Circulating cell-free *BRAF* V600E during chemotherapy is associated with prognosis of children with Langerhans cell histiocytosis

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Table S1. Treatment Protocol for children with Langerhans cell histiocytosis.

Treatment Element/Drug	Single or Daily dose	Days of Administration
First - line therapy		
Initial induction treatment course 1 [a]		
Prednisone	40 mg/m^2/day, orally, in two divided doses	Day1-28 (4 weeks), afterwards weekly reduction for 2 weeks
Vincristine	1.5 mg/m^2/dose (max. 2 mg), i.v. bolus	Day1, 8, 15, 22, 29, 36 (once a week) for 6 weeks
Initial induction treatment course 2 [b]		
Prednisone	40 mg/m^2/day, orally, in two divided doses	Day1-3, weekly for 6 weeks
vincristine	1.5 mg/m^2/dose (max. 2 mg), i.v. bolus	Day1, 8, 15, 22, 29, 36 (once a week) for 6 weeks
Maintenance treatment [c]		
Prednisone	40 mg/m^2/day, orally, in two divided doses	Day1-5 every 3 weeks
Vincristine	1.5 mg/m^2/dose (max. 2 mg), i.v. bolus	Day1 every 3 weeks
6-mercaptopurine [d]	50mg/m^2/day, orally	Daily, every night
Second-line therapy [e]		
Intensification treatment A: every 4 weeks, for 4 courses		
Cladribine	9mg/m^2/day, i.v.gtt	Day2-4 for RO^-, Day2-6 for RO^+
Cytarabine	150mg/m^2/day, i.v.gtt /H.	Day1-5
Vincristine	1.5 mg/m^2/dose (max. 2 mg), i.v. bolus	Day1
Dexamethasone	6mg/m^2/day, i.v.	Day1-5
Intensification treatment B : every 3 weeks, for 4 courses		
Cytarabine	150mg/m^2/day, i.v.gtt /H.	Day1-5
Vincristine	1.5 mg/m^2/dose (max. 2 mg), i.v. bolus	Day1
Dexamethasone	6mg/m^2/day, i.v.	Day1-5
Maintenance treatment		
Prednisone	40 mg/m^2/day, orally, in two divided doses	Day1-5, every 3 weeks
Vincristine	1.5 mg/m^2/dose (max. 2 mg), i.v. bolus	Day1 every 3 weeks
6-mercaptopurine	50mg/m^2/day, orally	Daily, every night

[a] After the first initial treatment (six weeks), response to treatment was evaluated. Non-active disease (NAD), active disease (AD)/better, and AD/intermediate were defined as complete resolution, continuous regression of disease, or unchanged disease respectively. AD/worse was disease progression or appearance of some new lesions. Patients responding to therapy were those who had NAD or AD/better

response. Patients with NAD were directly admitted to maintenance therapy. Patients with AD/better or AD/intermediate response were given the second induction treatment course. Patients with AD/worse response, or those with no significant improvement in risk organs, pituitary, or lung were shifted to the second-line therapy.

[b] Patients with NAD, RO^- patients with AD/better response after the second course of initial treatment (12 weeks) were then given maintenance therapy. Patients with AD/intermediate or AD/worse response, or those with no significant improvement in risk organs, pituitary, or lung were shifted to second-line therapy.

[c] The overall treatment duration was 12 months. Patients who assessed progression of lesions in dangerous organs, central nervous system (CNS) - risk sites, CNS or lung during maintenance or after discontinuation of drugs were given the second-line treatment, while those with progression or new lesions in other sites were given the first-line treatment again.

[d] 6-mercaptopurine for MS RO^+ patients.

[e] Intensification treatment of the second-line therapy included two protocol B or one A and one B, according to patient's financial situation.

Table S2. Primers and probes for detection of *BRAF*^{V600E} mutation by a Droplet Digital PCR assay.

Primers / probes*	Sequences(5'-3')
Forward primer	CATGAAGACCTCACAGTAAAAATAGGTGAT
Reverse primer	TGGGACCCACTCCATCGA
Wild-type probe (HEX)	CTAGCTACAGTGAAATC
Mutant probe (FAM)	TAGCTACAGAGAAATC

*Probes with a 5' fluorophore and a 3' quencher were synthesized by Thermo Fisher Scientific Inc (Shanghai, China). Amplifications were carried out in a reaction volume of 20μl containing 1×ddPCR Supermix for probes (No dUTP, Bio-Rad), 250 nM of wild-type (WT) or mutant probe, and 900 nM of each primer plus template (gDNA:12ng, cfDNA:10ng). The amplifications were carried out at 95ºC for 10 min, followed by 40 cycles of 95ºC for 50 sec and 58°C for 90 sec, and 1 cycle of 70°C for 5min incubation . The temperature ramp increment was 2.5°C/sec for all steps. Results were analyzed with Quantasoft Analysis software (Bio-Rad) according to manufacturer's instructions. Tru-Q7 (1.3% Tier) Reference Standard DNA (Horizon Discovery, Lafayette, USA) was used as positive control and gDNAs from white blood cells of healthy donors were used as negative control. For a given patient sample, the assay reported *BRAF*^{V600E} mutation fragments detected as a percentage of detected wild-type *BRAF*. All samples were tested at least in duplicate. To evaluate the limit of detection of the ddPCR assay, Tru-Q7 (1.3% Tier) Reference Standard DNA was serially diluted into gDNAs from healthy donors to achieve from 8% to 0.01% mutant alleles.

Table S3. Clinical characteristics and prognosis of patients with *BRAF*^{V600E} positive LCH (n=88) compared with patients without *BRAF*^{V600E} mutation (n=29) in the study cohort.

Variables	Total (n=117)	Tissue *BRAF*^{V600E}		*P*
		With (n=88)	Without (n=29)	
Gender, n (%)				
Male	62 (53.0)	43 (48.9)	19 (65.5)	0.137
Female	55 (47.0)	45 (51.1)	10 (34.5)	
Age (years) at diagnosis, n (%)				
< 3 years	70 (59.8)	58 (65.9)	12 (41.4)	0.028
≥ 3 years	47 (40.2)	30 (34.1)	17 (58.6)	
Median (range)	2.4 (0.1-16.0)	1.7 (0.2-11.6)	4.0 (0.1-16.0)	0.078
Clinical classification, n (%)				
SS LCH	55 (47.0)	39 (44.3)	16 (55.2)	
MS RO$^-$ LCH	34 (29.1)	24 (27.3)	10 (34.5)	0.145
MS RO$^+$ LCH	28 (23.9)	25 (28.4)	3 (10.3)	
Involvement, n (%)				
Bone	105 (89.7)	78 (88.6)	27 (93.1)	0.728
Skin	41 (35.0)	35 (39.8)	6 (20.7)	0.074
Liver	24 (20.5)	21 (23.9)	3 (10.3)	0.184
Spleen	13 (11.1)	13 (14.8)	0	0.036
Hematologic	8 (6.8)	8 (9.1)	0	0.197
Pituitary	11 (9.4)	10 (11.4)	1 (3.4)	0.288
Central nervous system	12 (10.3)	11 (12.5)	1 (3.4)	0.289
Lung	23 (19.7)	17 (19.3)	6 (20.7)	1.000
Lymph nodes	13 (11.1)	10 (11.4)	3 (10.3)	1.000
Ear	35 (29.9)	30 (34.1)	5 (17.2)	0.104
Eye	22 (18.8)	17 (19.3)	5 (17.2)	1.000
Oral cavity	21 (17.9)	15 (21.6)	2 (6.9)	0.096
2-year progression-free survival (%)	56.8 ± 5.2	53.4 ± 6.1	66.6 ± 10.5	0.163
2-year overall survival (%)	98.0 ± 1.4	97.3 ± 1.9	100	0.399

LCH: Langerhans cell histiocytosis; SS: single-system; MS: multiple system; RO: risk organ.

Table S4. Comparison of clinical characteristics in 151 children with Langerhans cell histiocytosis included (n=102) or excluded (n=49) in this study.

Variables	Total (n=151)	Included (n=102)	Excluded (n=49)	*P*
Gender, n (%)				
Male	85 (56.3)	57 (55.9)	28 (57.1)	1.000
Female	66 (43.7)	45 (44.1)	21 (42.9)	
Age (years) at diagnosis, n (%)				
< 3 years	89 (58.9)	64 (62.7)	25 (51.0)	0.216
≥ 3 years	62 (41.1)	38 (37.3)	24 (49.0)	
Median (range)	2.4 (0.1-16.0)	1.8 (0.2-13.4)	2.9 (0.1-16.0)	0.079
Clinical classification, n (%)				
SS LCH	73 (48.3)	46 (45.1)	27 (55.1)	0.544
MS RO^- LCH	41 (27.2)	29 (28.4)	12 (24.5)	
MS RO^+ LCH	37 (24.5)	27 (26.5)	10 (20.4)	
Involvement, n (%)				
Bone	133 (88.1)	90 (88.2)	43 (87.8)	1.000
Skin	48 (31.8)	37 (36.3)	11 (22.4)	0.096
Liver	32 (21.2)	23 (22.5)	9 (18.4)	0.672
Spleen	19 (12.6)	13 (12.7)	6 (12.2)	1.000
Hematologic	8 (5.3)	8 (7.8)	0	0.054
Pituitary	13 (8.6)	10 (9.8)	3 (6.1)	0.549
Central nervous system	14 (9.3)	11 (10.8)	3 (6.1)	0.550
Lung	31 (20.5)	21 (20.6)	10 (20.4)	1.000
Lymph nodes	18 (11.9)	13 (12.7)	5 (10.2)	0.791
Ear	43 (28.5)	29 (28.4)	14 (28.6)	1.000
Eye	31 (20.5)	20 (19.6)	11 (22.4)	0.673
Oral cavity	29 (19.2)	20 (19.6)	9 (18.4)	1.000
2-year progression-free survival (%)	58.2 ± 4.5	54.1 ± 5.8	66.5 ± 6.9	0.410
2-year overall survival (%)	98.3 ± 1.2	97.6 ± 1.7	100	0.331

LCH: Langerhans cell histiocytosis; SS: single-system; MS: multiple system; RO: risk organ.

Table S5. Univariate and multivariate analysis of prognostic factors for progression-free survival in children with Langerhans cell histiocytosis.

	Univariate*		Multivariate#		
	Progression-free survival (%)	*P*	HR	95% CI	*P*
Sex:					
Male *vs* female	46.1±8.9 *vs* 59.7±8.2	0.510	1.659	0.574 - 4.794	0.350
Age at diagnosis:					
< 3 years *vs* ≥ 3 years	48.0±7.0 *vs* 64.4±11.5	0.064	1.216	0.424 - 3.486	0.716
Involvement *vs* noninvolvement					
Bone	48.1±6.8 *vs* 80.0±12.6	0.152	1.916	0.388 - 9.473	0.425
Skin	47.0±8.7 *vs* 56.9±8.5	0.180	0.557	0.225 - 1.380	0.206
Risk organs	14.0±8.3 *vs* 68.5±7.2	< 0.001	4.675	1.952 - 11.198	0.001
Pituitary	66.7±15.7 *vs* 50.8±6.6	0.635	0.408	0.110 - 1.515	0.181
Lung	35.3±13.6 *vs* 56.1±7.0	0.046	0.996	0.399 - 2.485	0.993
Lymph nodes	30.0± 6.9 *vs* 55.1±6.6	0.254	1.412	0.505 - 3.950	0.511
Ear	27.5±9.0 *vs* 64.7±7.7	< 0.001	3.500	1.175 - 10.424	0.024
Eye	56.8±14.3 *vs* 51.1±7.0	0.606	0.670	0.199 - 2.251	0.517
Oral	39.0±14.8 *vs* 56.0±6.7	0.720	0.994	0.318 - 3.105	0.992
CNS risk lesions	45.2±8.2 *vs* 63.1±9.3	0.136	1.018	0.295 - 3.519	0.977
Cell-free *BRAF*^{V600E} at diagnosis					
Positive *vs* negative	34.7 ± 7.3 *vs* 92.3 ± 5.2	< 0.001	5.263	1.134 - 24.425	0.034

HR, Hazard ratio; CNS, central nervous system.

* Univariate analysis for progression-free survival was performed by Kaplan-Meier log-rank test.

#All factors in the univariate analysis were selected in Cox regression of the multivariate analysis for progression-free survival.

Table S6. Combined assessment of cell-free *BRAF*^{V600E} during follow-up and outcomes of pediatric Langerhans cell histiocytosis.

cell-free *BRAF*^{V600E}	Total n	Events n
Frist-line therapy (total)	39	7
$Dx^{+}W6^{+}W12^{+}W52^{+}$	3	1
$Dx^{+}W6^{+}W12^{+}W52^{-}$	3	0
$Dx^{+}W6^{-}W12^{-}W52^{+}$ *	3	3
$Dx^{+}W6^{-}W12^{-}W52^{-}$	13	3
$Dx^{-}W6^{-}W12^{-}W52^{-}$	17	0
Second-line therapy (total)	19	16
$Dx^{+}W6^{+}C8^{+}$ #	11	11
$Dx^{+}W6^{+}C8^{-}$	5	3
$Dx^{+}W6^{-}C8^{-}$	2	1
$Dx^{-}W6^{-}C8^{-}$	1	1

$^{+}$ indicating cell-free *BRAF*^{V600E}positive; $^{-}$ indicating cell-free *BRAF*^{V600E} negative; Dx: at diagnosis; W6: week 6; W52: week 52; C8: course 8 of the second-line therapy.

* The prognosis of $Dx^{+}W6^{-}W12^{-}W52^{+}$ (relapse/total: 3/3) *vs.* that of other patients (4/19) with detectable cell-free *BRAF*^{V600E} detection at diagnosis, $P = 0.023$.

The prognosis of $Dx^{+}W6^{+}C8^{+}$ (relapse/total: 11/11) *vs.* that of other patients (4/7) with detectable cell-free *BRAF*^{V600E} detection at diagnosis, $P = 0.043$.

Table S7. Comparison of characteristics of patients in the Chinese cohort and those in the French cohort[#].

Characteristics	At diagnosis, n (%)		cell free *BRAF*^{V600E}					
			The Chinese cohort			The French cohort		
	The Chinese cohort	The French cohort	Negative (%)	Positive (%)	*P*	Negative (%)	Positive (%)	*P*
Total	81	48	32.1	67.9		56.8	43.2	
Sex								
Male	42 (52)	25 (52)	53.8	45.5	0.634	44.0	52.6	0.760
Female	39 (48)	23 (48)	46.2	54.5		56.0	47.4	
Age (years) at diagnosis, median (range)	1.5(0.2-11.6)	2.1(0.1-15.7)	3.3	1.3	0.038	2.8	1.0	0.009
Clinical classification								
SS LCH	33 (41)	21 (44)	76.9	23.6		60.0	15.8	
MS RO$^-$ LCH	23 (28)	12 (25)	15.4	34.5	<0.001	36.0	10.5	< 0.001
MS RO$^+$ LCH	25 (31)	15 (31)	7.7	41.8		4.0	73.7	
Involvement								
Bone	71 (88)	38 (79)	88.5	87.3	1.000	84.0	73.7	0.470
Skin	35 (43)	27 (56)	19.2	54.5	0.004	40.0	84.2	0.005
Liver	21 (26)	12 (25)	7.7	34.5	0.013	4.0	57.9	< 0.001
Spleen	13 (16)	12 (25)	3.8	21.8	0.052	4.0	57.9	< 0.001
Hematological	8 (10)	14 (29)	0	14.5	0.050	0	73.7	< 0.001
Pituitary	9 (11)	5 (10)	3.8	14.5	0.259	12.0	10.5	1.000
Central nervous system	3 (4)	2 (4)	0	5.5	0.547	4.0	5.3	1.000
Lung	17 (21)	2 (4)	11.5	25.5	0.242	0	5.3	0.430
Lymph nodes	10 (12)	6 (13)	11.5	12.7	1.000	4.0	26.3	0.070
Follow-up, years; median (range)	1.2 (0.1-2.34)	2.2 (0.4-11.7)						
2-year reactivation (%)						32.3	48.1	0.070
2-year progression-free survival (%)			92.3 ± 5.2	34.7 ± 7.3	< 0.001			

[#] Héritier S, Hélias-Rodzewicz Z, Lapillonne H, et al. Circulating cell-free BRAFV600E as a biomarker in children with Langerhans cell histiocytosis. Br J Haematol. 2017;178(3):457-67.

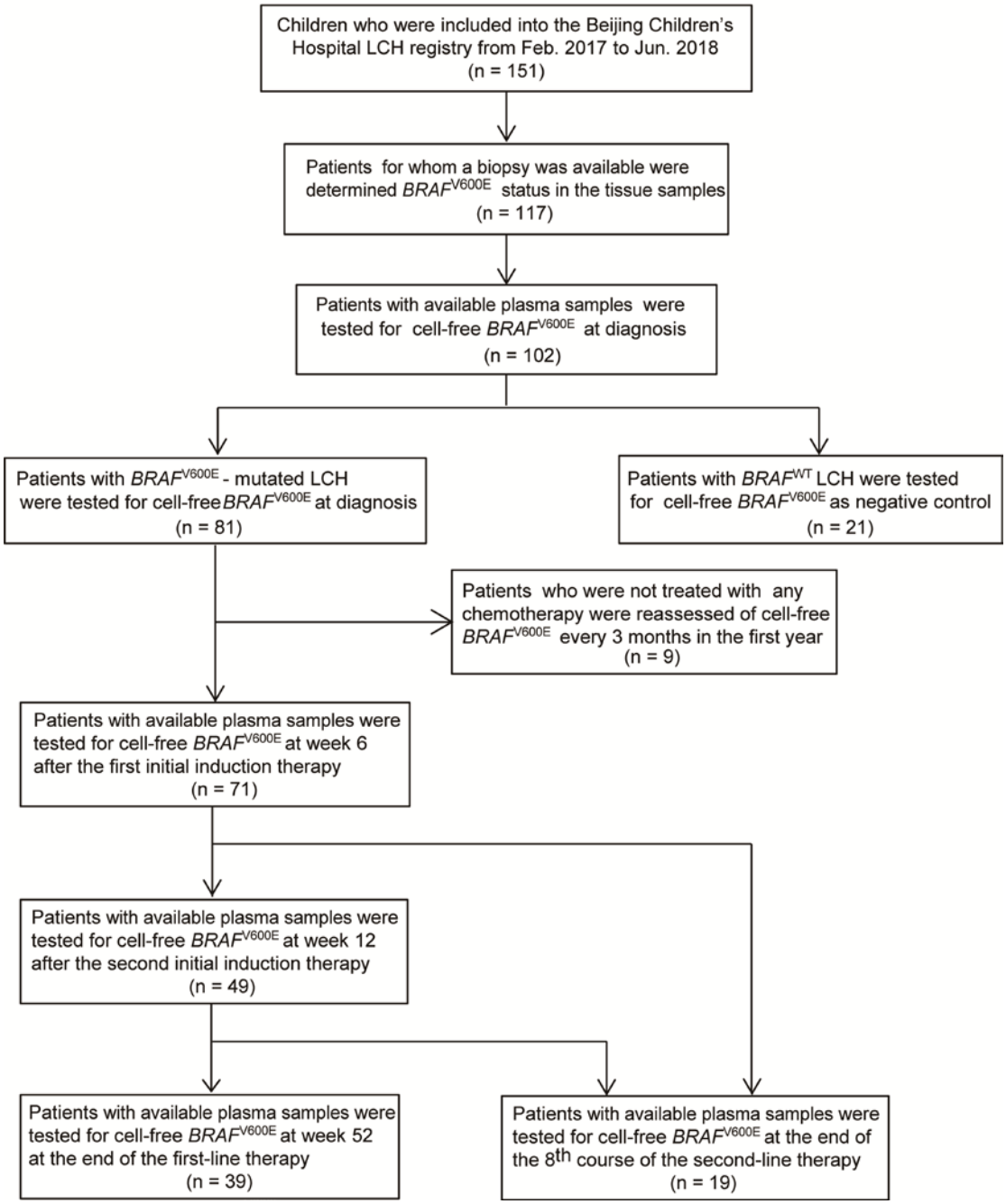

Figure S1. Study cohorts. Abbreviation: LCH, Langerhans cell histiocytosis; WT, wild type.

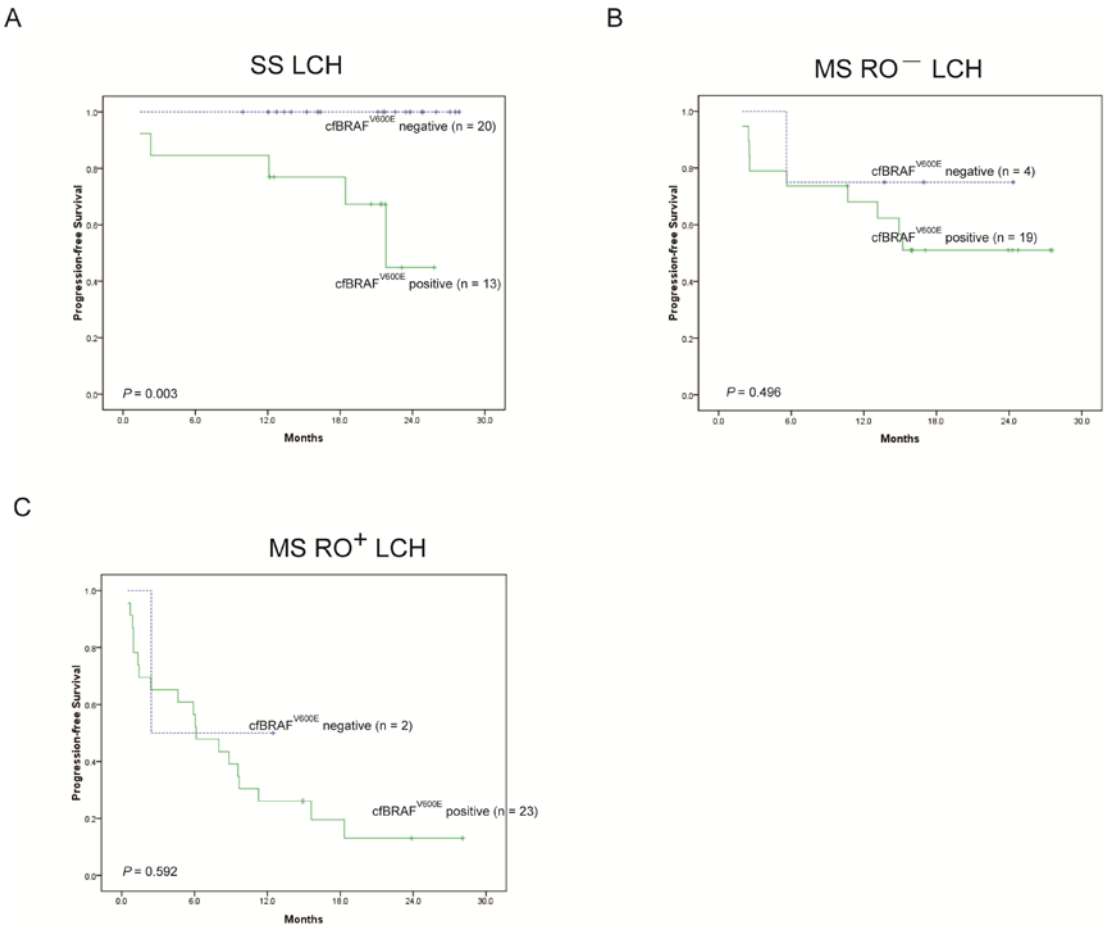

Figure S2. The prognostic significance of cf*BRAF*^{V600E} at diagnosis in patients with different clinical classifications of LCH. (A) Single-system (SS) LCH; (B) Multiple system (MS) risk organs negative (RO^-) LCH; (C) MS RO^+ LCH.

Statistical analysis

Differences between groups were tested with the Kruskal-Wallis or Mann-Whitney *U* test for quantitative variables and with Fisher's exact test for qualitative variables. progression-free survival (PFS) was estimated from the date of diagnosis until the date of one of the following events: progression, relapse, or death, whichever came first. The patients without event were censored at the date of last contact. Overall survival (OS) was defined as the time from the diagnostic date through the date of death due to any reasons, or the last follow-up. Survival rates were analyzed by the Kaplan-Meier method, and subgroups were compared with the log-rank test. Cox proportional hazards model was used for multivariate analyses. All tests were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).