Arch. Biol. Sci., Belgrade, 63 (3), 649-653, 2011 DOI:10.2298/ABS1103649J

NPM1 GENE MUTATIONS IN CHILDREN WITH MYELODYSPLASTIC SYNDROMES

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Abstract - Myelodysplastic syndromes (MDS) are rare in children and only a few studies have analyzed their molecular mechanisms. The NPM1 gene encodes for nucleophosmin (NPM) which regulates hematopoiesis. Mutations in exon 12 of the NPM1 cause the nucleophosmin cytoplasmic dislocation and disrupt its functions. We have analyzed mutations of the NPM1 gene in archival bone marrow samples from 17 children with MDS and detected, in one patient, transition C to T in codon 293. To the best of our knowledge, this is the first analysis of NPM1 mutations in childhood MDS and the very first missense mutation of the NPM1 gene reported so far.

Key words: Myelodysplastic syndromes, nucleophosmin, *NPM1* mutation, children

UDC 616.15.006.04:577.21

INTRODUCTION

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal disorders of the multipotent hematopoietic progenitor cell. MDS are rare in childhood and there are only a few studies analyzing the molecular mechanisms underlying these severe diseases.

Nucleophosmin (NPM) is an essential protein for the regulation of hematopoiesis. *NPM1*$^{+/-}$ heterozygous mice develop a hematological disorder with features of human myelodysplastic syndromes (Grisendi et al., 2005).

NPM is a highly conserved multifunctional phosphoprotein predominantly localized in the nucleolus. Nucleophosmin acts as a molecular chaperone, facilitates the transport of ribosomal proteins through the nuclear membrane and regulates the stability of various nuclear proteins (Chen et al., 2006). Additionally, the control of centrosomal duplication during the cell cycle and modulation of the activity of tumor-suppressors such as p53 (Colombo et al., 2002) are also attributed to NPM.

Mutations in exon 12 of the *NPM1* gene that cause the cytoplasmic dislocation of protein represent the most frequent genetic alterations in adult patients with karyotypically normal acute myeloid leukemia (Chou et al., 2006; Thiede et al., 2006).

MDS are referred to as preleukemias, because of their tendency to transform into acute myeloid leukemia (AML). Consequently, findings obtained from studies on AML patients may serve as a clue for molecular genetic analyses in MDS.

The aim of our study was to investigate the prevalence and the type of *NPM1* gene mutations in a cohort of Serbian children with MDS.

MATERIALS AND METHODS

Bone marrow samples

We analyzed archival bone marrow samples from 17 children (14 boys and 3 girls) with MDS, diagnosed and treated at the Belgrade Institute for Mother and Child Health of Serbia. The diagnosis and the type of MDS were established according to the FAB (French–American–British) criteria and bone marrow histology data after the exclusion of other causes of myelodysplasia.

The study was approved by the Ethical Committee of the School of Medicine, University of Belgrade.

Conventional cytogenetic investigations were performed by standard procedures and data were available for 10 patients. Seven patients showed karyotype abnormalities while three had normal karyotypes. Five of the seven patients had a single karyotype abnormality: chromosome 7 monosomy; chromosome 19 monosomy; t(3;5)(q23;q32); inv(9) (p11q13) and del(9)(p13), respectively. In the remaining two patients complex karyotypic abnormalities were observed (46,XY/46,XY del(12)(q22), del(5)(q12q13) and 44,XY del(5) del(7) del(11)-17,-18).

Five of the 17 MDS patients developed leukemia and four of them were from the group with karyotype abnormalities. Further progression of karyotypic complexity during the evolution to leukemia was not observed. Three of the five patients who developed leukemia, according to the FAB criteria, were classified as refractory anemia, refractory anemia with excess of blasts and refractory anemia with excess of blasts in transformation. The remaining two patients could not be classified at the time of diagnosis.

Genomic DNA was isolated from archival bone marrow smears using the standard phenol-chloroform method. Control DNA was taken from the blood of healthy volunteers.

PCR – SSCP analysis

For the screening of *NPM1* gene exon 12 mutations by the PCR-SSCP (polymerase chain reaction – single strand conformation polymorphism) method, we created primers flanking the region of interest. The forward and reverse primers were 5'- TGT-CTA-TGA-AGT-GTT-GTG-GTT-CC- 3' and 5'- GCA-TTA-TAA-AAA-GGA-CAG-CCA-GA-3', respectively. PCR amplifications were performed under the following conditions: initial denaturation at 95^0C for 5 min; 35 cycles of denaturation at 95^0C for 30 s, annealing at 64^0C for 45 s, extension at 72^0C for 1 min and final extension at 72^0C for 7 min. The PCR products were 179 bp long. The SSCP method was performed according to the standard procedure.

Direct sequencing

To confirm the results obtained by the PCR-SSCP method and to determine the type of mutation detected, we performed sequence analyses using the previously described primers. The PCR products were purified and directly sequenced using the ABI Ready Reaction Dye Terminator Cycle Sequencing Kit (Applied Biosystems).

RESULTS

PCR-SSCP analysis

According to PCR-SSCP analysis, the sample of one MDS patient showed a distinct pattern of mobility through polyacrylamide gel.

Sequence analysis

The sequence analyses confirmed that the patient previously indicated by the SSCP method as a carrier of mutation harbors transition T to C that results in the exchange of serine to proline in the WQWRK**S**L amino acid sequence of the NPM protein (Fig. 1).

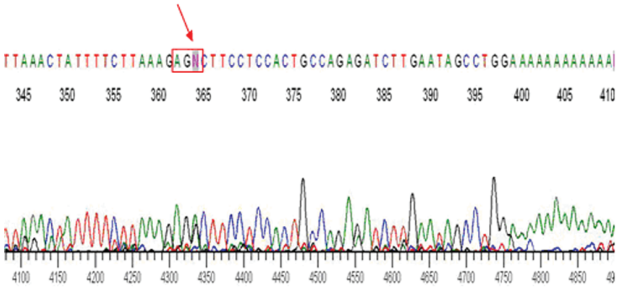

Fig. 1 Sequencing of a DNA sample from a patient with a mutation (transition T to C). The arrow indicates the position of the mutation.

Prediction of mutation effect

Three prediction methods were used to determine whether an amino acid substitution would affect protein function: the SIFT prediction method (http:// sift.jcvi.org/www/ SIFT_seq_submit2.html) (Ng and Henikoff, 2003), the PolyPhen program (http://www.bork.embl-heidelberg.de/PolyPhen/) (Ramensky et al., 2002) and the PMut program (http://mmb2.pcb. ub.es:8080/PMut) (Ferrer-Costa et al., 2005).

All prediction methods revealed that the substitution of S293P at the C - terminus of NPM could affect protein function.

DISCUSSION

MDS have been recognized as acquired genetic disorders and several genetic and epigenetic mechanisms have been pointed out as important for their etiology, but the molecular pathogenesis of MDS is still uncertain.

MDS are also characterized by an increased risk of developing acute myeloid leukemia (AML), thus indicating that MDS may represent a step in the evolution of AML. Additionally, AML and MDS have several features in common, such as some similar molecular lesions observed in both diseases. Hence, data obtained from AML cases may be a starting points for the elucidation of MDS.

The most common molecular alteration in AML is mutations of the *NPM1* gene (Gaidzik and Dohner, 2008). The *NPM1* encodes for nucleophosmin (NPM), a member of the family of chaperones predominantly found in the granular regions of nucleoli. Both tumor-suppressive and oncogenic features have been attributed to this protein. NPM has several domains that are crucial for its functions and one of these is a C-terminal nucleic acid-binding domain involved in ribosomal RNA processing (Hingorani et al., 2000) and for the nucleolar localization of nucleophosmin.

Mutations in exon 12 of the *NPM1* gene represent the most frequent molecular alterations in adult patients with acute myeloid leukemia (AML), detected in one third of all AML cases (Thiede et al., 2006). In AML patients with a normal karyotype the frequency of *NPM1* mutations is even higher and ranges from 45.7% to 63.8% (Chou et al., 2006; Suzuki et al., 2005). In childhood AML, these molecular events are less common and are detected in 2.1% (Chou et al., 2006), 6.5% (Cazzaniga et al., 2005), 8% (Brown et al., 2007) and 12% (Thiede et al., 2007) of all AML cases. Similarly to adult patients, in a karyotypically normal pediatric AML the occurrence of *NPM1* mutations is higher, accounting for 9% to 26.9% of cases. Only heterozygous *NPM1* mutations have been found so far.

Mutations of the nucleophosmin gene have been identified in 5.2% (2/38) of adults with MDS (Zhang et al., 2007). The detected mutations were of the same type and effects as the *NPM1* mutations found in AML.

We evaluated the prevalence of *NPM1* mutations in a group of 17 pediatric MDS patients. The *NPM1* mutation was detected in one case. To our knowledge, this is the first report of *NPM1* mutation in childhood MDS.

All AML-related mutations of the *NPM1* gene result in an insertion or an insertion associated with deletion. Despite genetic heterogeneity, all variants generate a frame shift in the region encoding for

the C-terminus of NPM protein resulting in the replacement of the last 7 amino acids (WQWRKSL) (Falini et al., 2006). Disruption of *NPM1* results in the generation of an additional leucine-rich nuclear exporting signal (NES) motif (Falini et al., 2006) and the loss of tryptophan residues 288 and 290 (Falini et al., 2005). As a consequence, nucleophosmin is dislocated and accumulated in the cytoplasm (Falini et al., 2007a) where it cannot execute its normal function.

The mutation detected in this study disrupts the same WQWRKSL protein sequence, but the type of mutation is the transition C to T in codon 293 that changes serine to proline.

This is the very first missense mutation of the *NPM1* gene reported so far. Our data may indicate that disruption of NPM has different roles in the evolution of MDS, comparable with AML. A Falini's immunohistochemical study, which showed on 50 patients with MDS only nucleus-restricted NPM expression (Falini et al., 2007b), favored the hypothesis that additional mechanisms, other than frameshift mutations, could be involved in the pathogenic role of NPM in MDS.

Although S293P substitution affects nucleophosmin functions according to all prediction programs, the interpretation of these predictions must be carefully correlated with the clinical impact in MDS. Prediction methods are not able to consider posttranslational modifications of the protein, as well as interactions with other molecules involved in cell cycle control. Hence, the effects of this mutation remain to be explored.

Acknowledgments - This work was supported by the Serbian Ministry of Science, Technology and Development (grant 175091). We are grateful to Dr. Milos Kuzmanovic for providing MDS samples and Aleksandra Kostic for cytogenetic analyses.

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