Molecular quantification of tissue diseaseburden is a new biomarker and independent predictor of survival in mastocytosis

Georg Greiner,[1,2] Michael Gurbisz,[1] Franz Ratzinger,[1] Nadine Witzeneder,[1,3] Svenja Verena Class,[4] Gregor Eisenwort,[2,3] Ingrid Simonitsch-Klupp,[4] Harald Esterbauer,[1,2] Matthias Mayerhofer,[5] Leonhard Müllauer,[4] Wolfgang R. Sperr,[2,3] Peter Valent,[2,3] and Gregor Hoermann[1,2,6]

[1]Department of Laboratory Medicine, Medical University of Vienna, Vienna; [2]Ludwig Boltzmann Institute for Hematology and Oncology, Medical University of Vienna, Vienna; [3]Department of Internal Medicine I, Division of Hematology and Hemostaseology, Medical University of Vienna, Vienna; [4]Department of Pathology, Medical University of Vienna, Vienna; [5]Ludwig Boltzmann Institute of Osteology, Hanusch Hospital, Vienna and [6]Central Institute of Medical and Chemical Laboratory Diagnostics, University Hospital Innsbruck, Innsbruck, Austria

 doi:10.3324/haematol.2019.217950

Received: January 29, 2019.

Accepted: April 18, 2019.

Pre-published: April 24, 2019.

Correspondence: *GREGOR HOERMANN* - gregor.hoermann@meduniwien.ac.at

Supplemental Tables

Treatment	Number of patients:
Interferon	14
Hydroxyurea	7
Cladribine	14
TKI	10
Midostaurin	5
Imatinib	4
Dasatinib	1
Antibodies	2
Alemtuzumab	1
Brentuximab Vedotin	1
Chemotherapy	15
HSCT	1

Supplemental Table 1. Cytoreductive treatment regiments. During the course of disease, 36 patients (31%) received a cytoreductive treatment with a median of 2 different regiments (range 1 – 5). TKI; tyrosine kinase inhibitor; HSCT, hematopoietic stem cell transplantation.

Control Patients	n=57
Age (median, range)	63 (19–88)
Sex (female | male)	21 | 36
***KIT* D816V positive**	0/57 (0%)

Supplemental Table 2. Characteristics of control patients. FFPE bone marrow sections from lymphoma patients undergoing a staging biopsy, and in whom no BM infiltration was detected, were used as control material for the assessment of the clinical specificity of *KIT* D816V testing.

Overall survival (OS)	Univariate		Multivariate	
	HR [95% CI]	p	HR [95% CI]	p
Molecular quantification of *KIT* D816V allele burden				
VAF in liquid specimen ≥2%	4.69 [1.84-11.98]	<0.001		n.s.
VAF in tissue ≥9%	12.79 [4.22-38.76]	<0.0001	18.12 [1.98-165.57]	0.01
Clinical characteristics				
Age > 65 years	1.06 [1.03-1.09]	<0.001		n.s.
Sex	2.54 [0.95-6.78]	n.s.		n.s.
B-findings				
MC infiltration in BM biopsy >30%	2.90 [1.17-7.22]	0.022		n.s.
Serum tryptase level >200 µg/l	2.43 [0.84-7.02]	n.s.		
Organomegaly without dysfunction *	4.50 [1.79-11.31]	0.001		n.s.
C-findings				
Hemoglobin <10 g/dl	20.98 [6.27-70.18]	<0.0001		n.s.
Platelets <100x10^9/l	20.68 [6.65-64.38]	<0.0001	29.28 [3.75-227.07]	0.001
Hepatomegaly with dysfunction #	7.71 [1.65-36.04]	0.009		n.s.
Alkaline phosphatase >150 U/l	3.59 [1.40-9.23]	0.008		n.s.
Weight loss	3.32 [1.31-8.41]	0.011		n.s.
Albumin levels <35 g/l	3.36 [0.97-11.68]	n.s.		n.s.

Supplemental Table 3. Parameters for overall survival (OS) in SM. Multivariate analyses regarding the prognostic impact of *KIT* D816V tissue VAF and clinical characteristics (including B- and C-findings) on overall survival of 103 patients with SM. HR, hazard ratio; CI, confidence interval; VAF, variant allele frequency; MC, mast cell; BM, bone marrow; n.s. not significant; * organomegaly including hepatomegaly, splenomegaly, or lymphadenopathy; # hepatomegaly with ascites and/or portal hypertension.

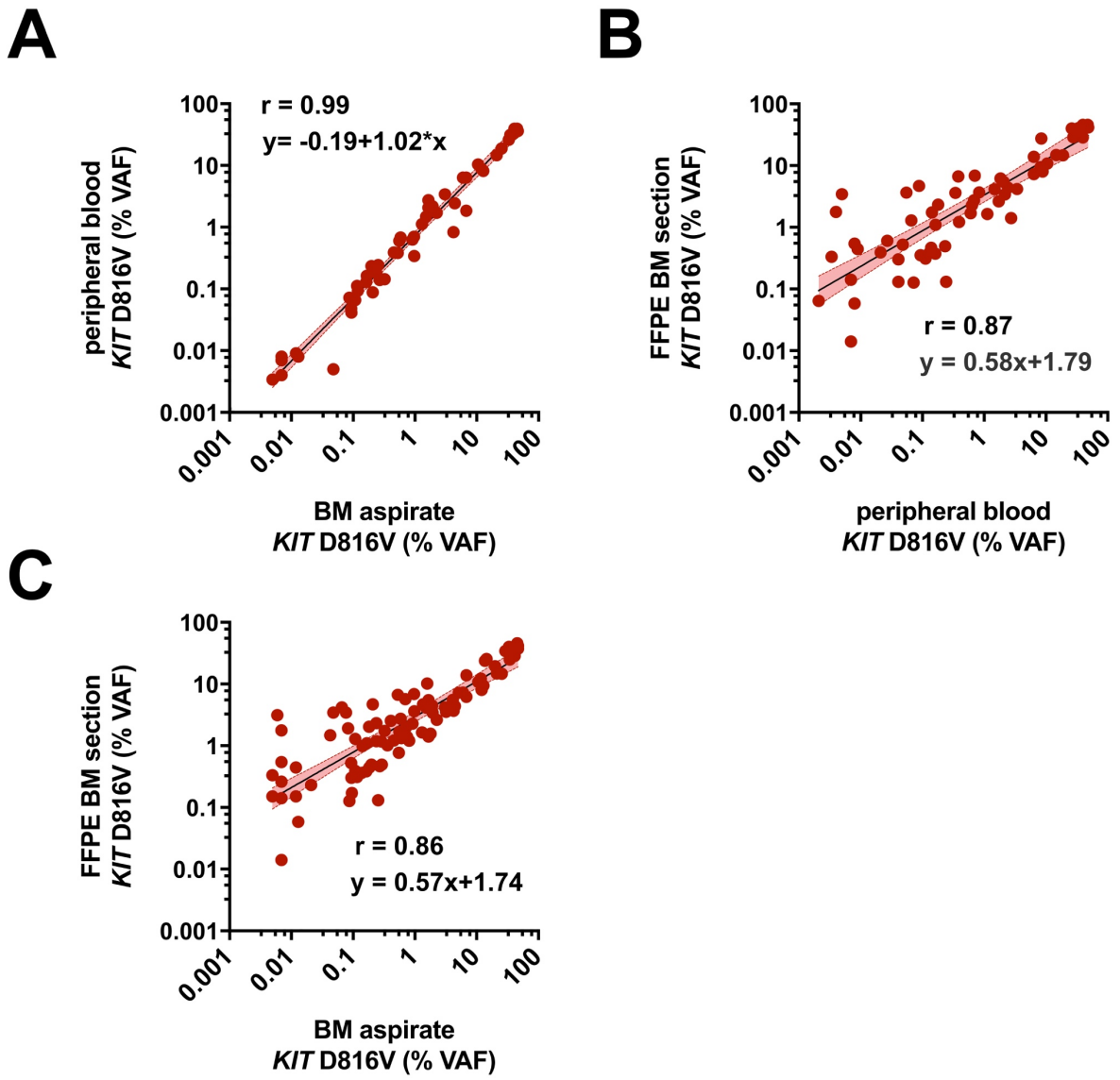
Sample ID	SM Subtype	MC (%) Flow	MC (%) Histology	*KIT* D816V (% VAF tissue)	*KIT* D816V (% VAF liquid)	CD2	CD25
2	ISM	0.010	5	0.06	n.t.	+	+
8	ISM	0.030	25	1.67	n.t.	+	+
16	ISM	0.060	25	40.90	46.60	+	+
22	ISM	0.070	10	1.72	0.32	+	+
25	ISM	0.050	20	2.70	0.59	+	+
32	ISM	0.001	5	0.13	0.04	+	+
51	ISM	0.030	20	1.28	0.11	+	+
53	ISM	0.050	50	1.08	0.17	+	+
54	ISM	0.320	10	0.06	0.01	n.t.	n.t.
86	ISM	0.005	3	0.15	0.01	+	+
88	ISM	1.180	30	13.60	6.82	+	+
90	ISM	0.030	5	0.52	0.04	+	+
100	ISM	0.160	40	12.00	11.69	+	+
102	ISM	0.080	20	36.90	41.10	n.t.	n.t.
108	ISM	0.470	20	7.21	6.02	-	+
109	ISM	0.006	5	0.98	0.14	-	+
110	ISM	0.004	5	1.20	0.46	-	+
134	ISM	0.140	10	24.60	34.20	+	+
141	ISM	0.020	5	1.16	0.29	+	+
150	ISM	0.020	10	2.30	0.24	+	+
153	ISM	0.030	20	3.40	0.05	+	+
154	ISM	0.010	5	0.23	0.02	+	+
192	ISM	0.010	30	5.40	1.66	+	+
199	ISM	0.010	3	0.03	n.t.	+	+
200	ISM	0.020	15	2.59	2.27	+	+
202	ISM	0.070	7	0.38	0.16	+	+
211	ISM	0.160	20	1.97	0.72	+	+
149	SSM	0.510	25	10.68	10.60	+	+
4	ASM	0.050	10	23.40	13.61	-	+
46	ASM	0.010	5	0.01	0.01	n.t.	n.t.
47	ASM	0.030	35	37.60	38.20	n.t.	n.t.
151	ASM	0.030	20	38.30	40.40	+	+
152	ASM	0.030	10	14.50	25.40	+	+
12	MCL	56.000	20	0*	n.t.	+	+
177	MCL	2.330	35	33.60	29.00	+	+
179	MCL	1.000	40	19.20	19.76	+	+
180	MCL	0.750	20	9.34	12.83	n.t.	n.t.
181	MCL	0.600	20	3.64	4.21	n.t.	n.t.
182	MCL	0.004	10	1.31	0.61	n.t.	n.t.
201	MCL	11.400	70	0*	0*	-	+
27	SM-AHN	0.050	15	1.46	0.04	-	+
164	SM-AHN	0.030	10	0	n.t.	+	+
209	SM-AHN	0.020	10	0.49	0.29	n.t.	n.t.

Supplemental Table 4. Assessment of mast cells by flow cytometry in a subset of systemic mastocytosis (SM) patients. Mast cell (MC) infiltration (%) determined by flow cytometry and histology, *KIT* D816V variant allele frequency (VAF) derived from tissue and liquid specimen, and aberrant surface expression of CD2 and CD25 on MC in 43 samples of 37 patients with SM. ISM, indolent SM; ASM, aggressive SM; MCL, mast cell leukemia; SM-AHN, SM with an associated hematological neoplasm; n.t., not tested; **KIT* D816H positive patient.

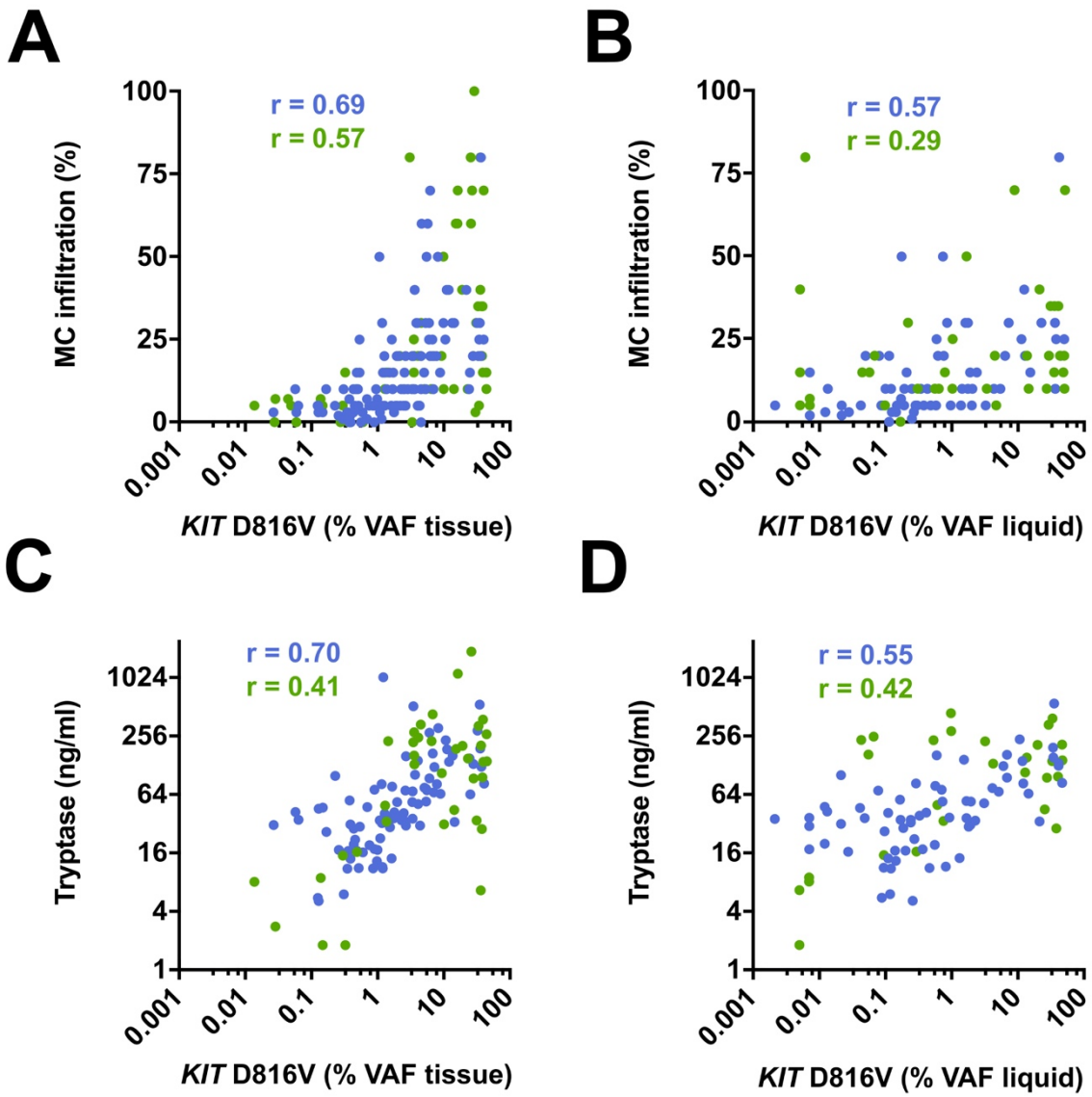
Turnaround-time	qualitative PCR	digital PCR
Total (min)	140	180
Hands-on (min)	33	33

Supplemental Table 5. Turnaround-time for molecular analyses of *KIT* D816V. Turnaround-time (TAT) for molecular analyses (qualitative PCR, *KIT* D816V mutation analysis using melting curve analysis after PNA-mediated PCR clamping; digital PCR) of batches of 8 samples is shown in minutes (min). Hands-on time as part of the total TAT is display separately.

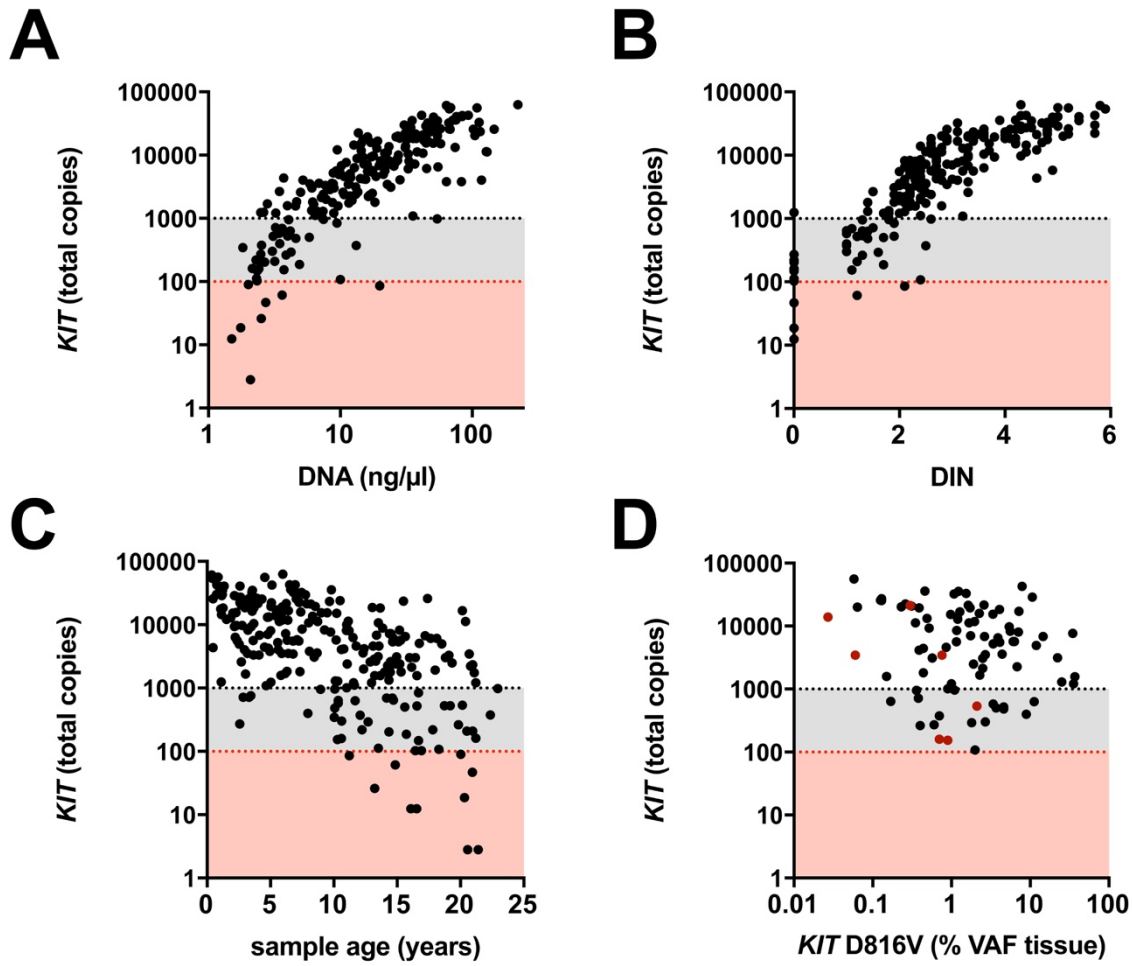
Supplemental Figures



Supplemental Figure 1. Relation between *KIT* D816V allele burden in PB, BM aspirate, and FFPE BM sections of SM patients. (A) Comparison of *KIT* D816V VAF in paired PB and BM aspirate samples showing no systematic deviation (r=0.99; slope: 1.02, intercept: 0.19 for log transformed data). (B-C) Comparison of *KIT* D816V VAF in paired samples from FFPE BM sections with PB (B) or BM aspirate (C) showing a systematic constant and proportional deviation to higher VAF in the tissue (r=0.87 and 0.86; slope: 0.58 and 0.57, intercept: 1.79 and 1.74 for log transformed data respectively). r indicates Spearman's correlation coefficient.



Supplemental Figure 2. Association of *KIT* D816V allele burden with biomarkers of disease burden in indolent and advanced SM. Correlation of *KIT* D816V mutation burden in FFPE BM sections (VAF tissue; A, C) and BM aspirate/PB samples (VAF liquid; B, D) with immunohistologically determined BM MC infiltration (A-B) and serum tryptase (C-D) of patients with ISM (blue) or advanced SM (green). r indicates the respective Spearman's correlation coefficients.



Supplemental Figure 3. Relation of DNA quality and assay sensitivity in FFPE BM sections. (A-C) Assay sensitivity represented by total *KIT* copy in dPCR of 211 FFPE BM sections from SM patients was correlated with DNA concentration (A), DNA integrity number (DIN; B), or sample age (C). Samples with a copy number <100 were excluded from further before analysis (n=11, red zone). Samples with a total number of *KIT* copies from 100 to 1000 were considered to have a limited sensitivity (n=35, grey zone). (D) *KIT* D816V VAF and assay sensitivity in diagnostic FFPE BM section of *KIT* D816V-positive ISM patients (n=91). Samples tested negative by melting curve analysis after PNA-mediated PCR clamping are depicted in red.

Supplemental Methods

Quantification of bone marrow mast cell infiltration

Formalin-fixed, paraffin-embedded (FFPE) bone marrow (BM) sections were stained with hematoxylin and eosin (H&E), Giemsa and Napthtol-AS-D-chloracetate-esterase stainings according to standard techniques, and immunohistochemistry with antibodies against tryptase, CD117 and CD25 was performed as described.[1] BM mast cell (MC) infiltration was quantified under light microscopy by a haemato-pathologist unaware of the results of molecular studies. The entire section was examined and all BM space with sufficient staining quality and assessability was used for MC quantification. Results were expressed as the percentage of MC involvement with respect to the total marrow cellularity and represent an average of the examined area.[2-4]

Flow cytometry

BM aspirates (n=43) from a subset of 37 patients with systemic mastocytosis (SM) were stained using fluorescent-labeled antibodies directed against CD45-APC/Cy7 (BioLegend, San Diego, CA, USA), CD117-PE/Cy7 (Invitrogen, Carlsbad, CA, USA), and CD34-Pacific Blue (BioLegend). After Red Blood Cell lysis using BD FACS Lysing Solution (BD Bisociences, Franklin Lakes, NJ, USA), cells were analyzed on a FACS Canto II flow cytometer (BD Biosciences). MC were defined as CD117hi/CD45+/CD34- cells. The percentage of MC was determined using FlowJo software (BD Biosciences). Results are shown in Supplemental Table 4.

Serum tryptase measurement

Total tryptase levels in serum were determined by a commercial fluoroenzyme-immunoassay (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden) on a Phadia 250 platform (Thermo Fisher Scientific) according to the recommendations of the manufacturer. The lower limit of detection amounted to 1 ng/ml.[5]

DNA extraction

Genomic DNA (gDNA) was extracted from FFPE BM sections using the EZ1 DNA Tissue Kit and the EZ1 Advanced XL Instrument (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. 20 BM sections à 10µm were isolated per sample and further subjected to dPCR analysis. DNA extraction of PB and BM was performed using the QIAsymphony Sp Instrument with the QIAsymphony DNA Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA from FFPE BM sections was quantified using the Qubit 3.0 fluorometer and the Qubit dsDNA HS assay kit (Thermo Fisher Scientific), and DNA Integrity Number (DIN) was assessed using the Agilent 2200 TapeStation system and the genomic DNA ScreenTape assay (Agilent Technologies, Santa Clara, CA).

Digital PCR for *KIT* D816V

dPCR was performed with the PrimePCR™ ddPCR mutation assay for *KIT* wild-type and the *KIT* D816V point mutation (Bio-Rad Laboratories GmbH, Munich, Germany) as described.[6] The wild-type and D816V alleles were labelled with FAM- and HEX-fluorochromes respectively. Reaction volume was 22 µL, consisting of 11 µL ddPCR supermix for probes (Bio-Rad Laboratories), 1.1 µL of each primer/probe mix, 1.1 µL

of HindIII restriction enzyme (20 units/µl; New England Biolabs, Ipswich, UK) and 7.7 µL genomic DNA (with a maximal concentration of 40 ng/µl). gDNA extracted from PB and/or aspirated BM cells was diluted to 40 ng/µl. gDNA isolated from FFPE BM sections was applied without dilution at a total volume of 7.7 µl. Automated droplet generator (Bio-Rad Laboratories) was used to partition the reaction mix into droplets followed by PCR reaction (95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 55°C for 1 min, and one final cycle of 98°C for 10 min). Cycled droplets were read on the QX-200 droplet-reader (Bio-Rad Laboratories) and analysis of the ddPCR data was performed using the QuantaSoft analysis software v1.7.4.0917 (Bio-Rad Laboratories). The threshold between the positive and negative droplet clusters was set for both fluorescence channels based on control samples.[6] The *KIT* D816V variant allele fraction (VAF) was calculated by dividing the number of mutated *KIT* D816V copies by the total number of *KIT* copies, and results were expressed as percent mutant alleles.

FFPE samples with a total number of *KIT* copies <100 were considered to have failed dPCR and were excluded from further analysis (n=11). Samples with a total number of *KIT* copies from 100 to 1000 were considered to have a limited sensitivity (n=35). The *KIT* copy number was found to correlate directly to the DNA concentration and DIN and inversely to the age of the FFPE block (Supplemental Figure 3A-C).

Sample processing from BM puncture, fixation, decalcification, embedding and DNA isolation of FFPE BM samples in a prospective setting is possible within 4 working days. However, processing of native BM biopsy material can be performed much faster within 24 hours. Assay turnaround time (TAT) and hands-on time of qualitative PCR or dPCR depends on the amount of samples used in the assay. TAT and hands-on time for 8 samples are depicted in Supplemental Table 5. Whereas, there is no major differences

in hands-on time between melting curve analysis after peptide nucleic acid-mediated PCR clamping and dPCR, total assay time of dPCR is slightly longer. When analyzing a larger amount of samples by dPCR, the total assay time is increased by approximately 2 minutes per sample due to the sequential acquisition of samples in the droplet-reader with no relevant effect on hands-on time.

Statistical analysis

Statistical analysis was performed using R (version 3.4.2, Vienna, Austria)[7] and GraphPad Prism (GraphPad Software, La Jolla, CA). Categorical data was assessed by McNemar's Chi-square test. Metric data is given as median and group differences were evaluated by the Mann-Whitney-U test. The correlation was assessed by applying Spearman's rank correlation coefficient (r). Differences between the dPCR results obtained from different specimen were graphically displayed using the Bland-Altman plot and statistically compared by applying Passing-Bablok regression.[8] The adjusted prognostic significance of *KIT* D816V tissue mutation burden with regard to overall survival (OS) and progression free survival (PFS) was analyzed by Cox proportional hazard regression. Optimal cut-off values were calculated by applying the maximally selected rank statistics method.[9] Kaplan-Meier survival plots were used for graphical representation, and difference between survivals curves were analyzed using the log-rank test. Differences were considered to be significant when the p-value was <0.05.

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