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# Prognostic impact of miR-196a/b expression in adult acute myeloid leukaemia: a single-centre, retrospective cohort study

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## Abstract

**Objective:** The prognostic effect of miR-196a/b expression in adult patients with leukaemia remains unclear. This study aimed to determine whether miR-196a/b expression can serve as a prognostic factor for patients with acute myeloid leukaemia.

**Methods:** We enrolled 124 patients with acute myeloid leukaemia. We measured miR-196a/b expression by real-time reverse transcription-polymerase chain reaction. We classified patients into high and low expression groups based on the median expression value. Cox regression analyses were carried out to assess the prognostic significance of miR-196a/b expression in the context of well-established predictors.

**Results:** Patients with high miR-196a/b expression were older in age, and had higher white blood cell and platelet counts than did patients with low miR-196a/b expression. Patients with high miR-196a/b expression were associated with the French–American–British classification M5 subtype and with the presence of nucleophosmin and FLT3 internal tandem duplication mutations, but were not associated with the favourable karyotype risk subgroup. Moreover, patients with high miR-196a/b expression had a shorter event-free survival rate compared with those with low miR-196a/b expression in univariate and multivariate analyses.

**Conclusion:** High miR-196a/b expression is associated with poor event-free survival.

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**Keywords**

Acute myeloid leukaemia, prognosis, microRNA, miR-196a/b, event-free survival, overall survival

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## Introduction

Acute myeloid leukaemia (AML) is a heterogeneous, haematological malignancy characterized by various genetic abnormalities.[1] Therefore, to improve overall survival, a better understanding of the biology of AML is required. Specific chromosomal aberrations and gene mutations are important for AML prognostic stratification. Moreover, aberrant expression of coding or noncoding genes is also of prognostic relevance. The prognosis of AML remains poor. One of the main reasons for this poor prognosis is the lack of a reliable biomarker for prognostic prediction in clinical practice.

MicroRNA (miRNA) signatures were first identified in leukaemia and came to be known as oncomirs.[2] Previous microarray studies have demonstrated a group of miRNAs that have the ability to predict clinical outcomes, and can help to understand the mechanisms of developing disease.[3,4] In fact, miRNAs are involved in a variety of biological and pathological processes of leukaemia transformation. By perfect or partial pairing with the 3′-untranslated region (3′-UTR) of target mRNAs, miRNAs regulate gene expression without the requirement for changes in DNA sequences. In addition to posttranscriptional gene silencing, miRNA is involved in epigenetic modifications, such as DNA methylation and histone modifications.[5] Importantly, some miRNAs that can play a role as either oncogenes or tumour suppressor genes are also observed in leukaemia. Notable examples of these miRNAs are miR-155, miR-29, miR-150, and miR-196b.[2] Recently, a few studies showed that miR-196b could act as a tumour suppressor by repressing the activity of oncogenes, such as *MEIS1*,[6] annexin A1 (*ANXA1*),[7] homeoboxA9 (*HOXA9*),[6] and *ERG*.[8,9] Additionally, miR-196b can provoke leukaemogenesis by downregulating tumour suppressor genes[10] and proapototic genes- (e.g., *FAS*),[6] or be involved in chemodrug resistance.[11] Dual roles of miR-196b add to the difficulty in defining its effect on the clinical outcome of disease. This difficulty is further compounded because results based on *in vitro* investigations might not be consistent with events *in vivo*. Therefore, investigation of the prognostic value of miR-196a/b in patients with AML could advance our understanding of how miRNA contributes to development of leukaemia. A recent study showed that miR-196b was associated with a poor outcome in paediatric patients with AML.[12] However, the effect of miR-196b in adult AML is unclear. Therefore, we conducted a retrospective study on the prognostic significance of miR-196b expression in adult AML. The main objective of this study was to evaluate the prognostic value of miR-196a/b expression on event-free survival (EFS) in these patients.

## Materials and methods

### Study subjects

In this study, we enrolled 124 patients with *de novo* AML who were diagnosed in our hospital between 2011 and 2016. Patients

with acute promyelocytic leukaemia were excluded from this cohort. All of the patients were treated with standard anthracycline and cytarabine (idarubicin 6–8 mg/m$^2$/day for 3 days, Ara-C 100 mg/m$^2$/day for 7 days) regimen for induction chemotherapy. For consolidation therapy, 114 patients were treated with a high-dose cytarabine-based chemotherapy, and 10 young patients received bone marrow stem cell transplantation. Informed consent was obtained from all of patients who were included in the study. All procedures followed were approved by the Institutional Review Board of our hospital on human experimentation. The research was conducted in accordance with the Helsinki Declaration. Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature.[13] Nucleophosmin (*NPM1*) and FLT3 internal tandem duplication (*FLT3-ITD*) mutations were assessed by DNA sequencing as previously described.[14,15]

## RNA extraction and real-time reverse transcription-polymerase chain reaction

Bone marrow mononuclear cells were purified by Ficoll density gradient centrifugation and total RNA was isolated using Trizol reagents (Invitrogen, Shanghai, China) according to the manufacturer's protocol. Quantification of miRNA was conducted using the ALL-in-one miRNA real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) detection kit (GeneCopeia, Rockville, MD, USA). The assay was carried out on an Applied Biosystems 7900 Real Time PCR machine (Applied Biosystems, Foster City, CA, USA). The primers used for qRT-PCR were miR-196a/b (5′-TAGGTAGTTTCCT GTTGTTGGG-3′) and U6 (5′-TTCGTGA AGCGTTCCATATTTT-3′). The reactions were incubated in a 96-well plate at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 10 s. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method[16] and U6 was used for normalization.

## Overexpression and knockdown of miR-196a/b in HL60 cells by lentiviral infection

Replication-deficient lentivirus encoding human miR-196a/b precursor or inhibitor sponge to overexpress or knockdown the endogenous miR-210 in HL60 was constructed by GenePharma Inc. (Shanghai, China). Virus with a nonspecific miRNA construct was used as control (Ctrl-miR-196a/b). After 12 hours of transfection, cells were replaced with fresh complete culture medium for another 24 hours to measure *HOXA9, MEIS1, ANXA1,* and *ERG* mRNA levels by qRT-PCR as described above. Primers for qRT-PCR were as follows: RT-HOXA9 forward, GATCCCAA TAACCCAGCAGCC; RT-HOXA9 reverse, TCACTCGTCTTTTGC TCGGT; ANXA1 forward, TGCTAGGT GTGGCTTCCTTT; ANXA1 reverse, CACAGTTTG AACATATTCCTGCTC; ERG forward CGCATTATGGCCAGC ACTATT; and ERG reverse, GAGAGTT CCTTGAGCCATTCA).

## Western blot analysis

The transfected HL60 cells were lysed by radioimmune precipitation buffer and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Protein was then transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA) and 5% skim milk was used to block proteins for 1 hour. The blots were detected with primary antibodies against β-actin (1: 1000; Abcam, Cambridge, MA, USA), HOXA9 (1: 1000; Cell Signaling Technology, Danvers, MA, USA), ANXA1 (1: 1000; Cell Signaling

Technology), and ERG (1: 1000, Abcam), at 4°C incubation overnight. After the primary antibodies were washed away, the corresponding secondary antibodies were added (1: 5000; Beyotime, Jiangsu, China) for at least 1 hour at room temperature. Enhanced chemiluminescence solution (Amersham Biosciences, Little Chalfont, UK) was used to detect the target bands.

## Cell viability assay

After transfection with indicated lentivirals, cells were replaced with fresh complete culture medium and 20,000 cells were seeded in 96-well culture plates for another 24 hours of culture. HL60 cell viability was evaluated by the Cell Counting Kit-8 assay (Beyotime) according to the manufacturer's instructions. An absorbance of 450 nm was used to reflect cell viability.

## Statistical analysis

We first subdivided our patients into four quartiles (Q1: < 25%, Q2: 25%–50%, Q3: 51%–75%, and Q4: > 75%) based on miR-196a/b expression levels to determine the group with the best EFS (Figure 1). EFS was used because it comprehensively evaluates the clinical outcome, including response to chemotreatment, the probability of disease relapse, and patients' survival. No significant difference was observed between Q1 and Q2. However, patients in Q3 and Q4 had poorer EFS compared with those in the first two quartiles. Therefore, we dichotomized these patients into high and low groups according to the median values. Patients' characteristics are shown using descriptive statistics, which included frequency counts, median, and range. The relationship between miR-196a/b expression and patients' characteristics was estimated by the nonparametric test and the chi-square test. Overall survival (OS) was measured as the time from diagnosis of disease to death from any cause, or censoring for patients alive at their last follow-up. EFS was defined as the time from the date of diagnosis to removal from the study because of the absence of complete remission, relapse, or death. OS and EFS were censored on days for patients with bone marrow transplantation. The Kaplan–Meier method was used in univariate analysis and the Cox proportional hazard regression model in multivariate analysis to determine the prognostic value

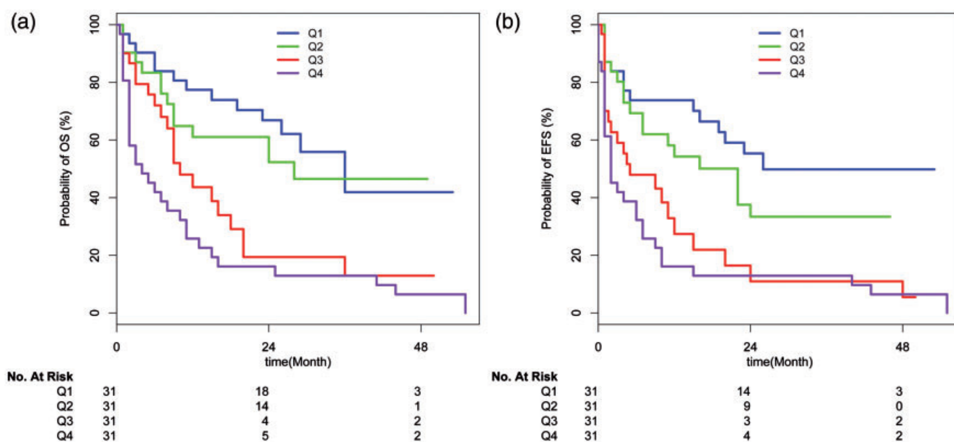


**Figure 1.** Kaplan–Meier estimates of overall survival (a) and event-free survival (b) by four quartiles (Q1: < 25%, Q2: 25%–50%, Q3: 51%–75%, and Q4: > 75%) of miR-196b expression levels

of miR-196a/b expression. All statistical analyses were conducted with R statistic package, version 3.3.1 (www.r-project.org). $P < 0.05$ indicates a statistical difference.

## Results

### Patient characteristics

Clinical features of 124 patients with high and low miR-196a/b expression are shown in Table 1. Patients with high miR-196a/b expression had an older age ($P = 0.002$) and higher white blood cell and platelet counts ($P = 0.007$, $P = 0.041$, respectively) compared with those with low expression. Additionally, patients with high miR-196a/b expression more frequently had the French–American–British (FAB) classification M5 subtype ($P = 0.022$), and were more associated with the presence of *NPM1* and *FLT3-ITD* mutations (both $P < 0.001$) compared with those with low miR-196a/b expression. Patients with high miR-196a/b expression also had a significantly lower frequency of a favourable karyotype ($P = 0.004$) and FAB M2 subtype ($P = 0.022$) compared with those with low miR-196a/b expression. There were no significant associations between miR-196a/b expression and other

**Table 1.** Characteristics of patients with acute myeloid leukaemia and high or low miR-196a/b expression

| | Low expression | High expression | P value |
|---|---|---|---|
| Number of patients (%) | 62 (50%) | 62 (50%) | |
| miR-196a/b, mean (SD) | 0.31 (0.11) | 1.17 (0.78) | |
| Age, mean (range), years | 36.5 (26–53) | 47.5 (37–61) | 0.002 |
| Female sex, n (%) | 21 (33.9%) | 30 (48.4%) | 0.144 |
| WBC, mean (SD), $\times 10^9$/L | 46.30 (36.21) | 71.37 (61.40) | 0.007 |
| HB, mean (SD), g/L | 82.56 (19.84) | 86.50 (21.98) | 0.297 |
| PLT, mean (SD), $\times 10^9$/L | 47.01 (68.72) | 70.22 (55.43) | 0.041 |
| BM blast, mean (SD), % | 70.17 (18.27) | 72.68 (22.43) | 0.497 |
| FAB classification, n (%) | | | 0.022 |
| M0 | 0 (0%) | 1 (1.6%) | |
| M1 | 13 (21.0%) | 17 (27.4%) | |
| M2 | 30 (48.4%) | 13 (21.0%) | |
| M4 | 10 (16.1%) | 11 (17.7%) | |
| M5 | 6 (9.7%) | 17 (27.4%) | |
| M6 | 2 (3.2%) | 3 (4.8%) | |
| Unclassified | 1 (1.6%) | 0 (0%) | |
| Karyotype risk, n(%) | | | 0.004 |
| Favourable | 14 (22.6%) | 2 (3.2%) | |
| Intermediate | 43 (69.4%) | 56 (90.3%) | |
| Unfavourable | 5 (8.1%) | 4 (6.5%) | |
| Gene mutations, n (%) | | | |
| *NPM1*+/*FLT3*-ITD− | 0 (0%) | 17 (24.7%) | <0.001 |
| *NPM1* | 1 (1.6%) | 33 (53.2%) | <0.001 |
| *FLT3-ITD* | 4 (6.5%) | 31 (50.0%) | <0.001 |

Abbreviations: WBC, white blood cell; HB, haemoglobin; PLT, platelet count; BM, bone marrow; FAB, French–American–British classification; *NPM1*, nucleophosmin; *FLT3*-ITD, FLT3 internal tandem duplication; SD, standard deviation.

**Table 2.** Multivariable analyses for overall survival and event-free survival in patients with acute myeloid leukaemia

| | Overall survival | | Event-free survival | |
|---|---|---|---|---|
| | P value | HR (95% CI) | P value | HR (95% CI) |
| High vs. low miR-196a/b | 0.052 | 1.845 (0.996–3.417) | 0.027 | 1.955 (1.078–3.545) |
| Age | < 0.001 | 1.034 (1.017–1.051) | 0.023 | 1.018 (1.002–1.034) |
| White blood cell count | 0.69 | 1.001 (0.996–1.006) | 0.597 | 1.001 (0.996–1.006) |
| Karyotype | | | | |
| Intermediate vs. favourable | 0.701 | 1.17 (0.525–2.608) | 0.894 | 1.052 (0.499–2.22) |
| Poor vs. favourable | 0.023 | 3.487 (1.19–10.216) | 0.035 | 2.968 (1.078–8.169) |
| Gene mutations (mutation vs. wild-type) | | | | |
| *FLT3-ITD* | 0.056 | 1.702 (0.987–2.935) | 0.185 | 1.433 (0.842–2.44) |
| *NPM1* | 0.617 | 1.16 (0.648–2.074) | 0.865 | 1.051 (0.592–1.867) |

Abbreviations: HR, hazard ratio; CI, confidence interval; *NPM1,* nucleophosmin; *FLT3*-ITD, FLT3 internal tandem duplication.

variables, including sex, haemoglobin, and the percentage of bone marrow blasts (Table 1).

## *miR-196a/b expression is associated with a poor outcome*

In this cohort of patients, patients with high miR-196a/b expression (n = 62) had poorer OS and EFS than did those with low miR-196a/b expression (n = 62) (Figure 1). White blood cell counts tended to predict a poor outcome, but this was not significant. In multivariate analysis, miR-196a/b expression was still an independent prognostic factor for EFS and an association with OS was found after analysis was adjusted for age, white blood cell count, karyotype risk groups, and *FLT3-ITD* and *NPM1* gene mutations (EFS: hazard ratio [95% confidence interval], 1.955 [1.078–3.545]; P = 0.027; OS: hazard ratio [95% confidence interval], 1.845 [0.996–3.417]; P = 0.052; Table 2).

## *Overexpression of miR-196a/b enhances cellular proliferation*

To gain insight into the function of miR-196a/b, we overexpressed or knocked down miR-196a/b in HL60 cells through

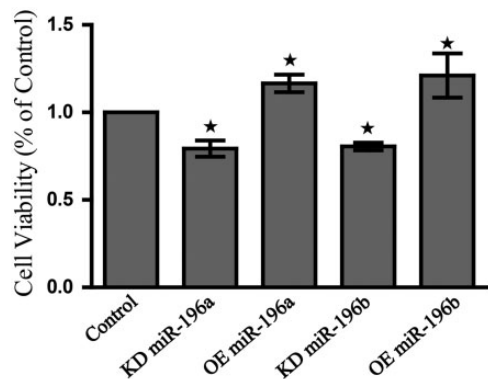


**Figure 2.** Overexpression of miR-196a/b enhances cellular proliferation, while knockdown of miR-196a/b inhibits HL60 cellular proliferation

lentivirals. Transient overexpression of miR-196a/b significantly promoted cellular proliferation (Figure 2), while knockdown of miR-196a/b inhibited proliferation of HL60 cells (both P < 0.05).

## *miR-196a/b regulates target genes*

To verify that miR-196a/b regulates expression of *HOXA9, ANXA1,* and *ERG*, the miR-196a/b precursor was transfected into HL-60 by lentivirals. As shown in Figure 3a,

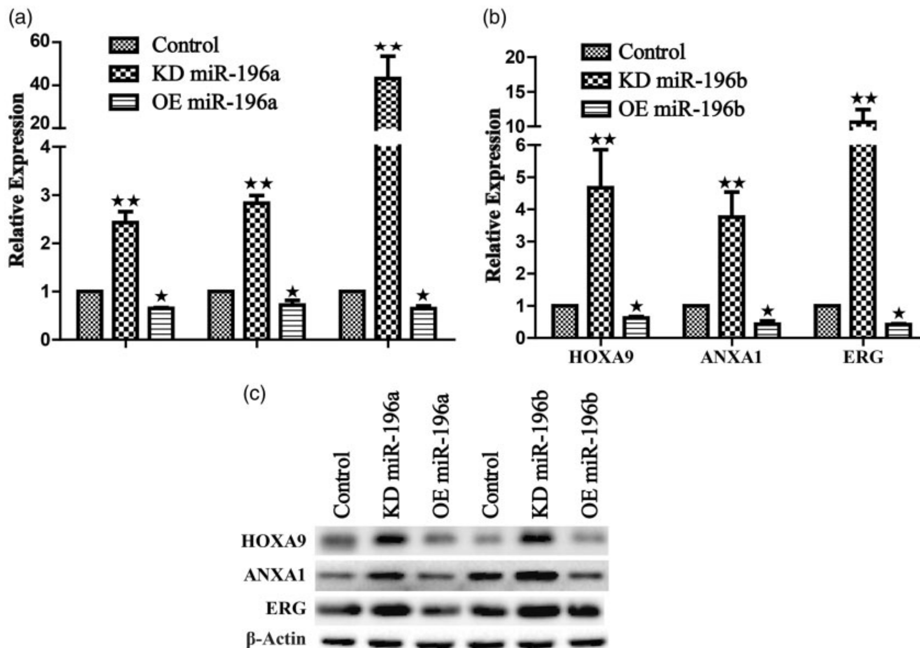


**Figure 3.** miR-196a/b regulates expression of *HOXA9, ANXA1,* and *ERG*. Real-time reverse-transcription polymerase chain reaction data for relative expression of *HOXA9, ANXA1,* and *ERG* are shown with miR-196a (a) and miR-196b (b) overexpression or knockdown. Protein expression levels of HOXA9, ANXA1, and ERG were detected by western blot. The data are presented as mean ± SD ($^{*}P < 0.05$, $^{**}P < 0.01$). Abbreviations: *HOXA9,* homeoboxA9; *ANXA1*. annexin A1

knockdown of miR-196a resulted in a significant increase in *HOXA9* mRNA by 2.43 fold, *ANXA1* mRNA by 2.83 fold, and *ERG* mRNA by 43.17 fold compared with controls (all $P < 0.01$). Knockdown of miR-196b also increased the levels of *HOXA9, ANXA1,* and *ERG* mRNA by 4.67, 3.76, and 10.61 fold, respectively (all $P < 0.01$). However, expression levels of *HOXA9, ANXA1,* and *ERG* mRNA in miR-196a/b overexpressed cells were lower compared with those in controls (all $P < 0.05$). Western blotting also showed that *HOXA9, ANXA1,* and *ERG* were target genes of miR-196a/b (Figure 3c).

## Discussion

The discovery of miRNAs may be one of the most major advances in biological and medical sciences in the last decade.[17] Most of these miRNAs have been implicated in cancer development through regulating either oncogenes or tumour suppressor genes.[8] In this study, we aimed to investigate the clinical significance of miR-196a/b in adult patients with AML.

Mature miR-196a and miR-196a/b belong to the miR-196 family and differ from each other by only one nucleotide. The gene encoding miR-196a/b is located at the mammalian homeobox (HOX) clusters, and several *HOX* genes are targets of miR-196a/b.[18] Functionally, miR-196a/b, but not miR-196a, has been reported to play crucial roles in the pathogenesis of disease,[10] particularly in AML.[17] Additionally, downregulation of miR-196a/b has been reported in several tumours, including cervical cancer,[19] B- and T-leukemia[10] and

melanoma.[20] These studies indicated that miR-196a/b might function as a tumour suppressor gene in tumour development. In contrast, upregulated expression of miR-196a/b was observed in pancreatic adenocarcinoma, breast cancer, oesophageal adenocarcinoma, and colonic cancer.[21] This finding suggests that miR-196a/b expression acts as an oncogene in tumour development. Taken together, these results indicate that miR-196a/b expression may function differently in the development of different cancers. Notably, an *in vivo* study showed that high miR-196a/b expression levels were associated with adverse survival in patients with AML.[20] However, in animal models, miR-196a/b expression significantly delayed leukaemogenesis, and resulted in prolonged overall survival.[10] These contradictory results from animal models and a cohort of patients regarding survival need to be validated in an independent study. Therefore, we conducted a retrospective study to focus on this issue.

In this study, we found that miR-196a/b expression levels were positively associated with some well-established adverse predictors, such as age and the presence of the *FLT3-ITD* mutation. Additionally, high miR-196a/b expression was negatively associated with favourable prognostic factors, such as *NPM1* gene mutation and higher platelet counts. These contradictory findings might reflect the dual roles of miR-196a/b in regulating oncogenes and tumour suppressor genes in blasts. However, in multivariate analysis after adjusting for favourable and unfavourable predictors, we found that miR-196a/b expression was only an independent predictor for EFS. This finding might be explained by the fact that high miR-196a/b expression is a biomarker for chemoresistance.[22] Patients with high miR-196a/b levels might obtain multiple chemoresistance,[22] leading to relapse. Our findings suggested that miR-196a/b expression was associated with a shorter EFS in patients with AML.

There are a few limitations to this study. First, our study was a retrospective design. Further, large-scale, prospective research is required to clarify the exact roles of miR-196a/b expression in AML. Second, we did not study the functional roles of miR-196a/b expression in AML blasts, but our results had some implications for miR-196a/b acting as an oncogene in development of AML. Finally, we used qRT-PCR to measure expression of miR-196a/b. Therefore, we may not have discriminated the high homology between miR-196a and miR-196a/b because there is only one nucleotide difference in the sequence.

In conclusion, our study shows that miR-196a/b expression is associated with an adverse EFS in patients with AML.

## Declaration of conflicting interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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