Detection and prognostic value of recurrent exportin 1 mutations in tumor and cell-free circulating DNA of patients with classical Hodgkin lymphoma

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Camus et al.

Supplementary Methods

Patients, Samples and DNA extraction

Blood samples were collected in EDTA tubes and processed within two hours after collection, by centrifuging once at 2,000g (10 min) at 4°C to isolate plasma, which was then stored at -80°C. DNA was extracted from 1-3ml of EDTA plasma using a QIAmp Circulating Nucleic Acid Kit® (Qiagen, Hilden, Germany) following the manufacturer's instructions. Extracted cfDNA from each plasma sample was eluted in 40 µL of AVE buffer and then stored at -80°C. Double-stranded DNA quantification was performed by a fluorimetric method using Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). DNA was also extracted from frozen or FFPE tumor tissues using proteinase K, followed by salt and ethanol precipitation and stored at -20°C in 10 mM Tris-Cl and 1 mM EDTA (pH 8) buffer.

All tissue slides were reviewed by experienced pathologists and cHL subtypes were confirmed according to usual histological criteria. Blood samples and biopsies at diagnosis were taken before treatment.

dPCR experiments

Due to the limited amount of cfDNA material available, preamplification of 4ng of cfDNA was performed using our Custom TaqMan® primers and probes and TaqMan® Universal PCR Master Mix (no UNG). In our experiments we used normal genomic DNA (Promega® Corporation, Madison WI) and wild type pre-amplified DNA as negative controls. Total amount of DNA used for dPCR reaction was 4ng of cfDNA (~1200 genome equivalents) with a pre-amplification step for plasma samples and 30ng of genomic DNA (~9000 genome equivalents) for biopsy samples.

The Variant Allele Fraction (VAF) was defined as the proportion of mutant DNA copies relative to the sum of mutant (MT) and wild-type (WT) DNA copies obtained by dPCR. dPCR experiments were performed on both platforms in duplicate.

Ion torrent personal genome machine™ (PGM) sequencing

Amplified libraries (Ion AmpliSeq™ Library Kit 2.0) were submitted to emulsion PCR with the Ion OneTouch™ 200 Template Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) using the Ion OneTouch™ System (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. The templated Ion Sphere™ Particles (ISPs) were enriched with the Ion OneTouch™ Enrichment System and loaded and sequenced on an Ion 316™ v2 Chip (Thermo Fisher Scientific).

After alignment, the presence of the E571K (chr2:61719472C>T) mutation was assessed by an exact multinomial test, comparing the number of reads containing the mutated base (T) to the pseudo-counts of reads containing noise bases (Gs and As observed plus 1 read). This test is able to identify a mutated read count significantly higher than what can be observed for known noise (G and A), assuming that the noise level is the same over the three non-reference bases. False discovery rates (FDR) were computed to account for the numerous tested samples, and samples yielding a FDR < 10% were considered mutated. As non-significant FDRs observed in samples with low sequencing depth can also result from a lack of power, we considered as non-interpretable every sample with a non-significant FDR and a sequencing depth lower than 1000x. This threshold was set to reflect the minimal VAF threshold observed in dPCR, as a mutation with such a VAF sequenced less than 1000x would only be represented by a single mutated read, and thus could never be distinguished from the pseudo-counted noise reads.

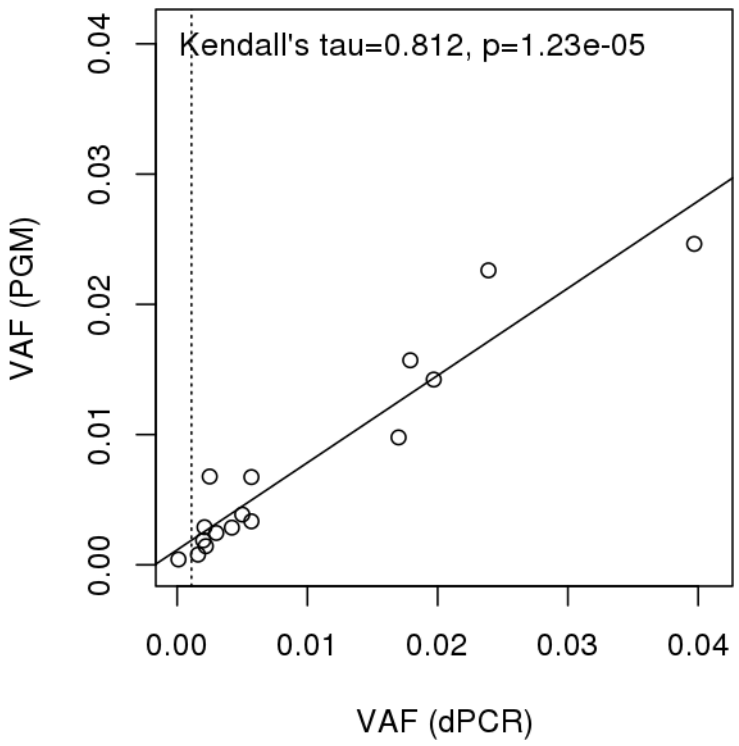
Response evaluation, survival analysis and PET-CT

The uptake in the involved area was visually compared with mediastinum and liver as reference organs (score 1, no residual uptake; score 2, uptake ≤ mediastinum; score 3, uptake > mediastinum but ≤ liver; score 4, uptake moderately >liver; score 5, uptake markedly increased and/or progression of the lesions) [28].

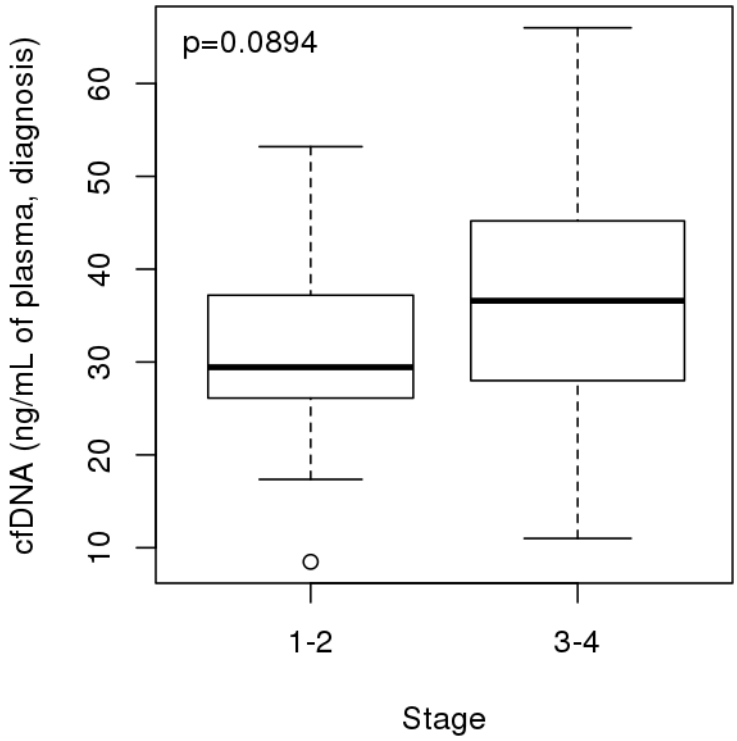
PET was considered positive if residual activity ≥ 4. Complete Response (CR) was defined as PET negativity with or without a residual mass. Relapsed disease (RD) was defined as partial response (PR), stable disease (SD) or progressive disease (PD) with persistence of any residual PET-positive lesion or any new appearance of PET-positive findings, according to the IWG criteria. PET-CT reviews were performed by two experienced nuclear medicine physicians (PV, SB). The overall survival (OS) was calculated from the date of diagnosis to death from any cause or last patient follow-up. The progression-free survival (PFS) was calculated from the date of enrollment until disease progression, relapse, and death from any cause or last patient follow-up. Survival curves were estimated using the Kaplan-Meier method and compared using the log-rank test.

Supplementary Figure

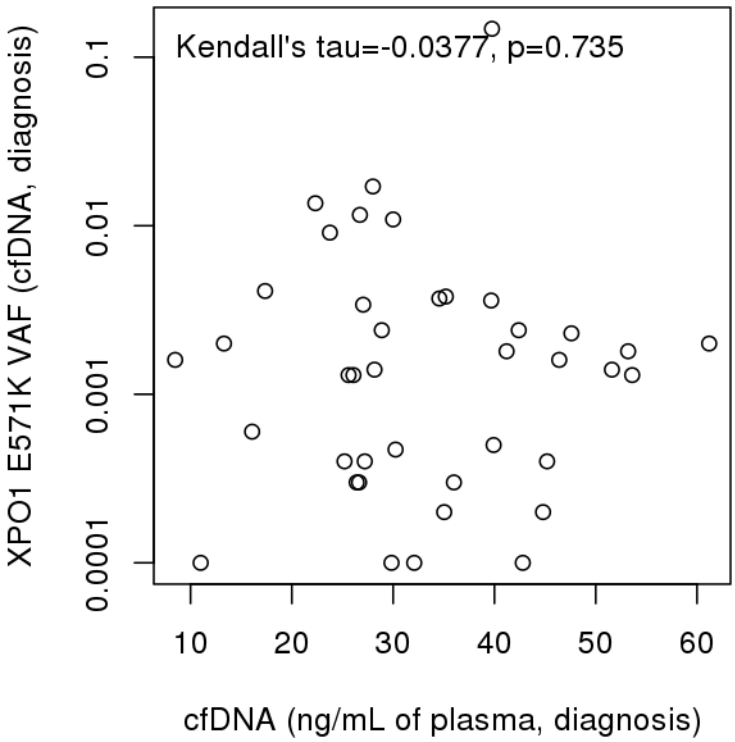
Supplementary Figure 1: Correlation between *XPO1* E571K VAF by digital PCR and PGM. The dotted line represents the dPCR detection limit (0.001).



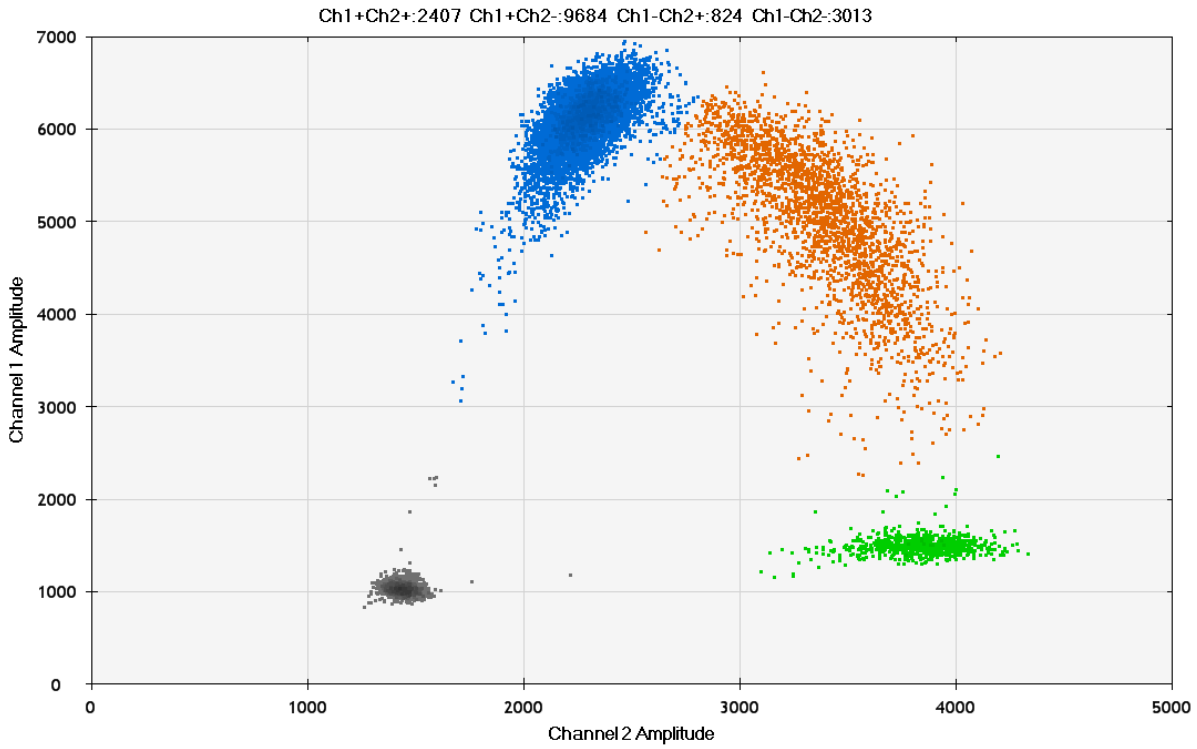
Supplementary Figure 2: Correlation between plasma cfDNA concentration and disease stage.



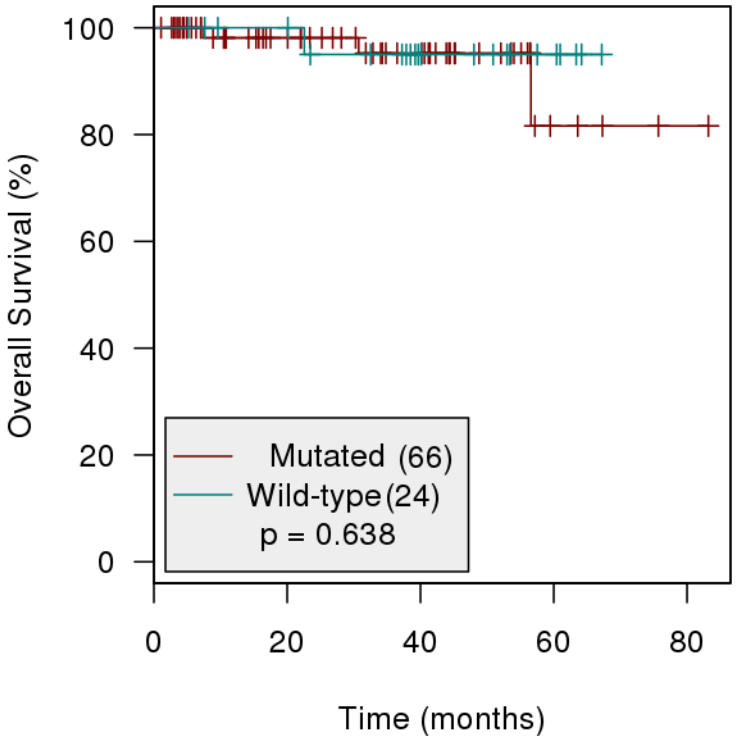
Supplementary Figure 3: Correlation between *XPO1* E571K VAF in cfDNA and plasma cfDNA concentration at diagnosis.

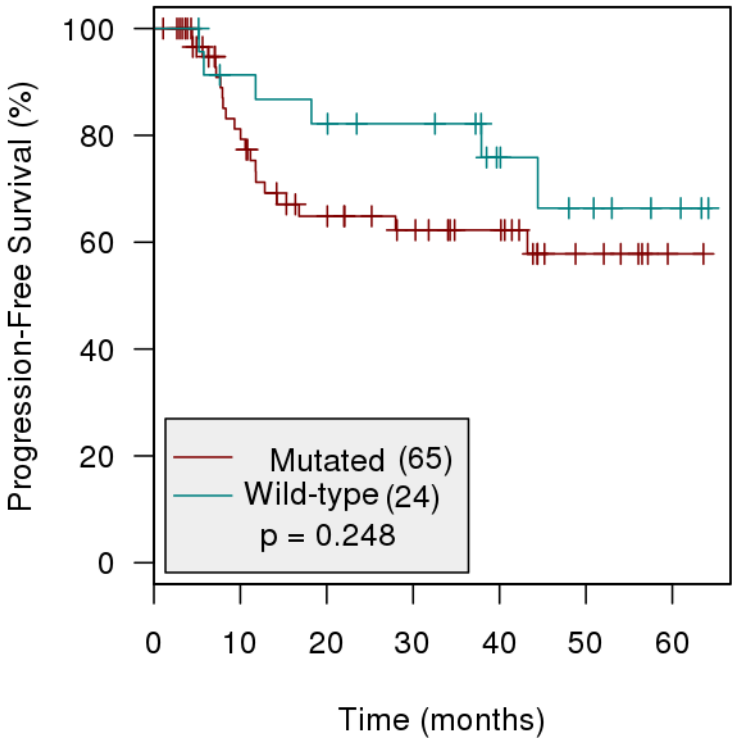


Supplementary Figure 4: droplet digital PCR (ddPCR, Biorad) scatter plot representing *XPO*1 E571K results in U-H01 cell line DNA.

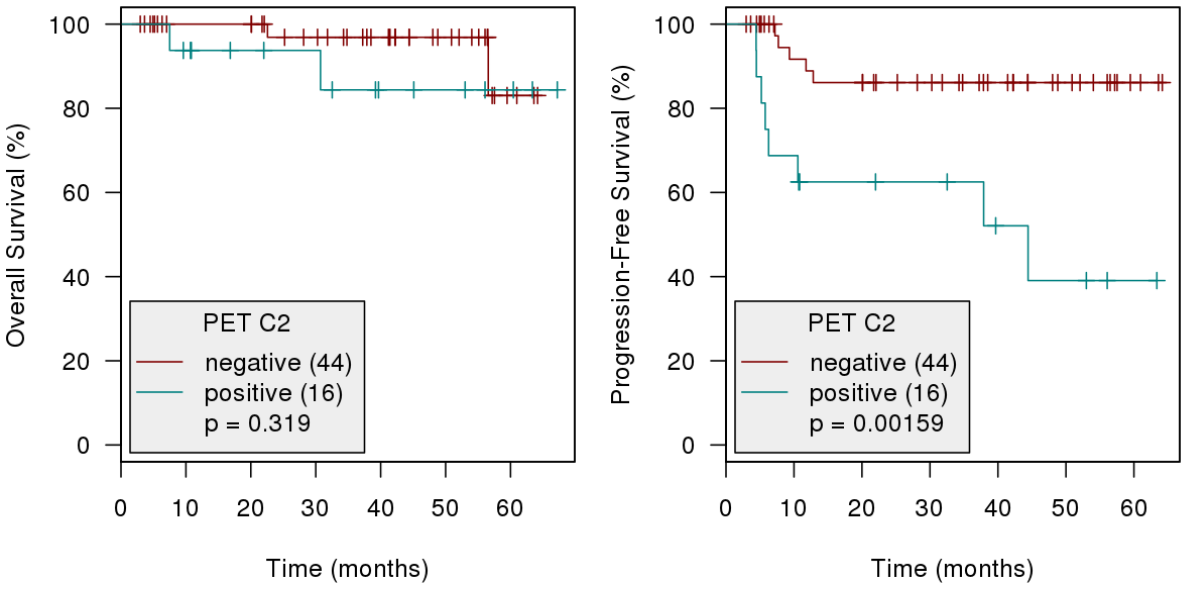


Supplementary Figure 5: OS and PFS according to the *XPO1* E571K biopsy results by digital PCR.

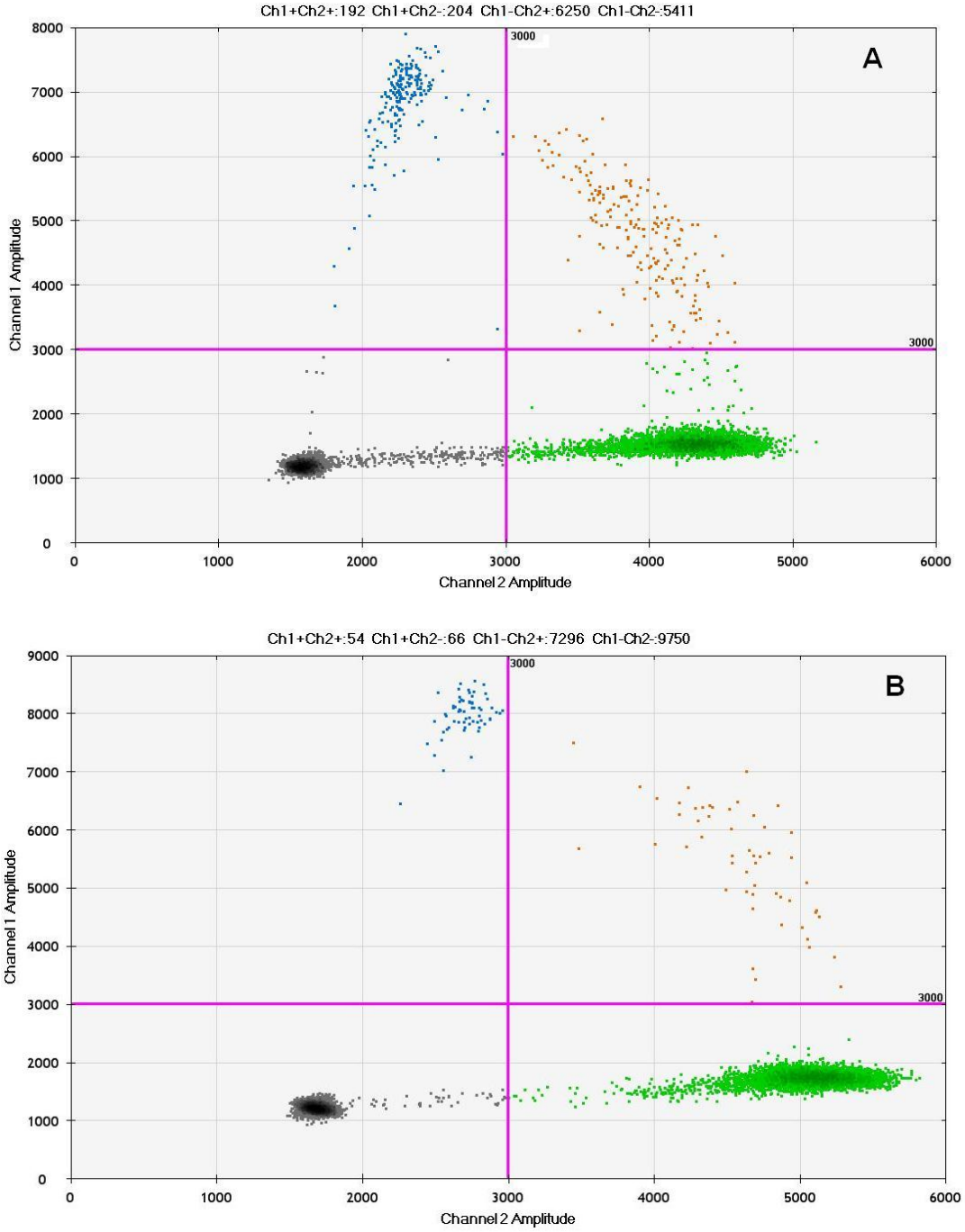




Supplementary Figure 6: OS and PFS according to the PET C2 results



Supplementary Figure 7: Representative views of digital PCR plots (mutant samples) for *XPO1* E571K quantification in tumor (VAF = 3.97%, **A**) and plasma (VAF = 0.91%, **B**) for a cHL patient at diagnosis (patient N°42) by ddPCR (Biorad). Data are displayed in a scatter plot based on color of FAM and VIC events. Green, blue, orange and black plots represent wild-type alleles, mutant alleles, both alleles in the same droplet and no amplifications, respectively. VAF: Variant Allele Fraction.



Supplementary Tables

Supplementary Table 1: *XPO1* E571K digital PCR primer and probe sequences

XPO1 E571K	**Primer/probe ID**	**Sequence**
dPCR design	XPO1_E571K_F	5'-TCTCTAACAAGACAAAAACATTCATTTATTTTCTTCA-3'
	XPO1_E571K_R	5'-GCTCACTGGAAATTTCTGAAGACTGT-3'
	XPO1_E571K-wt probe	5'-VIC-AGCTGTTCGAATTCAT-MGB-3'
	XPO1_E571K-mt probe	5'-FAM-AGCTGTTC**A**AATTCAT-MGB-3'

Supplementary Table 2: digital PCR (dPCR) cycling conditions

Step	PCR cycling	Number of cycles	Reagent composition
pre-amplification	95°C for 10 min	1	TaqMan universal PCR master mix, no UNG : 12.5 µL
	95°C for 15 sec	10	Custom TaqMan dPCR Assay 20X : 0.25 µL
	60°C for 4 min		DNA : 4ng
			Water, PCR grade : QSAD total volume : 25 µL
dPCR QuantStudio 3D	96°C for 10 min	1	QuantStudio 3D Digital PCR Master Mix 2X : 7.5 µL
	55°C for 2 min	39	Custom TaqMan dPCR Assay 20X : 0.75 µL
	98°C for 30 sec		cfDNA : 2 µL of 10-fold diluted pre-amplified DNA; tumor tissue DNA : 30ng
	60°C for 2 min	1	Water, PCR grade, QSAD Total volume : 15 µL
ddPCR Qx200	95°C for 10 min	1	Bio-Rad ddPCR Supermix for probes (No dUTP) 2X : 10µL
	94°C for 30 seconds	40	Custom TaqMan dPCR Assay 20X : 1µL
	55°C for 1 min		cfDNA : 9µL of 10-fold diluted pre-amplified DNA; tumor tissue DNA : 30ng
	98°C for 10 min	1	Water, PCR grade, QSAD Total Volume 20µL

Supplementary Table 3: Detailed results of dPCR control experiments in plasma cfDNA.

SAMPLE ID	Frequency of mutation by ddPCR (Replicate 1)	Frequency of mutation by ddPCR (Replicate 2)	Mean	Standard Deviation (SD)	Limit of Blank [Mean + (1.645 x SD)]
CONTROL 1	0.02%	0.04%	0.04%	0.04%	**0.1%**
CONTROL 2	0.01%	0.01%			
CONTROL 3	0.03%	0.04%			
CONTROL 4	0.02%	0.04%			
CONTROL 5	0.01%	0.05%			
CONTROL 6	0.03%	0.11%			
CONTROL 7	0.02%	0.02%			
CONTROL 8	0.01%	0.00%			
CONTROL 9	0.01%	0.03%			
CONTROL 10	0.03%	0.04%			
CONTROL 11	0.09%	0.02%			
CONTROL 12	0.03%	0.00%			
CONTROL 13	0.02%	0.00%			
CONTROL 14	0.06%	0.04%			
CONTROL 15	0.04%	0.05%			
CONTROL 16	0.05%	0.04%			
CONTROL 17	0.12%	0.12%			
CONTROL 18	0.11%	0.11%			
CONTROL 19	0.14%	0.12%			
CONTROL 20	0.00%	0.00%			

Supplementary Table 4: Association between *XPO1* mutation detection in biopsy and cfDNA by digital PCR, p=0.0179 (Fisher's exact test)

	cfDNA *XPO1* E571K mutation detection		
Biopsy status	negative	positive	NA
Wild-type	20	10	0
Mutated	5	12	0
NA	0	3	0

Supplementary Table 5: Association between *XPO1* mutational status at diagnosis, according to dPCR or PGM, p=4.11e-09 (Fisher's exact test)

	dPCR	
PGM	Wild type	Mutated
Wild type	26	1
Mutated	1	14

Supplementary Table 6: Detailed results of *XPO1* PGM experiments

Patient number	PGM.depth	PGM.mutated base (n=)	PGM.pvalue	PGM.FDR	PGM.VAF.diag.biopsy	PGM.Status.diag.biopsy
1	10307	6	0.059	0.167	0.058%	WILD TYPE
2	8481	1	1.000	1.000	0.012%	WILD TYPE
3	8087	127	0.000	0.000	1.570%	XPO1 E571K
4	5410	2	0.630	1.000	0.037%	WILD TYPE
5	7041	3	0.383	0.875	0.043%	WILD TYPE
6	11881	4	0.136	0.326	0.034%	WILD TYPE
7	6744	1	1.000	1.000	0.015%	WILD TYPE
8	19240	8	0.004	0.015	0.042%	XPO1 E571K
9	6747	2	0.630	1.000	0.030%	WILD TYPE
10	1076	1	1.000	1.000	0.093%	WILD TYPE
11	403	0	1.000	1.000	0.000%	NI
12	995	0	1.000	1.000	0.000%	NI
13	2223	0	1.000	1.000	0.000%	WILD TYPE
14	110	0	1.000	1.000	0.000%	NI
15	7690	1	1.000	1.000	0.013%	WILD TYPE
16	11098	4	0.136	0.326	0.036%	WILD TYPE
17	4551	13	0.000	0.000	0.286%	XPO1 E571K
18	4627	2	0.630	1.000	0.043%	WILD TYPE
19	2175	0	1.000	1.000	0.000%	WILD TYPE
20	3263	1	1.000	1.000	0.031%	WILD TYPE
21	1284	1	1.000	1.000	0.078%	WILD TYPE
22	2161	1	1.000	1.000	0.046%	WILD TYPE
23	1898	27	0.000	0.000	1.423%	XPO1 E571K
24	1505	0	1.000	1.000	0.000%	WILD TYPE
25	5194	35	0.000	0.000	0.674%	XPO1 E571K
26	3108	12	0.000	0.001	0.386%	XPO1 E571K
27	9194	2	0.630	1.000	0.022%	WILD TYPE
28	6148	139	0.000	0.000	2.261%	XPO1 E571K
29	3759	0	1.000	1.000	0.000%	WILD TYPE
30	7711	4	0.136	0.326	0.052%	WILD TYPE
31	2644	0	1.000	1.000	0.000%	WILD TYPE
32	8177	0	1.000	1.000	0.000%	WILD TYPE
33	5966	0	1.000	1.000	0.000%	WILD TYPE
34	4803	1	1.000	1.000	0.021%	WILD TYPE
35	2126	1	1.000	1.000	0.047%	WILD TYPE
36	4891	7	0.027	0.085	0.143%	XPO1 E571K
37	3392	23	0.000	0.000	0.678%	XPO1 E571K
38	6883	23	0.000	0.000	0.334%	XPO1 E571K
39	11026	27	0.000	0.000	0.245%	XPO1 E571K
40	4263	8	0.012	0.040	0.188%	XPO1 E571K
41	135	0	1.000	1.000	0.000%	NI
42	487	12	0.000	0.000	2.464%	XPO1 E571K
43	6385	5	0.059	0.167	0.078%	WILD TYPE
44	1957	2	0.630	1.000	0.102%	WILD TYPE

45	920	9	0.002	0.006	0.978%	XPO1 E571K
46	182	0	1.000	1.000	0.000%	NI
47	490	1	1.000	1.000	0.204%	NI
48	4500	13	0.000	0.000	0.289%	XPO1 E571K

NI : non interpretable

Supplementary Table 7: Detailed results of *XPO1* dPCR experiments.

In this study, 0.1% rate of mutated copies was considered the relevant threshold to discriminate positive versus negative samples (See Patients and Methods section). Positive results appear in red. VAF : Variant allele frequency.

Patient	Gender	Age	Diagnosis	Ann Arbor Disease stage	*XPO1* E571K VAF Diagnostic biopsy	*XPO1* E571K VAF Diagnostic plasma cfDNA	diagnostic cfDNA concentration (ng/mL of plasma)	*XPO1* E571K VAF End of Treatment plasma cfDNA	End of Treatment cfDNA concentration (ng/mL of plasma)
1	Male	26	NODULAR SCLEROSIS cHL	1	0.00%	1.16%	26.72	0.00%	11.76
2	Male	25	NODULAR SCLEROSIS cHL	4	0.01%	0.00%	37.2	NA	NA
3	Male	41	NODULAR SCLEROSIS cHL	2	1.79%	0.41%	17.36	0.10%	9.97
4	Male	25	NODULAR SCLEROSIS cHL	4	0.01%	0.02%	44.8	NA	NA
5	Female	34	NODULAR SCLEROSIS cHL	3	0.01%	0.00%	58.4	NA	NA
6	Male	39	NODULAR SCLEROSIS cHL	2	0.01%	0.00%	39.36	NA	NA
7	Female	31	NODULAR SCLEROSIS cHL	4	0.00%	0.01%	42.8	NA	NA
8	Male	18	NODULAR SCLEROSIS cHL	2	0.01%	0.00%	26.8	NA	NA
9	Female	39	NODULAR SCLEROSIS cHL	4	0.01%	0.14%	28.16	0.31%	4.69
10	Female	22	NODULAR SCLEROSIS cHL	4	0.00%	0.00%	22	NA	NA
11	Female	41	NODULAR SCLEROSIS cHL	3	0.00%	0.00%	52	NA	NA
12	Female	41	NODULAR SCLEROSIS cHL	3	0.00%	0.03%	26.4	NA	NA
13	Male	20	NODULAR SCLEROSIS cHL	2	0.00%	0.13%	25.6	0.40%	9.6
14	Female	18	NODULAR SCLEROSIS cHL	2	NA	0.16%	8.48	0.08%	2.85
15	Male	43	NODULAR SCLEROSIS cHL	2	0.00%	0.04%	27.2	NA	NA
16	Female	21	NODULAR SCLEROSIS cHL	2	0.00%	0.37%	34.56	0.00%	8.69
17	Female	25	NODULAR SCLEROSIS cHL	2	0.42%	1.36%	22.32	0.09%	9.07
18	Female	35	NODULAR	4	0.00%	0.01%	11	0.04%	11.04

			SCLEROSIS cHL						
19	Female	23	NODULAR SCLEROSIS cHL	2	0.00%	0.24%	28.88	0.07%	5.73
20	Female	57	NODULAR SCLEROSIS cHL	2	0.03%	0.03%	26.64	NA	NA
21	Female	34	NODULAR SCLEROSIS cHL	2	0.00%	0.04%	25.2	NA	NA
22	Female	18	NODULAR SCLEROSIS cHL	2	0.00%	0.01%	32.08	NA	NA
23	Male	35	MIXED CELLULARITY cHL	2	1.97%	14.74%	39.76	0.08%	11.44
24	Male	22	NODULAR SCLEROSIS cHL	3	0.00%	0.24%	42.4	0.00%	4.27
25	Male	34	NODULAR SCLEROSIS cHL	4	0.57%	0.06%	16.08	0.10%	4.99
26	Male	22	MIXED CELLULARITY cHL	2	0.50%	0.14%	51.6	NA	NA
27	Male	42	NODULAR SCLEROSIS cHL	4	0.00%	0.04%	45.2	NA	NA
28	Male	21	NODULAR SCLEROSIS cHL	4	2.39%	1.71%	28	0.00%	5.31
29	Female	43	NODULAR SCLEROSIS cHL	3	0.00%	0.00%	34.08	NA	NA
30	Female	24	NODULAR SCLEROSIS cHL	4	0.00%	0.03%	36	NA	NA
31	Male	30	NODULAR SCLEROSIS cHL	2	0.02%	0.34%	27.04	0.00%	9.95
32	Male	44	NODULAR SCLEROSIS cHL	3	0.00%	0.00%	66	0.04%	15.2
33	Male	19	NODULAR SCLEROSIS cHL	4	0.00%	0.20%	13.3	0.00%	7.49
34	Female	42	MIXED CELLULARITY cHL	4	0.00%	0.05%	39.92	0.04%	9.68
35	Male	31	NODULAR SCLEROSIS cHL	4	0.01%	0.23%	47.6	NA	NA
36	Male	29	MIXED CELLULARITY cHL	3	0.22%	0.13%	26.08	0.08%	8.05
37	Male	30	NODULAR SCLEROSIS cHL	2	0.25%	0.02%	35.04	0.03%	3.92
38	Female	21	NODULAR SCLEROSIS cHL	2	0.57%	1.09%	30	0.12%	8.21
39	Female	62	NODULAR SCLEROSIS cHL	4	0.30%	0.13%	53.6	0.18%	18.67
40	Male	32	MIXED	2	0.20%	0.00%	31.68	0.04%	11.47

			CELLULARITY cHL						
41	Male	36	unclassified cHL	3	0.00%	0.20%	61.2	0.13%	3.87
42	Female	28	NODULAR SCLEROSIS cHL	2	3.97%	0.91%	23.76	0.19%	7.15
43	Male	23	NODULAR SCLEROSIS cHL	3	0.16%	0.05%	30.24	0.00%	8.75
44	Male	27	NODULAR SCLEROSIS cHL	4	0.00%	0.01%	29.84	NA	NA
45	Female	32	MIXED CELLULARITY cHL	3	1.70%	0.18%	41.2	0.02%	27.2
46	Female	55	NODULAR SCLEROSIS cHL	2	0.33%	0.18%	53.2	0.02%	10.99
47	Male	25	unclassified cHL	2	0.45%	0.00%	34.48	0.01%	16
48	Female	27	NODULAR SCLEROSIS cHL	1	0.21%	0.36%	39.68	0.32%	22.2
49	Male	38	MIXED CELLULARITY cHL	2	NA	0.16%	46.4	0.08%	13.6
50	Female	60	NODULAR SCLEROSIS cHL	4	NA	0.38%	35.2	0.00%	8.43
51	Male	63	MIXED CELLULARITY cHL	3	0.00%	NA	NA	NA	NA
52	Male	46	NODULAR SCLEROSIS cHL	4	0.00%	NA	NA	NA	NA
53	Male	44	NODULAR SCLEROSIS cHL	4	0.01%	NA	NA	NA	NA
54	Female	25	unclassified cHL	4	0.01%	NA	NA	NA	NA
55	Female	77	MIXED CELLULARITY cHL	4	0.01%	NA	NA	NA	NA
56	Female	24	NODULAR SCLEROSIS cHL	3	0.02%	NA	NA	NA	NA
57	Female	31	NODULAR SCLEROSIS cHL	2	0.01%	NA	NA	NA	NA
58	Female	21	NODULAR SCLEROSIS cHL	2	0.01%	NA	NA	NA	NA
59	Female	33	LYMPHOCYTE -RICH cHL	2	0.03%	NA	NA	NA	NA
60	Male	53	NODULAR SCLEROSIS cHL	4	0.00%	NA	NA	NA	NA
61	Male	55	NODULAR SCLEROSIS cHL	1	0.01%	NA	NA	NA	NA
62	Male	35	MIXED CELLULARITY cHL	2	0.01%	NA	NA	NA	NA
63	Male	40	NODULAR SCLEROSIS cHL	4	0.13%	NA	NA	NA	NA
64	Male	57	NODULAR	4	0.00%	NA	NA	NA	NA

			SCLEROSIS cHL						
65	Male	47	NODULAR SCLEROSIS cHL	4	0.01%	NA	NA	NA	NA
66	Male	25	NODULAR SCLEROSIS cHL	4	0.18%	NA	NA	NA	NA
67	Male	54	NODULAR SCLEROSIS cHL	4	0.02%	NA	NA	NA	NA
68	Male	78	NODULAR SCLEROSIS cHL	4	0.01%	NA	NA	NA	NA
69	Male	35	NODULAR SCLEROSIS cHL	4	0.00%	NA	NA	NA	NA
70	Female	57	NODULAR SCLEROSIS cHL	2	0.00%	NA	NA	NA	NA
71	Female	52	unclassified cHL	2	0.00%	NA	NA	NA	NA
72	Female	20	NODULAR SCLEROSIS cHL	3	0.00%	NA	NA	NA	NA
73	Male	38	NODULAR SCLEROSIS cHL	2	0.00%	NA	NA	NA	NA
74	Male	50	NODULAR SCLEROSIS cHL	2	0.01%	NA	NA	NA	NA
75	Female	18	NODULAR SCLEROSIS cHL	2	0.00%	NA	NA	NA	NA
76	Male	21	NODULAR SCLEROSIS cHL	2	0.00%	NA	NA	NA	NA
77	Male	83	NODULAR SCLEROSIS cHL	4	0.00%	NA	NA	NA	NA
78	Female	33	NODULAR SCLEROSIS cHL	4	0.01%	NA	NA	NA	NA
79	Male	51	NODULAR SCLEROSIS cHL	2	1.42%	NA	NA	NA	NA
80	Male	25	NODULAR SCLEROSIS cHL	2	0.02%	NA	NA	NA	NA
81	Male	32	NODULAR SCLEROSIS cHL	4	0.27%	NA	NA	NA	NA
82	Male	21	NODULAR SCLEROSIS cHL	2	0.00%	NA	NA	NA	NA
83	Female	36	NODULAR SCLEROSIS cHL	4	0.01%	NA	NA	NA	NA
84	Male	46	NODULAR SCLEROSIS cHL	2	0.00%	NA	NA	NA	NA
85	Male	26	NODULAR SCLEROSIS cHL	4	0.00%	NA	NA	NA	NA
86	Female	32	NODULAR SCLEROSIS cHL	2	4.78%	NA	NA	NA	NA

87	Male	22	NODULAR SCLEROSIS cHL	2	0.00%	NA	NA	NA	NA
88	Female	28	NODULAR SCLEROSIS cHL	2	0.00%	NA	NA	NA	NA
89	Female	55	NODULAR SCLEROSIS cHL	3	0.00%	NA	NA	NA	NA
90	Female	24	NODULAR SCLEROSIS cHL	4	0.01%	NA	NA	NA	NA
91	Male	32	NODULAR SCLEROSIS cHL	2	0.01%	NA	NA	NA	NA
92	Female	22	NODULAR SCLEROSIS cHL	4	0.01%	NA	NA	NA	NA
93	Male	25	NODULAR SCLEROSIS cHL	4	0.01%	NA	NA	NA	NA
94	Male	42	MIXED CELLULARITY cHL	4	0.00%	NA	NA	NA	NA