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# Lenalidomide treatment induced the normalization of marker protein levels in blood plasma of patients with 5q-myelodysplastic syndrome

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**Abstract.** A specific type of myelodysplastic syndrome (MDS) is associated with isolated deletion on the long arm of chromosome 5, i.e., 5q-syndrome (del(5q)). The treatment approaches for MDS del(5q) include the immunomodulating drug lenalidomide (LEN). Thirteen MDS del(5q) patients were included in this study. We found elevated activities of lactate dehydrogenase (LDH) and matrix metalloproteinase 9 (MMP-9) in the blood plasma of MDS del(5q) patients as compared with healthy controls. This was stabilized to control values after LEN treatment. Similar behavior we registered also for the thioredoxin and calnexin contents in BP. Peripheral blood mononuclear cells (PBMC) from patients with MDS del(5q) prior to and after treatment with LEN did not exhibit any detectable amount of P-glycoprotein (P-gp) gene transcript. However, we detected a measurable amount of multidrug resistance associated protein 1 (MRP1) mRNA in PBMCs from three patients prior to LEN treatment and in one patient during LEN treatment but it was not present prior to treatment. These data indicated on usefulness of applied protein markers estimation for monitoring of MDS del(5q) patient treatment effectiveness by LEN. Expression of MRP1 seems to be independent on LEN treatment and reflects probably the molecular variability in the ethiopathogenesis of MDS del(5q).

**Key words**: Myelodysplastic syndrome — 5q-syndrome — Lenalidomide — Lactate dehydrogenase — Matrix metalloproteinases — ABC transporters

## Introduction

The myelodysplastic syndromes (MDSs) are a group of clonal disorders that are characterized by the presence of ineffective hematopoiesis, peripheral cytopenias, and an increased risk of transformation to acute myeloid leukemia (AML) (Abdel-Wahab and Figueroa 2012). MDS manifests in older adults with a median age at diagnosis of approximately 70 years (Stone 2009). Chromosomal deletions are common molecular events in myeloid malignancies (Ebert 2010). *De novo* MDS shows cytogenetic abnormalities in 30–50% of cases. The most common karyotypic abnormality in MDSs is a deletion on the long arm of chromosome 5 (5q-) (Ebert 2010), which occurs in 10–15% of patients (Giagounidis et al. 2004). The 5q-syndrome (del(5q)) was described in the 1970s and represents the first chromosomal deletion to be associated with a specific hematologic phenotype (Ebert 2010), which is characterized by refractory hypoproliferative anemia, dysplastic megakaryocytes with preserved or increased thrombopoiesis, and indolent clinical course (Wei

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et al. 2009). Patients are usually transfusion-dependent with a relatively low rate of transformation to AML (Syed and Scott 2013).

Over recent years, attention has been paid to immunomodulatory-acting drugs such as thalidomide analogues (IMiDs). The effect of these drugs against MDS involves antiproliferative effects, downregulation of crucial cytokines, and co-stimulatory effects on T and NK cells (Quach et al. 2010). The thalidomide structural backbone was used as a template by chemists to design and synthesize compounds with increased immunological and anticancer properties, but lacking the toxicity associated with the parent compound (Bartlett et al. 2004). Lenalidomide (LEN), obtained by this way (LEN, known as Revlimid) was proven to be effective in the treatment of patients with low-risk MDS, particularly in MDS del(5q) (List et al. 2005). However, it is important to identify prognostic factors to better risk-stratify patients for more effective treatment (Ma 2012).

A number of previous studies have shown that elevated serum lactate dehydrogenase (LDH) levels are associated with poor prognosis in MDS (Wimazal et al. 2001). Aul et al. (1992) have shown that an elevated LDH level (more than 3.4 μkat/l) indicates a significantly shorter survival when compared to a lower LDH level (less than 3.4 μkat/l). However, Wimazal et al. (2001) showed that an LDH blood activity of 5.1 μkat/l indicated a borderline activity that was usable for the assessment of MDS patient prognosis.

The two matrix metalloproteinases, MMP-2 ($M_r$ 72,000) and MMP-9 ($M_r$ 92,000) were described as additional factor for improved reliability of diagnosis and prognosis of MDS patients and is a possible target for experimental treatments (Travaglino et al. 2008).

In the current paper, we aimed to study the effects of LEN treatment on levels of blood plasma (BP) prognostic factors in patients with MDS del(5q). The activities of LDH, MMP-2, and MMP-9 were used as proven prognostic markers for MDS (Wimazal et al. 2001; Travaglino et al. 2008; Wu et al. 2010). Moreover, we also measured the BP levels of two acidic intracellular proteins, thioredoxin and calnexin, which represent proteins involved in redox equilibrium and intracellular calcium homeostasis. To determine if LEN treatment induced any changes in the expression of drug efflux pumps, which are members of the ABC transporters family (P-glycoprotein and multidrug resistance associated protein), was another aim of our paper.

## Materials and Methods

### *Patients*

BP was obtained from 13 patients with MDS del(5q) (8 female, 5 male) median age (range: 55–75, mean: 67 ± 7) prior to and during the LEN treatment. Peripheral blood from 11 healthy individuals aged 42–69 (mean 61 ± 8) was used as controls. Samples from patients were collected between years 2009–2012 at Hematologic Outpatient Department, 1st Internal Clinic, General University Hospital of Charles University, Prague. LEN was administered as recommended at 10 mg/day for 21 days, with a 1-week interruption (Belickova et al. 2012). All of the patients gave informed consent, and this study was approved by an institutional review board. BP and fractions of peripheral blood mononuclear cells (PBMCs) were collected from their respective layers after Ficoll Paque Plus (ProScience Tech. s.r.o.) gradient centrifugation of patient and control peripheral blood.

### *Determination of LDH activity in BP*

LDH activities in BP were determined at the time of diagnosis and after each cycle of LEN treatment. LDH activities were determined spectrophotometrically at 340 nm as the initial velocity of NADH oxidation during the conversion of pyruvate to lactate (Bohacova et al. 1998).

### *Direct estimation of MMP-2 and MMP-9 activity using Gelatin Zymography in an electrophoretic gel*

BP samples were used to directly estimate the MMP-2 and MMP-9 activities using gelatin zymography in gels after SDS-polyacrylamide electrophoresis by protocol published elsewhere (Barancik et al. 2012).

### *Estimation of MMP-2, MMP-9, thioredoxin, and calnexin protein levels by Western blotting*

For Western blot analysis, 30 μg of BP proteins were separated using SDS-PAGE under reducing conditions in 10% (14% in the case of thioredoxin) polyacrylamide gels. Proteins were then transferred onto nitrocellulose membranes. Rabbit anti-MMP-2 polyclonal antibody (Santa Cruz Biotechnology Inc.), rabbit anti-MMP-9 polyclonal antibody, rabbit anti-thioredoxin polyclonal antibody (both from Cell Signaling Technology Inc.), rabbit anti-calnexin polyclonal antibody (Merck & Co., Inc.) and peroxidase-labeled anti-rabbit immunoglobulins (Cell Signaling Technology Inc.) were used as primary and secondary antibodies, respectively. Loading of proteins to the gel were controlled by Coomassie blue staining of protein band in parallel SDS-PAGE. Peroxidase reactions were visualized using the enhanced chemiluminescence (ECL) system (GE Healthcare USA) and a Kodak Image Station 440 CF (USA). Optical density of protein bands was evaluated by ImageQuant™ image analysis software (GE Healthcare USA).

*Reverse transcription PCR*

Total RNA was isolated from PBMCs using TRI reagent (Sigma-Aldrich) following the manufacturer´s protocol and was quantified using UV spectrophotometry. Reverse transcription reactions were performed using a RevertAid First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer´s protocol. Amplification of cDNA was achieved using PCR with cDNA template, DreamTaq Green PCR Master Mix (2x) (Fermentas), and the following primers: for multidrug resistance associated protein 1 (MRP1): 5'-AGAAGTCTGGACGTCCCTG-3' and 5'-ACACCAAGCCGGCGTCTTT-3', which generated products of 404 bp; for P-glycoprotein (P-gp): 5'-AAGTTGTATATGGTGGTGGGAACT-3' and 5'-AATTTTGTCACCAATTCCTTCATT-3', which generated products of 429 bp; for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control: 5'-GACCACTTTGTCAAGCTCATTTC-3' and 5'-AGCACAGGGTACTTTATTGATGGT-3', which generated products of 291 bp. Annealing temperatures for the primers were as follows: GAPDH 57.2°C, MRP1 57.3°C, and P-gp 57.0°C. These PCR products were separated by electrophoresis on a 1.7% agarose gel, visualized using GelRed (Biotium) using Typhoon 9210 imaging system (GE Healthcare, USA). Densitometric evaluation was processed with the aid of ImageQuant™ image analysis software (GE Healthcare USA).

*Statistical analysis and data processing*

Numerical data are expressed as the mean ± S.E.M. Statistical significance was assessed using an unpaired Student's *t*-test using SigmaPlot Graphing Software (version 8.00).

The intercorrelation of protein markers were evaluated by linear regression using SigmaPlot Graphing Software (version 8.00). Statistical significance of correlation were evaluated using online statistic calculator: (http://www.danielsoper.com/statcalc3/calc.aspx?id=44).

## Results

*Effect of LEN treatment on LDH activity in the BP of MDS patients*

MDS patients differed from controls by a significant elevation of BP LDH activity of greater than four times (Fig. 1). This elevation was normalized after the first period of LEN treatment to a value of approximately 7.5 μkat/l. However, this value still differed significantly from those we obtained from healthy individuals. The second period of LEN treatment induced an additional decrease in the patients BP LDH activity to a value that was equal to that of the control group. Six patients initially had strongly elevated LDH activities in BP that were normalized after LEN treatments. The LDH activities of other patients oscillated at values that were moderately higher than those of the control independent of LEN treatment. This behavior points to the importance of the LDH activity in BP as an indicator of the effectiveness of LEN treatment of MDS del(5q) patients.

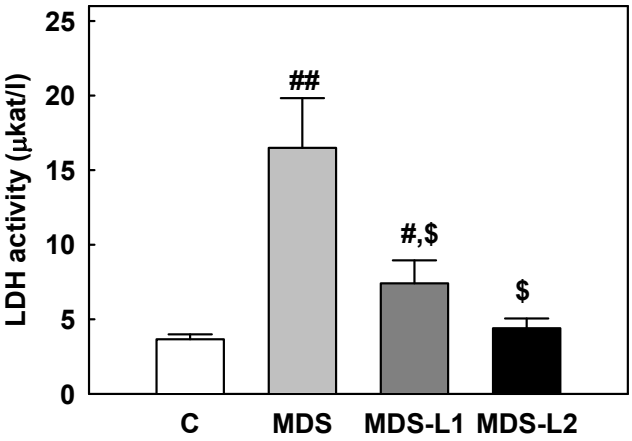


**Figure 1.** LDH activities in the BP of MDS del(5q) patients. The data represent LDH activities in the BP of patients prior to (MDS) and after the first (MDS-L1) and second (MDS-L2) periods of LEN treatment in comparison to healthy controls (C). Data are expressed as the means ± S.E.M. obtained for 13 patients and 11 healthy individuals. # $p < 0.05$ *vs.* C; ## $p < 0.01$ *vs.* C; $ $p < 0.05$ *vs.* MDS.

*Effects of LEN treatment on MMP-2 and MMP-9 activity in the BP of MDS del(5q) patients*

The MMP-9 and MMP-2 activity in the BP were determined by evaluating gelatinase activity directly in SDS-PAGE gels (Fig. 2B). The respective protein bands were identified by Western blotting methods (Fig. 2A). MMP-9 activity was found to be at least two times higher in the BP of MDS del(5q) patients compared to that of healthy controls (Fig. 2D). The normalization of BP MMP-9 activities in MDS del(5q) patients was observed after the first period of LEN treatment, and this value continued to be normalized during all additional periods of LEN treatments. While MMP-2 activity in the BP of MDS del(5q) patients seemed to exceed the value that was obtained for healthy individuals, this increase was not statistically significant (Fig. 2C). However, significant downregulation of MMP-2 activity was observed when untreated and LEN-treated patients with MDS del(5q) were compared.

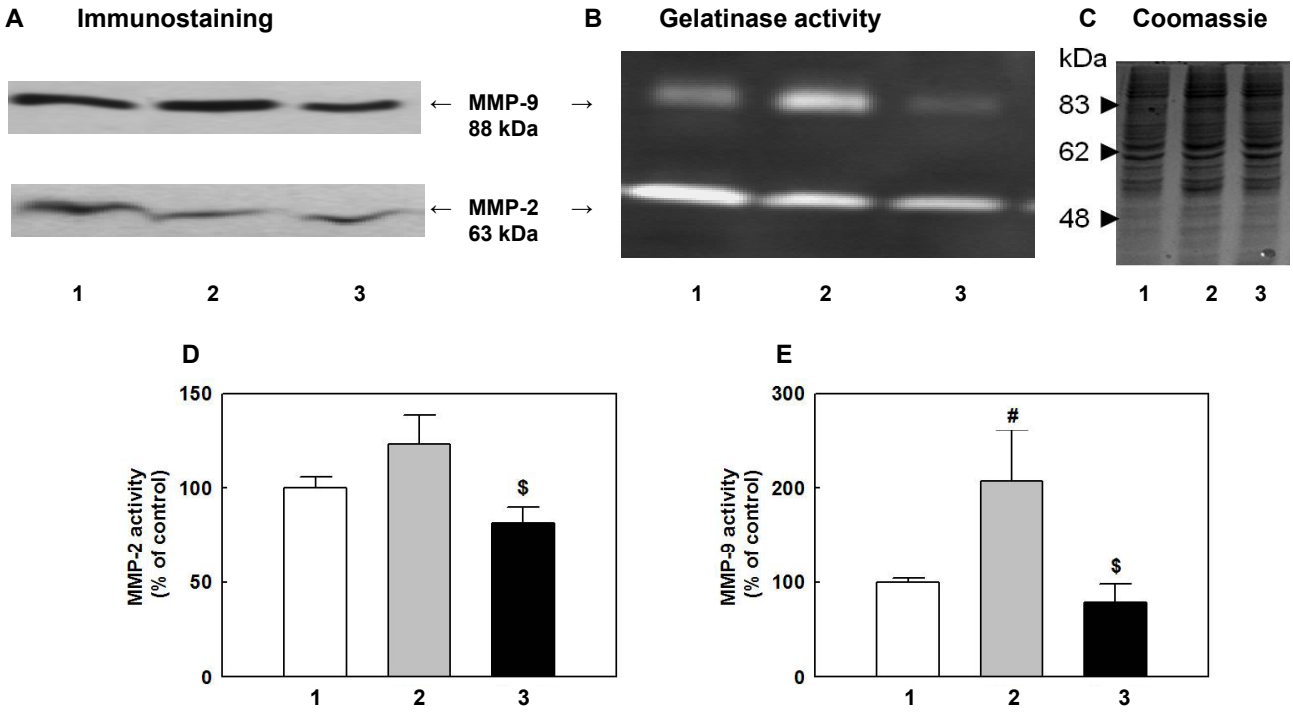


**Figure 2.** Gelatinase activities of MMP-9 and MMP-2 in the BP of MDS del(5q) patients. **A.** Estimation of the MMP-2 and MMP-9 protein contents in the BP of a 63-year-old male MDS del(5q) patient prior to (2) and after the first period (3) of LEN treatment in comparison with that of a healthy control (1). Similar estimations were applied to 13 MDS del(5q) patients and 11 healthy controls. **B.** The detection of MMP-2 and MMP-9 activities using gelatin zymography in the BP of the same patients as in panel A prior to (2) and after the first period (3) of LEN treatment in comparison to that of a healthy control (1). Loading of protein to the lane were controlled by Coomassie staining in parallel SDS-PAGE gel (panel **C**, lane description is same as in A and B). Similar estimations were applied for 13 patients and 11 healthy controls and were quantified using densitometry. The results for MMP-2 are summarized in panel **D** and, for MMP-9, in panel **E**. Meaning of 1, 2 and 3 is same as in panel A, B and C. The data represent the means ± S.E.M. # $p < 0.05$ *vs.* C; $ $p < 0.05$ *vs.* MDS.

*Effects of LEN treatment on the protein levels of calnexin and thioredoxin in the BP of MDS patients*

We found several changes in BP proteins that stained blue on SDS-PAGE gels when the cationic dye Stains-all was used for protein detection in samples of BP from patients with MDS del(5q) and healthy controls in a panel of preliminary experiments (not shown). Proteins that gave blue signals using this stain are considered acidic, in contrast to other proteins that gave pink signals (Goldberg and Warner 1997). Therefore, we compared the protein levels in the BP of control and MDS del(5q) patients for the two following proteins: i) calnexin – a typical acidic protein that is involved in calcium homeostasis and protein quality control in the ER (Seres et al. 2008, 2010); and ii) thioredoxin – a typical acidic protein that is involved in redox status control (Stefankova et al. 2005). We found that the protein levels of calnexin and thioredoxin were higher in the BP of untreated MDS del(5q) patients compared to those of healthy controls (Fig. 3). LEN treatment induced the normalization of the contents of both proteins to the control value.

*Intercorrelations between protein markers in BP of MDS del(5q) patients prior and after first period of LEN treatment*

Levels of proteins markers of thirteen MDS del(5q) patients prior and after first period of LEN treatment were intercorrelated using linear regression. Our finding indicated on statistically significant correlation of all pairs of applied markers with exception of MMP-9 activity *vs*. thioredoxin content that gave probability higher than 0.05 (Table 1). Highest significance were observed when MMP-2 and MMP-9 activities were correlated that gave two-tailed probability at the level $p < 0.001$.

*Effects of LEN treatment on the expression of multidrug resistance markers in fractions of mononuclear cells*

P-gp transcripts were not detected in PBMCs isolated from control or patients prior to or after LEN treatment (not shown). To prove that our RT-PCR methods were running correctly, mice leukemia L1210 cells transfected with a human gene encoding the full-length P-gp, which were shown

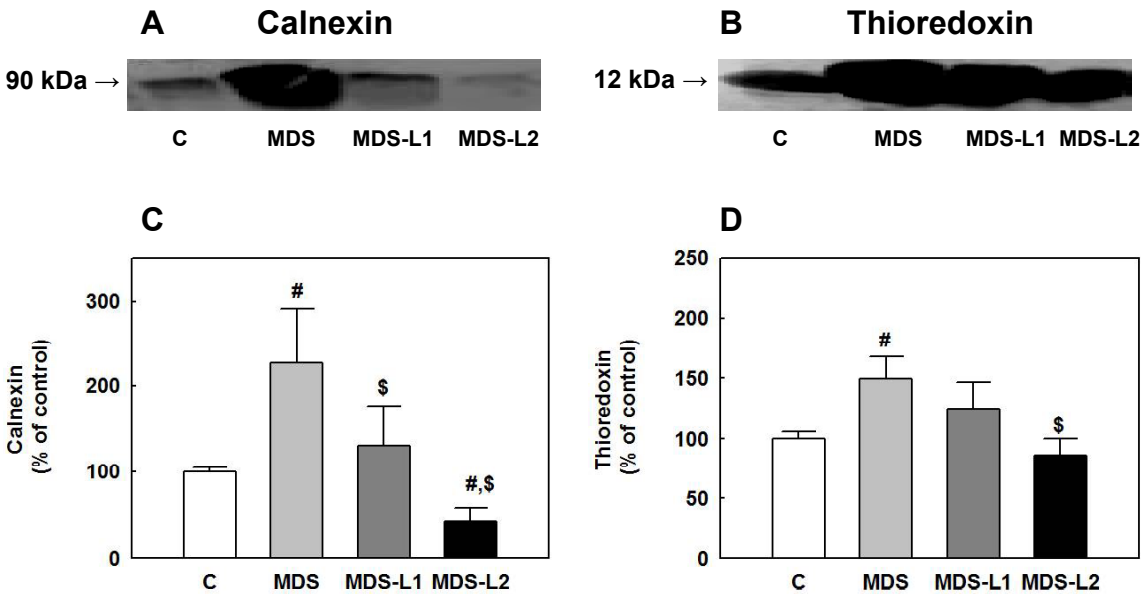


**Figure 3.** Calnexin and thioredoxin contents in the BP of MDS del(5q) patients estimated by Western blotting. **A.** Estimation of calnexin protein contents in the BP of a 63-year-old male MDS del(5q) patient prior to (MDS) and after the first (MDS-L1) and second (MDS-L2) periods of LEN treatment in comparison with that of a healthy control. Loading of protein to the lane were controlled by Coomassie staining in parallel SDS-PAGE gel (see Fig. 2C). Similar estimations were applied for 13 patients and 11 healthy controls, were quantified by densitometry, and are summarized in panel **C**. **B.** Estimation of the thioredoxin protein content in the BP of a 63-year-old male MDS del(5q) patient prior to (MDS) and after the first (MDS-L1) and second (MDS-L2) periods of LEN treatment in comparison with that of a healthy control. Similar estimations were applied for 13 patients and 11 healthy controls, were quantified by densitometry, and are summarized in panel **D**. The data represent the means ± S.E.M. # $p < 0.05$ *vs.* C; \$ $p < 0.05$ *vs.* MDS.

to contain a large amount of human P-gp gene transcript (Sulova et al. 2010), were used as a positive control. Therefore, we may conclude that the expression of P-gp in the PBMCs of controls and patients is so negligible that it cannot be detected by the applied RT-PCR method. In contrast to P-gp, measurable gene transcripts of MRP1 were found in PBMCs from three MDS del(5q) patients (Fig. 4) prior to LEN treatment. In these cases, the MRP1 gene transcripts seem to be downregulated during LEN treatment. In one case, a detectable amount of the MRP1 gene transcript was found during several periods of LEN treatment, but it was not present in the patient's PBMCs prior to treatment. We did not detect any measurable amount of MRP1 gene transcript in any of the other patient or control samples.

## Discussion

In MDS ethiopathogenesis, quantitative or qualitative/functional defects of the pluripotent progenitor cell compartment represent a major cause for the cytopenia in MDS-patients (Geissler et al. 1988). The ultrastructural defects in the membranes of blood cells, including erythrocytes, may have a role in their early destruction within circulation (Basu et al. 2010) and during anemia development. The elevation of early cell destruction is associated with an increase in the BP levels of cell intracellular proteins such as LDH and serves as a known measure of cell destruction. We observed elevated LDH activity prior to LEN treatment in six of the thirteen MDS del(5q) patients who were involved in this study. These elevated activities were normalized to the control value after the first two periods of LEN treatment and were retained at the stabilized value during all additional periods of LEN treatment. The LDH activity in seven other MDS del(5q) patients oscillated near control values prior to and during all periods of LEN treatment. Elevated values of MMP-9 activity, calnexin, and thioredoxin contents, when compared with those of healthy controls, were registered in the BP of patients with MDS del(5q) prior to LEN treatment. Therapy with LEN depressed these parameters to values equal to those of controls, which indicated the importance of measuring the LDH, MMP-2, MMP-9 activities and the contents of other intracellular proteins (such as calnexin and thioredoxin) in the BP of MDS del(5q) patients during LEN treatment.

MDS patients with isolated del(5q) are considered to have good prognosis as compared to other MDS subtypes. Most patients suffered of anemia and half of them required transfusions at diagnosis. It is known that for patients with MDS del(5q) in transfusion dependence, LEN is the first choice treatment (Rojas et al. 2014). The ultrastructural defects in erythrocyte

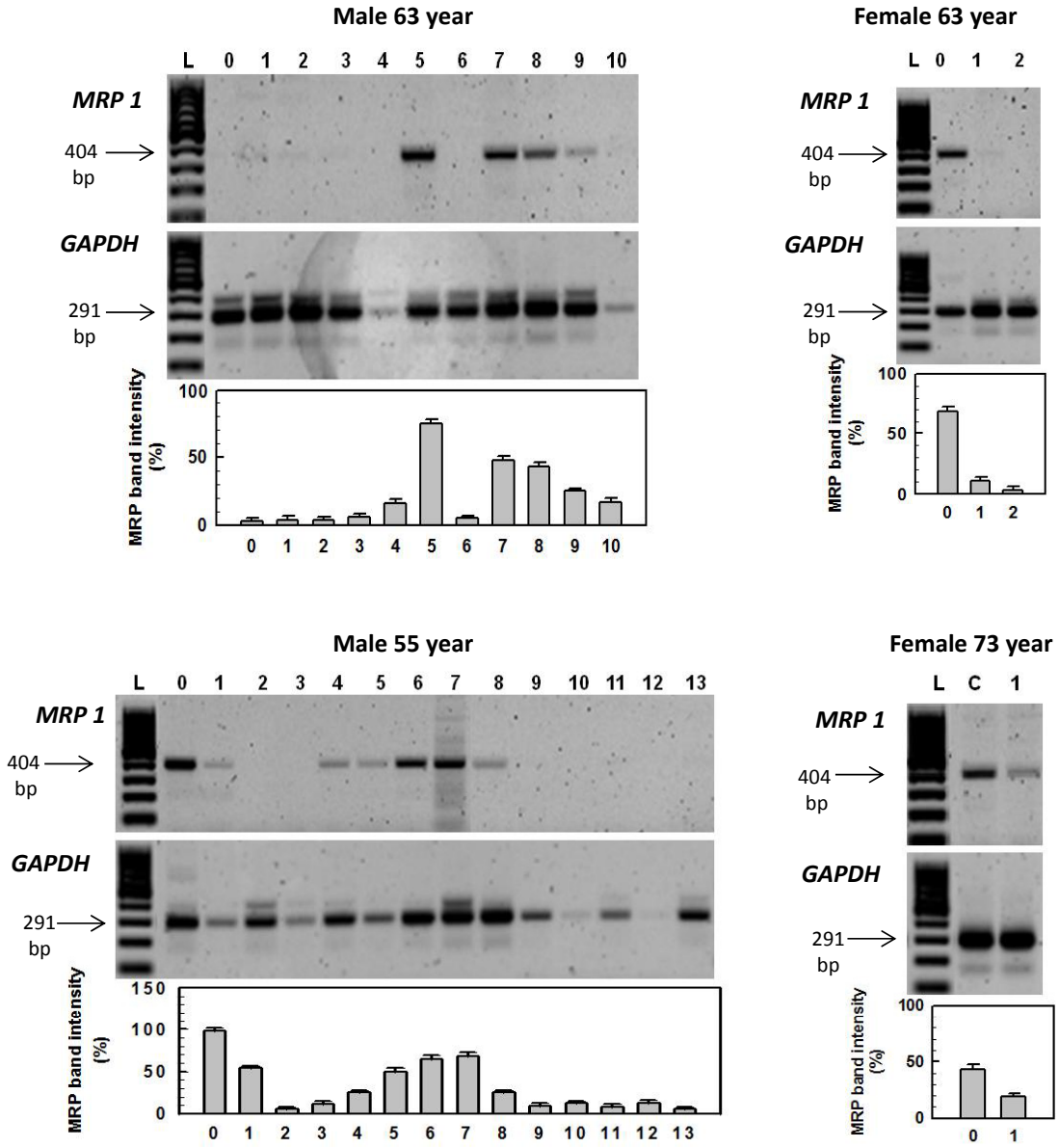


**Figure 4.** Detection of MRP1 mRNA in fractions of mononuclear cells isolated from peripheral blood of four MDS del(5q) patients using RT-PCR. The GAPDH signal was used as an internal standard. The amounts of the PCR products were quantified using densitometry and are summarized in bar plots. The data represent the means ± S.E.M. for triplicate of PCR reaction. Weak signals of GAPDH in several samples are caused by limited amount of isolated PBMC. L, ladder; 0, patient isolated prior LEN treatment; 1–13, period of LEN treatment. Any detectable MRP-1 transcript contents were not detected in PBMC of other nine patients either prior or after LEN treatment.

membranes may have a role in early red cell destruction within circulation (Basu et al. 2010). The early destruction of cells is associated with elevated levels of intracellular proteins like LDH in BP, which serves as a known indicator of cellular damage. We can assume that reduced levels of LDH and other markers in the BP and thus reduced early destruction of red blood cells in circulation after LEN treatment led to transfusion independence and improving clinical status. Statistically significant intercorrelations were observed between estimated markers with exception of MMP-9 activity *vs.* thioredoxin content that gave $p > 0.05$ (Table 1). This indicated common cause (at least partially) of these protein markers elevated contents in MDS del(5q) patients BP as compared with healthy controls (Figs. 1–3). Blood cells damage within circulation enhanced in MDS del(5q) seems to be responsible for increased contents of these proteins in BP. Depression of these values indicated effectiveness of LEN treatment of patients with this diagnosis. However, the mechanism by which LEN depressed the early cell destruction

**Table 1.** Correlations between protein markers in blood plasma of patients with 5q-myelodysplastic syndrome

| | Correlation *versus* | Correlation coefficient | Two-tailed probability |
|---|---|---|---|
| LDH activity | MMP2 activity | 0.437 | 0.0256 |
| | MMP9 activity | 0.391 | 0.0483 |
| | calnexin content | 0.478 | 0.0135 |
| | thioredoxin content | 0.459 | 0.0183 |
| MMP2 activity | MMP9 activity | 0.613 | 0.00087 |
| | calnexin content | 0,431 | 0.0279 |
| | thioredoxin content | 0.417 | 0.0340 |
| MMP9 activity | calnexin content | 0.405 | 0.0401 |
| | thioredoxin content | 0.382 | 0.0541 |
| Thioredoxin content | calnexin content | 0.597 | 0.0013 |

Protein markers were estimated in blood plasma of patients with 5q-myelodysplastic syndrome prior and after first period of lenalidomide treatment. Correlation were evaluated by Sigmaplot 8.0 grafic software. Two-tailed probabilities were accounted using online statistic calculator (http://www.danielsoper.com/statcalc3/calc.aspx?id=44) for 26 measurement (D.F. = 24).

is rather unclear. Future study will be necessary for better understanding the beneficial effect of LEN in MDS del(5q).

As in other malignancies, increased expression of drug resistance genes, such as P-gp and MRP1, is involved with in multi-drug resistance in MDS (Vidal et al. 2007). LEN represents a weak substrate, but not an inhibitor of P-gp (Chen et al. 2012), and failed to be a substrate or inhibitor of MRP1 (Kumar et al. 2008). We did not register measurable expression of P-gp in PBMCs isolated from patients with MDS del(5q) prior to or during LEN treatment, which indicated that, while LEN is a weak P-gp substrate (Chen et al. 2012), its capability to induce P-gp expression (a typical feature of P-gp substrates (Breier et al. 2013)) was not exhibited. We found detectable amounts of MRP1 gene transcripts in PBMCs of three MDS del(5q) patients prior to LEN treatment, which were depressed during LEN treatment. In the PBMCs of one MDS del(5q) patient, we registered the expression of MRP1 during LEN treatment, while, prior to LEN treatment, measurable expression was not detected. MDS patients having progressed to acute myeloid leukaemia treated with intensive chemotherapy exert about 70% incidence MRP1 expression in contrast to MDS patients having 36% incidence (Poulain et al. 2000). We registered about 30% incidence of MRP1 expression in our group of patients. However, these data are not sufficient to conclude that MRP1 expression in mononuclear cell fractions of MDS del(5q) patients could be altered by LEN treatment. Therefore, expression of this protein most likely reflects the molecular variability in the ethiopathogenesis of del(5q) subgroup of MDS.

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