in detecting sequence variations can be obtained employing NGS. In many cases this will have a major impact on diagnosis, risk stratification and follow-up for the individual patient.

The approach presented in this study was conducted in an average run-time from DNA extraction to final sequencing results of approximately five days, leaving room for further acceleration employing automated sample preparation approaches, thus rendering next-generation sequencing fit for diagnostic requirements in hematopathological practice.

The detection of mutations in *TET2*, *KRAS* and *CBL* genes may in many instances assist in patient management, e.g. planning revaluation of bone marrow or cytogenetic analysis or for the decision to initiate cytoreductive therapy as they ultimately aid in discriminating reactive and neoplastic bone marrow lesions.

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Disclosure of conflict of interest

None declared.

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Supplementary Table 1. Excluded TET2 single nucleotide variations outside of evolutionary conserved Regions

Sample	Diagnosis	Variation 1	Variation 2	Variation 3
5	CMML2	p.N715D		
8	CMML2	p.N715D		
14	CMML1	p.S1708N	p.G1754S	p.G1697E
23	CMML2	p.G613W		
26	CMML2	p.L406P		
28	CMML1	p.A575S		
33	CMML1	p.S86P	p.P851L	p.S1775N