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RESEARCH

Expression of Eph A4, Eph B2 and Eph B4 Receptors in AML

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Abstract Eph receptors represent the largest subfamily of receptor tyrosine kinases (RTKs). The up- regulation of Eph receptors has been documented in various solid tumors, where it often correlates with poor prognosis. Their significance in hematologic malignancies is still unclear. This study aimed to investigate the expression of Eph A4, Eph B2, and Eph B4 mRNA in non - M3 AML patients and determine their prognostic significance. Bone marrow samples from 101 newly diagnosed non - M3 AML patients and 26 healthy controls for comparison were quantified by real time reverse transcriptase polymerase chain reaction (RT-PCR), and the comparative cycle threshold (Ct) method was used to determine their relative expression levels to GUS control gene. The results showed that expression of all selected Eph receptors was significantly lower in AML patients comparing to controls. It also differed according to FAB subtypes. The decreased expression levels of Eph A4 were associated with higher leukocytes (p=0.022) and blast cell counts (p=0.001), and unfavorable FLT3-ITD mutation. Our study revealed significant correlation between lower EphB2 expression levels, and higher complete remission rate (p=0.009724) and longer overall survival. Additionally, we found that patients with shorter RFS had decreased EphB4 expression (p=0.00). In conclusion, the results suggest the prognostic impact of decreased expression levels of some Eph receptors in AML patients.

Keywords Acute myeloid leukemia · Eph A4-Eph B2-Eph B4 · Prognostic impact

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Introduction

Eph receptors represent the largest subfamily of receptor tyrosine kinases (RTKs), containing 14 distinct receptors in humans, named for its expression in an erythropoietin – producing human hepatocellular carcinoma cell line [1]. The Eph receptors bind to specific ligands, called ephrins [1]. Both Eph receptors and their ligands – ephrins can be divided into two subclasses: A and B, based on their sequence similarity, structure, and ligand binding affinity [2, 3]. Their unique feature is bidirectional signaling, via cell-cell contact [4–6]. Eph receptors are key regulators of physiological and pathological processes, by regulating cytoskeleton organization, cell shape, cellular adhesion – repulsion and migration [4]. The best known biological processes regulated by Eph receptors and ephrins are: the development of the nervous and vascular system [7, 8]. The up - regulation of Eph receptors has been reported in various solid tumors, including breast, lung, colorectal, gastric and esophageal cancers [9–15]. This over-expression correlates with tumor invasiveness, vascularization, metastatic potential and poor prognosis [10, 14, 16, 17]. Many studies have also reported their role in tumor suppression [18, 19]. The significance of Eph receptors in hematologic malignancies is still unclear. Some previous studies have shown decreased expression of Eph A3 in acute myeloid leukemia (AML) patients, but without regarding clinical data [20].

Materials and Methods

Patients

Bone marrow aspirated samples from 101 newly diagnosed non - M3 AML patients (58 males, 43 females, median age =49 years, range=19–84 years) and 26 healthy controls (17 males, 9 females, median age =46 years, range=22–74 years) were collected between 2000 and 2009 at the Department of Hematology, Blood Neoplasms and Bone Marrow Transplantation, Medical University in Wroclaw. The subtypes of AML were classified based on French-American-British classification criteria because patients were mainly diagnosed before 2008, when the new WHO classification was introduced, therefore some cytogenetic data necessary for this classification were missing [21]. According to European Leukemia Net (ELN) patients were categorized into 3 risk groups: standard (favorable - t(8:21)(q22:q22) RUNX1-RUNX1T1; inv(16)(p13.1q22) or t(16:16)(p13.1:q22) CBFB-MYH11; mutated NMP1 without FLT3-ITD; mutated CEBPα), intermediate (I- mutated NMP1 and FLT3-ITD; Wild-type NMP1 and FLT3-ITD; Wild-type NMP1 without FLT3-ITD, II-t(9:11)(p22:q23) MLLT3-MLL, cytogenetic abnormalities not classified as favorable or adverse) and high risk (adverse-inv(3)(q21q26.2) or t(3:3)(q21:q26.2) RPN1-EVI1; t(6:9)(p23:q34) DEK-NUP214; t(v;11)(v;q23) MLL rearranged; -5 or del5q; -7; abnormalities (17p); complex karyotype) [22]. According to treatment the group was heterogeneous. Patients ≤60 year (n=76) were treated according to the Polish Adult Leukemia Group (PALG) induction protocols based on daunorubicin and conventional dose of cytarabine arabinoside (Ara-C) [23–25]. Patients >60 year (n=25) were treated according to the PALG or Cancer and Leukemia Group B (CALGB) protocols [26, 27]. In three patients older than 60 years we applied a low dose of Ara-C and in two patients decitabine. 7 of 76 patients ≤60 years and 3 of 25 patients >60 years received only palliative therapy due to poor performance status and comorbidities, their distribution between the groups was regular, so it did not influence the results. Response criteria were assessed based on European Leukemia Net recommendations [22]. The study was approved by the local ethical committee and the informed consents were obtained. Patients' data are summarized in Table 1.

Methods

We used frozen bone marrow samples. Mononuclear cells were isolated from bone marrow by gradient separation using Gradsiol L (Aqua Medica, Łódź). Total RNA was extracted from the cells by using TriReagent (Ambion/Applied Biosystems, Warsaw) following the manufacturer's instructions. The concentration of RNA was measured by a spectrophotometer. The complementary DNA (cDNA) was synthesized with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warsaw). The samples were quantified by real-time reverse transcriptase polymerase chain reaction (RT-PCR), based on TaqMan Gen Expression Assay (Applied Biosystems): respectively Hs00177847_m for EphA4, Hs00362096_m1 for EphB2, Hs00174752 for EphB4 and Hs99999908_m1 for GUS (www.appliedbiosystems.com). The comparative cycle threshold (Ct) method was used to determine the relative expression for Eph A4, Eph B2, Eph B4 mRNA to GUS control gene ($\Delta Ct = C_{T\ \text{analyzed gene}} - C_{T\ \text{control gene GUS}}$). The relative expression levels of analyzed genes were calculated as $2^{-\Delta Ct}$ according to Applied Biosystems instruction.

Statistical Methods

Results were evaluated using the STATISTICA 9.0 software. To study the relationship between two categorical variables, the independence χ^2 test was used. In the case of small number of groups exact Fisher's test and Yates's correction test were used. Pearson's correlation was used to evaluate correlation between paired values. Due to the presence of outliers, in some cases the Spearman rang correlation coefficient was used. Statistical comparisons between groups were performed also by means of the Mann-Whitney U-test (non-parametric analysis) and ANOVA rang Kruskal-Wallis test. P values less than 0.05 were considered statistically significant. The Kaplan-Meier method was used to estimate overall survival.

Results

The relative expression levels of Eph A4, Eph B2 and Eph B4 mRNA were significantly lower in AML patients comparing to healthy controls ($p \leq 0.05$). The expression levels of Eph receptors also differed according to FAB subtypes. In spite of introducing WHO classification in 2008, we also present these data, because patients were mainly diagnosed before this period. Patients were divided into 4 groups, using morphological features according to the FAB criteria: Group 1 – AML M0+M1; Group 2 – AML M2, Group 3 - AML M4+M5, Group 4 – AML M6. The statistical analysis does not include patients with subtype M6 (group 4), due to the limited number of patients (n=3). The Eph A4 and B4 expression was the highest among the patients with M0 and M1 AML subtypes. The expression of Eph B2 receptor was the highest in M4 and M5 subtypes. The results are shown in Table 2. The expression levels of Eph A4 and Eph B4 corresponded with higher amount of CD34 positive blast cells. The was no correlation between expression of Eph B2 and CD34 positive blast cells count. Next we correlated the results with some clinical data. We found that lower expression levels of Eph A4 correlate

Table 1 Clinical characteristic of AML patients

Number of patients	101
Age	49 (19–84)
Sex	F- 43, M -58
FAB, *n*	
M0	8
M1	20
M2	23
M4	31
M5	16
M6	3
AML de novo, *n*	96
AML secondary to MDS, *n*	5
Risk, *n*	
SR	21
IR	48
HR	32
Molecular and cytogenetic changes, *n*	
FLT3-ITD	22
NPM1-mut	31
t(8:21) (q22:q22) RUNX1-RUNX1T1	7
inv(16)(p13.1q22) or (16:16)(p13.1:q22) CBFB MYH 11	5
t(9:11)(p22::q23) MLLT3- MLL	2
del 7	4
del 5	3
Median white blood cells count (10^9/L)	30 (0.52–509)
Median platelet count (10^9/L)	52 (5–433)
Median hemoglobin level (g/dL)	9 (5.5–15.1)
LDH (U/l)	709 (180–13534)
Blast cells in bone marrow (%)	75 (20,5–97)
CD 34^+ , *n*	61
Treatment, *n*	
a) ≤60 year (*n*=76)	
DA	35
DAC	25
DAF	9
Palliative treatment	7
b) >60 year	(*n*=25)
DA	15
Ara-C + mitoxantron	2
Low dose of Ara-C	3
Low dose of decitabine	2
Palliative treatment	3
Response to treatment, *n*	
CR	57
NR	44

F female; *M* male; *LDH* lactic acid dehydrogenase; *SR* standard risk; *IR* intermediate risk; *HR* high risk; *MDS* myelodysplastic syndrome, *FAB* French, American, British Co-operative group; *CR* complete remission; *NR* non response; *Ara-C* cytarabine arabinoside; *DA* daunorubicin + cytarabine arabinoside; *DAF* daunorubicin + cytarabine arabinoside + fludarabine; *DAC* daunorubicin + cytarabine arabinoside + cladribine

with higher leukocytes (p=0.022) and blast cell counts (p=0.001). The expression levels of Eph receptors did not appear to be related with cytogenetic risk group but lower Eph A4 expression did relate to FLT3-ITD mutation (p=0.07970, FLT3-ITD: 0.015±0.01, FLT3-WT: 0.063±0.11). The patients with NPM1 mutation displayed significantly lower levels of Eph B2 and Eph B4 (respectively p=0.004229 and p=0.001079) compared to patients with wild-type form of this

Table 2 EphA4, B2 and B4 mRNA expression according to FAB classification

	n	*EphA4 mRNA* (x ± SD)	*p*-value		
Group 1	28	0.0805 ± 0.11244			
			]	0.1126	
Group 2	23	0.0416 ± 0.0913			**0.0025**
			]	1.0000	
Group 3	47	0.0385 ± 0.0926			
	n	*EphB2 mRNA* (x ± SD)	*p*-value		
Group 1	28	0.0161 ± 0.0281			
			]	0.5330	
Group 2	23	0.0151 ± 0.0389			**0.0339**
			]	**0.0003**	
Group 3	47	0.0408 ± 0.0474			
	n	*EphB4 mRNA* (x ± SD)	*p*-value		
Group 1	28	0.1785 ± 0.2031			
			]	**0.0257**	
Group 2	23	0.0681 ± 0.0705			**0.000016**
			]	0.5194	
Group 3	47	0.0482 ± 0.0507			

x- mean; SD- standard deviation; Group 1- M0 + M1; Group 2- M2; Group 3 – M4 + M5

gene. We did not took into account other molecular or cytogenetic changes as a single factor due to the limited number of patients. The another goal of this study was to evaluate the expression of Eph A4, Eph B2 and Eph B4 receptors in the context of clinical outcome: complete remission rate (CR), overall survival (OS) and relapse free survival (RFS). Our study revealed significant correlation between lower Eph B2 expression levels, and higher complete remission rate, compared to patients without remission (p=0.009724). The lower Eph B2 expression correlated also with longer overall survival (p=0.04961) (Fig. 1). In overall survival analysis expression levels below median were considered as lower. Additionally, patients with shorter RFS showed decreased Eph B4 expression (p=0.00).

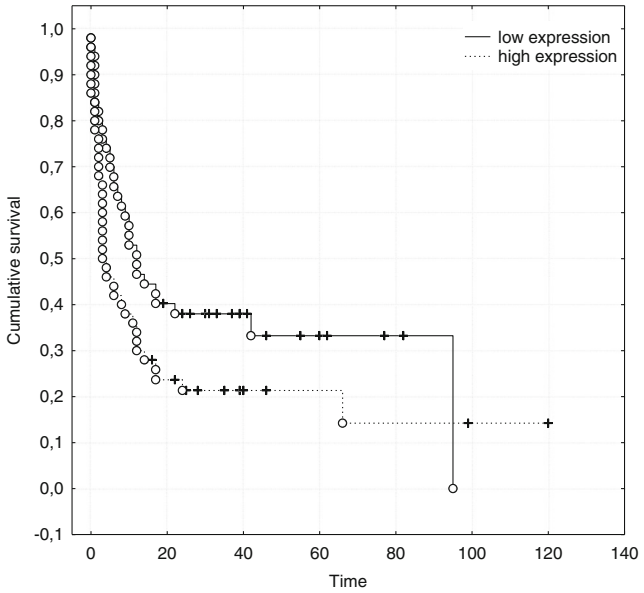

Fig. 1 Kaplan-Meier analysis of OS according to Eph B2 expression levels (low expression below median)

Discussion

Acute myeloid leukemia is a phenotypically and genetically heterogeneous hematological malignancy, associated with malignant transformation, autonomous proliferation and impaired differentiation of hematopoietic progenitor cells. The development of AML is caused by accumulation of acquired genetic abnormalities and epigenetic changes [28]. From 60 to 80 % of patients with AML achieve a complete remission (CR). However, the majority of these patients subsequently relapse and 5-year survival rate is only 30 % for younger adults and 15 % for elderly patients [29]. Even if the patients with AML belong to the same risk group, the course of the disease can vary. This confirm the need for new prognostic factors and novel treatment options in AML.

Eph receptors and their ligands – ephrins are known to play important roles in carcinogenesis and tumor growth, progression and vascularization. Many studies suggest that they can function as a prognostic factor or potential treatment target. There are only a few reports on their expression in hematologic malignancies. Initially we had screened samples of 8 patients with AML for 11 different Eph receptors to identify these with marked expression variability. Based on this screening we have chosen 3 Eph receptors with the biggest variation for further investigation. We studied the expression of Eph A4, Eph B2, and Eph B4 mRNA in bone marrow of non - M3 AML patient. Moreover we correlated our results with some clinical data and course of the disease. The results were analyzed taking into account: AML subtype according to FAB classification, the CD34 expression, molecular and cytogenetic prognostic factors and clinical data such as: complete remission rate, relapse free survival and overall survival.

Our analyses identified a significantly reduced expression of all selected Eph receptors in AML patients comparing to healthy controls. Comparable results have been obtained in previous studies. Guam et al. found deletion of Eph A3 in AML patients, but this study did not regard clinical data [20]. The lower expression of EphB4 has been also observed in another hematologic malignancy – acute lymphoblastic leukemia (ALL). Where it was caused by methylation and was associated with worse outcome. This study showed that EphB4 function as a tumor suppressor in ALL [30]. However, the underlying mechanisms of down - regulation are still unclear. Some studies suggest that decreased Eph expression apart from hypermethylation is due to mutations of Eph receptors' and ephrins' genes [31, 32]. Hypermethylation of DNA is frequently observed in myelodysplastic syndromes (MDS) and acute myeloid leukemia where it frequently results in decreased expression of tumor suppressor genes. This changes are potentially reversible and can be an attractive therapy target. Demethylating agents in elderly patients with MDS and AML have proved efficiency [32]. The cause of the lowered Eph expression in AML needs further research, as they could theoretically serve as target for demethylating agents.

There was no statistical association between the expression levels of Eph A4, Eph B2, and Eph B4, and patients age and sex.

We observed significant variability in receptors' expression according to FAB classification. The expression levels of Eph A4 and Eph B4 depended on maturation of blast cells. It was higher among patients with undifferentiated AML and AML with minimal maturation and corresponded with higher amount of CD34 positive blast cells. The highest expression of Eph B2 was found in patients with myelo-monocytic leukemia, without correlation with CD34 positive blast cells count. Variable expression of selected Eph receptors among AML subtypes can be due to the disease heterogeneity, which derive from different stages of myelopoiesis. Another heterogenic hematologic malignancy – chronic lymphocytic leukemia (CLL) also characterize different Eph expression depend on disease stage. Early, favorable stages of CLL associate with EphB1 expression and advanced ones with the expression of EphB6 [33].

Another important observation of our study is that Eph A4 expression correlates inversely with "tumor mass". The expression was lower, the higher was leucocytes and blast cell count. Our results are consistent with those in previous reports in colorectal tumors, where decreased expression of Eph B2 correlated with tumor growth and liver metastases [34].

Furthermore, we found that decreased expression of Eph A4 was related to unfavorable FLT3-ITD mutation, with slight tendency towards shorter overall survival but the difference was not statistically significant. Moreover, the favorable NPM1 mutation correlated with decreased expression of Eph

B2 and Eph B4. The association between low expression of Eph B2 and coexistence of favorable mutation was consistent with better outcome in this group (CR rate and OS). In contrast, the decreased expression of Eph B4 related to worse prognosis - shorter RFS. Which also associates with worse outcome in ALL [30].

In summary, all these evidences suggest that Eph B2 and Eph B4 may function as prognostic factors for predicting outcome and aid in therapeutic decision making.

Conclusions

The study showed that AML patients had significantly lower expression of Eph A4, Eph B2 and Eph B4. The results suggest the prognostic impact of decreased expression levels of some Eph receptors in AML patients.

Disclosures No relevant conflicts of interest to declare.

References

1. Eph Nomenclature Committee (1997) Unified nomenclature for Eph family receptors and their ligands, the ephrins. Cell 90:403–404
2. Gale NW, Holland SJ, Valenzuela DM (1996) Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. Neuron 17:9–19
3. Pasquale EB (2005) Eph receptor signaling casts a wide net on cell behavior. Nat Rev Mol Cell Biol 6:462–475
4. Himanen JP, Nicolov DB (2003) Eph receptors and ephrins. IJBCB 35:130–134
5. Bruckner K, Klein R (1998) Signaling by Eph receptors and their ephrin ligands. Curr Opin Neurobiol 8:75–82
6. Cowan CA, Henkemeyer M (2002) Ephrins in reverse, park and drive. Trends Cell Biol 12:339–346
7. Wang HU et al (1998) Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor EphB4. Cell 93:741–753
8. Flanagan JG, Vanderhaegen P (1998) The ephrins and Eph receptors in neural development. Annu Rev Neurosci 21:309–345
9. Andeas AC, Ziemiecki A (2003) Eph and ephrin signaling in mammary gland morphogenesis and cancer. J Mammary Gland Biol Neoplasia 8:475–485
10. Ji XD, Li G, Feng YX (2011) EphB3 is overexpressed in non-small-cell lung cancer and promotes tumor metastasis by enhancing cell survival and migration. Cancer Res 71(3):1156–1166
11. Kinch MS, Moore MB, Harpole DH Jr (2003) Predictive value of the EphA2 receptor tyrosine kinase in lung cancer recurrence and survival. Clin Cancer Res 9(2):613–618
12. Stephenson SA, Slomka S, Douglas EL (2001) Receptor protein tyrosine kinase EphB4 is up-regulated in colon cancer. BMC Mol Biol 2:15
13. Yuan W, Ge J, Chen Z (2009) Over-expression of EphA2 and EphrinA-1 in human gastric adenocarcinoma and is prognostic value for postoperative patients. Dig Dis Sci 54:2410–2417
14. Yuan W, Chen Z, Wu S (2009) Expression of EphA2 and E-cadherin in gastric cancer: correlated with tumor progression and lymphogenous metastasis. Pathol Oncol Res 15(3):473–478
15. Tachibana M, Tonomoto Y, Hyakudomi M (2007) Expression and prognostic significance of EFNB2 and EphB4 genes in patients with oesophageal squamous cell carcinoma. Dig Liver Dis 39(8):725–732
16. Wang J, Dong Y, Wang X (2010) Expression of EphA1 in gastric carcinomas is associated with metastasis and survival. Oncol Rep 24(6):1577–1584
17. Yu G, Gao Y, Ni C (2011) Reduced expression of EphB2 is significantly associated with nodal metastasis in Chinese patients with gastric cancer. J Cancer Res Clin Oncol 137(1):73–80
18. Alazzouzi H, Davalos V, Kokko A (2005) Mechanisms of inactivation of the receptor tyrosine kinase EPHB2 in colorectal tumors. Cancer Res 65(22):10170–10173
19. Huusko P, Ponciano-Jackson D, Wolf M (2004) Nonsense-mediated decay microarray analysis identifies mutations of EPHB2 in human prostate cancer. Nat Genet 36(9):979–983
20. Guan M, Liu L, Zhao X (2011) Copy number variations of EphA3 are associated with multiple types of hematologic malignancies. Clin Lymphoma Myeloma Leuk 11(1):50–53
21. Bennett JM, Catovsky D, Daniel MT et al (1985) Proposals for the classification of the acute leukaemias. A report of the French-American-British (FAB) co-operative group. Ann Intern Med 103: 620–625
22. Döhner H, Estey EH, Amadori S et al (2010) Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European Leukemia Net. Blood 115:453–474
23. Hołowiecki J, Grosicki S, Robak T et al (2004) Polish Adult Leukemia Group (PALG). Addition of cladribine to daunorubicin and cytarabine increases complete remission rate after a single course of induction treatment in acute myeloid leukemia. Multicenter, phase III Study. Leukemia 18:989–997
24. Hołowiecki J, Grosicki S, Kyrcz-Krzemien S et al (2008) Daunorubicin, cytarabine and fludarabine (DAF) for remission induction in relapsed or refractory acute myeloid leukemia. Evaluation of safety, tolerance and early outcome–Polish Adult Leukemia Group (PALG) pilot study. Ann Hematol 81:361–367
25. Hołowiecki J, Grosicki S, Robak T et al (2001) Daunorubicin, cytarabine, and 2CdA (DAC-7) for remission indiction in "de novo" Adult acute myeloid leukemia patients. Acta Haematol Pol 2002: 839–847
26. Wrzesień-Kus A, Robak T, Jamroziak K et al (2002) The treatment of acute myeloid leukemia with mitoxantrone, etoposide and low-dose cytarabine in elderly patients - a report of Polish Acute Leukemia Group (PALG) phase II study. Neoplasma 49:405–411
27. Yates J, Glidewell O, Wiernik P et al (1982) Cytosine arabinoside with daunorubicin or adriamycin for therapy of acute myelocytic leukemia: a CALGB study. Blood 60: 454–462
28. Christ O, Feuring-Buske M, Hiddemann W et al (2007) Pathobiology of acute myeloid leukemia. Med Clin 102: 290–295
29. Sekeres MA (2008) Treatment of older adults with acute myeloid leukemia: state of the art and current perspectives. Haematologica 93: 1769–1772
30. Kuang SQ, Bai H, Fang ZH (2010) Aberrant DNA methylation and epigenetic inactivation of Eph receptor tyrosine kinases and ephrin ligands in acute lymphoblastic leukemia. Blood 115(12):2412–2419
31. Davalos V, Dopeso H, Velho S (2007) High EPHB2 mutation rate in gastric but not endometrial tumors with microsatellite instability. Oncogene 26(2):308–311

32. Oba SM, Wang YJ, Song JP, Li ZY, Kobayashi K, Tsugane S, Hamada GS, Tanaka M, Sugimura H (2001) Genomic structure and loss of heterozygosity of EPHB2 in colorectal cancer. Cancer Lett 164(1):97–104
33. Alonso-C LM, Trinidad EM, de Garcillan B, Ballesteros M, Castellanos M, Cotillo I, Muñoz JJ, Zapata AG (2009) Expression profile of Eph receptors and ephrin ligands in healthy human B lymphocytes and chronic lymphocytic leukemia B-cells. Leuk Res 33(3):395–406
34. Oshima T, Akaike M, Yoshihara K (2008) Overexpression of EphA4 gene and reduced expression of EphB2 gene correlates with liver metastasis in colorectal cancer. Int J Oncol 33(3): 573–577