ORIGINAL ARTICLE

LYVE-1 upregulation and lymphatic invasion correlate with adverse prognostic factors and lymph node metastasis in neuroblastoma

Pramila Ramani · Josiah V Dungwa · Margaret T. May

Abstract Neuroblastoma (NB) accounts for 15% of all childhood cancer deaths. The majority of patients have widespread lymphatic and/or haematogenous metastases at diagnosis, but lymphangiogenesis has not been well documented. Sixty-seven NBs were immunostained for the lymphatic endothelial marker, LYVE-1, and the lymphatic density (LD) and lymphatic invasion (LI), were counted in LYVE-1-expressing lymphatics. LYVE-1-stained lymphatic vessels and LI were present in 26/67 (39%) and 14/67 (21%) of the NBs, respectively. Central LD (CLD) and LI were higher in NBs from stage 4 ($p=0.012$, $p=0.004$, respectively), high-risk group ($p=0.030$, $p=0.002$), NBs with high mitosis karyorrhexis index (MKI) ($p=0.011$, $p=0.005$), unfavourable histology group ($p=0.040$, $p=0.017$) and distant lymph node metastasis (LNM) ($p<0.001$ for each). Marginal LD (MLD) was higher in patients with LNM ($p<0.001$). CLD and MLD correlated with LI ($p<0.001$ each). Total LYVE-1 protein levels, quantified by a sensitive enzyme-linked immunosorbent assay ($n=55$), were also higher in NBs from patients with stage 4 disease ($p=0.046$), high-risk group ($p=0.028$), MYCN-amplified NBs ($p=0.034$) and LNM ($p=0.038$). Kaplan–Meier analysis showed that the presence of CLD was associated with both worse OS at 5 years (77% [95% CI: 62–87%] versus 60% [95% CI: 32–80%], $p=0.062$) and EFS (74% [95% CI: 58–85%] versus 43% [95% CI: 15–69%], $p=0.070$) and LI with OS (71% [95% CI: 57–81%] versus 56% [95% CI: 26–78%], $p=0.055$). Significant upregulation of LYVE-1 and the presence of LI in patients with stage 4 and high-risk disease, MYCN-amplification and LNM suggests that LYVE-1 may have value as predictors of outcome.

Abbreviations

NB	Neuroblastoma
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor-1
LD	Lymphatic density
CLD	Central lymphatic density
MLD	Marginal lymphatic density
LNM	Lymph node metastasis
LI	Lymphatic invasion

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Introduction

Neuroblastoma (NB) is the most common extra-cranial solid paediatric cancer and is responsible for approximately 15% of all childhood cancer deaths, as reviewed by Maris [1].

NBs arise from tissue of the sympathetic nervous system and frequently originate in the adrenal medulla or the sympathetic abdominal ganglia. However, NBs can also arise in the neck, chest and pelvis. The International Neuroblastoma Staging System defines five stages of NBs: stages 1 through 4 and 4-special (4 s) [1]. Stage 4 s tumours occur in children <18 months old and are widely disseminated but have a good prognosis and are capable of spontaneous regression. Adverse prognostic factors include an age ≥18 months at diagnosis, an abdominal location of the primary tumour [2] and specific genetic abnormalities, such as MYCN oncogene amplification, the loss of chromosome 1p or the gain of chromosome 17q [1]. Pathology is also an important prognostic indicator [2, 3]. Differentiated ganglioneuromas (GNs) lie at the benign end of the spectrum, and undifferentiated or poorly differentiated NBs with a high mitosis karyorrhexis index (MKI) lie at the most malignant end of the spectrum [3]. The majority of patients with NBs have widespread lymphatic and/or haematogenous metastases at diagnosis. Whereas angiogenesis has been studied extensively (reviewed by Rossler) [4], few reports have examined lymphangiogenesis in NBs [5–11].

Increased lymphatic density (LD) and lymphatic invasion (LI) are correlated with the incidence of lymph node metastasis (LNM) and poor prognosis in some human cancers (comprehensively reviewed by Sleeman) [12]. Specific staining for lymphatic vessels has been facilitated by the discovery of several proteins that recognise lymphatic endothelial cells (LECs), such as podoplanin and the transcription factor prospero-related homeobox gene-1 (PROX1) [13, 14]. The hyaluronan receptor LYVE-1 has been widely used for the detection of tumour-associated lymphatic vessels in different types of tumours [12, 15, 16]. However, the only published investigation of LYVE-1 in NB reports on only a small number of samples [6]. Moreover, the presence or absence of LI was not commented on, and no correlative data on LI, clinicopathological or biological variables are available. We performed a comprehensive expression analysis of LYVE-1 in a series of malignant neuroblastic tumours to determine the following: (1) the sensitivity of LYVE-1 in detecting LD and LI by immunohistochemistry, (2) the potential role of LD and LI based on their associations with clinicopathological and biological features and (3) the value of LD and LI as predictors of outcome.

Patients and methods

Patients

The study cohort consisted of 85 consecutive patients with NBs (including ganglioneuroblastomas, GNBs)

treated at Bristol Children's Hospital between January 1994 and June 2009. The study was approved by the South Bristol and North Somerset Research Ethics Committee (09/H0106/06). Written informed consent was obtained from parents, legal guardians or subjects for clinical information and use of tumour samples surplus to diagnostic requirements. Staging was based on the International Neuroblastoma Staging System [17]. Subjects were treated according to the appropriate clinical protocols based on clinicopathological and biological characteristics. Clinical information was extracted from the medical records, and all patient data were de-identified. The tumours were classified into prognostic groups according to the International Neuroblastoma Pathology Classification (INPC) system [3]: 79 NBs and 6 GNBs (4 intermixed and 2 nodular subtypes).

Tissue samples

Samples were obtained from the Paediatric Pathology files of the Bristol Royal Infirmary. All of the samples were obtained at primary resection (stages 1 and 2) or before chemotherapy was administered (stages 3 and 4). For immunohistochemistry, paraffin-embedded tissues were available for 67 NBs (including all GNBs). Paraffin-embedded sections were not available from 18 NBs because of insufficient viable tumour material remaining in the tissue blocks. For LYVE-1 protein assays, snap-frozen tissue samples, comprising at least 70% viable tumoural tissue, were available for 55 NBs (including the 18 NBs that did not have paraffin-embedded tissue sections).

Enzyme-linked immunosorbent assay (ELISA)

Snap-frozen tissue samples were homogenised in an ice-cold lysis buffer composed of 1% Triton X-100 buffer containing 1:100 dilutions of protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Dorset, UK). After homogenisation, the samples were centrifuged at $12,000 \times g$ for 15 min at 4°C. Total protein was quantified using the Bradford assay following the manufacturer's instructions (Sigma-Aldrich, Dorset, UK).

The concentration of LYVE-1 total protein was measured using the human LYVE-1 Duoset ELISA kit (DY2089, R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Standard curves were constructed using two-fold serial dilutions of recombinant LYVE-1 protein (range 0–2,000 pg/ml). Optical densities were determined using a spectrophotometer at $\lambda = 450$ nm with a corrective reading at $\lambda = 570$ nm. Each test was performed in triplicate, and the mean values were used. The intraassay and interassay variation were both <10%.

Immunohistochemistry

Four-micrometer-thick consecutive sections from paraffin-embedded blocks were stained using a BOND-III automated immunostainer (Leica Biosystems, Milton Keynes, UK). Rabbit anti-human LYVE-1 polyclonal antibody (5 μg/ml, Abcam, Cambridge, UK) was applied to the sections for 1.5 h and detected using the Bond^{TM} Polymer Refine Detection system (DS9800, Leica Novocastra, Milton Keynes, UK). Omission of the primary antibody was used as a negative control.

Assessment of lymphatic vessel density (LD)

LYVE-1-stained sections were scanned at low magnification (×10 eyepiece, ×10 objective) to identify the 'hot spots' showing the maximum number of lymphatics. Three 'hot spots' within each tumour section were counted at ×200 magnification (×10 eyepiece, ×20 objective) with a surface area of 1.23 mm^2. The sum of lymphatic vessels was divided by the sum of the surface area of all counted fields to obtain the mean lymphatic density (LD)/mm^2. Vessels with a clearly defined lumen or well-defined linear vessel shape, but not single endothelial cells, were taken into account for LD determination. Intratumoural lymphatic hotspots from the central (CLD) and marginal (MLD) (<2 mm from the margin of tumour) regions of the tumour were counted as previously described [18] by two independent observers blinded to the clinical data. LYVE-1-stained lymphatic vessels were assessed for the presence or absence of lymphatic invasion (LI).

Statistical analysis

Patients were divided into high-risk and non-high-risk groups. Patients with non-amplified *MYCN* stage 1, 2 and 3 tumours and children <18 months at presentation with stage 4 and 4 s NBs that lacked *MYCN* amplification were classified as non-high-risk. Patients with stage 3 *MYCN*-amplified tumours and children ≥18 months of age with stage 4 disease were classified as high risk. Only two tumour-related deaths occurred among stages 1, 2 and 3, so we combined these stages.

Associations between the LYVE-1-LDs and LYVE-1 total protein levels and the clinicopathological and biological variables were compared using the Mann–Whitney or Cuzick test for trend (MKI and stage) tests. χ^2 tests were used to test whether the presence or absence of LI was independent of other variables. Spearman's rank correlation was used to test for correlations between CLD, MLD and LYVE protein.

Overall survival (OS) was determined from the time of diagnosis to the last follow-up or tumour-specific-related death. Event-free survival (EFS) was calculated from the time of diagnosis until the time of first occurrence of relapse, progression, secondary malignancy or death or until the time of last contact if none of these occurred. Patients with poor or no response to therapy were excluded from the EFS analysis (n=7, all stage 4). A patient with co-incidental stage 2 NB and stage 4 alveolar rhabdomyosarcoma died.

For comparison of groups in survival analyses, LYVE-1-LDs were categorised as absent versus present because the medians were zero. LYVE-1 total protein level was dichotomised at 2 pg/μg protein, which was close to the median value. OS and EFS were estimated using the Kaplan–Meier (KM) method, and the differences in survival of the LYVE-1 subgroups were compared using the log-rank test. We used

Table 1 Clinicopathological and biological characteristics of the NB cohort (n=85)

Variable	Frequency (%)
Stage	
1	6 (7)
2	15 (18)
3	16 (19)
4	45 (53)
4 s	3 (4)
Age (months)	
<18	36 (42)
≥18	49 (58)
Risk group	
Non-high-risk	42 (49)
High-risk	43 (51)
Primary tumour site	
Extra-abdominal	17 (20)
Abdominal	68 (80)
MKI	
High	38 (45)
Intermediate	18 (21)
Low	29 (34)
INPC	
Favourable	35 (41)
Unfavourable	50 (59)
MYCN status	
Non-amplified	59 (69)
Amplified	21 (25)
Unknown	5 (6)
Deletion 1p	
Present	22 (26)
Absent	46 (54)
Unknown	17 (20)
17q gain	
Present	35 (41)
Absent	23 (27)
Unknown	27 (32)

NB neuroblastoma including ganglioneuroblastomas, *INPC* International Neuroblastoma Pathology Classification, *MKI* mitosis-karyorrhexis index

Schoenfeld residuals to test the proportional hazards assumption for all Cox models fitted to the data and found no evidence that this assumption was not valid. Univariable Cox regression models were used to estimate the unadjusted hazard ratio for tumour-related death and relapse for each of the prognostic factors.

The statistical analyses were performed using the Stata 11.0 software package (Stata for Windows, StataCorp, Texas, US).

Results

Clinicopathological characteristics of the cohort

The clinicopathological characteristics of the 85 NB patients (including GNBs) are summarised in Table 1. The median age at presentation was 20.14 months (range 0.03–188.3 months), and the median follow-up time of all the survivors was 75 months (range 14.0–195.0 months).

LYVE-1 expression in neuroblastic tumours

Lymphatic density

LYVE-1 expression was present mainly in the LECs but was also seen in scattered macrophages in the tumoural and stromal compartments. Lymphatics in the central part of the tumour were generally collapsed (Fig. 1a), and the marginal lymphatics were larger with wider lumens (Fig. 1b). However, lymphatics in the central part of the tumour were easily visualised when they contained tumour emboli (Fig. 1c).

The lymphatic count was 0 for 41/67 (61%) of patients. However, a significantly higher proportion of NBs exhibited MLD compared to NBs with CLD (39% vs. 26%, respectively, $p<0.001$) (Table 2). The CLD could not be assessed in two NBs because the biopsies contained peripheral tumoural tissue only and the biopsies were 2 mm in depth.

LYVE-1 total protein expression

As a result of the relatively low LD counts, the number of NBs stained by immunohistochemistry and the commercial availability of a sensitive ELISA, LYVE-1 protein were quantified in tumour lysates. LYVE-1 protein was detected in all 55 NBs analysed (median 1.62 pg/μg, range 0.0005–61.59 pg/μg total protein).

Lymphatic invasion (LI)

Immunostaining with LYVE-1 highlighted the presence of lymphatic invasion (LI) by tumour emboli in 14/67 (21%) NBs and nodular GNBs (Table 2), but none of the intermixed GNBs were positive for LI. The lymphatics in the marginal as well as central regions showed tumour emboli (Fig. 1b, c).

Fig. 1 Immunohistochemical expression of LYVE-1 in NBs. a Collapsed lymphatics (black arrow) in the central regions of the NB. b Large, dilated lymphatics in the marginal areas of NB containing tumour emboli. c LYVE-1-expressing lymphatics in the central region of NB show luminal occlusion by NB tumour emboli
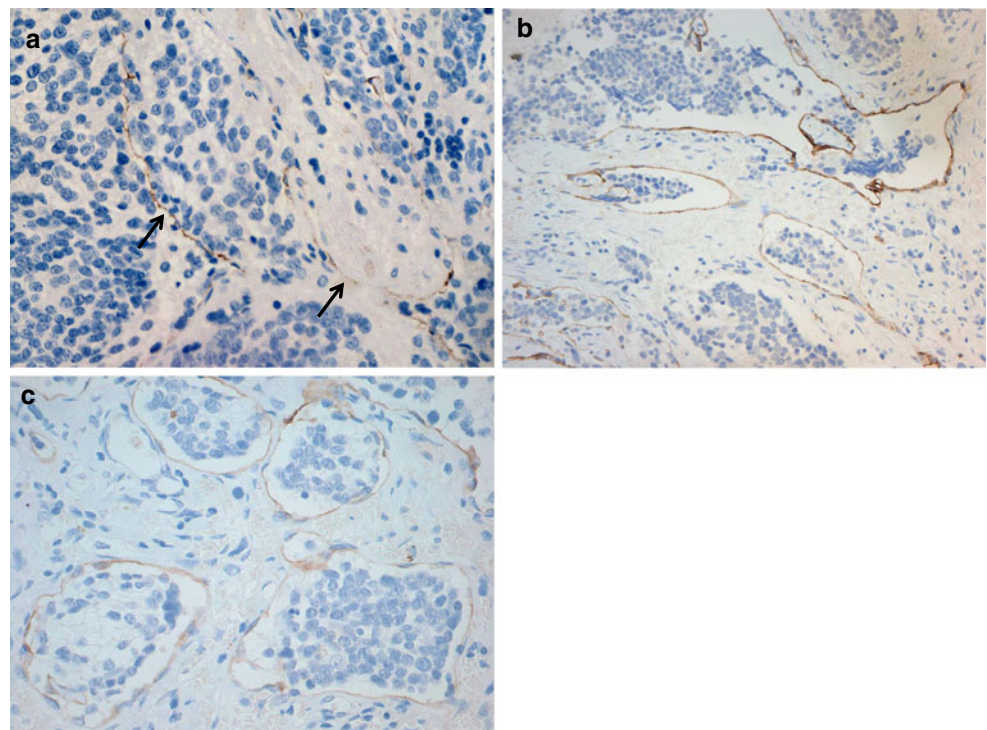
Table 2 Relationship between LD and LI with clinicopathological and biological variables

	CLD			MLD			LI present	
All NBs								
Frequency	26% (17/65)			39% (26/67)			(14/67) 21%	
Mean (SE)	0.518 (0.195)			0.987 (0.276)				
Score range	0–9.00			0–11.6				
Variables	Number	Mean/mm^2 (SE)	p-value	Number	Mean/mm^2 (SE)	p-value	Percent	p-value[a]
Age (months)								
<18	28	0.098(0.043)		31	0.392(0.110)		6/31 (19)	
≥18	37	0.903(0.378)	0.320	36	1.428(0.475)	0.790	8/36 (22)	>0.999
Stage								
1, 2, 4 s	23	0.022(0.015)		24	0.469(0.187)		1/24 (4)	
3	12	0.208(0.126)		12	0.583(0.272)		1/12 (2)	
4	30	1.094(0.454)	**0.012**[b]	31	1.528(0.551)	0.558[b]	12/31(39)	**0.004**
Risk group								
Non-high-risk	37	0.095(0.045)		39	0.513(0.143)		3/39 (8)	
High-risk	28	1.150(0.483)	**0.030**	28	1.625(0.604)	0.920	11/28(39)	**0.002**
Site								
Extra-abdominal	14	0.087(0.090)		14	0.261(0.154)		1/14 (7)	
Abdominal	51	0.560(0.282)	0.260	53	0.943(0.342)	0.810	13/53(25)	0.269
MKI								
Low	24	0.093(0.067)		25	0.382(0.195)		0/25	
Intermediate	14	0.087(0.073)		15	1.301(0.796)		5/15 (33)	
High	27	0.946(0.499)	**0.011**[b]	27	0.911(0.486)	0.940[b]	9/27 (33)	**0.005**
INPC								
Favourable	33	0.080(0.050)		34	0.541(0.238)		3/34 (9)	
Unfavourable	32	0.831(0.430)	**0.040**	33	1.064(0.497)	0.990	11/33(33)	**0.017**
MYCN status								
Non-amplified	47	0.207(0.085)		49	0.824(0.301)		9/49 (18)	
Amplified	13	1.595(1.060)	0.440	13	1.033(0.905)	0.540	4/13 (31)	0.444
1p deletion								
Absent	37	0.143(0.070)		39	0.677(0.262)		8/39 (21)	
Present	14	1.800(0.965)	**0.020**	13	1.759(1.151)	0.650	6/13 (46)	0.086
17q gain								
Absent	19	0.118(0.075)		21	0.721(0.518)		5/21 (24)	
Present	21	1.229(0.676)	0.220	20	1.113(0.675)	0.510	7/20(35)	0.506
LNM								
Absent	51	0.063(0.034)		53	0.324(0.110)		4/53 (8)	
Present	14	1.843(0.878)	**<0.001**	14	2.672(1.100)	**<0.001**	10/14(71)	**<0.001**

All p-values were calculated using Mann–Whitney test unless otherwise stated

[a] χ^2 test

[b] Cuzick test for trend

SE standard error, *CLD* central lymphatic density, *MLD* marginal lymphatic density, *INPC* International Neuroblastoma Pathology Classification, *MKI* mitosis karyorrhexis index, *LNM* lymph node metastasis, *LI* lymphatic invasion

The association of the LDs, LI and total protein with clinicopathological and biological variables

The relationships between LD and LI with clinicopathological and biological variables are shown in Table 2.

LYVE-1-LD

CLD was higher in NBs from patients with stage 4 disease ($p=0.012$), high-risk group NBs ($p=0.030$), NBs with high MKI ($p=0.011$), unfavourable histology ($p=0.040$) and 1p

deletion ($p=0.020$) (Table 2). The data also show that CLD is increased in children ≥18 months at age of diagnosis, NBs from the primary abdominal site, *MYCN* amplification and chromosome 17q gain (Table 2). Similarly, MLD was higher in NBs with each of the adverse clinicopathological and biological variables, although *p* values indicated that there was insufficient power to exclude the possibility that differences may have been due to chance (Table 2).

LI

LI was higher in patients with stage 4 disease ($p=0.004$), high-risk group ($p=0.002$) and in NBs with high MKI ($p=0.005$) and unfavourable histology ($p=0.017$) (Table 2). LI also appeared to be increased in children ≥18 months at age of diagnosis, NBs from primary abdominal site, *MYCN* amplification and chromosome 17q gain (Table 2).

LYVE-1 total protein

The relationship between LYVE-1 protein expression in 55 NBs and the clinical, pathological and biological variables is shown in Fig. 2 and Supplementary File 1. There was an increase in LYVE-1 total protein levels in NB biopsies from stage 4 patients (Fig. 2a, $p=0.046$), high-risk group (Fig. 2b, $p=0.028$), LNM (Fig. 2c, $p=0.035$) and those with *MYCN* amplification (Fig. 2d, $p=0.034$). LYVE-1 total protein was higher in tumours with a high compared with intermediate or low MKI (median, 2.95 vs. 1.10 and 0.59 pg/μg total protein, respectively, $p=0.157$), tumours with unfavourable compared with favourable histology NBs (median, 2.30 vs. 0.51 pg/μg total protein, respectively, $p=0.357$), tumours with 1p deletion versus NBs without (median, 1.59 vs.

0.88 pg/μg total protein, respectively, $p=0.626$) and tumours with a gain of the 17q chromosome versus NBs without (median, 3.56 vs. 0.44 pg/μg total protein, respectively, $p=0.126$). There was no evidence of association between LYVE-1 protein levels and age at diagnosis, primary tumour location, INPC pathology prognostic group, MKI, deletion 1p or 17q gain (Supplementary File 1).

Correlation between LD, LI and LNM

CLD and MLD were strongly correlated ($n=65$, rho=0.605, $p<0.001$). CLD correlated with LI (rho=0.611, $p<0.001$) as did MLD (rho=0.638, $p<0.001$). Both CLD and MLD were higher in patients with LNM ($p<0.001$ for each, Table 2). A higher proportion of patients with LI showed LNM ($p<0.001$, Table 2).

Survival analysis

At the last follow-up, 26 of 85 patients (31%) had died, and 9 patients (10%) were alive with recurrent disease. All eight of the patients with poor or no response to therapy died.

In the univariable Cox proportional hazard regression analyses, age ≥18 months at diagnosis ($p=0.038$), clinical stage 4 ($p<0.001$), high-risk group ($p<0.001$), high MKI value ($p=0.001$), *MYCN*-amplification ($p=0.001$), 1p deletion ($p<0.001$) and 17q gain ($p=0.032$) were associated with lower OS (Table 3).

Similarly, age ≥18 months at diagnosis ($p=0.015$), clinical stage 4 ($p<0.001$), high-risk group ($p<0.001$), high MKI value ($p=0.004$), INPC pathology unfavourable prognostic group ($p<0.001$), 1p deletion ($p<0.001$) and 17q gain ($p=0.022$) were associated with worse EFS. There was a

Fig. 2 Increased LYVE-1 total protein levels in NBs from **a** stage 4 patients, **b** high-risk group, **c** LN metastasis and **d** those with *MYCN* amplification. Each data point (*open circle*) represents protein concentration for each sample, and the *horizontal line* represents the median values. All *p*-values calculated using Mann–Whitney U-test
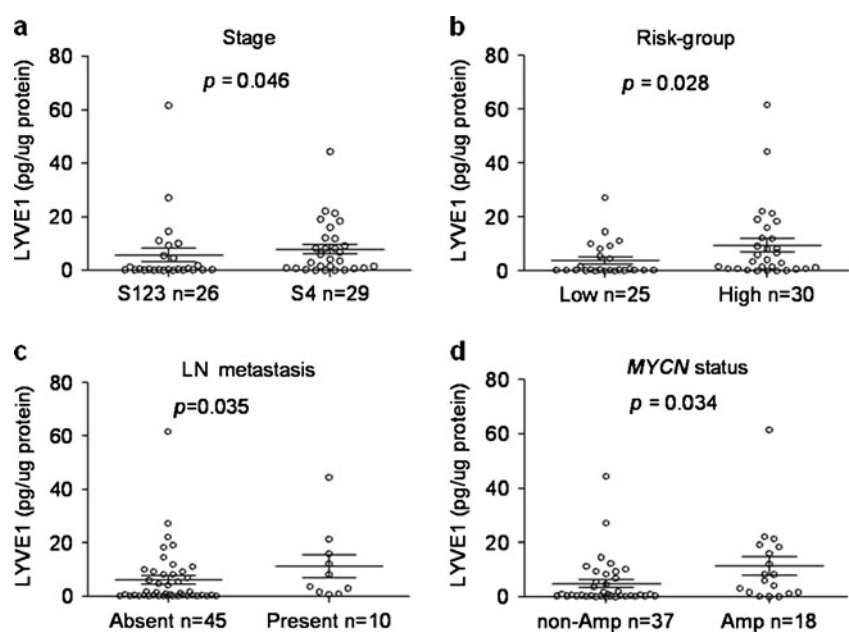
Table 3 Univariate Cox proportional hazards regression for NBs and GNBs

Variable		Deaths/n	Overall survival		Events/n	Event-free survival	
			HR (CI)	p-value		HR (CI)	p-value
Age (months)	<18	6/36	1		6/34	1	
	≥18	20/49	2.64 (1.06–6.59)	**0.038**	22/44	3.04 (1.24–7.59)	**0.015**
Stage	1–3,4 s	2/40	1		7/40	1	
	4	24/45	19.15 (4.46–82.17)	**<0.001**	21/38	5.22 (2.17–12.56)	**<0.001**
Risk group	Non-high-R	2/42	1		7/42	1	
	High-R	24/43	19.68 (4.59–84.29)	**<0.001**	21/36	5.41 (2.26–12.98)	**<0.001**
Site	Extra A	3/17	1		6/17	1	
	Abdo	23/68	2.76 (0.82–9.29)	0.101	22/61	1.26 (0.51–3.13)	0.613
MKI	Low/Interm	8/47	1		11/45	1	
	High	18/38	4.31(1.84–10.12)	**0.001**	17/33	3.10 (1.42–6.76)	**0.004**
INPC	FH	0/33			4/35	1	
	UH	26/32		0.072[a]	24/43	8.79 (2.97–25.99)	**<0.001**
MYCN status	Non-A	12/59	1		17/56	1	
	Amplified	11/21	3.88 (1.69–8.92)	**0.001**	9/18	2.15 (0.95–4.85)	0.066
1p deletion	Absent	5/46	1		7/45	1	
	Present	14/22	9.75 (3.47–27.41)	**<0.001**	13/18	7.91 (3.11–20.07)	**<0.001**
17q gain	Absent	2/23	1		2/22	1	
	Present	13/35	5.13 (1.15–22.81)	**0.032**	14/32	5.69 (1.29–25.12)	**0.022**
MLD	0	10/41	1		13/39	1	
	>0	7/26	1.12 (0.42–2.93)	0.824	6/23	0.83 (0.31–2.18)	0.700
CLD	0	10/49	1		12/46	1	
	>0	7/16	2.43 (0.93–6.40)	0.071	7/14	2.32 (0.91–5.92)	0.078
LYVE-1 protein	<2	9/30	1		8/29	1	
	≥2	7/25	1.12 (0.42–3.02)	0.818	5/23	0.92 (0.38–2.26)	0.863
LI	Absent	19/69	1		22/65	1	
	Present	7/16	2.29 (0.96–5.46)	0.062	6/13	1.87 (0.76–4.64)	0.175
LNM	Absent	19/70	1		20/65	1	
	Present	7/15	2.15 (0.90–5.13)	0.086	8/13	2.62 (1.14–6.01)	**0.023**

[a] Pearson χ^2 test. Presence or absence of LD was used as the cut-off for LD. Total LYVE-1 protein threshold of 2 pg/μg protein was used as the cut-off for protein

GNB ganglioneuroblastoma, *R* risk, *Extra-A* extra-abdominal, *Abdo* abdominal, *INPC* International Neuroblastoma Pathology Classification, *FH* favourable histology prognostic group, *UH* unfavourable pathology prognostic group, *Non-A MYCN* non-amplified, *MKI* mitosis karyorrhexis index, *Interm* intermediate, *CLD* central lymphatic density, *MLD* marginal lymphatic density, *LI* lymphatic invasion, *LNM* lymph node metastasis, *HR* hazard rate, *CI* confidence interval

strong association between distant LNM and shorter EFS and a weaker association with worse OS (Table 3).

The estimated overall survival (OS) at 5 years was 68% (95% CI: 56–77%) (Fig. 3a), and the estimated 5-year event-free survival (EFS) was 62% (95% CI: 50–72%) (Fig. 3b). Kaplan–Meier analysis showed that the presence of CLD was associated with both worse OS at 5 years (77% [95% CI: 62–87%] versus 60% [95% CI: 32–80%], $p=0.062$) (Fig. 3c) and EFS (74% [95% CI: 58–85%] versus 43% [95% CI: 15–69%], $p=0.070$) (Fig. 3d) and LI with OS (71% [95% CI: 57–81%] versus 56% [95% CI: 26–78%], $p=0.055$). However, as the sample size is small and there are few events, confidence intervals for survival estimates are wide, and therefore, observed differences in survival may be due to chance. There was no evidence of an association between MLD or total protein and OS or EFS (Table 3).

Discussion

This study represents the first detailed expression analysis of the lymphatic endothelial marker LYVE-1 in biopsies of NB patients and is the first examination of the relationship
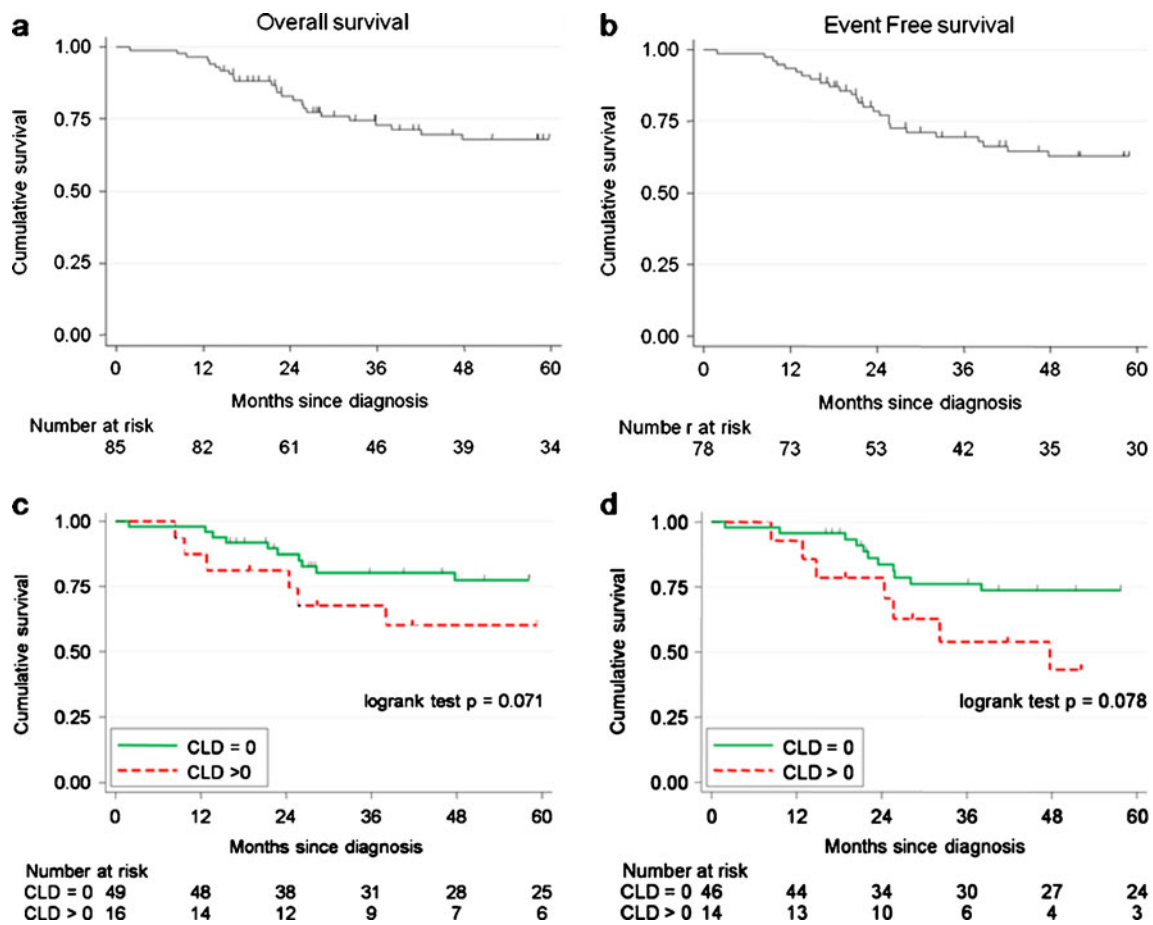
Fig. 3 Kaplan–Meier curves showing **a** overall survival (OS) and **b** event-free survival (EFS) of all patients in the study (85 NBs); worse **c** OS and **d** EFS rates in patients with LYVE-1 central lymphatic density (CLD >0) than those without

between the expression levels of this marker, clinicopathological and biological features and the prognosis of the patients.

LYVE-1 detected intratumoural lymphatics in up to 39% of 67 NBs, in contrast to 72% reported in the only previous study of 25 NBs [6]. Variations in the sensitivity of antibodies from different sources may provide a possible explanation for this difference. However, similar to the previous study in NBs [6], we noted LYVE-1 positive intratumoural lymphatics in the central and peripheral regions of the NBs. Most of the LYVE-1-expressing lymphatics in the central, deeper parts of the tumour were small and collapsed, although they were easily visualised when tumour emboli were present. In contrast, the marginal lymphatics in the peripheral parts of the NBs were larger with widely patent lumina. There was also a significant difference in the lymphatic count between the central and marginal areas of the NBs, which is in line with previous studies of other cancers [15, 16, 18, 19].

In our cohort, CLD and MLD were highly correlated with each other, and all of the associations for MLD were similar to those with CLD, but a little less strong. Increased CLD was associated with key adverse prognostic factors, such as inclusion in the high-risk group. We also observed a significant increase in NBs from patients with stage 4 tumours, in line with those of the previous study of 25 NBs [6].

The primary significance of the increased number and size of lymphatic vessels is that they provide a possible conduit for the spread of NB cells. Intravasation into tumoural vessels is an essential step in the metastatic cascade, and the presence of LI is an important pathological feature for assessing the risk of recurrence and metastasis. Immunostaining by LYVE-1 confirmed the presence of LI by NB tumour emboli and also showed a strong relationship between LI and various adverse prognostic features. Moreover, increased CLD and MLD strongly correlated with LI and LNM. As expected, the presence of LI correlated with distant LNM. Furthermore, both LD and LI showed a trend towards a poorer OS, whereas LNM showed a trend towards poorer OS and EFS. Our results are in line with recent findings on melanomas [20], tumours that share an embryonic origin with NBs, and various epithelial cancers, as

reviewed by Sleeman [12]. It is likely that a study including a larger cohort would show a significant relationship between LI and survival.

Quantification of total LYVE-1 protein using a sensitive ELISA assay also showed that LYVE-1 levels were significantly higher in the NBs of patients in an advanced stage, in the high-risk group or those with *MYCN*-amplified NBs. To our knowledge, LYVE-1 total protein, which measures expression in LEC and a subset of macrophages, has not been utilised in any other published clinical studies. The results obtained by immunohistochemistry were concordant with the data obtained by ELISA; increased LYVE-1 protein levels were also seen in NBs with similarly adverse prognostic features and distant LNM. However, LYVE-1 total protein levels did not show a significant association with OS or EFS in patients. Based on these findings, prospective studies including a larger number of samples from patients with NB may be useful to determine whether ELISA assays for LYVE-1 protein levels have clinical utility in providing prognostic data on the biological behaviour of NBs.

In summary, our results show for the first time that increased LYVE-1 (both LD and total protein levels) and LI are closely associated with key adverse risk factors and LNM. Distant LN involvement by NB is significantly correlated with EFS. These findings suggest that LYVE1 LD and LI may predict the outcome.

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