Supplementary complete methods

Patient specimens

Formalin–fixed, paraffin-embedded (FFPE) specimens from 128 patients younger than 18 years of age were analyzed. The cases were selected from the archives of the Department of Pathology, Hematopathology Section and Lymph Node Registry, University of Kiel, on the basis of whether sufficient biopsy material was available to construct a tissue microarray (TMA). 8 patients were excluded from the final analysis due to lack of ALK-expression (n=2) or because the patients were not treated according to the NHL-Berlin-Frankfurt-Münster (BFM) protocol (n=6). Since for one patient a morphological subtype was not assessable, the final cohort was composed of 119 patients, all treated in the subsequent trials NHL-BFM 90, NHL-BFM V95, NHL-BFM 95 and ALCL99. These trials have a comparable BFM-type chemotherapeutic backbone. For all cases informed consent was obtained from the patients and/or parents at registration in the respective study of BFM study group. A subset of cases (n=36) were part of a previous publication (1). The cohort was composed of 85 (71%) male and 34 (29%) female patients. All clinical data of the cohort are shown in table 1. ALK-antibody titer and minimal disseminated disease were measured as described previously (2;3).

Histology and tissue microarrays (TMA)

The TMA was constructed by using 2 cores, 1 mm in diameter, from each case containing tumor rich areas, which were selected with light microscopy on whole hematoxin and eosin stained slides. 102 cases (86%) showed nuclear and cytoplasmic ALK staining suggestive of an underlying NPM-ALK fusion and 16 cases (13%) showed staining restricted to the cytoplasm (4). The histological subtype was assessed according to previous publications including the guidelines of the WHO classification and the European Pathology Panel for Pediatric Lymphomas(5). We distinguished the pure common type of ALCL (n=64, 54%) from all other non-common morphological variants: lymphohistiocytic, small cell variants, and mixed variants (n=55, 46%).

Fluorescence double staining and immunohistochemistry

Tumor cells were detected using an ALK1 mouse monoclonal antibody (mAb) (Dako, 1:10 dilution) or ALK/p80 rabbit mAb (Epitomics, 1:50 dilution). To determine the tumor cell immunophenotype, the ALK1 stainings were combined with a second marker using the following antibodies: CD30 mouse mAb (self-produced clone BerH2,1:5 dilution), CD3 mouse mAb (1:100 dilution), CD5 mouse mAb (1:25, Novocastra, Newcastle, United Kingdom), CD8 rabbit mAb (1:25, NeoMarkers, Freemont, CA, USA), anti-pSTAT3 rabbit mAb (1:25, Epitomics, 1:100, Burlingame, CA, USA) and Ki67 mouse mAb (self-produced clone Ki-S5 (6)). The secondary fluorescence labeled antibodies were: Alexa Fluor^{R} 555 and 488 donkey anti-mouse IgG (1:100) and Alexa Fluor^{R} donkey anti-rabbit IgG 555 and 488 (1:100) (Molecular Probes, Eugene, OR, USA). In our hands, the immunofluorescence staining for CD56 was much less sensitive than the conventional immunohistochemistry. Therefore, CD56 was stained with a mouse mAb (1:20 dilution, Novocastra, Newcastle, United Kindom) using an automated staining system (Bond, Leica, Germany).

Digital image analysis of tumor cell immunophenotypes

Photos of the stainings were made with an Axioplan 2 microscope (Zeiss, Jena, Germany) and a digital camera SPOT RT^{TM} slider (Diagnostic Instruments Inc., Burroughs, Sterling Heights, MI, USA) and VisisView 1.7.2 software (Visitron Systems, Puchheim, Germany). Individual photos from a representative area were obtained for ALK to identify tumor cells, DAPI to identify cell nuclei and the second immuno-marker, respectively (see above). The pictures were subsequently transformed into black and white and further analyzed with TissueQuest 2.2 software (TissueGnostics, Vienna, Austria). The number of cells analyzed ranged from a minimum of 18 to 2577. The mean number of ALK-positive lymphoma cells analyzed was 728 for ALK-CD30, 637 for ALK-CD3, 556 for ALK-CD5, 642 for ALK-CD8, 604 for ALK-Ki67 and 554 for ALK-pSTAT3 staining combinations. The conventional staining for CD56 was scored as positive and negative by one observer (DA) using a light microscope.

Statistical analysis

Event-free survival (EFS) was calculated from date of diagnosis to last follow-up or first event (relapse, secondary malignancy or death of any cause). Overall survival (OS) was calculated from date of diagnosis to death of any cause or last follow-up. Probabilities of survival were estimated by the method of Kaplan and Meier, with standard errors (SE) according to Greenwood, and were compared using the log-rank test (7). Differences in the distribution of individual parameters among patient subsets were analyzed using the t-test for continuous variables (Graph Pad Prism, GraphPad Software, Inc., La Jolla, CA, USA) or Fisher's exact test for contingency tables.

Reference List

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6. Klapper W, Hoster E, Determann O, Oschlies I, van der Laak J, Berger F, Bernd HW, Cabecadas J, Campo E, Cogliatti S, et al. Ki-67 as a prognostic marker in mantle cell lymphoma-consensus guidelines of the pathology panel of the European MCL Network. J.Hematop. 2009 Jun 16.

7. Kalbfleisch J, Prentice RL. The statistical analysis of failure time data. New York: John Wiley; 2005.

Supplementary tables and figures

	All	Common type	Non-common type	
CD30	81/109 (74.3%)	49/61 (80.3%)	32/48 (66.7%)	p=0.125
CD3	15/95 (15.8%)	6/49 (12.4%)	9/46 (19.6%)	p=0.404
CD5	59/97 (60.8%)	38/51 (74.5%)	21/46 (45.7%)	p=0.006
CD8	20/102 (19.6%)	3/54 (5.6%)	17/31 (35.4%)	p=0.0002
CD56	8/114 (7%)	4/63 (6.3%)	4/51 (7.8%)	p=1.000

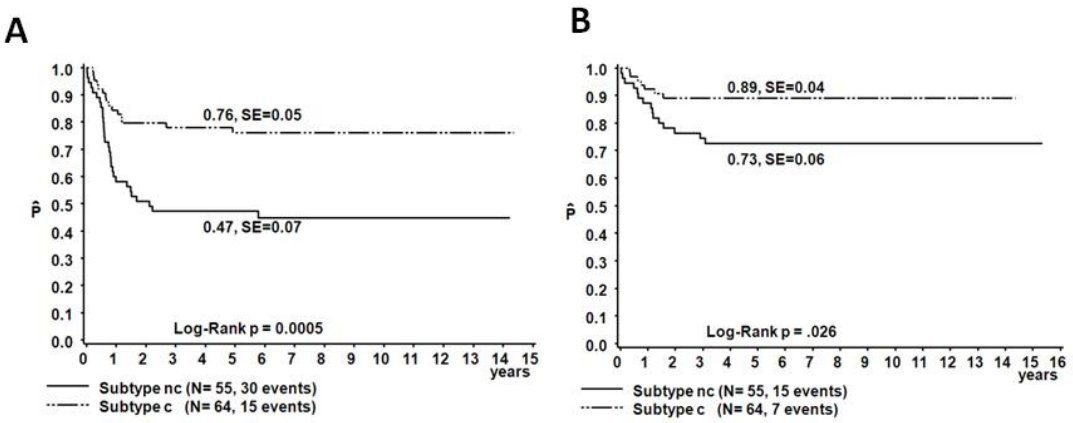
Supplementary table 1: Immunophenotype of ALK-positive lymphoma cells (number of positive cases/cases with evaluable result and percentage of all ALCL analyzed). Any expression of CD3, CD5, CD8, CD56 was scored as positive; for CD30, >80% positive cells was scored as positive.The p-values were calculated according to Fisher's exact test.

	Flow cytometry		conventional immunohistochemistry[3]		this study	
	all[1]	all[2]	common	non-common	common	non-common
CD3	32%	45%	24%	56%	12%	20%
CD5	26%	14%	49%		75%	46%
CD8	21%	14%	24%		6%	35%

Supplementary table 2: **Expression of markers in ALK-positive ALCL in two studies using flow cytometry, one study aplying conventional immunohistochemistry and the current study.** The expression is indicated as percent of positive cases as far as reported. [1] Jucco et al. (2003), [2]Muzzafar et al. (2009), [3]Lamant et al. (2011)

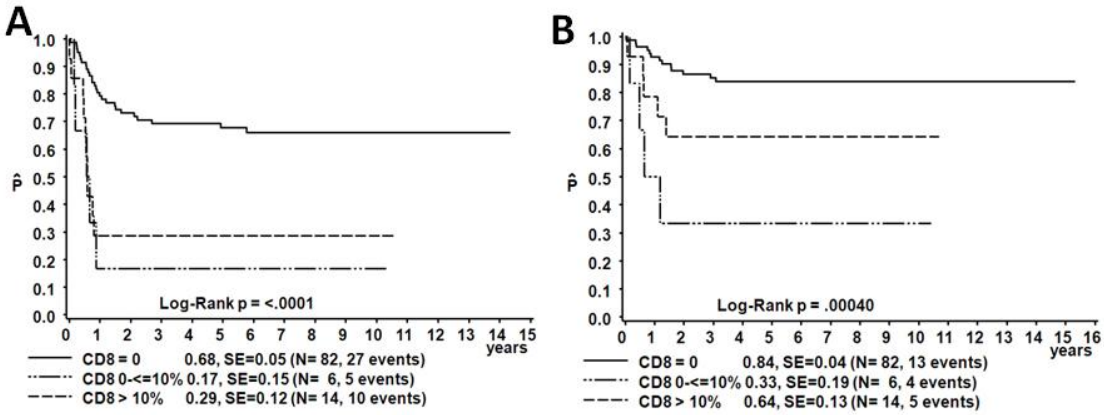
Patient Number	subtype summary	subtype details	ALK staining pattern	CD30 (%)	CD3 (%)	CD5 (%)	CD8 (%)	Ki67 (%)	pSTAT3 (%)	CD56
1	non-common type	lymphohistiocytic/small cell	nuclear and cytoplasmic	93,8	0,0	0,0	3,3	na	100,0	na
2	non-common type	lymphohistiocytic/small cell	nuclear and cytoplasmic	18,6	16,5	0,0	5,4	na	na	negative
3	non-common type	not further specified	nuclear and cytoplasmic	81,2	0,0	46,9	7,7	na	4,7	na
4	non-common type	common/small cell	nuclear and cytoplasmic	83,8	na	22,5	7,9	21,6	na	negative
5	common type	common	nuclear and cytoplasmic	60,4	na	na	8,3	36,3	na	negative
6	non-common type	not further specified	nuclear and cytoplasmic	39,2	16,0	0,0	8,5	na	na	na
7	non-common type	lymphohistiocytic/small cell	nuclear and cytoplasmic	na	27,6	0,0	11,2	15,5	na	negative
8	common type	common	nuclear and cytoplasmic	61,6	0,0	9,9	11,6	70,6	71,1	negative
9	non-common type	not further specified	nuclear and cytoplasmic	95,9	69,5	61,6	19,8	33,1	90,0	negative
10	non-common type	lymphohistiocytic	nuclear and cytoplasmic	89,0	0,0	0,0	21,2	56,2	70,8	negative
11	non-common type	lymphohistiocytic	nuclear and cytoplasmic	74,0	0,0	36,0	21,4	57,8	80,8	negative
12	non-common type	lymphohistiocytic/small cell	nuclear and cytoplasmic	66,1	0,0	46,2	25,7	22,9	47,2	negative
13	non-common type	common/small cell	nuclear and cytoplasmic	86,4	na	na	30,5	80,0	50,3	negative
14	non-common type	not further specified	nuclear and cytoplasmic	90,3	0,0	na	49,4	59,5	21,9	negative
15	common type	common	nuclear and cytoplasmic	88,7	0,0	39,6	52,3	80,4	53,1	negative
16	non-common type	not further specified	nuclear and cytoplasmic	66,5	38,7	41,5	58,0	85,5	88,8	negative
17	non-common type	lymphohistiocytic/small cell	nuclear and cytoplasmic	46,1	0,0	0,0	62,0	30,0	21,6	negative
18	non-common type	lymphohistiocytic	nuclear and cytoplasmic	79,3	81,1	0,0	67,3	36,1	64,0	negative
19	non-common type	lymphohistiocytic	nuclear and cytoplasmic	75,4	0,0	0,0	67,8	49,4	na	negative
20	non-common type	common/lymphohistiocytic/small cell	nuclear and cytoplasmic	86,6	87,1	0,0	86,1	54,6	78,5	positive

Supplementary table 3: Morphological and immunophenotypical details of the CD8 positive ALCL. na= not available.



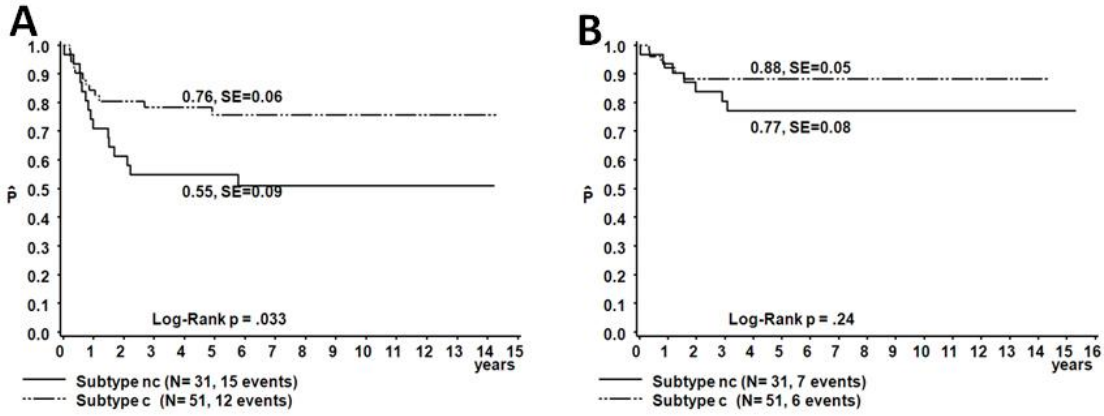
Supplementary Figure 1

Event free survival (EFS, A) and overall survival (OS, B) according to the histological type. c= common type, nc= non-common type.



Supplementary Figure 2:

Event free survival (A) and overall survival (B) according to a 10% cut-off for CD8 expression.



Supplementary Figure 3:

Event free survival (A) and overall survival (B) according to the morphological subtype for CD8-negative ALCL only. nc= non common type, c= common type.