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Polo-like kinase 2 (*SNK/PLK2*) is a novel epigenetically regulated gene in acute myeloid leukemia and myelodysplastic syndromes: genetic and epigenetic interactions

Leonidas Benetatos¹, Aggeliki Dasoula¹, Eleftheria Hatzimichael¹, Nelofer Syed², Maria Voukelatou¹, George Dranitsaris³, Konstantinos L. Bourantas¹, Tim Crook²

¹Department of Hematology, University Hospital of Ioannina, Greece

²Imperial College, Charing Cross Hospital, London, United Kingdom

³Department of Medical Oncology, Princess Margaret Hospital, Toronto, Canada

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Corresponding author

Leonidas Benetatos

Department of Hematology, University Hospital of Ioannina

Niarchos Avenue, 45500

Ioannina

GREECE

Tel: +302651099729

Fax: +302651046617

e-mail: benetatosleon@yahoo.com

me01532@cc.uoi.gr

Abstract

Polo-like kinase 2 (SNK/PLK2), a transcriptional target for wild-type p53 and is hypermethylated in a high percentage of multiple myeloma and B-cell lymphomas patients. Given these data, we sought to study the methylation status of the specific gene in AML and MDS, and to correlate it with clinical and genetic features. Using methylation specific PCR (MSP), we analyzed the methylation profile of 45 cases of acute myeloid leukemia (AML) and 43 cases of myelodysplastic syndrome (MDS). We also studied the distribution of MTHFR A1298C and MTHFR C677T polymorphisms and FLT3 mutations in AML patients, and correlated the results with hypermethylation in the SNK/PLK2 CpG island. The SNK/PLK2 CpG island was hypermethylated in 68.9% and 78.4% of AML and MDS cases respectively. Cases with hypermethylation had a trend towards more favourable overall survival (OS). There was no association between different MTHFR genotypes and susceptibility to develop AML. **SNK/PLK2 hypermethylation combined with the MTHFR AA1298 genotype was associated with a tendency for a better OS. Similarly, patients with SNK/PLK2 hypermethylation combined with the MTHFR CT677 polymorphism had a better OS (HR=0.34, p= 0.017). SNK/PLK2 methylation associated with unmutated FLT3 cases had a trend for better OS compared to patients with mutated FLT3 gene. SNK/PLK2 is a novel epigenetically regulated gene in AML and MDS and methylation occurs at high frequency in both diseases. As such SNK/PLK2 could represent a potential pathogenetic factor, although additional studies are necessary to verify its exact role in disease pathogenesis.**

Key words: *SNK/PLK2*, methylation, AML, MDS, *MTHFR* polymorphisms

Introduction

DNA cytosine methylation is the best characterized epigenetic marker, and is involved in the control of gene expression and the nuclear architecture of the cell [1]. Dynamic changes in promoter methylation are important for the timely and appropriate expression of growth factors and their cognate receptors, cytokines and other molecules necessary for normal myeloid development [2]. Aberrant hypermethylation in the CpG islands frequently results in the transcriptional silencing of tumour suppressor genes and is an important event in carcinogenesis [1,3]. Genes from multiple functional classes are subject to silencing in human cancer, including those involved in DNA repair, apoptosis, angiogenesis, cell cycle regulation, cell-to-cell interaction, and in metabolism of carcinogens. CpG methylation is established and maintained by the DNA methyltransferases [4], and aberrant expression or recruitment of the DNA methyltransferases may result in hypermethylation of specific target genes, thus contributing to oncogenesis-leukemogenesis [5]. In hematopoietic neoplasia, DNA hypermethylation is a common gene inactivating mechanism and disease-specific methylation patterns for individual genes have been described [6]. In acute myeloid leukemia (AML), aberrant hypermethylation is common, with 70% of AML cases having at least one methylated gene at diagnosis, and hypermethylated genes have clinical implications. Methylation of genes involved in cell cycle control and apoptosis may also play a role in the pathogenesis of myelodysplastic syndromes (MDS) [6].

The Polo-like kinases (*PLK*) are a highly conserved family of proteins implicated in regulation of the G2-M transition and mitotic entry during the cell cycle. *PLK* also play a role in bipolar spindle formation during mitosis, centrosome maturation and

separation, and M-phase exit through destruction of cyclin B and inactivation of *p34^{cdc2}* [7].

Polo-like kinase 2 (*SNK/PLK2*) is expressed in a tissue specific manner in neurons, heart and lung [8]. The protein has a role in regulating cell proliferation in G1 phase [9], is required for centriole duplication and centrosome function during S phase [9,10] and is also a cellular stress responsive gene [11]. *SNK/PLK2* may have a role in embryogenesis as *SNK/PLK2*^{-/-} mouse embryos show a delay in skeletal development and retarded growth [12]. *SNK/PLK2* is transcriptionally induced by *p53* and its silencing in the presence of cytotoxic drugs such as taxol results in increased apoptosis [13]. *SNK/PLK2* protein may possess oncogenic activity [8] as its deregulation results in multinucleated phenotypes, one of the hallmarks in carcinogenesis [14]. It is known that centrosome abnormalities are present early in the development of aplastic anemia, AML and MDS, including numerical and structural centrosome aberrations which positively correlate with numerical chromosome changes [15]. *SNK/PLK2* also has tumour suppressor functions which are regulated by the tumor microenvironment and may inhibit mTOR pathway through interaction with the TSC1 and TSC2 complexes [8,16]. *SNK/PLK2* has been found to be hypermethylated and consequently silenced in a high percentage of multiple myeloma [17] and B-cell lymphomas patients but not in breast, and in head and neck carcinomas [18], suggesting a physiological function in regulation of B-cell proliferation and differentiation. As these results imply that *SNK/PLK2* hypermethylation is hematopoietic tissue-specific, we sought to study the methylation status of the specific gene in AML and MDS, and to correlate it with clinical and genetic features.

Materials and methods

Patients

Eighty-eight patients with myeloid malignancies who presented at the Department of Haematology of the University Hospital of Ioannina were included in the study. Bone marrow and peripheral blood genomic DNA from 45 AML patients (51.1%) and 43 MDS patients (48.9%) was obtained at diagnosis. None of the patients had received any prior treatment. There were 30 female patients (34.1%) and 58 male patients (65.9%), with mean age 71.5 ± 13.7 years (range 16-92 years). All patients were classified according to the revised 2008 WHO classification [19] and MDS diagnosis was based on the recently published diagnostic criteria [20]. The WHO classification-based prognostic scoring system for MDS (WPSS) [21] was used for risk stratification of MDS patients. Conventional cytogenetic analysis was performed in all but 12 AML patients and all patients were subdivided into good, intermediate and poor karyotypes as described in the WPSS definition for MDS, and the SWOG/ECOG cytogenetic classification system for AML patients [22]. MDS patients with no available cytogenetic analysis and patients who were lost to follow-up were excluded from the study. All patients were further evaluated, for statistical purposes, according to their haematological parameters as having anaemia (haemoglobin <100 g/l), leukocytosis or leukopenia (white blood cell count < $1.5 \times 10^9/l$ or $>30 \times 10^9/l$), and thrombocytopenia (platelet count < $100 \times 10^9/l$). Treatment in AML patients consisted of Ara-C and idarubicin, followed by consolidation with intermediate/high dose Ara-C, idarubicin \pm etoposide or azacytidine for patients not suitable for intensive chemotherapy. Treatment in MDS patients consisted of red blood cell and/or platelet transfusions as required, recombinant human erythropoietin, granulocyte-colony stimulating factor administration, and azacytidine. Patient characteristics are shown in Table 1.

Bone marrow samples obtained from 15 patients who underwent diagnostic procedures for borderline cytopenias, and were proved to have no hematological malignancies or solid tumor infiltration were controls.

Informed consent was obtained from all patients and control subjects, and the study was approved by the Scientific Committee of the University Hospital of Ioannina.

SNK/PLK2 methylation analysis

DNA extraction from bone marrow nucleated cells and bisulphite treatment were performed using the QIAamp DNA blood mini Kit (Qiagen), and the EZ-DNA methylation kit (Zymo Research Inc.) respectively, according to the manufacturers' protocols. The methylation status of the *SNK/PLK2* CpG island was determined by using methylation-specific PCR (MSP) using the HotStarTaqMaster Mix kit (Qiagen). All PCR reactions were run in duplicates with positive controls DNA (CpG GenomeTM Universal Methylated, Chemicon International), and blanks (samples containing no DNA).

Methylenetetrahydrofolatereductase (MTHFR) A1298C and C677T genotyping

Genotyping for both *MTHFR* polymorphisms used genomic DNA extracted from peripheral blood with the QIAamp DNA mini blood kit (Qiagen), as described previously [23]. For PCR, the HotStarTaq Master Mix kit was used according to the manufacturer's instructions (Qiagen). PCR reactions were carried out using the HotStarTaq DNA polymerase (Qiagen). Amplified *MTHFR* A1298C PCR products (163bp) were digested with MboII (New England BioLabs), resolved on 4% agarose gels and visualised after ethidium bromide staining. Wild type (1298AA) produced

fragments of 56, 31, 30, 28 and 18bp, heterozygotes (1298AC) produced fragments of 84, 56, 31,30, 28 and 18bp and homozygotes (1298CC) produced fragments of 84, 31,30, and 18bp. *MTHFR* C677T-amplified PCR products (198bp) were digested with the *HinfI* enzyme (New England BioLabs), producing a single band at 198bp for wild type 677CC, fragments of 198, 175, and 23bp for 677CT heterozygotes, and fragments of 175 and 23bp for homozygous 677TT.

FLT3 mutation analysis

Genomic DNA was isolated from bone marrow samples using the QIAamp DNA mini blood kit (Qiagen) according to the manufacturer's instructions. Mutational analysis of internal tandem duplication (ITD) of the *FLT3* gene was restricted to exons 11 and 12. Primers used in the experimental procedures are shown in Table 2.

Statistical analysis

Demographic and clinical data were analyzed descriptively as means, medians, or proportions. Survival curves were generated using the method of Kaplan-Meier and compared with the log-rank test. Patients alive or lost to follow up as of after March 1, 2010 were censored. In an exploratory analysis the relative risk of death between various subgroups such as *SNK/PLK2* was estimated using Cox Proportional Hazard regression with only a single predictor variable remaining in the final model. All of the statistical analyses were performed using Stata, release 9.0 (Sata Corp., College Station, Texas, USA).

Results

SNK/PLK2 is methylated in MDS

Using MSP, we analyzed methylation in the CpG island of *SNK/PLK2* in a series of 88 bone marrow samples from patients with myeloid malignancies. Methylation in *SNK/PLK2* was observed in 69/88 cases (78.4%) and in none of the control samples that were examined ($p=0.0004$) (Figure 1). Methylation was detected in 38 of 43 (88.4%) MDS patients, including 6 patients (14%) with refractory anemia, 2 patients (4.7%) with refractory neutropenia, 1 patient (2.3%) with refractory thrombocytopenia, 16 patients (37.2%) with refractory cytopenias with multi-lineage dysplasia, 5 patients (11.6%) with refractory anemia with excess of blasts-I, and 6 patients (14%) with refractory anemia with excess of blasts-II. Twenty-nine (76.3%) presented good prognosis karyotype, 3 (7.9%) had intermediate risk karyotype, and 6 (15.8%) had poor prognosis karyotype. With regard to the WPSS prognostic system 6 patients (15.8%) belonged to the very low risk group of patients, 12 patients (31.6%) belonged to the low risk group. *SNK/PLK2* methylation was detected in 8 (21.1%), 9 (23.7%), and 3 (8%) patients of the intermediate risk, high risk, and very high risk group respectively. No significant association was observed between blood cell counts, WHO subtype, WPSS score, cytogenetic features and methylation. MDS patients' data presenting *SNK/PLK2* methylation are shown on Table 3. Median overall survival (OS) for the entire group of patients was 8 months and 21 months for the MDS patients. Patients with methylated *SNK/PLK2* had a median OS of 10 months while individuals lacking methylation had a median OS of 6 months, a difference that was not significant ($HR=0.86$, $p=0.62$). MDS patients had a median OS of 21 months and of 19.5 months, for those patients with abnormally methylated

and unmethylated gene respectively. However, the difference in OS was not significant (HR=1.35, p=0.62).

SNK/PLK2 is methylated in AML

SNK/PLK2 methylation was detected in 31 of 45 (68.9%) AML patients, of which 2 (6.5%), 15 (48.4%), and 4 (12.9%) patients presented good, intermediate, and bad prognosis karyotype respectively. According to the WHO classification 1 patient (2.2%) was characterized by the t(8;21) genetic abnormality, 1 patient (2.2%) had the t(15;17) characteristic abnormality, 10 patients (23.3%) had AML with myelodysplasia-related changes, 1 patient (2.2%) had therapy related AML, 4 patients (8.9%) had AML with minimal differentiation, 6 patients (13%) had AML without maturation, 3 patients (6.7%) had AML with maturation, 3 patients (6.7%) had acute myelomonocytic leukemia, and finally 2 patients (4.4%) had acute monoblastic/monocytic leukemia. Patients' data are shown on Table 4. As with the MDS patients, no significant association between *SNK/PLK2* methylation, karyotype, and clinical parameters was observed. Median OS was 3.5 months for all AML patients, 3 and 5 months for those with unmethylated and methylated *SNK/PLK2* respectively. Kaplan-Meier **curves** for AML and MDS are shown in table 5. The difference among the two groups was not significant (HR=1.03, p=0.9).

MTHFR A1298C and C677T in AML

The distribution of *MTHFR* A1298C and C677T polymorphisms was determined in all AML patients. First, for the *MTHFR* 1298AA, 1298AC, and 1298CC

polymorphisms we observed 27 (60%), 14 (31.1%), and 4 (8.9%) AML cases respectively. Of the cases with *SNK/PLK2* methylation, 19 patients, (63.1%) were of AA genotype, while 9 patients (29%) had the AC polymorphism and 3 patients (9.7%) had the CC polymorphism. There was no significant association between distribution of polymorphisms and blood cell counts, WHO subtypes and karyotype. Median OS was 3 months, 7 months, and 2 months for the AA, AC, and the CC polymorphisms respectively. Cox regression analysis relative to the AA polymorphism demonstrated that the difference in OS between patients with the various A1298C genotypes was not significant (HR=1.44, p=0.32) (Table 6a). Comparison among patients with *SNK/PLK2* methylation and the A1298C polymorphisms demonstrated that those patients who had both hypermethylated *SNK/PLK2* and the AA genotype had a tendency for a better OS (HR=1.80, p=0.37) in comparison with those individuals with the AC and CC genotypes (Table 6b).

The distribution, of the C677T polymorphisms was as follows: 9 patients (20%) presented the CC genotype, 31 patients (68.9%) had the CT genotype, and 5 patients (11.1%) had the TT genotype. *SNK/PLK2* methylation was observed in 7 patients (22.6%), in 22 patients (71%), and in 2 patients (6.4%) with the CC, CT, and TT genotypes respectively. Again, no significant association between distribution of polymorphisms and blood cell counts, WHO subtypes and karyotype was observed. Median OS was 2 months for the CC genotype, 7 months for the CT genotype, and 6 months for those patients with the TT genotype. Survival analysis relative to the CC genotype shown that AML patients with the CT polymorphism had a better OS (HR=0.34, p= 0.017) (Table 6c). In respect to the methylation status of the *SNK/PLK2* and C677T subtypes, patients with both *SNK/PLK2* methylation and the CT genotype had a better OS (HR=0.3, p=0.05) (Table 6d).

FLT3 mutation analysis in AML

FLT3-ITD was detected in only 7 (15.6%) AML patients and there were no significant findings in comparison to their various clinical features. *SNK/PLK2* methylation was detected in 4 (57.1%) out of the 7 patients. Median OS was 2 months and 6 months for patients who were positive and negative for FLT3-ITD respectively. Individuals with the FLT3-ITD had a shorter OS although not significant ($p=0.08$ by the long rank test). Those patients with both methylated *SNK/PLK2* and unmutated *FLT3* gene had a trend for better OS (HR=0.38, $p=0.1$) in comparison to the patients harbouring *FLT3* mutations and *SNK/PLK2*/ methylation. No impact on OS between association of the FLT3-ITD, *MTHFR* C677T and *MTHFR* A1298C was observed.

Discussion

Acute myeloid leukemia is a heterogeneous group of clonal hematopoietic progenitor cell disorders with a diverse range of morphologic, cytogenetic, immunophenotypic and molecular features [24]. The MDS are characterized by stem cell clonal myelopoiesis, commonly a hypercellular-dysplastic bone marrow, peripheral blood cytopenias and a tendency to progression to AML [25,26]. The molecular pathogenesis of both disorders is characterized by genetic changes commonly targeting genes encoding proteins functioning in cell proliferation and myeloid differentiation [22,27]. In addition to the genetic changes, DNA methylation and associated gene silencing play an important role in the pathogenesis of AML and MDS [28]. Elegant work by Figueroa et al [29] showed that CpG methylation is not randomly distributed in leukemic blasts and so AML can be categorized and grouped according to the methylation profile. In the same study, it was also shown that oncoproteins can drive methylation resulting in leukemogenesis and that clinical outcome can be predicted independent of cytogenetic class. A common epigenetic signature implicated in leukemogenesis was also described [29]. Moreover, aberrant promoter methylation, in terms of frequency and density, correlate with clinical outcome and is also increased at disease relapse [30,31]. As in AML, DNA methylation occurs in MDS, is associated with biological and clinical features of the disease, and appears to be a progressive process already present in low-risk MDS that significantly increases as the disease evolves [32-34]. A prognostic model based on the “methylotype” has recently been proposed for MDS in an effort to predict overall and progression-free survival probabilities, adding a useful prognostic tool [35].

In the present study we report the identification of *SNK/PLK2* as a novel hypermethylated gene in a high percentage of patients with myeloid malignancies.

The frequency of methylation was higher in MDS (88.4%) than in AML (68.9%). We also observed that aberrant methylation was detected in 26 (68.4%) low and intermediate risk MDS patients in comparison to the 12 (31.6%) higher risk groups of patients ($p=0.04$) (same results were obtained when the IPSS was used instead of the WPSS). This finding is a potential explanation, at least in part, for the increased apoptosis that characterizes early stage MDS. Although we did not demonstrate transcriptional silencing of the gene, it is well known that CpG islands methylation is associated with aberrant transcription silencing and inactivation of tumour suppressor genes [36], and as drug induced silencing of the *SNK/PLK2* gene results in accentuated apoptosis [13], aberrant cytosine methylation of the specific gene can be a potential mechanism of increased apoptosis in early MDS. Methylation-dependent transcriptional silencing of *SNK/PLK2* has previously been demonstrated in Burkitt (and other) B-cell lymphomas [18]. The present data further extend the involvement of down-regulation of this gene in hematological neoplasia. An interesting finding was also a tendency for improved survival for both AML and MDS patients who displayed hypermethylation of the *SNK/PLK2* gene, suggesting that *SNK/PLK2* may have utility as a prognostic biomarker in myeloid malignancies.

MTHFR activity plays an important role in DNA synthesis and methylation reactions, and its reduced activity has been associated with abnormal DNA synthesis and repair, chromosomal instability, and alterations of the key pathway of the methylation resulting in an increased cancer risk [37]. We were therefore interested to establish the distribution of the two most common polymorphisms, *MTHFR* A1298C and *MTHFR* C677T, in the AML group of patients and to determine whether correlations exist between methylation in the *SNK/PLK2* CpG island and specific *MTHFR* genotypes. The frequencies of the C677C, C677T, and T677T polymorphisms were 20%, 68.9%,

and 11.1% in the AML cases, while A1298A, A1298C, and C1298C were present in 60%, 31%, and 8.9% of cases respectively. There were no significant differences in allele frequencies between cases and controls (we studied 70 samples of healthy blood donors-data not shown), arguing against association between different genotypes and the susceptibility to develop AML. Our data are consistent with those of other studies who evaluated the two *MTHFR* polymorphisms in adult AML patients [38-40]. Patients with the C677T genotype had a better OS with a relative risk of death reduced by 66% (HR=0.34, p= 0.017), while the *MTHFR* A1298C polymorphism had no impact on OS. However, the CC genotypes (for both polymorphisms) were positively associated with *SNK/PLK2* methylation (Table 4). *SNK/PLK2* methylation was associated with a trend for better OS when considered together to A1298A genotype (HR=1.80, p=0.37), and with a better OS (HR=0.3, p=0.05) and a 64% relative reduction in the risk of death when considered with the C677T genotype, in comparison to all other polymorphisms. Our results indicate that although *MTHFR* polymorphisms do not play a direct role in the risk of AML development, they may be indirectly implicated in the pathogenesis of the disease as they do correlate with the aberrant methylation of the *SNK/PLK2* CpG islands, and that their association could predict prognosis.

The tyrosine kinase receptor *FLT3* controls normal hematopoiesis through regulation of transcription, proliferation, metabolism and apoptosis, and is one of the most commonly mutated genes in AML. The FLT3-ITD type of mutation is seen in 15-35% of AML patients and is associated with an overall poor prognosis disease [41]. FLT3-ITD was detected in 15.6% of AML cases consistent with published literature. FLT3-ITD positive patients had shorter OS in comparison with the FLT3-ITD negative individuals, a difference that was not significant. We also found that

AML patients harbouring both the *FLT3* mutation and *SNK/PLK2* methylation had a trend for worse survival in relation to those patients who were *FLT3* wild-type and *SNK/PLK2* methylated. *FLT3*-ITD positive patients are characterized by cytokine-independent cellular proliferation, and one can argue that reduced survival is expected in these subjects, but given the statistic results, we can hypothesize that *SNK/PLK2* methylation, and consequent gene silencing might affect the apoptosis/proliferation ratio.

In conclusion, we have identified *SNK/PLK2* as a novel **epigenetically regulated gene** in AML and MDS and we show that methylation occurs at high frequency in both diseases. We have studied possible interactions between *SNK/PLK2* methylation and the molecular marker *FLT3* (an established genetic change in AML) and with germline *MTHFR* polymorphisms which are important for appropriate DNA methylation. We have also tested the possibility that analysis of these molecular genetic variables might impact on clinical outcome. **Drug-induced *SNK/PLK2* silencing has been determined to lead in increased cell apoptosis rate [13], which is also a characteristic of early stage MDS. Thus, we assume that reactivation of *SNK/PLK2* expression with hypomethylating agents used for the treatment of MDS could be of help, probably by decreasing the pronounced apoptosis rate observed in this subset of MDS. Moreover, given that *SNK/PLK2* methylation has been demonstrated to be involved in the pathogenesis of B-cell lymphomas [18], we hypothesize that it might also be associated with the pathogenesis of myeloid malignancies. It will clearly be of interest to extend our finding to a larger patient population to verify these initial observations.**

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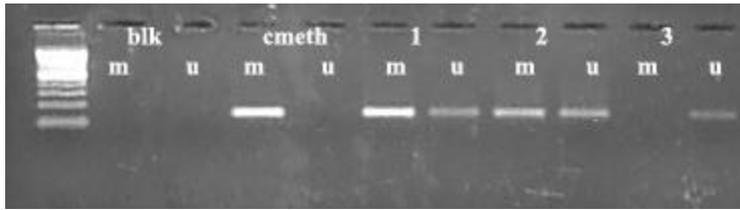
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Figure 1. Methylation specific PCR on SNK/PLK2 methylation. Sample blk (blank) indicates a sample containing no DNA. Sample cmeth (methylated control) is a commercially available universally methylated sample. Samples 1 and 2 belong to patients with abnormal methylation of the SNK/PLK2 gene. Both bands for the unmethylated and methylated allele are generated. Sample 3 belong to a subject with normal methylation profile. m: methylated, u: unmethylated, L: ladder



Patients' Characteristics

Gender

Male	58 (65.9%)
Female	30 (34.1%)

Age (range) **71.5±13.2 (16-92)**

Blood cell count

Hb gr/dl (mean-range)	9±1.7 (3.4-13.2)
WBC x10 ⁹ /l (mean-range)	18.9±36.2 (0.2-200)
Plt x10 ⁹ /l (mean-range)	105.9±120.5 (1-650)

Cytogenetics

Favorable	35 (46%)
Intermediate	23 (30.3%)
Adverse	18 (23.7%)

Acute Myeloid Leukemia **45(51.1%)**

t(8;21)	1 (1.1%)
t(15;17)	1 (1.1%)
with myelodysplasia related changes	16 (18.2%)
therapy-related myeloid neoplasms	1 (1.1%)
with minimal differentiation	5 (5.7%)
without maturation	9 (10.2%)
with maturation	5 (5.7%)
acute myelomonocytic leukemia	3 (3.4%)
acute monoblastic/monocytic leukemia	4 (4.6%)

FLT3-ITD

Positive	7 (15.6%)
Negative	38 (84.4%)

MTHFR A1298C

AA	27 (60%)
AC	14 (31.1%)
CC	4 (8.9%)

MTHFR C677T

CC	9 (20%)
CT	31 (68.9%)
TT	5 (11.1%)
<i>Myelodysplastic syndromes</i>	43 (48.9%)
RA	8 (9.1%)
RN	2 (2.3%)
RT	1 (1.1%)
RCMD	17 (19.3%)
RAEB-I	7 (8%)
RAEB-II	6 (6.8%)
del 5q	2 (2.3%)
WPSS	
Very low	7 (16.3%)
Low	13 (30.2%)
Intermediate	10 (23.3%)
High	10 (23.3%)
Very high	3 (7%)

Table 1: Patients' characteristics participating in the study

Hb: hemoglobin, WBC: white blood cell count, Plt: platelet count, FLT3-ITD: FLT3-internal tandem duplication, MTHFR: methylenetetrahydrofolate reductase, RA: refractory anemia, RN: refractory neutropenia, RT: refractory thrombocytopenia, RCMD: refractory cytopenias with multilineage dysplasia, RAEB: refractory anemia with excess blasts, WPSS: WHO based classification prognostic scoring system

Gene	Primers	T_{ann}/Cycles
<i>SNK/PLK2</i>	MF: 5'-GAT-A-GG-GTT-CGT-TTG-TAG-TTG-TTT-C-3' MR: 5'-TAA-AAT-CCT-AAC-ACG-ATT-AAC-CGA-T-3' UF: 5'-TAG-GGT-TTG-TTT-GTA-GTT-GTT-TTG-T-3' UR: 5'-AAT-AAA-ATC-CTA-ACA-CAA-TTA-ACC- AAT-3'	56°C/40
<i>MTHFRA1298C</i>	F: 5'-CTT-TGG-GGA-GCT-GAA-GGA-CTA-CTA-3' R: 5'-CAC-TTT-GTG-ACC-ATT-CCG-GTT-TG-3'	62°C/30
<i>MTHFR C677T</i>	F: 5'-TGA-AGG-AGA-AGG-TGT-CTG-CGG-GA-3' R: 5'-AGG-ACG-GTG-CGG-TGA-GAG-TG-3'	60°C/38
<i>FLT3-ITD</i>	11F: 5'- GCA-ATT-TAG-GTA-TGA-AAG-CCA-GC-3' 12R: 5'-CTT-TCA-GCA-TTT-TGA-CGG-CAA-CC-3'	62°C/30

Table 2: Primers used in experimental procedures

T_{ann}: annealing temperature, MF: Methylated forward, MR: methylated reverse, UF: unmethylated forward, UR: unmethylated reverse

Patients No 88	SNK/PLK2 methylation 69/88 (78.4%)
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<i>MDS</i>	<i>38/43 (88.4%)</i>
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Cytogenetics

Favorable	29/33 (87.9%)
Intermediate	3/3 (100%)
Poor	6/7 (85.7%)
<i>WHO subtype</i>	
RA	6/8 (75%)
RN	2/2 (100%)
RT	1/1 (100%)
RCMD	16/17 (94.1%)
RAEB-I	5/7 (71.4%)
RAEB-II	6/6 (100%)
del 5q	2/2 (100%)
<i>WPSS</i>	
Very low	6/7 (85.7%)
Low	12/13 (92.3%)
Intermediate	8/10 (80%)
High	9/10 (90%)
Very high	3/3 (100%)

Table 3: Clinical findings of MDS patients with SNK/PLK2 methylation.

RA: refractory anemia, RN: refractory neutropenia, RT: refractory thrombocytopenia, RCMD: refractory cytopenias with multilineage dysplasias, RAEB: refractory anemia with excess blasts, WPSS: WHO classification based prognostic scoring system

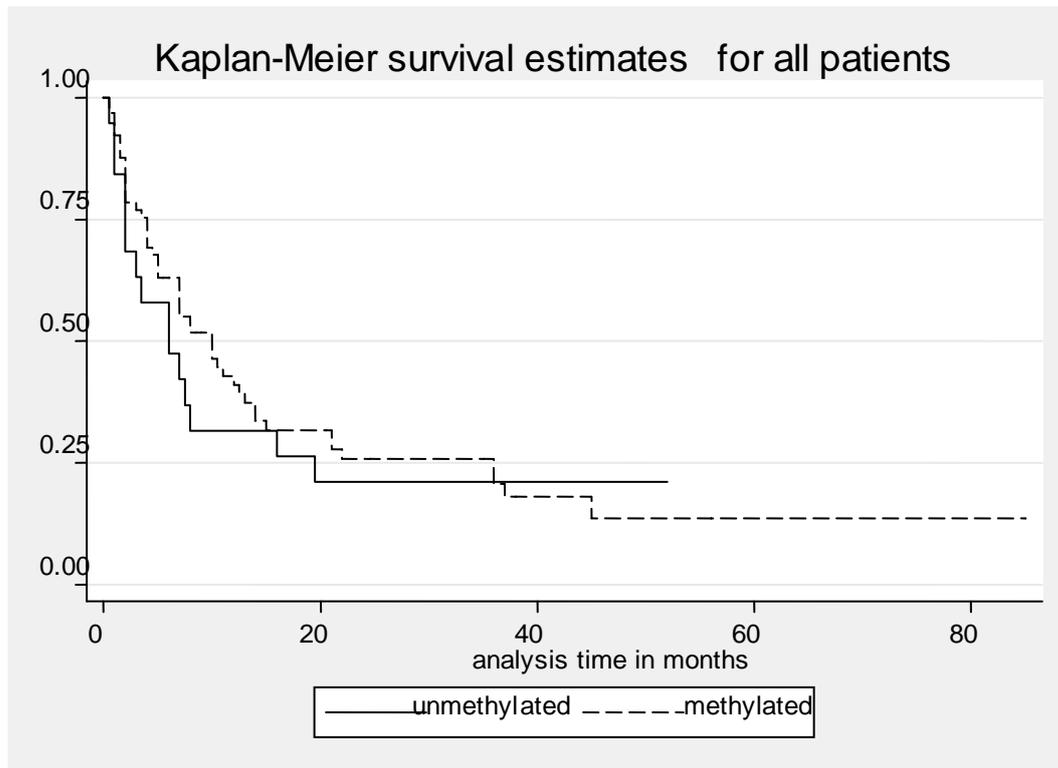
Patients No 88	SNK/PLK2 methylation 69/88 (78.4%)
<i>AML</i>	<i>31/45 (68.9%)</i>
<i>Cytogenetics</i>	
Favorable	2/2 (100%)
Intermediate	15/20 (75%)
Poor	4/11 (36.4%)
<i>WHO subtype</i>	
t(8;21)	1/1 (100%)
t(15;17)	1/1 (100%)
with myelodysplasia related changes	10/16 (62.5%)
therapy-related myeloid neoplasms	1/1 (100%)
with minimal differentiation	4/5 (80%)
without maturation	6/9 (66.7%)
with maturation	3/5 (60%)
acute myelomonocytic leukemia	3/3 (100%)
acute monoblastic/monocytic leukemia	2/4 (50%)
<i>MTHFR A1298C</i>	
AA	19/27 (70.4%)
AC	9/14 (64.3%)
CC	3/4 (75%)
<i>MTHFR C677T</i>	
CC	7/9 (77.8%)
CT	22/31 (71%)

TT	2/5 (40%)
<i>FLT3-ITD positive</i>	4/7 (57.1%)

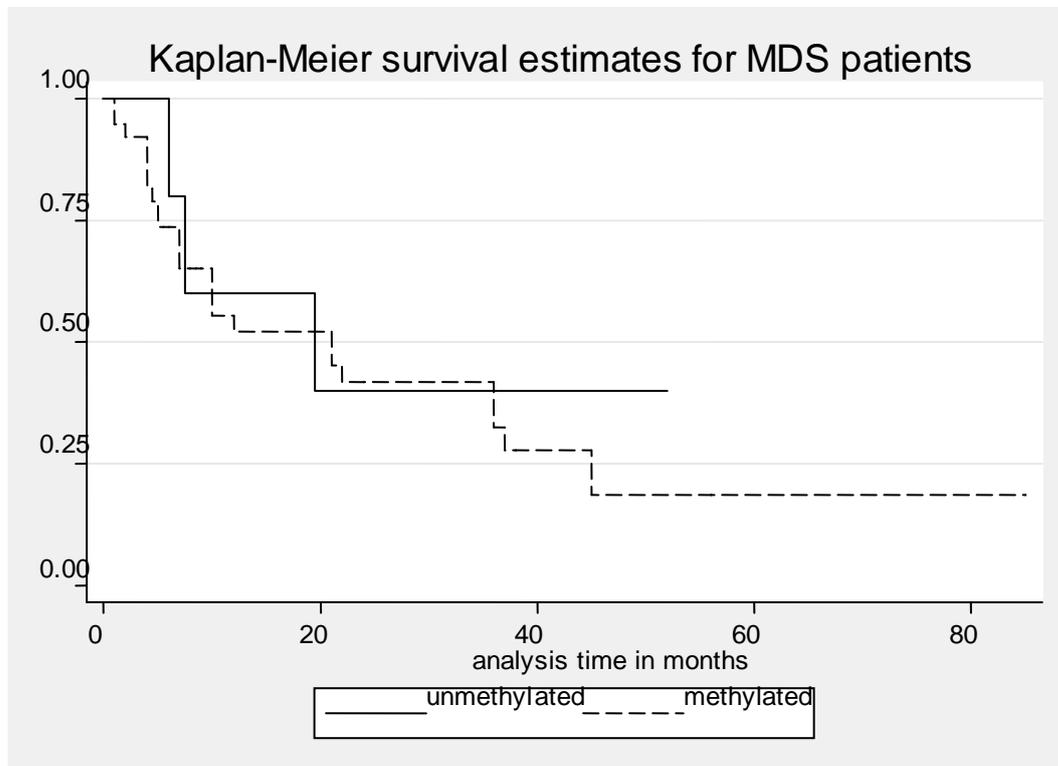
Table 4: Clinical findings of acute myeloid leukemia (AML) patients with SNK/PLK2 methylation

FLT3-ITD: FLT3-internal tandem duplication, MTHFR: methylenetetrahydrofolate reductase

A



B



C

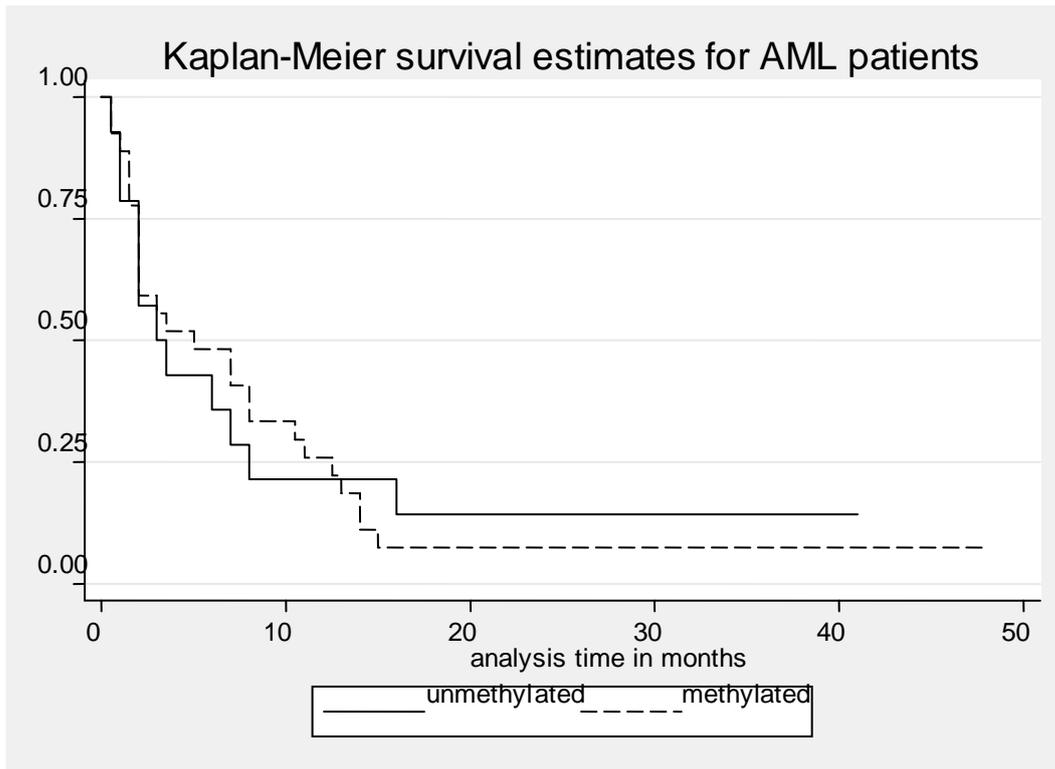
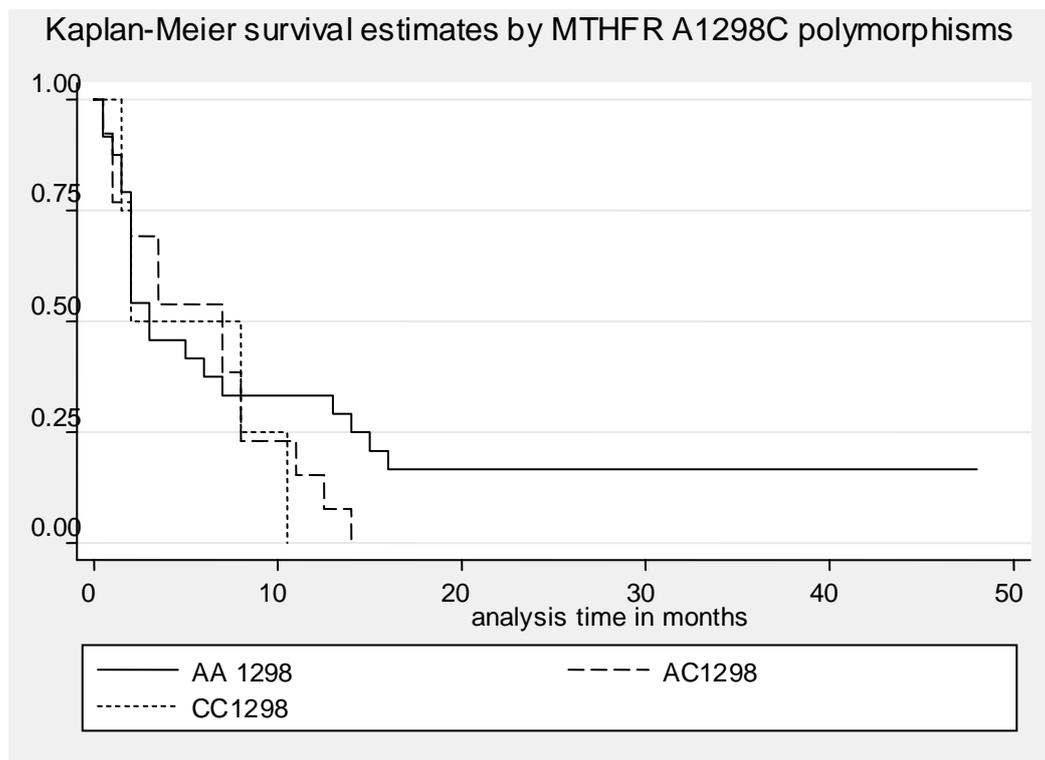


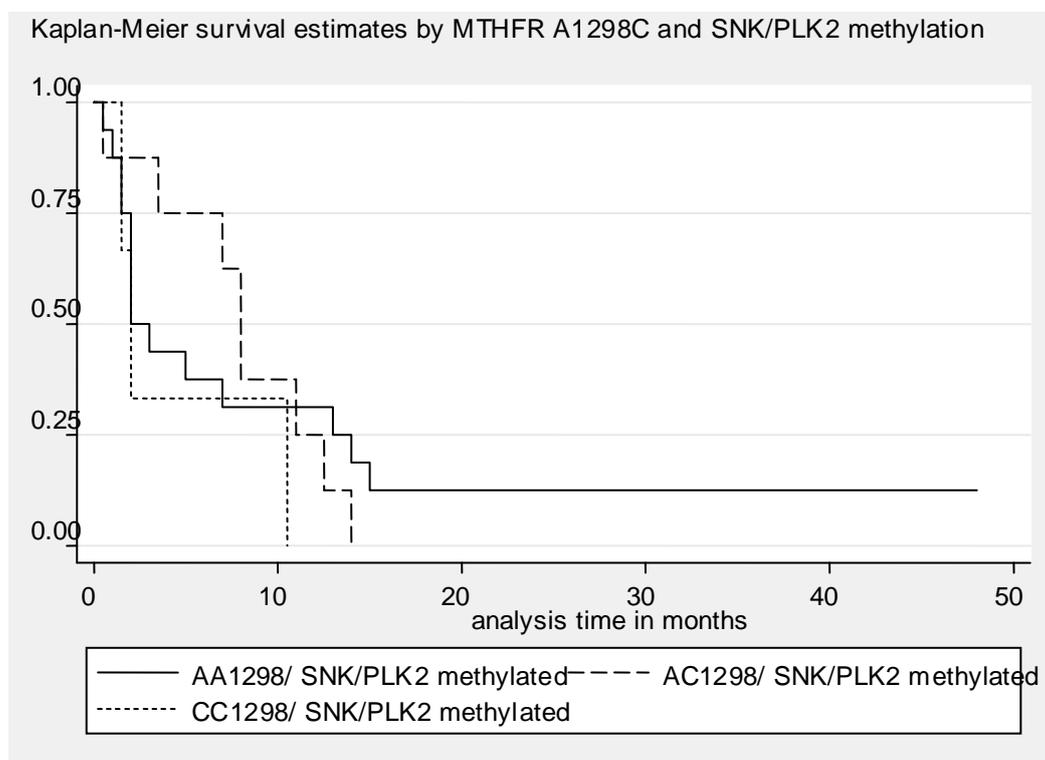
Table 5: Kaplan-Meier survival estimates in respect of the SNK/PLK2 methylation status

A: all patients, B: Myelodysplastic syndrome (MDS) patients, C: Acute myeloid leukemia (AML) patients

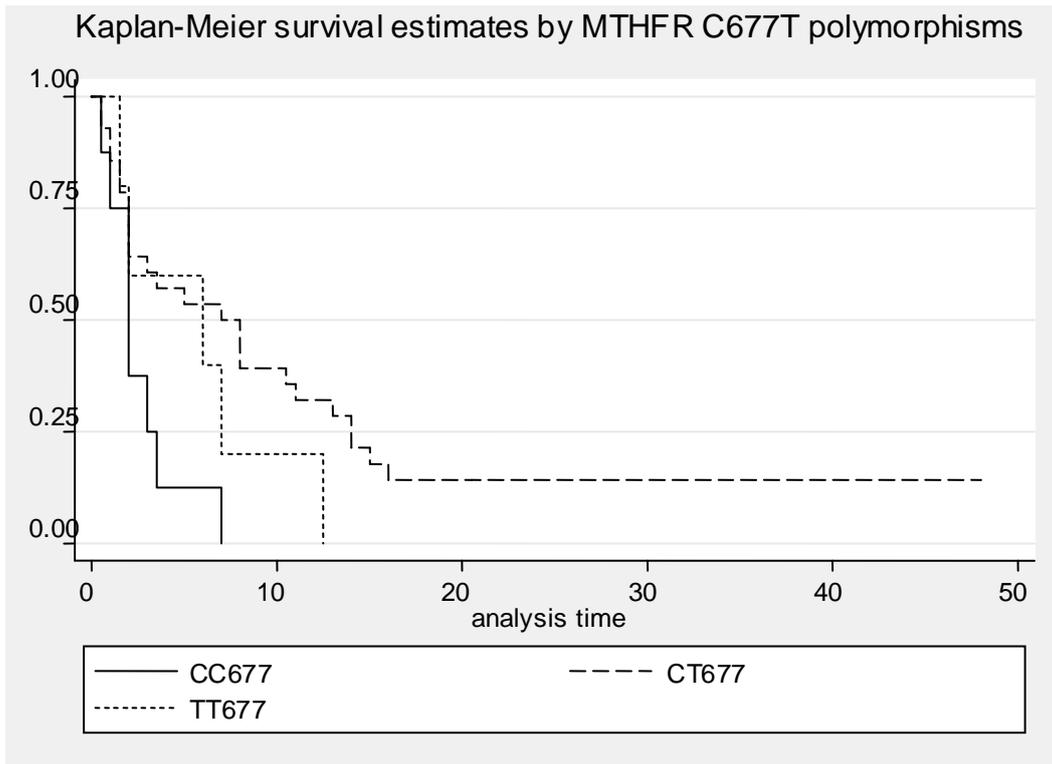
A



B



C



D

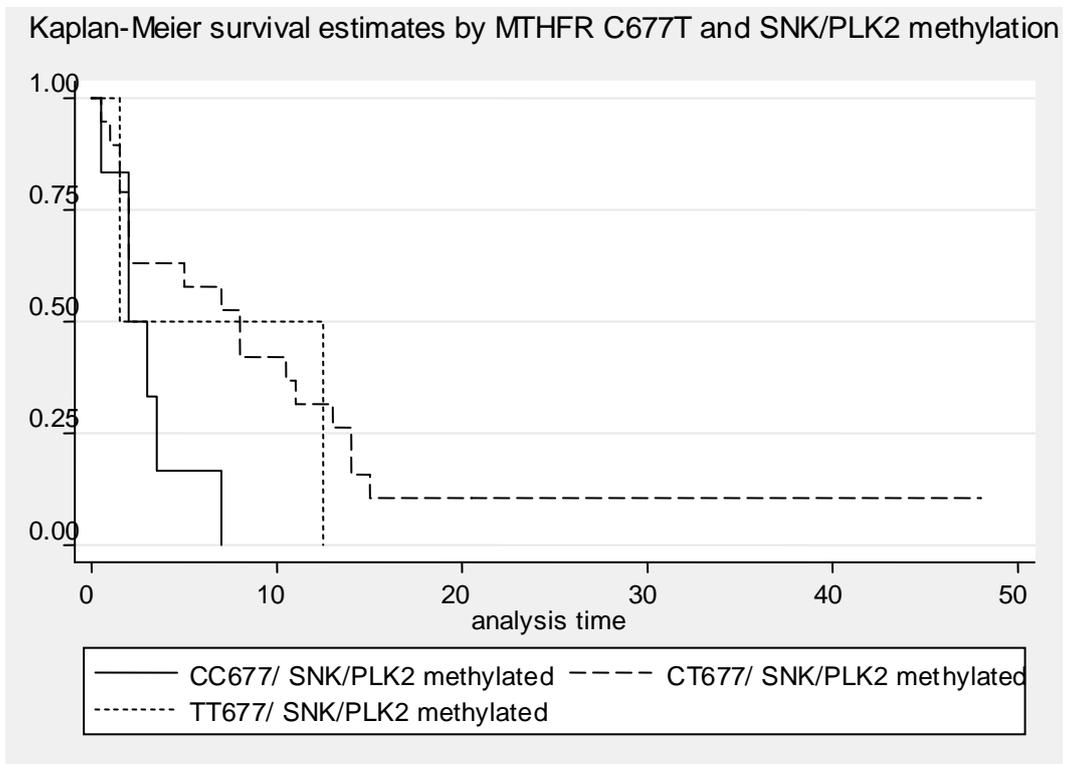


Table 6: Kaplan-Meier survival estimates presentinh MTHFR polymorphisms in acute myeloid leukemia patients

A: survival estimates by AA1298, AC1298, and CC1298 polymorphisms. Cox regression analysis was relative to the AA polymorphism. B: survival estimates in respect to patients' A1298C genotypes and combined with SNK/PLK2 methylation, C: survival estimates by CC677, CT677, and TT677 polymorphisms. Cox regression analysis was relative to the CC polymorphism, D: survival estimates in respect to patients' C677T genotypes and combined with SNK/PLK2 methylation