Over-expression of *MIR9* indicates poor prognosis in acute lymphocytic leukemia

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Running title: *MIR9* in acute lymphocytic leukemia

Key words: *MIR9*; microRNA; acute lymphocytic leukemia; prognostic factor; overall survival; relapse-free survival

Abstract

Aberrant expression of *MIR9* predicts poor prognosis in acute myelogenous leukemia. To evaluate its clinical significance in acute lymphocytic leukemia, we analyzed expression levels of *MIR9* in bone marrow samples from patients with acute lymphocytic leukemia and compared them to those in normal bone marrow cells. Approximately 20% of them showed higher expression compared with controls. There was a tendency that patients who showed over-expression of *MIR9* underwent worse clinical courses but without a statistical significance. However, when the analyses were restricted to patients who did not receive stem cell transplantation, over-expression of *MIR9* was significantly associated with worse overall survival. Interestingly, exaggerated *MIR9* expression as well as higher white blood cell count at presentation were independent unfavorable prognostic factors in the entire patients for overall survival by multivariate analyses. The presence of higher *MIR9* expression could be one of useful indicators for treatment stratification.

Introduction

Although many progress in the treatment for adult acute lymphocytic leukemia (ALL) has been made during the past several decades, prognosis of adult ALL patients still remains poor. Even in those patients without Philadelphia (Ph1) chromosome, 5-year overall survival (OS) and 5-year relapse rate are reported to be 45% and 49-63%, respectively, when they are not treated with hematopoietic stem cell transplantation (HSCT) [1]. It is well known that standard chemotherapy alone is insufficient to improve the poor prognosis. High-risk ALL patients with one or more adverse prognostic factors are recommended to undergo allogeneic HSCT. Adverse prognostic factors identified so far in ALL are older age, higher white blood cell (WBC) count, poor response to 1st remission induction therapy, and the presence of specific cytogenetic abnormalities such as t(9;22) and t(4;11) [2].

MicroRNA (miRNA) is a class of 18-24 nucleotides small non-coding RNAs that induce mRNA degradation and negatively modulate protein translation by pairing with their target sequences in the 3' UTR of mRNA [3-5]. miRNA affects various pathways in cell proliferation, differentiation, survival and apoptosis and also plays vital roles in normal and abnormal hematopoiesis. Developmentally controlled expression of miRNA regulates each step of hematopoiesis starting at a level of hematopoietic stem cell and continuing during differentiation process of both myeloid and lymphoid lineages. On the other hand, dys-regulated expression of miRNA results in the development of hematological malignancies including leukemia, lymphoma and myeloma [6,7].

Expression changes of a lot of miRNAs that show pathogenetic significance have been reported in ALL. Examples whose altered expression could constitute molecular basis in ALL include *MIR19*, *MIR142-3p*, *MIR16*, *MIR150* and *MIR451*. Up-regulation of *MIR19* and

MIR142-3p that target deubiquitinase-encoding *CYLD* and *glucocorticoid receptor a* (*GRA*) mRNAs causes activation of NF-κB signaling and glucocorticoid resistance, respectively [8,9]. Down-regulation of *MIR16*, *MIR150* and *MIR451* results in activation of anti-apoptotic proteins such as BCL2, MCL1 and CDK6, NOTCH3 and MYC, respectively [10-12]. Clinical significance has been also emphasized in abnormal expression of several miRNAs. Increased levels of *MIR33, 215, 369-5p, 496, 518d* and *599* are associated with an unfavorable long-term clinical outcome, while those of *MIR10a, 134, 214, 484, 572, 580, 624* and *627* with a favorable prognosis [13]. Further, low expression of *MIR454* is found to co-relate with L-asparaginase (L-Asp)-resistance, and high expression of *MIR7*, *MIR198* and *MIR663* as well as low expression of *MIR126, MIR345, MIR222* and *MIR551A* with relapse in the central nervous system [14].

MIR9, whose function in hematopoietic system still remains unknown, works as an oncogene or a tumor suppressor in a context-dependent manner. We recently have reported that about 20% of acute myelogenous leukemia (AML) patients show exaggerated expression of *MIR9*, and that abnormal expression of *MIR9* is an independent predictor for poor OS and relapse-free survival (RFS) in AML [15]. In this study, we examined *MIR9* expression levels in bone marrow mononuclear cells from ALL patients and evaluated clinical significance of over-expression of *MIR9*. Over-expression of *MIR9* could be another independent prognostic factor for poor prognosis in ALL patients who do not receive HSCT.

Materials and Methods

Patients

A total of 32 bone marrow samples from ALL patients at diagnosis were analyzed for *MIR9* expression. All these ALL patients were sequentially diagnosed in Dokkyo Medical University Hospital from 2001 to 2011 and selection of samples used in this study was strictly based on their availability. Written informed consent for these studies was obtained from all the patients. Clinical characteristics of each patient are shown in Supplementary Table I. Patients received various kinds of induction chemotherapy depending on the subtype of leukemia and their age. About half of the Ph1-positive ALL patients (54%: 7 patients among 13 patients) received induction therapies consisting of cyclophosphamide (CPA), doxorubicin (DXR), vincristine (VCR), and prednisolone (PSL) (hyper-CVAD regimen) [16,17] with (4 patients) or without (2 patients) conventional dose (600mg/body/day) of imatinib mesilate [18], or imatinib mesilate alone (1 patients), while the other Ph1-positive patients (46%: 6 patients among 13 patients) received L-Asp, anthracycline（DXR or daunorubicin）, VCR, PSL and ± CPA (L-AdVP ± CPA regimen [19]). All the Ph1-negative ALL patients (59%: 19 patients among 32 patients) received L-AdVP ± CPA or hyper-CVAD regimen as induction therapies. Patients who achieved complete remission (CR) also received two to four courses of consolidation therapies such as high-dose cytarabine (Ara-C) + mitoxantrone (MIT) regimen [20] or hyper-CVAD regimen with alternating high-dose methotrexate and Ara-C protocol. When patients failed to achieve CR, salvage therapies such as intermediate-dose Ara-C and MIT with etoposide (MEC regimen [21]) were employed.

RNA isolation and quantitative reverse transcriptase-PCR

Total RNA was isolated from bone marrow mononuclear cells using the mirVana miRNA Isolation kit (Ambion, Austin, TX). Quantitative reverse transcriptase (qRT)-PCR to evaluate expression levels of mature *MIR9* was performed using the TaqMan MicroRNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix, TaqMan MicroRNA Assay primers (Applied Biosystems, Foster City, CA) and the 7700 real-time PCR system (Applied Biosystems). *MIR9* level was quantified by measuring cycle threshold (CT) that is defined as a fractional cycle number at which fluorescence encounters a fixed threshold. The CT value of *MIR9* was normalized to the mean CT value of two control small nuclear RNAs (*RNU6-2 and SNORA74A*) that were amplified with purchased primers (Applied Biosystems).

The presence or absence of *NOTCH1* mutations was determined in the pretreatment samples as described previously. In brief, PCR was performed with complementary DNA (cDNA) using PCR primers shown in Supplementary Table II. Nucleotide sequences of the heterodimerization (HD) domain in *NOTCH1* were amplified by PCR using primers HD-fw and HD-rev [22]. In some cases, used were additional sets of PCR primers, HD-N1-F/HD-N1-R, HD-N2-F/HD-N2-R, HD-C1-F/HD-C1-R, and HD-C2-F/HD-C2-R. To fully sequence the C-terminal region of the HD domain, genomic PCR was also performed using PCR primers, HD-C-P5F, HD-C-P5R, and subsequently nested primers HD-C-P6F, HD-C-P6R in some cases. Nucleotide sequences of the proline-glutamate-serine-threonine-rich (PEST) domain in *NOTCH1* were amplified by using primers PEST-fw and PEST-rev. The PCR products were subjected to direct sequence analysis with the same primers used for PCR amplification.

Statistical methods

SPSS 19.0 (IBM, Armonk, NY) was used for all statistical analyses. Pretreatment clinical features of patients with or without *MIR9* expression were compared using Pearson's chi-square and Mann-Whitney tests. Estimated probabilities of OS and RFS were calculated using the Kaplan-Meier method, and the Log-Rank test evaluated differences of survival distribution between patients with and without *MIR9* expression. Cox proportional hazard models were used to estimate hazard ratios (HRs) for univariate and multivariate analyses for OS and RFS. In univariate analyses, we evaluated the presence of *MIR9* over-expression in addition to known prognostic factors listed in Tables III and IV as predictors. In multivariate analyses using Cox proportional hazard models with a stepwise forward likelihood ratio method, we incorporated the same candidates of prognostic factors that were employed in univariate analyses. RFS was defined as the time for patients having achieved CR from the end of induction chemotherapy to relapse or death due to progressive disease, in which deaths from non-progressive disease were censored.

Results

Over-expression of *MIR9* in acute lymphocytic leukemia

We recently reported that about 20 % of AML samples showed aberrant expression of *MIR9* and patients with high expression of *MIR9* significantly showed poor prognosis compared with those without the expression [15]. To investigate the prevalence and prognostic significance of over-expression of *MIR9* in ALL patients, we studied expression levels of *MIR9* by the mean of real-time RT-PCR method in a cohort of adult ALL patients (32 patients; 19 men and 13 women) diagnosed in our institute from 2001 to 2011 (Supplementary Table I). Median age of the 32 patients was 41.25 years (range; 15-78 years). Twelve among 32 patients (37%) had normal karyotype. Thirteen patients were classified as Ph1-positive leukemia. As in AML patients, some fraction of bone marrow samples from these ALL patients showed increased levels of *MIR9*. We have already reported by analyzing 23 samples that normal bone marrow cells show hardly detectable levels of *MIR9* expression, but have not presented their relative expression values in the previous paper [15]. We thus here provide with the data from controls along with them from ALL samples (Figure 1). Expression levels of *MIR9* were changing rather continuously in both the controls and leukemic samples. Range of the relative expression values of normal control was from 0.005 and 0.377. We calculated two standard deviations above the mean value in the controls (relative expression value, 0.212) and employed it as a cut-off value in leukemic samples. We then divided the ALL patients into two groups based on their expression levels of *MIR9*; positive and negative groups of *MIR9* expression (*MIR9* (+) and *MIR9* (-) groups). Six patients (19%) belonged to the positive group and 26 (81%) to the negative group. Demographic, laboratory and clinical characteristics were compared between these two

groups. Any parameter including sex, age, WBC count, percentage of blasts in the peripheral blood or bone marrow, lactate dehydrogenase (LDH) value, the presence of Ph1 chromosome was not significantly different between these two groups (Table I). There was no positive co-relationship between expression levels of *MIR9* and percentage of blasts in the bone marrow (correlation coefficient=0.070), suggesting that levels of *MIR9* were not necessarily affected by remaining normal bone marrow cells. On the other hand, over-expression of *MIR9* tended to be more frequent in T-ALL (43%: 3 patients among 7 patients) than in B-ALL (12%: 3 patients among 25 patients) (p=0.064). We also analyzed the presence or absence of mutation in the *NOTCH1* gene in T-ALL patients. Because *NOTCH1* mutations in T-ALL are recurrent in the HD (exons 26 and 27) and PEST (exon 34) domains, we directly sequenced the corresponding exons of *NOTCH1* gene in the samples. We observed two mutations that were novel in two of seven T-ALL patients (28%); one (c.4818-4822 delCAAGCinsGAACTT) in HD domain and the other (c.7021-7025 delTCCTinsGGAGAA) in PEST domain. Interestingly, mutations of *NOTCH1* were detected only in the *MIR9* (-) T-ALL patients.

Over-expression of *MIR9* has adverse prognostic impact

Half of the Ph1(+) ALL patients (54%: 7 patients among 13 patients) received hyper-CVAD regimen with or without imatinib mesilate or imatinib mesilate alone as induction therapies, while the other Ph1(+) patients (46%: 6 patients among 13 patients) received L-AdVP ± CPA regimen. Ph1(-) ALL patients (59%: 19 patients among 33 patients) received L-AdVP ± CPA or hyper-CVAD regimen as induction therapies. Differences of treatment regimens between the *MIR9* (+) and *MIR9* (-) groups in the entire cohort were not

statistically significant (p=0.614) (Table II). The CR rate was investigated for all patients after the first course of induction therapy. Patients with over-expression of *MIR9* seemed to have a lower rate of CR (67%) compared with those without the expression (81%), but this difference was not statistically significant (p=0.451) (Table II). In the Ph1(+) patients, either of two patients in the *MIR9* (+) group did not receive imatinib-containing regimen, while five out of eleven patients in the *MIR9* (-) group did. However, there was no difference in the CR rate to 1st induction therapy between the *MIR9* (+) and *MIR9* (-) groups (p=0.522). There was no difference in implementation rate of HSCT between the *MIR9* (+) and *MIR9* (-) groups (p=0.865) (Table I). We analyzed characteristics of patients who received and did not receive HSCT (Supplementary Table III). The only difference between them was that patients who received HSCT were significantly younger than those who did not. We then analyzed whether OS and RFS differed between the *MIR9* (+) and *MIR9* (-) groups by Kaplan-Meier method and Log-Rank test. The median observation period was 838.5 days. As a result, although the presence of over-expression of *MIR9* had no impact on RFS (p=0.718), the *MIR9* (+) group tended to be associated with inferior OS compared with the *MIR9* (-) group (p=0.259: Figure 2). When analyses were restricted to the patients who did not receive HSCT, the *MIR9* (+) group exhibited significantly inferior OS compared with the *MIR9* (-) group (P=0.001) (Figure 3A). RFS also seemed to be worse for those with *MIR9* over-expression than those without it, but this difference was not statistically significant (p=0.105) (Figure 3C). In contrast, on the cohort who received HSCT, neither OS nor RFS showed any significant difference between *MIR9* (+) and *MIR9* (-) patients (P=0.953 or P=0.386) (Figure 3B and D). These data suggested that over-expression of *MIR9* may cause worse OS in ALL patients, especially in those who do not receive HSCT. We speculated that HSCT would overcome

inferior OS in *MIR9* (+) ALL patients. It is interesting to know clinical consequences of three patients who showed the extremely high levels of *MIR9*. One patient is still alive after HSCT, while two died without receiving HSCT. Thus, as expectedly, there observed was the same tendency in patients with the highest levels of *MIR9*.

Prognostic factor

We performed univariate Cox regression analyses on the entire cohort to evaluate the status of *MIR9* over-expression as well as known prognostic factors listed in Tables III and IV as predictors of OS and RFS. As a result, only WBC count greater than 30 x 10^9/L was found to be a predictor for poor OS with a statistical significance (HR=10.017, p=0.003) (Table III). The presence of *MIR9* over-expression was not a strong predictor of decreased OS (HR=1.914, p=0.267). On the other hand, any analyzed factors including the presence of *MIR9* over-expression (HR=0.760, p=0.719) but except WBC count greater than 30 x 10^9/L (HR=3.821, p=0.017) were not predictors of increased risk of relapse (Table IV). Of note, the presence of Ph1 was not extracted as a prognostic factor. Among thirteen Ph1-positive patients, seven are still alive, while six passed. Thus, prognosis of Ph1-positive patients was relatively fair in this cohort. Among seven patients still alive, three of them underwent HSCT, and two received imatinib-including regimen for their induction, which made their prognosis improved.

We also performed multivariate analyses for OS and RFS incorporating the same candidates of prognostic factors in the univariate analyses, age over 65, WBC count greater than 30 x 10^9/L, LDH greater than 600 IU/L, and the presence of Ph1 chromosome and *MIR9* over-expression on the whole cohort (Tables III and IV). It is well known that high initial

WBC count correlates with worse OS and RFS, and our data confirmed it with a statistical significance (OS: HR=21.175, p=0.001, RFS: HR=3.821, p=0.017). Surprisingly, the presence of *MIR9* over-expression was an independent prognostic factor for OS (HR=7.042, p=0.008) as well, whereas it was not a predictor for RFS.

Because our multivariate analyses showed that initial WBC count and *MIR9* over-expression were only two independent prognostic factors in predicting OS, we performed the Kaplan–Meier analysis for OS in patients stratified by *MIR9* over-expression and WBC count at diagnosis. We categorized all patients into three groups; patients who had both *MIR9* over-expression and WBC count greater than 30 x 10^9/L, those who had either *MIR9* over-expression or WBC count greater than 30 x 10^9/L, and those who had neither of them. Amazingly, survival curves of the three groups were separated each other with statistical significances (Figure 4). The both-negative patients showed the best OS, while the both-positive patients showed the worst. These results demonstrated that *MIR9* over-expression is a prognostic factor independent of initial WBC count.

Discussion

MIR9 is potentially transcribed from three different genomic loci; 1q22 (*MIR9*-1), 5q14.3 (*MIR9*-2) and 15q26.1 (*MIR9*-3) (http://www.mirbase.org/cgi-bin/query.pl?terms=*MIR9*). Although a lot of miRNAs are known to be crucial in the maintenance of stem cells and proliferation and differentiation of lineage-committed cells in hematopoiesis [6,7], there are no reports on roles of *MIR9* in hematopoietic system, which may be concordant with our previous observation that normal bone marrow cells do not express detectable levels of *MIR9*. *MIR9* works as oncogene [23,24] or tumor suppressor [25,26] in various kinds of solid tumors. Interestingly, significance of expressional changes of *MIR9* in human leukemia also appears to be context-dependent. We have recently reported over-expression of *MIR9* in AML patients [15], while the other group showed hypermethylation in its promoter in ALL patients [27]. Hypermethylation is usually associated with down-regulation of gene expression [28]. However, both the alterations have been found to be an independent poor prognostic factor in each type of leukemia. Thus, regard to leukemia, *MIR9* seemed to be oncogenic for AML, while anti-oncogenic for ALL. However, in this report, we clearly demonstrated by using real-time PCR method that 19 % of ALL patients (6 among 32 patients) showed markedly increased expression of *MIR9* associated with poor clinical outcome, which is rather contradictory with the previous report [27]. We divided patients based on the expression level of *MIR9*, while they on the methylation status. We paid attention to patients with higher expression of *MIR9* compared with controls, and did not further divide patients with a similar range of *MIR9* expression to controls. Some of them may have had non-methylated promoters, and others hypermethylated ones. On the other hand, hypomethylated group in the previous

report from the other group should have contained patients with various levels of *MIR9* expression from rather high levels to significantly high levels. We consider that the different ways to group patients could have resulted in somehow inconsistent results between the two reports. Both of higher and lower expression of *MIR9* could play a role in the development and/or progression of ALL, and be clinically relevant.

Expression of *MIR9* family genes is epigenetically regulated through methylation of CpG island and surrounding histone modification in their promoter regions. Hypermethylation of CpG island and specific histone marks such as high levels of H3K9me2 and/or low levels of H3K4me3 that cause a closed chromatin structure are found to be associated with repressed expression of *MIR9* in ALL samples [28]. On the other hand, molecular mechanisms underlying aberrant expression of *MIR9* remain unknown yet. To understand a molecular basis of the increased *MIR9* expression, it is necessary to compare methylation status and histone modification of the *MIR9* genes between *MIR9* (+) and *MIR9* (-) patients. Further, transcription factors, MYC/MYCN [24,29], RE1-silencing transcription factor (REST) and cAMP-responsive element binding protein (CREB) [30], are shown to directly bind to and thereby enhance the promoter activity of the *MIR9* genes. MYC controls cell cycle progression and apoptosis, and causes the development of Burkitt lymphoma when overexpressed by the t(8;14) translocation in precursor B-cells [31]. CREB is postulated to play a role in T-cell differentiation and function [32], while functions of REST are unknown in hematopoietic cells. It is also interesting to know whether expression levels of these transcription factors are correlated with those of *MIR9* in ALL cells. However, unfortunately, no more leukemic samples are available in this study.

We speculate that ectopic expression of *MIR9* leads to the development of leukemia

possibly through repressing translation of its target mRNAs. The reported targets of *MIR9* include *PRDM1* [33,34], *E2F1* [35], *FGFR1* and *CDK6* [28] in lymphoid malignancies. Down-regulation of *PRDM1* through activation of *MIR9* may contribute to maintaining immature phenotype in *MIR9* (+) ALL cells, while up-regulation of *E2F1, FGFR1* and *CDK6* through suppression of *MIR9* could cause aberrant proliferation. Further, it is interesting to note that *RUNX1* mRNA possesses a seed sequence of *MIR9* in its 3' UTR. RUNX1 is known to be essential in T-cell differentiation [36] and be dys-regulated in precursor B-cell ALL by TEL-RUNX1 chimera that is generated by t(12;21) translocation and inhibits function of wild-type RUNX1 in a dominant-negative manner [37]. Loss of function of RUNX1 through over-expression of *MIR9* also may play some roles in the development of ALL of both B and T cell origin. By combining information of protein expression profiles and *MIR9* expression signature in ALL samples, it could be possible to identify additional *MIR9* targets whose altered translation causes leukemia.

Because prognosis of adult ALL patients is significantly poor if treated only with chemotherapy, risk stratification is necessary to distinguish patients who need HSCT to be cured. Unfavorable prognostic factors reported so far include older age, higher WBC count at presentation, specific chromosomal abnormalities such as t(9;22)/*BCR-ABL* and t(11q23)/*MLL* chimera, immunophenotype of null or B cells, and time till CR achievement. We have shown that *MIR9* (+) patients undergo worse clinical courses than *MIR9* (-) patients in ALL, as similarly in AML. Although over-expression of *MIR9* had no impacts on remission induction rate or RFS, it conferred inferior OS with a statistical significance in multivariate analysis incorporating other known prognostic factors (HR=7.042, p=0.008). This is the first report demonstrating that over-expression of *MIR9* remains independently

predictive of OS in ALL. Further, we have shown that concomitant presence of WBC count more than 30 x 10^9/L at presentation and *MIR9* over-expression predicts poorer prognosis than single presence of either one of the two factors. Notably, *MIR9* expression and *NOTCH1* mutation (a possible favorable prognostic factor in adult T-ALL) [38-40] were observed exclusively mutually in T-ALL patients, while *MIR9* expression and Ph1 chromosome (an unfavorable prognostic factor in B-ALL) in an overlapping way in B-ALL. Considering that there was no positive co-relation between *MIR9* over-expression and these known genetic markers, over-expression of *MIR9* could be one of independent genetic markers of poor prognosis in ALL. Because of a relatively low number of patients, we could not investigate clinical significance of *MIR9* over-expression in each immunophenotypic and cytogenetic subtype. Thus, we could not deny a possibility that there might be potential confounding factors in our analysis. Most importantly, when analysis was restricted to patients who did not receive HSCT, the presence of *MIR9* over-expression was a strong prognostic factor in predicting OS with a statistical significance (p=0.001). Because the presence of *MIR9* over-expression was not predictive of OS in patients who underwent HSCT, this observation suggested that poor prognosis caused by *MIR9* over-expression could be overcome by HSCT. In this meaning, information of dys-regulated expression of *MIR9* is useful to stratify patients to be cured by HSCT. Recent accumulating evidence suggests that various kinds of dys-regulation of miRNAs could constitute molecular basis in leukemogenesis and refine molecular risk. Down-regulation of *MIR124a* [41], *27A*, *223*, *708* [42] and up-regulation of *MIR142-3P* [8], *146A*, *181A/C* [43] are shown to have a predictive value of poor prognosis in ALL. In the purpose of progressing therapeutic approaches for ALL, it is required to determine what kinds of combination of dys-regulated miRNA expression could predict poor

clinical course with the strongest power.

Acknowledgements

We thank Ms. A. Okada for her special technical assistance.

This work was financially supported by the Grants-in-Aid from the Ministries in Japan of Education, Culture, Sports, Science and Technology (17016068), and Health, Labour and Welfare, Japanese Society for the Promotion of Science (20390275, 18591086, 50337391) and Seki Minato Award from Dokkyo Medical University School of Medicine.

Declaration of Interests

All authors have no conflict of interest to report.

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Figure Legends

Figure 1. *MIR9* is expressed in a fraction of ALL patients.

Bone marrow samples from a total of 32 ALL patients were subjected to expressional analysis of *MIR9*. The expression values were normalized to the mean expression of two control small nuclear RNAs, *RNU6-2 and SNORA74A*. Data of normal controls were derived from the analysis described in the previous paper [ref. 15]. ALL patients were divided into *MIR9* (+) (n=6) or *MIR9* (-) (n=26) group by setting two standard deviations above the mean in the normal controls (relative expression value, 0.212) as a cut-off value.

Figure 2. Clinical significance of *MIR9* expression in whole ALL patients.

Kaplan-Meier estimates show that *MIR9* expression tends to indicate shorter overall survival in whole ALL patients.

Figure 3. Clinical significance of *MIR9* expression in patients with or without HSCT.

Kaplan-Meier estimates show that *MIR9* expression exhibits significantly shorter overall survival (A) and tends to indicate worse relapse-free survival (C) in ALL patients who did not receive HSCT. In ALL patients who received HSCT, *MIR9* expression shows no impact on overall survival (B) and relapse-free survival (D).

Figure 4. Kaplan-Meier analysis with initial WBC count and aberrant *MIR9* expression for overall survival.

When ALL patients are stratified into three groups; patients with both aberrant *MIR9* expression and high initial WBC count, patients with either aberrant *MIR9* expression or high initial WBC count, and patients with neither of them, Kaplan-Meier estimate shows that these three groups are separated each other with statistical significances.