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Original Article

Significance of Neuropilin-1 (CD 304) Expression in Paediatric B-Lineage Acute Lymphoblastic Leukemia (ALL)

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Abstract

Background: To determine the prognostic significance of NRP-1 (CD304) expression in paediatric B-lineage Acute Lymphoblastic Leukaemia (ALL) patients.

Methods: In this comparative study newly diagnosed cases (aged 1-15 years) of B-ALL were selected. Age and sex matched, 21 healthy controls were also included in the study to assess the NRP-1 expression on peripheral blood lymphocytes. A minimum 1ml of blood and bone marrow aspirate samples were taken in EDTA vacutainer and immunophenotyping was done on gated blast cells using an extensive panel of antibodies including myeloid markers (CD13, CD33 and cytoplasmic anti-MPO) and lymphoid markers (CD34, CD2, CD3, CD5, CD7, CD4, CD8, HLA DR, CD10, CD19, CD22; cytoplasmic CD3, CD22, CD79a and nuclear anti-TdT). The sample was considered NRP-1 positive if 20% or more of the gated blast cells expressed it . Man-Witney U test and Kruskal Wallis test were used for non-parametric data. The *p* value <0.05 was considered significant.

Results: Out of 66 B-ALL patients, 53% were males. There were 20 (30%) NRP-1 positive and 46 (70%) NRP-1 negative patients. The prognosis of NRP-1 positive group was poor as compared to NRP-1 negative group with high blast percentage (80%) (*p*= 0.042), low morphological remission rate (21%) (p=0.004) and low survival rate (29%) (p=0.009). The mean survival days in dead patients was also less (22.75 days).

Conclusion: NRP-1 over expression is associated with disease progression and severity in paediatric B-ALL patients.

Key Words:NRP-1/CD304, Pediatric B-lineage ALL Patients,Minimal Residual Disease(MRD), Flow Cytometry

Introduction

Acute lymphoblastic leukaemia (ALL) is the most common leukaemia accounting for 26% of all cancer incidences in children.[1] Globally, the cure rate of childhood ALL is above 80%.[2] The vascular endothelial growth factor A (VEGFA) has a key role in regulation and development of blood vessels and lymphatics both in health and disease.[3] Semaphorin (SEMA) is a family of proteins involved in the development of nervous system, repulsive axon guidance, neuronal connectivity, organogenesis, angiogenesis and cancer progression.[4] Angiogenesis is an important requirement for the development and progression of haematological and non-haematological malignancies.[5] Anti-angiogenic drugs are used to control angiogenesis hence control malignancy and reduce both morbidity and mortality.[6] Due to better diagnosis and improved treatment, the survival rate of childhood ALL patients has considerably improved worldwide and also in Pakistan.[7]

Neuropilins (NRP) are non-tyrosine kinase receptors. They bind to class III semaphorin family and are co-receptors for VEGF. NRP is upregulated in many types of tumors because it regulates angiogenesis. There are two types of Neuropilins; Neuropilin-1 and Neuropilin 2.[8] Neuropilin-1(BDCA4/NRP-1/CD304)is a 130 kDa trans-membrane non-tyrosine kinase glycoprotein.[9] NRP-1 gene is of 112kb and located on chromosome 10q12. It is expressed by plasmacytoid dendritic cells, vascular endothelial cells, osteoblasts, T cells, glomerular epidermal cells and tumor cells.[10] It binds to VEGFR2 to mediate angiogenesis.[11] It also binds to other growth factors including hepatocyte growth factor (HGF), fibroblast growth factor (FGF), placental growth factor (PGF), platelet derived growth factor (PDGF) and fibronectin (FN) to mediate cellular functions.[12] NRP-1 activation of PDGFR pathway leads to upregulation of RAD51 which leads to resistance of cancer cells to therapy.[13] So, the drugs targeting NRP-1 can control tumor resistance against

therapy. NRP-1/VEGF interaction leads to infiltration of tumor with regulatory T cells (T-regs). These T-regs express NRP-1 and play a role in suppressing immune response against tumor. Drug targeting, NRP-1 can cause down regulation of T-reg infiltration of tumor cells and prevent immunosuppression. [14-16]

NRP-1 is over-expressed in many types of cancers because involved cancer increased the vascularization.[17] It is over-expressed in prostatic carcinoma, nasopharyngeal carcinoma, breast carcinoma, neuroblastoma, leukaemia and lymphomas.[18-21] In ALL it is expressed more on pre-B ALL blasts. Relapse cases show higher expression of NRP-1 and representing poor prognosis. Relapse of ALL can be assessed by specialized techniques including PCR and flow cytometry which detect leukemia associated phenotype.[22] NRP-1 also acts as a useful marker to monitor minimal residual disease (MRD). [23]

NRP-1 association with ALL makes it an important diagnostic and prognostic marker because these patients have short survival and high blast count on bone marrow biopsy. Therapy aiming at targeting of NRP-1 to control angiogenesis can be a new step to treat the different type of cancers, leukemia and lymphomas.[24,25] Targeting NRP-1 may prevent unwanted side effects of aggressive chemotherapy. Anti-NRP-1 drugs are underway subsequently, targeted approach can be achieved and this will be helpful to improve the overall survival of the diseased patents. [25]

Patients and Methods

Present study was a cross sectional comparative and conducted on 87 subjects. Newly diagnosed cases (aged 1-15 years) of B-ALL were selected in the studyon basis of clinical presentation, morphology, cytochemistry and immunophenotyping. Age and sex matched, 21 healthy controls were also included in the study to assess the NRP-1 expression on peripheral blood lymphocytes. A minimum 1ml of blood and bone marrow aspirate samples were taken for immune-phenotyping. Immunophenotyping was done on gated blast cells using an extensive panel of antibodies including myeloid markers (CD13, CD33 and cytoplasmic anti-MPO) and lymphoid markers (CD34, CD2, CD3, CD5, CD7, CD4, CD8, HLA DR, CD10, CD19, CD22; cytoplasmic CD3, CD22, CD79a and nuclear anti-TdT). The sample was considered NRP-1 positive if 20% or more of the gated blast cells expressed it (figure-1). The patients were treated according to standard protocols. Follow up was also done for the period of 6 months. For quantitative variables,Shapiro Wilk test was used to differentiate parametric data from non-parametric.The mean ±SD and student t-test were used for parametric data. Median and IQR, Man-Witney U test and Kruskal Wallis test were used for non-parametric data. The *p* value <0.05 was considered significant.

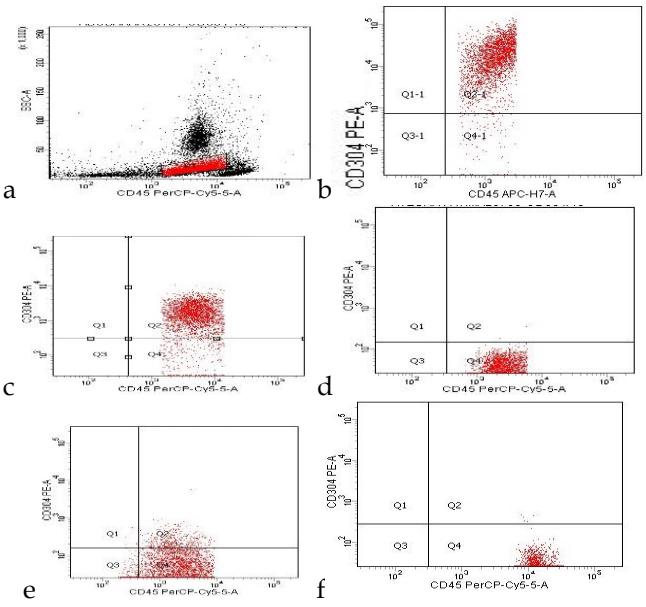

Figure- 1:Expression of Neuropilin-1 (NRP-1/CD304) in B-ALL patients and healthy control: a.Lymphoblast population (P1 area) in the B-ALL sample was gated using CD45 on x-axis and SSC (side scatter) on y axis. B-lymphoblast exhibit incomplete maturation spectrum and present as a single immature population;b.NRP-1 positive case of B-ALL: NRP-1 expression was examined on the gated cell population using PE conjugtaed CD304 on y axis and per CP conjugated CD45 on x axis. Dot plot showNRP-1 positive case with high expression of 90.1% on blasts;c.NRP-1 positive case of B-ALL:NRP-1 expression of 64.4% onblast;d.NRP-1 negative case of B-ALL: NRP-1 expression of 0.1% on blast;e.NRP-1 negative case of B- ALL: mild NRP-1 expression of 8.4% on blasts;f.Negative control. NRP-1 expression was evaluated in peripheral blood lymphocytes of a healthy control. NRP-1 expression was minimal (0%) while CD45 was bright.

Results

Out of 87, 21 were healthy controls and 66 were B-lineage ALL patients. The median age of ALL patients was 60 months (range= 12-180 months). There were 20 (30%) NRP-1 positive patients and 46 (70%) were NRP-1 negative (staticitcally significant differences;$p<0.001$) (Table-1).Regarding gender, presence of consanguinity of parents, fever, bleeding tendency, hepatomegaly, lymphadenopathy and CNS involvement, no significant difference was found between the above mentioned two groups ($p>0.05$)(Table-2).Blast percentage was significantly high in NRP-1 positive group ($p = 0.042$) (Table-3).A highly significant difference of NRP-1 expression was observed between ALL cases and healthy controls

(p<0.001). The mean expression of NRP-1 in ALL cases was 14.26% (range = 0-90.10%) while in healthy control it was 0.11% (range: 0-0.90%). The ALL patients were categorised into high risk and standard risk group according to National Cancer Institute (NCI) criteria. High risk group included those patients with WBC count ≥ 50 x 10^9/l and age ≥10 years. The standard risk group included the patients with WBC count <50 x 10^9/l and age <10 years. There was no significant difference in NRP-1 expression in the high risk and standard risk group (p = 0.23). The mean expression of NRP-1 in standard risk patients (n=39) was 12.24% (range= 0.0-90.1%) while in high risk patient (n=1), the NRP-1 expression was 2.1%.Follow up of ALL patients was done for a duration of 6 months and 07 out of 66 were lost to follow up. In the remaining 59 ALL patients, three prognostic groups were made after the day 28th of induction therapy. The patients having blasts <5% on bone marrow were considered to be in morphological remission, those having blasts >5% on bone marrow were considered not to be in morphological remission and the last one group was the patients with early death (death during induction therapy). Out of 59 follow up patients, 19 were NRP-1 positive and 40 were NRP-1 negative (Table-4).

Twenty four patients achieved morphological remission after day 28th of induction therapy, out of these, 5 (21%) were NRP-1 positive and 19 (79%) were NRP-1 negative. The prognosis of NRP-1 positive group was poor with morphological remission in only 5 (21%) of patients (p=0.004). Nineteen patients (19) did not achieve morphological remission, out of these, 7 (37%) were NRP-1 positive and 12 (63%) were NRP-1 negative (p = 0.251). Sixteen patients who died during induction therapy included 7 (44%) NRP-1 positive and 9 (56%) NRP-1 negative patients (p = 0.617) (Table- 4). A total of 21/59 patients (36%) died after 6 months of follow up. The cause of death ranged from renal failure, liver failure, septic shock, drug toxicity and cardiac arrest. The remaining 36/59 (60%) patients were on maintenance therapy who suffered mild infections and fever. Only 2/59 (4%) patients left the treatment.Out of total patients who survived (38/59), 27 (71%) were NRP-1 negative and 11 (29%) were NRP-1 positive. So, there was statistically significant difference in survival of the two groups (p= 0.009). In NRP-1 positive group, the mean survival days in dead patients were less(22.75 days) as compared with NRP-1 negative group (40.46 days) (Figure-2). The deaths were comparatively higher in males than in females, out of 21 died patients, 12 (57%) were males and 9 (43%) were females. Also, deaths were significantly more in patients having history of consanguinity of parents (81%) (p= 0.013).

Table 1:Comparison of NRP-1 expression in the two groups (n=66)

NRP-1 expression	Mean %	Median %	p value
NRP-1(positive group;n = 20)	39.52	36.35 (22.82- 50.55)	<0.001
NRP-1(negative group;(n = 46)	3.28	0.95 (0.20 – 5.35)	

Table- 2: Demographic features of NRP-1 positive and negative groups

Parameter		NRP-1 Positive Group (n = 20)		NRP-1 Negative Group (n = 46)		p value
		Frequency	Percentage	Frequency	Percentage	
Gender	Male	8	40	27	59	0.162
	Female	12	60	19	41	
Consanguinity of parents	Present	14	70	26	57	0.35
	Absent	6	30	20	43	
Fever	Present	20	100	42	91	0.083
	Absent	0	0	4	9	
Bruising/ Bleeding:	Present	8	40	23	50	0.454
	Absent	12	60	23	50	
Lymphadenopathy	Present	13	65	28	61	0.751
	Absent	7	35	18	39	
Hepatomegaly	Present	17	85	43	93	0.275
	Absent	3	15	3	7	
Splenomegaly	Present	12	60	38	83	0.049
	Absnet	8	40	8	17	
CNS infiltration	Present	1	5	3	7	0.813
	Absent	19	95	43	93	

Table- 3: Baseline laboratory findings in NRP-1 positive/negative group

Parameter	NRP-1 positive group	NRP-1 negative group	p value
	Median (IQR)		
Hemoglobin (g/dl)	8.9 (6.3- 9.57)	7.7 (5.57-9.35)	0.419
WBC count (x10^9/l)	16.85 (4.47-55.05)	17.69 (4.65 – 51.7)	0.807
Blast %	80 (70-90)	70 (30-83.50)	0.042
Platelet (x10^9/l)	42.500 (25.0 - 155.7)	57.250 (17.000 – 111.0)	0.660
LDH (U/L)	724.0 (398.0-1748.2)	494.00 (332.00 – 1213.0)	0.213
Uric acid(mg/dl)	4.25 (2.47 - 6.55)	3.85 (2.87 – 6.10)	0.958

Table- 4: Prognosis of patients of NRP-1 positive/negative groups after day 28 of induction therapy (n=59)

Group	NRP-1 positive group (n = 19)		NRP-1 negative group (n = 40)		*p* value
Morphological remission(n=24)	5	21%	19	79%	0.004
No morphological remission(n=19)	7	37%	12	63%	0.251
Early death(n=16)	7	43%	9	56%	0.617

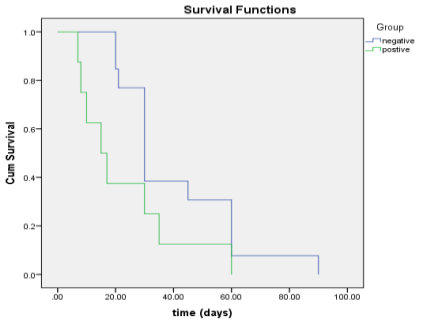

Figure-2:Kaplan Meyer estimate of overall survival in pediatric B-lineage ALL patients. The NRP-1 positive group (green) and NRP-1 negative group (blue) are shown with 95% confidence interval. Follow up was done for a period of 180 days. There was statistically significant difference in prognosis of NRP-1 negative and NRP-1 positive groupwith short overall survival in NRP-1 positive group as compared to NRP-1 negative group (Log rank value of 3.917; p = 0.048).

Discussion

Worldwide, ALL is more common in males than in females. According to International Classification of Childhood Cancer (ICCC), 1.9/100,000 males and 1.5/100,000 females are affected by ALL.[26] Our data showed similar male preponderance in incidence of ALL.In present study, the median age for paediatric ALL subjects were 5 years. These findings were comparable to another Pakistani study which showed median age of 6 years. [27] However, according to National Cancer Institute, the median age at the time of diagnosis of ALL was 15 years in U.S.A.[28] Majority of patients in our study were less than 5 years old (53%). This can be explained because incidence of ALL peaks between ages 2 to 5 years.[29]

In present study, 59% ALL patients were in standard risk group. This outcome was nearly consistent with another study where 62.2% patients were in standard risk group (30). Many patients (94%) in our study presented with fever at the time of diagnosis. The bruising/bleeding tendency was seen in 45% of our study patients. This finding goes hand in hand with a previous study which showed fever and bruising in 81% and 46.3% ALL patients respectively (31). In current study subjects, the mean haemoglobin level of ALL patients was 7.79 ± 2.84g/dl. This value was nearest to another study which shows the mean hemoglobin level of 8.0 g/dl in ALL patients.[32]

In our study, 20 (30%) out of 66 B-ALL cases were positive for NRP-1 expression. This value was low as compared to another study which showed 48% NRP-1 positive cases in B-ALL patients.[33] In our study, NRP-1 expression in bone marrow blasts of ALL patients was 39.52%. This value was nearest to the findings by a previous study where NRP-1 expression in pediatric B lineage ALL patients was 36.86%. [34] Many other studies have been conducted to evaluate the significance of NRP-1 in B-ALL patients and the mean expression of NRP-1 in these previous studies ranged from 36.86% to 80% (Table-5).

Table-5:NRP-1 expression in BM blasts of B-ALL cases in different populations

Study conducted by	Year	Mean NRP-1 expression in BM blasts
Elaine Coustan-Smith et al	2011	80%
Solly et al	2012	48%
Meyerson et al	2012	71%
Nosair and Hagag	2014	36.86%
Hagag and Nosair	2015	62%
Current study	2017	39.52%

In present study population, the NRP-1 positive group was associated with high blast percentage (p = 0.042). This finding was in accordance with previous studies where mean blast percentage was high in patients with NRP-1 overexpression. [35,36] In our study, there was no significant correlation between NRP-1 expression and WBC count, haemoglobin level and platelet count. This finding was in concordance with a previous study which showed similar results.[37] However, contrary to these results, some other studies have shown high WBC count and high LDH levels in NRP-1 positive group. [34,38] In our research results, 5 out of 20 (25%) NRP-1 positive patients presented with leucopenia. The bone marrow of these patients was hypoplastic. Due to decreased turnover of cells, the LDH level was low in these patients. If we keep aside these 5 cases, in the remaining 15 NRP-1 positive patients, the WBC count and serum LDH level was high (mean WBC and LDH = 61.7 x 10^9/L and 1390.7 U/L respectively) which was comparable to past studies. [34]

The NRP-1 expression on peripheral blood lymphocytes of normal healthy control was 0.11%. It was in accordance with the previous studies which showed low to absent NRP-1 expression on peripheral blood B-lymphocytes. [34, 39] The prognosis of ALL patients in our study was poor as compared with international statistics. The suboptimal outcome of our

pediatric ALL patients can be explained by the socioeconomic status of the patients, lack of parental education, late diagnosis of disease, malnourishment and failure to control infections as stressed by another study on Pakistani population.[40] Another Pakistani study has shown mortality rate of 24% in childhood ALL cases (74/304 patients). The main cause of death in these patients was infection. [41] Contrary to these results, Idris et al. showed better prognosis of ALL with complete remission in 94% patients after induction therapy and also with no mortality. [42]

Results of present study highlight the association of NRP-1 over expression with disease severity and its biological progression. These findings were consistent with earlier studies. [34,35] Younan et al. has suggested that the NRP-1 expression was significantly associated with disease progression in acute leukemia. Hagag et al. has suggested that NRP-1 was a bad prognostic marker in children with B-lineage ALL. Many other studies have been conducted which highlight the association of NRP-1 expression with MRD in acute lymphoblastic leukemia .[33, 37, 39, 43] NRP-1, co receptor of VEGF can act as target for drug therapy and in this way it can control angiogenesis and tumor growth.[5, 44]

There were 21 deaths in 6 months, out of which, 12 (57%) were in standard risk group. The findings by Hunger at el. has shown 36% deaths in standard risk group of ALL and emphasized the need for efforts to improve the survival in standard risk group along with efforts to improve survival in high risk group. [45] Our findings call for similar efforts to improve survival in our setting as well. The patients in NRP-1 negative group had comparatively better prognosis. The morphological remission on day 28 of induction therapy was achieved by 19 out of 24 (79%) NRP-1 negative patients and after 6 months tenure, 27 out of 38 (71%) NRP-1 negative patients survived. These findings were concordant with the previous studies that have shown better prognosis of NRP-1 negative group of ALL patients.[33, 34,39] Among 21 dead patients, 17 (81%) had history of consanguinity of parents. So, consanguinity of parents was significantly high in dead patients (p = 0.013). This finding was consistent with those of Nasir et al, which showed similar high incidence of leukemia in children born to people having cousin marriages. [46]

Conclusion

NRP-1 over expression is associated with disease progression and its severity in pediatric B-ALL patients.Incorporation of NRP-1 as bad prognostic marker can help stratify high risk patients so that they can be intensively treated.

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Key for Contribution of Authors : A= Conception/ Study/ Designing /Planning; B= Experimentation/Study conduction;C=Analysis/Interpretation/ Discussion; D= Manuscript writing;E= Critical review;F= Facilitated for reagents/Material/Analysis