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Expression analysis of proteins involved in the non homologous end joining DNA repair mechanism, in the bone marrow of adult de novo myelodysplastic syndromes

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Abstract Myelodysplastic syndromes (MDS) are characterized by genetic instability which is associated with abnormal DNA repair mechanisms. The most lethal type of DNA damage are double strand DNA breaks (DSBs), which are mainly repaired by Non Homologous End Joining Mechanism (NHEJ), whose core enzyme components include the Ku70/Ku80 heterodimer, DNA-PKcs, XRCC4 and DNA Ligase IV. The aim of the present study was the analysis of expression of proteins required for NHEJ in bone marrow cells of adult de novo MDS and their association with clinical characteristics and prognosis. Our analysis included 48 cases of MDS; 19 RA, 5 RARS, 19 RAEB, 3 RAEB-T, 1 CMML, 1 transformation to AML according to FAB classification. The expression of the enzymes Ku70, Ku80, XRCC4, DNA-PKcs and Ligase IV was determined by Western Blotting. The mean Ligase IV expression value was significantly lower in MDS patients compared to normal

controls (0.53 vs. 0.78, $p=0.03$). A negative correlation was found between karyotype risk group and Ligase IV values. ($p=0.05$). Moreover, Ku70 expression levels were significantly lower in patients with a good prognosis karyotype ($p=0.04$). Furthermore, a negative correlation between Ku70 expression values and Hb levels was observed ($p=0.04$). Finally, a positive correlation was observed between enzyme Ku70 expression values and level of blasts ($p=0.04$). Our findings support a potential role of NHEJ enzyme Ligase IV in the pathogenesis of MDS. Larger numbers of cases need to be screened in order to draw definite conclusion.

Keywords DNA repair mechanism ·
Non homologous end joining · Ligase IV · Ku70 · MDS

Introduction

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal stem cell disorders characterized by ineffective hemopoiesis and an increased risk (30%) of transformation to acute myeloid leukemia (AML) [1, 2]. Cytogenetic abnormalities are seen in approximately 50% of cases of de novo MDS. These abnormalities, that carry strong prognostic value, generally consist of partial or complete chromosomal deletion or addition such as del(5q), monosomy 7, trisomy 8, -Y, del(20q), whereas balanced or unbalanced translocations are rarely encountered [3].

Evidence supports that chromosomal rearrangements can arise through perturbations in the pathways involved in DNA repair [4]. Although all DNA lesions can threaten genomic integrity, DNA double strand breaks (DSBs) are

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considered to be the most lethal form of DNA damage [5]. They occur as the result of two simultaneous nicks in opposite strands of the DNA helix. It is known that DSBs are generated in response to ionizing radiation, radiomimetic drugs such as bleomycin, neocarzinostatin and etoposide, and topoisomerase inhibitors [6]. They can also arise as a consequence of natural processes, such as V(D)J recombination (a lymphoid specific process required for gene rearrangement and maturation of T and B cells) and are necessary or accidental intermediates in a number of normal cellular transactions [7]. If not repaired prior to DNA replication or mitosis, DSBs can induce cell death. If misrepaired, they have the potential to lead to chromosomal breakage and cancer predisposition [7].

Mammalian cells depend on two separate mechanisms for DSB repair, homologous recombination (HR) and non homologous end joining (NHEJ) [8]. HR requires a homologous DNA molecule which serves as a template and functions only in the S/G2 phases of the cell cycle, when a sister chromatid is available. On the other hand, NHEJ is a potentially less accurate, homology-independent mechanism, in which broken ends are rejoined after being processed in order to be compatible [8–10]. It is effective in all phases of the cell cycle and is considered to be the predominant repair pathway in mammalian cells. The main factors that participate in NHEJ are the DNA binding Ku70/Ku80 heterodimer, which initially recognizes the DNA break, a protein kinase DNA-PKcs that signals the presence of the break, activates and recruits repair proteins involved in DNA processing, and the XRCC4-Ligase IV complex that re-ligates the broken ends [8, 9].

Given the fact that cytogenetic abnormalities are associated with the appearance and evolution of the pre-malignant clone, we consider the developing understanding of NHEJ essential, with the view to elucidate its contribution to the pathogenesis of MDS. The aim of the present study was to examine the expression of proteins involved in the NHEJ mechanism in bone marrow cells of adult de novo MDS and their association with clinical characteristics and prognosis.

Design and methods

Patients

Our analysis included 48 cases of newly diagnosed adult de novo MDS. The samples were obtained by bone marrow aspiration at the time of diagnosis before the onset of any treatment. Bone marrow mononuclear cells from 28 cases of lymphoma patients without bone marrow involvement were used as controls. All samples were obtained after informed consent from the individuals. The patients were diagnosed and treated at the Hematology-Oncology unit of

the Second Department of Internal Medicine of Athens University and the diagnosis was established according to the French-American-British (FAB) [11] and the World Health Organization (WHO) criteria [12]. They were 34 males and 14 females with a median age of 68 years. They were classified as follows: a) according to FAB classification: 19 RA, 5 RARS, 19 RAEB, 3 RAEB-T, 1 CMML, 1 transformation to AML and b) according to WHO classification: 18 RA, 5 RARS, 1 5q (-) syndrome, 11 RAEB-1, 8 RAEB-2, 1 myelodysplastic/myeloproliferative disorder, 4 AML. The International Prognostic Scoring System (IPSS) was defined as follows: low (score: 0) n=22, Intermediate-1 (score: 0.5–1) n=13, Intermediate-2 (1.5–2) n=9, and high- risk (score: >2) n=3. Their clinical and hematological features are summarized in Table 1.

Cytogenetic analysis

Cytogenetic analysis was performed by direct culture of bone marrow cells and G-banding technique. Chromosomal aberrations were described according to the International System of Human Cytogenetic Nomenclature (ISCN 1995) recommendations [13].

Protein isolation

Protein was isolated from the bone marrow samples as follows: firstly, mononuclear cells were extracted using

Table 1 Clinical characteristics of patients

No of patients	48
Sex	34 males/14 females
Age	68 years (range: 51–88)
Hb	10,03 g/dl (range: 7,5–14,2)
WBC	7696,04 /mm ³ (range: 1430–21250)
PLT	182863 (range: 6800–607000)
FAB classification	WHO classification
RA : 19	RA : 18
RARS : 5	RARS : 5
	5q(-) syndrome : 1
RAEB : 19	RAEB-1 : 11
RAEB-T: 3	RAEB-2 : 8
CMML :1	Myelodysplastic/Myeloproliferative dis. : 1
Transformation to AML :1	Transformation to AML: 4
IPSS score	
Low risk (score 0)	22
Intermediate-1 risk (score 0,5-1)	13
Intermediate-2 risk (score 1,5-2)	9
High risk (score >2)	3

Histopaque buffer. Lysing buffer (BD Pharm Lyse) diluted to a 1x concentration was added for the lysis of the remained red blood cells. Then, protein was purified from mononuclear cells using the Nucleospin DNA/RNA and RNA/protein buffer sets (Macherey–Nagel) according to the manufacturer's protocol. The protein pellet was dissolved in Protein Loading Buffer (PLB) containing the reducing agent TCEP. Protein was then quantified after a Bovine Serum Albumin (BSA) dilution series and addition of trichloacetic acid, upon which protein precipitates and causes turbidity. Following measure of absorbance at 570 nm, the degree of turbidity was used for quantification relative to a sample with known protein concentration using computer software system Digwin 3.

Western blot analysis

The expression of the enzymes Ku70, Ku80, DNA-PKcs, XRCC4 and Ligase IV was determined by Western Blot analysis. Thirty µg of each protein sample were loaded on 10% or 6% polyacrylamide Trig/Glycine gels and blotted onto nitrocellulose membrane (0.45 µm, Biorad, CA, USA) using Tris/Glycine transfer buffer under the recommended conditions. Non specific binding sites were blocked by incubation with 10% non fat dry milk in TBS-T, for 1, 15 hour at room temperature. Then, membranes were incubated overnight at 4°C in antibody buffer (non fat dry milk in TBS-T) containing primary antibody: Ku70 clone 2C3.11, 1:1000 (Abcam, Cambridge, UK), Ku80, Ab-7, 1:2000 (Cell Signaling), DNA-PKcs, ab230, 1:3000 (Abcam), XRCC4, 1:6000 (Serotec, Oxford, UK), Ligase IV, ab6145, 1:2000 (Abcam) and Actin clone AC-15, 1:1000 (Sigma, Gillingham, UK). After three 15 min washes in TBS-T, blots were incubated with secondary antibody (Alexafluor 680-conjugated goat anti-rabbit or goat anti-mouse, Molecular Probes, Leiden, the Netherlands) 1:10000 or 1:15000 respectively. Following four 15 min washes in TBS-T, immunoreactivity was visualized with Super Signal West Pico Chemiluminescent kit (Pierce, IL, USA) and exposed to Kodak film for 1–2 min. Labeled proteins were detected and quantified using the Vision Works, Image Analysis Software System (UVP).

Immunocytochemistry studies

A double immunohistochemical staining was applied on 4 µm paraffin-embedded bone marrow sections, using the following monoclonal antibodies: anti-Ku70 (clone 2C3.11, Abcam, Cambridge, UK) at a dilution of 1:100 and anti-CD34 (clone QBEnd 10, Vision Biosystems) at a dilution of 1:100. Deparaffinization, rehydration and antigen retrieval for both epitopes was achieved by treating the slides in a bond max for

20 minutes in ER1 pH=6. After quenching at endogenous peroxidase, anti Ku70 was applied overnight at 4°C and the localization of Ku70 was visualized using DAKO REAL™ Envision™ detection system (DAKO) and DAB (3, 3'-diaminobenzidine) as chromogen. Subsequently, anti-CD34 was applied for 25 minutes at room temperature with a bond polymer AP Red detection in Bond autostainer. Fast red was used as a chromogen. Subsequently, sections were counter-stained with hematoxylin for the identification of nuclei and mounted.

The reliability of double stainings was achieved by correlating the staining pattern with that of single stainings with anti-Ku70 and anti-CD34 on serial sections.

Stained sections were inspected in a two headed microscope by a pathologist (P.Foukas) and a trained oncologist (P.Economopoulou). Ku70 immunolocalization was identified by brown (DAB) color and CD34 granular cytoplasmic staining by red [Fast red] color. All CD34 (+) cells with blastic morphology were interpreted for Ku70 immunopositivity in the entire bone marrow area.

Statistical analysis

Statistical analysis was performed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). For comparison of mean and median expression values of enzymes Ku70, Ku80, DNA-PKcs, XRCC4 and Ligase IV between patients and controls as well as between different sex, age, IPSS, karyotype, PLT, WBC, Hb, blast percentage, FAB and WHO classification patient groups Mann-Whitney U test for independent samples was used. Spearman correlation coefficient test was used to evaluate the strength of association between continuous variables, i.e. enzymes expression values and IPSS categories, age, Hb, WBC, PLT and % blast values. Differences in clinical characteristics subgroups distribution between patients over or under expressing the enzymes were evaluated using Pearson χ^2 test. Mann-Whitney U test was used for comparison of mean and median expression values between patients over or under expressing the enzymes among different age, WBC, Hb, PLT, IPSS, and blast percentage groups.

We then performed the same statistical analysis by replacing the absolute expression values of the enzymes with their transformed log10 values. P- Values <0.05 were considered significant for both the analyses.

Results

Cytogenetic analysis

Cytogenetic analysis was successful in 39/48 patients. As shown in Table 2, structural or numerical abnormalities were found in 17 patients (43, 5%).

Table 2 Structural and numerical cytogenetic abnormalities of patients

8 cases with RA	46,XX[12]/46,XX,-5+mar1[18] 46,XY[19],46,XY,del(5)(q14q31)[1] 46,XX[2]/46,XX,del(5)(q13q33)[18] 46,XY[5],45,X,-Y[15] 46,XX,21ps+[20] 46,XX[17]46,XX,del(11)(q23)[3] 46,XY[17],45,X,-Y[3] 45,X,-Y[20]
2 cases with RARS	46,XY[16],45,X,-Y[4] 47,XX,+8[20]
7 cases with RAEB	46,XY[9],45,XY,-7,del(20)(q12)[3],46XY,-7,+11,del(20)(q12)[4], 46,XY,add(6p),-7,+11,del(20)(q12)[4] 46,XY[2]/47,X,-Y,der(5)t(5q;?11q),+11,-16,-18,+21x2,+mar1[18] 46,XX,inv(9)(q12q13)[20] 46,XX[16],46,XX,del(7)(p13)[4] 46,XY,13ps+[20] 46,XY[7],42-44,XY,del(2p),del(3q),-5,add(7)(p22),-9,-10,-13,add(16q),-20,+mar`1,+mar2,+mar3,inc[13] 46,XY[14],47,XY,+8[4],46,XY,del(9p)[2]

Expression of proteins

In the present study, we examined the expression of the enzymes-components of the NHEJ pathway in 48 cases of MDS patients.

Fig. 1 Expression analysis of enzymes Ku70, XRCC4 and Ligase IV by Western blotting. M: marker Pageruler Prestained Protein Ladder (Fermentas) N: normal control, 1-2: protein samples from MDS patients **Fig. 2** Expression analysis of enzymes DNAPKcs and Ku80 by Western blotting. M: marker Pageruler Prestained Protein Ladder (Fermentas) N: normal control, 3-8: protein samples from MDS patients

Ku70 was expressed in 32/35, Ku80 in 27/29, DNAPKcs in 25/31, XRCC4 in 29/29, and Ligase IV in 23/27 of all cases examined (Figs. 1 and 2). All enzymes were expressed in all cases of normal controls. We then performed a quantitative analysis of the expression of all enzymes. When the log10 values of enzymes' expression were used, the mean Ligase IV expression value was significantly lower in MDS patients compared to normal controls (0, 53 vs. 0, 78, $p=0.03$). All other enzymes' expression values were not significantly different between patients and controls.

Furthermore, we divided the patients' population into subgroups based on clinical parameters (age, sex, Hb, WBC, PLT, % blast percentage, karyotype, IPSS, FAB and WHO classification) and tested for significant differences of enzyme expression, among different clinical characteristic groups.

A positive correlation was observed between enzyme Ku70 expression values and level of blasts ($p=0.04$). This result was confirmed by immunochemistry using Ku70 and CD34+ cells double staining. All CD34+ cells with blastic morphology were positive for Ku70 expression in the entire bone marrow area of each sample, as shown in Fig. 3. On the contrary, we found a negative correlation between Ku80 expression values and level of blasts although statistically not significant ($p=0.07$). Moreover, a statistically significant negative correlation was found between Ku70 expression values and Hb levels, suggesting higher Ku70 expression values in patients with anemia ($p=0.05$) (Fig. 4).

Furthermore, we examined whether clinical characteristics and outcome were different between cases over or under expressing all the enzymes. We performed the

Fig. 3 Immunohistochemistry studies on a bone marrow sample of a patient with MDS RAEB-II. Ku70 immunolocalization was identified by brown (DAB) color and CD34 granular cytoplasmic staining by red [Fast red] color. All CD34 (+) cells with blastic morphology were interpreted for Ku70 immunopositivity in the entire bone marrow area

analysis two times. Each time, we defined over and under expression by comparing for each patient, the mean value of each enzyme with mean control value and mean patient value. As shown in Fig. 5, a negative correlation was found between karyotype risk group and Ligase IV values, suggesting that patients with higher Ligase IV expression values had low risk karyotype (100% vs. 53,3%, $p=0.05$). In addition, Ku70 expression levels were significantly lower in patients with a good prognosis karyotype (87, 5% of patients with lower vs. 50% with higher expression levels, $p=0.04$) (Fig. 6). The same phenomenon was observed for Ku70 levels and low risk IPSS score group, although statistically not significant ($p=0.07$). Finally, a negative correlation between Ku70 expression values and Hb levels was observed, suggesting that 33,3% of patients with Ku70 expression value lower than mean control value had low Hb levels compared to 69,2% of patients

Fig. 4 Correlation between Ku70 and Hb levels. ($p<0.04$)

Fig. 5 Correlation between Ligase IV levels and karyotype group. ($p<0.05$)

with Ku70 expression value higher than mean control value ($p=0.04$).

Discussion

Chromosomal instability due to deficiencies in DNA repair could explain the underlying genetic alterations in MDS. Given that the most dangerous DNA lesions are DSBs, the elucidation of leukemogenesis secondary to defects of NHEJ mechanism, which is the optimal DSB repair pathway, is crucial for the understanding of MDS pathogenesis.

In our study, we examined the expression of NHEJ enzymes in 48 patients with adult de novo MDS before the initiation of treatment. We demonstrated for the first time a significantly lower expression of Ligase IV protein in MDS patients compared to normal controls. Ligase IV is a crucial NHEJ component that has been linked to predisposition to chromosomal instability disorders. O'Driscoll et al. [14] reported four patients with mutations in the Ligase IV gene. He demonstrated that one of them had myelodysplasia and three of them had pancytopenia, two of the main characteristics of the myelodysplastic syndromes. Furthermore, two

Fig. 6 Correlation between Ku70 levels and karyotype group. ($p<0.04$)

other groups [15], [16], reported two patients with a mutation in Ligase IV gene that had developed T-cell acute lymphoblastic leukemia. In addition, Nijnik et al. [17], described a mouse with a Ligase IV mutation characterized by pancytopenia that developed a progressive, age dependent defect in hemopoiesis, defined as reduced multipotent hematopoietic progenitors and reduced total bone marrow cell count. Taken together, these data support our finding, and indicate that patients with a Ligase IV deficiency are likely to be at an increased risk for hematological malignancies, like MDS and acute leukemia.

In our study, we also examined the association between the expression of proteins and clinical characteristics of patients. A negative correlation was observed between karyotype risk group and Ligase IV expression. More specific, half of the patients with low Ligase IV expression (expression values lower than the mean patient value) belonged to the intermediate-high karyotype risk group, compared to all patients with high Ligase IV expression (expression values higher than the mean patient value) who had low risk karyotype ($p=0.05$). This result proposes a potential role of reduced Ligase IV expression in the induction of genetic instability, and, as a consequence, complex cytogenetic abnormalities and poor prognosis in MDS. This suggestion is supported by published data from mouse models or cells indicating that defects in that NHEJ component, is associated with complex chromosomal aberrations. In Ligase IV deficient mice, there is an accumulation of DNA damage in haematopoietic stem cells [17] which may give rise to karyotype abnormalities. Furthermore, Sharpless et al. [18] demonstrated that Ligase IV heterozygosity promotes the development of tissue sarcomas displaying clonal amplifications, deletions, and translocations in a tumor-prone mouse strain. Moreover, increased cytogenetic aberrations have been detected in murine fibroblasts heterozygous for Ligase IV gene [19]. Similarly, Ferguson et al. found that Ligase IV^{-/-} mouse embryonic fibroblasts (MEFs) have dramatic chromosomal instability compared to wild type controls [20]. Finally, Ribalo et al. [15] described a cell line derived from a human leukemia patient with inherited hypomorphic mutation in Ligase IV and high frequencies in DSBs.

Moreover, we observed a positive correlation between enzyme Ku70 expression values and the level of blasts ($p=0.04$). We also found that Ku70 expression levels were significantly higher in patients with high risk cytogenetic abnormalities ($p=0.04$). Ku70 binds to the pro-apoptotic protein BAX (member of the bcl-2 family) and inhibits BAX-mediated apoptosis, functioning as an anti-apoptotic protein [21]. Increased apoptosis has been found in patients with early MDS stages, while patients with high risk MDS have increased number of blasts and are characterized by reduced level of apoptosis and high proliferation rate [22].

Therefore, it could be hypothesized that as the expression level of Ku70 increases, the degree of apoptosis is decreased due to suppression of BAX function, leading to an increased level of blasts that escape apoptosis, and progression of the disease.

Finally, a negative correlation between Ku70 expression values and hemoglobin level was described, suggesting a significantly higher protein expression level in patients with anemia. It is known that the level of Hb reflects oxygen concentration. As a consequence, anemia is linked to hypoxia. It has been reported that hypoxia could up regulate expression of Ku70 in mouse and human hepatoma cells [23]. In addition, Lynch et al., demonstrated up regulation of Ku70 and Ku80 expression in cervical carcinoma, histiocytic lymphoma and human breast carcinoma cells [24]. Therefore, since the expression of the enzyme Ku70 is shown to be induced in malignant cells under hypoxic conditions, we could claim that up regulation of Ku70 in MDS patients with low levels of Hb is a result of hypoxia.

In summary, we found that enzymes involved in the NHEJ mechanism are expressed in the majority of cases with MDS. Ligase IV expression is significantly lower in MDS compared to controls. On the contrary, no significant differences were observed in the expression of all other enzymes. In addition, we correlated Ku70 expression with more aggressive disease. The analysis of the integrity of the mechanism as well as the expression of all these enzymes following exposure to DNA damaging signal is under way in order to clarify the pathogenetic significance of this key DNA repair mechanism in MDS.

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