The Role of the Iron Transporter ABCB7 in Refractory Anemia with Ring Sideroblasts

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Abstract

Refractory Anemia with Ring Sideroblasts (RARS) is an acquired myelodysplastic syndrome (MDS) characterized by an excess iron accumulation in the mitochondria of erythroblasts. The pathogenesis of RARS and the cause of this unusual pattern of iron deposition remain unknown. We considered that the inherited X-linked sideroblastic anemia with ataxia (XLSA/A) might be informative for the acquired disorder, RARS. XLSA/A is caused by partial inactivating mutations of the ABCB7 ATP-binding cassette transporter gene, which functions to enable transport of iron from the mitochondria to the cytoplasm. Furthermore, ABCB7 gene silencing in HeLa cells causes an accumulation of iron in the mitochondria. We have studied the role of ABCB7 in RARS by DNA sequencing, methylation studies, and gene expression studies in primary CD34+ cells and in cultured erythroblasts. The DNA sequence of the ABCB7 gene is normal in patients with RARS. We have investigated ABCB7 gene expression levels in the CD34+ cells of 122 MDS cases, comprising 35 patients with refractory anemia (RA), 33 patients with RARS and 54 patients with RA with excess blasts (RAEB), and in the CD34+ cells of 16 healthy controls. We found that the expression levels of ABCB7 are significantly lower in the RARS group. RARS is thus characterized by lower levels of ABCB7 gene expression in comparison to other MDS subtypes. Moreover, we find a strong relationship between increasing percentage of bone marrow ring sideroblasts and decreasing ABCB7 gene expression levels. Erythroblast cell cultures confirm the low levels of ABCB7 gene expression levels in RARS. These data provide an important link between inherited and acquired forms of sideroblastic anemia and indicate that ABCB7 is a strong candidate gene for RARS.

Introduction

The myelodysplastic syndromes (MDS) are a heterogeneous group of hematopoietic malignancies, characterized by blood cytopenias, ineffective hematopoiesis and a hypercellular bone marrow [1]. Refractory Anemia with Ring Sideroblasts (RARS) is a subtype of MDS in which excess iron accumulates in the form of aberrant mitochondrial ferritin in the mitochondria of the erythroid precursors [2,3]. RARS is a myeloid malignancy and transforms to acute leukemia in approximately 10–20% of cases [4]. The molecular genetic basis of RARS remains unknown.

The hereditary sideroblastic anemias are a heterogeneous group of disorders characterized by the presence of ring sideroblasts in the bone marrow, microcytic hypochromic anemia and typically show X-linked inheritance [5–7]. Many of the reported cases of inherited X-linked sideroblastic anemia have been found to be caused by mutations in the erythroid-specific delta-aminolevulinate synthase gene (ALAS2) [8]. A rare inherited X-linked sideroblastic anemia with ataxia (XLSA/A) is caused by mutations of the mitochondrial ATP-binding cassette transporter ABCB7 [9,10]. The ABCB7 gene is the functional orthologue of the yeast Atm1p gene [11], which is required for mitochondrial iron homeostasis [12] and has been implicated in the transport of a component required for the maturation of iron-sulfur (Fe-S) cluster proteins out of mitochondria [13]. It has been demonstrated that XLSA/A is due to partial loss of function mutations in ABCB7 that directly or indirectly inhibit heme biosynthesis [13]. The anemia in XLSA/A is caused by the accumulation of iron in a form that is not readily usable for heme synthesis [14]. Moreover, ABCB7 has been demonstrated to be essential for hematopoiesis in experiments using conditional gene targeting in mice [13]. In an important experiment, ABCB7 has been silenced in HeLa cells by sequential transfection experiments with siRNAs [14]. The phenotype of the ABCB7-deficient cells was characterized by a reduction in proliferation rate that was not rescued by iron supplementation, by signs of iron deficiency, and by a large...
increase of iron accumulation in the mitochondria that was poorly available to mitochondrial ferritin. We have investigated the expression levels of the ABCB7 gene using data from our large microarray dataset on CD34+ cells from MDS patients and healthy controls in order to determine whether the ABCB7 gene is implicated in the common acquired sideroblastic anemia, RARS.

Materials and Methods

Sample collection and cell separation

122 patients with MDS and 16 healthy controls were included in the study. Classification of MDS patients was according to the French-American-British (FAB) criteria [15] and the patients were selected solely on the basis of having MDS. The MDS patient samples were obtained from several sources: Oxford, Bournemouth and Dundee (United Kingdom), Stockholm (Sweden), Duisburg (Germany) and Pavia (Italy). At the time of investigation, 35 patients had Refractory Anemia (RA), 33 RARS and 54 Refractory Anemia with Excess Blasts (RAEB). The study was approved by the ethical committees of the John Radcliffe Hospital (Oxford), the Karolinska Institutet (Stockholm), the IRCCS Policlinico S. Matteo (Pavia), the Royal Bournemouth Hospital (Bournemouth), the St Johannes Hospital (Duisburg) and the Ninewells Hospital (Dundee); written informed consent was obtained from study participants. CD34+ cells were isolated from the bone marrow samples of MDS patients and healthy controls using MACS magnetic cell separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s recommendations.

Erythroblast cell cultures

CD34+ cells isolated from the bone marrow of 8 patients with RARS, 9 patients with RA and 8 healthy controls were cultured according to a method developed to study the generation of erythroblasts [16,17]. Gene expression profiling experiments were performed on cultured cells at day 7.

Microarray experiments and data analysis

Gene expression profiling experiments and data analysis were performed as previously described [18]. Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays (47,000 transcripts) were used. Gene correlations were calculated using Pearson correlation.

Real-time quantitative PCR

Real-time quantitative PCR for the ABCB7 gene was performed using cDNA made from the unamplified RNA from CD34+ cells of 7 patients with RARS, 8 patients with RA, 6 patients with RAEB and 7 healthy controls. Beta-2-microglobulin was used to normalize for differences in input cDNA. Reactions were performed on cultured cells at day 7. We confirmed the low levels of ABCB7 expression in RARS erythroblasts in comparison to those of RA and healthy controls (Table 1; Figure 1G).

Statistical analysis

Statistical analysis and box plots were created using StatView software. Kruskal-Wallis test was used for comparison between three or more groups and Mann-Whitney U test for comparisons between any two groups. A P-value <0.01 was considered significant.

DNA sequencing

Direct sequencing of ABCB7 was performed on DNA from 13 RARS patients using Applied Biosystems Big dye terminator kit v1.1.

Promoter methylation studies

We looked at promoter methylation of ABCB7 in DNA samples from 6 RARS patients (2 bone marrow, 2 neutrophil and 2 CD34+ samples) and from 5 healthy controls (1 bone marrow, 1 neutrophil, 1 mononuclear cell and 2 CD34+ samples). The ABCB7 promoter was predicted using PromoterScan and CpGPlot software. For investigation of methylation status we used bisulphite sequencing. Bisulphite modification of the genomic DNA was carried out using the EpiTect Bisulphite kit (Qiagen). Primers to amplify the modified DNA for bisulphite sequencing were designed using Primer3 software and were as follows: 5'-GTTTGGGGTTTTGGGAAAAAT-3' (ABCB7 forward primer) and 5'- TTCCATATCAATAACATCTTCCTAA-3' (ABCB7 reverse primer). The PCR products were purified with the Wizard PCR Clean-Up System and Vacuum Manifold (Promega, Madison, WI) according to the manufacturer’s instructions. PCR products were cloned using the pGEM-T Easy cloning system (Clontech) and sequenced with Applied Biosystems Big dye terminator kit v1.1 and the ABCB7 forward primer.

Results

ABCB7 expression levels in MDS subtypes

We have determined the ABCB7 gene expression levels from our microarray dataset on CD34+ cells in 122 MDS patients and 16 healthy controls. We have compared the gene expression levels of ABCB7 between 35 patients with refractory anemia (RA), 33 patients with RARS, 34 patients with RA and excess blasts (RAEB), and 16 healthy controls (Table 1; Figure 1A). The expression levels of ABCB7 are lowest in the RARS group compared to RA (p<0.0001), RAEB (p<0.0001) and healthy controls (p<0.0001). No significant difference was observed between the RA, RAEB and healthy control groups.

ABCB7 expression levels and percentage of bone marrow ring sideroblasts

The 101 of 122 MDS cases, where data was available on the exact percentage of bone marrow ring sideroblasts, have been divided in three groups by percentage of bone marrow ring sideroblasts (0–14%, 15–40% and >41%) (Table 1; Figure 1B). It can be seen that the median expression level of ABCB7 falls progressively across the three groups. As shown in Table 1, the differences in ABCB7 expression between the three groups are all statistically significant.

ABCB7 expression levels in erythroblast cultures

In addition, we have studied the ABCB7 gene expression levels in erythroblast cultures derived from the CD34+ cells of 8 patients with RARS, 9 patients with RA and 8 healthy controls. Cells were cultured according to a method developed to study the generation of erythroblasts [16,17] and gene expression profiling experiments were performed on cultured cells at day 7. We confirmed the low levels of ABCB7 gene expression levels in RARS erythroblasts in comparison to those of RA and healthy controls (Table 1; Figure 1G).

Confirmation of ABCB7 expression data

The microarray-generated expression data of the ABCB7 gene were validated and confirmed in a subset of 21 MDS patients (7 patients with RARS, 8 patients with RA, 6 patients with RAEB) and 7 healthy controls using real-time quantitative PCR (Figure 2). A high concordance was observed between the expression levels obtained with Affymetrix chips and with real-time quantitative PCR, indicating a good level of agreement between the two assays.
ABCB7 gene sequencing and promoter methylation

Direct sequencing of ABCB7, including the promoter region, was performed on DNA from 13 RARS patients and did not show any mutations in any of the patients. We have investigated the methylation status of the ABCB7 promoter region in 6 patients with RARS and 5 healthy controls, and the results showed 80% methylation in all samples, with no significant difference between RARS patients and healthy controls.

ABCB7 gene correlation

No genomic abnormality in the ABCB7 gene was identified in our cases. Therefore, we examined our microarray dataset for genes with expression levels correlated either strongly positively ($\geq 0.8$) or inversely ($\leq -0.8$) with ABCB7 gene expression levels. The expression of two genes showed a positive correlation with ABCB7 expression: ELP2 (0.82) and RTF1 (0.80). No genes were found whose expression correlated inversely with ABCB7.

Table 1. Descriptive statistics of ABCB7 expression levels in CD34$^+$ cells according to FAB group, in CD34$^+$ cells according to percentage of bone marrow ring sideroblasts, and in cultured erythroblasts at day 7.

| CD34$^+$ CELLS - FAB | MEAN | STD DEV | Kruskal-Wallis p | Controls | RA | RAEB |
|----------------------|------|---------|------------------|----------|----|------|
| Controls (n = 16)    | 0.98 | 0.36    | —                | Controls | RA | RAEB |
| RA (n = 35)          | 0.88 | 0.34    | 0.3610           | —        | —  | —    |
| RAEB (n = 54)        | 0.94 | 0.44    | 0.5476           | 0.7211   | —  | —    |
| RARS (n = 33)        | 0.49 | 0.19    | <0.0001          | <0.0001  | <0.0001 | — |

| % SIDEROBLASTS       | MEAN | STD DEV | Kruskal-Wallis p | 0–14 (n = 57) | 15–40 (n = 26) | 41+ (n = 18) |
|----------------------|------|---------|------------------|---------------|---------------|-------------|
| Controls (n = 16)    | 1.03 | 0.39    | —                | 1.03          | 0.62          | 0.42        |
| RA (n = 35)          | 0.62 | 0.26    | <0.0001          | 1.5–40        | <0.0001       | —           |
| RAEB (n = 54)        | 0.42 | 0.07    | <0.0001          | <0.0001       | 0.0022        | —           |

| DAY 7 ERYTHROBLASTS  | MEAN | STD DEV | Kruskal-Wallis p | Controls (n = 8) | RA (n = 9) | RARS (n = 8) |
|----------------------|------|---------|------------------|-----------------|------------|--------------|
| Controls (n = 8)     | 1.05 | 0.11    | —                | Controls (n = 8) | RA (n = 9) | RARS (n = 8) |
| RA (n = 9)           | 0.73 | 0.24    | 0.0071           | 0.0008          | 0.0021     |

|                      | Controls | RA | RARS |
|----------------------|----------|----|------|
| Controls (n = 8)     | 1.06     | 0.12 | Controls (n = 8) |
| RA (n = 9)           | 0.74     | 0.24 | RA (n = 9) |
| RARS (n = 8)         | 0.34     | 0.08 | RARS (n = 8) |

Figure 1. ABCB7 expression levels. (A) ABCB7 expression levels in the CD34$^+$ cells from healthy controls and MDS patients with RA, RAEB and RARS. (B) ABCB7 expression levels and percentage of ring sideroblasts in the bone marrow. (C) ABCB7 expression levels in cultured erythroblasts from healthy controls and MDS patients with RA and RARS. The boxes represent the 25th, 50th and 75th percentiles, the bars correspond to the 10th and 90th percentiles.

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Discussion

We have examined the role of ABCB7, the gene involved in the hereditary XLSA/A, in the acquired RARS syndrome. The hypothesis under consideration is that XLSA/A and acquired RARS may have a shared molecular basis. Interestingly, there is an excess of erythrocye protoporphyrin in XLSA/A (in contrast with other types of genetic sideroblastic anemia) as also found in RARS [20]. We have determined the ABCB7 gene expression levels from our microarray dataset on CD34+ cells in 122 MDS patients and 16 healthy controls. The expression levels of ABCB7 are significantly lower in the RARS group than in healthy patients and 16 healthy controls. The expression levels of ABCB7 were validated and confirmed in a subset of MDS patients and healthy controls using real-time quantitative PCR. Direct sequencing of ABCB7, including the promoter region, was performed on DNA from 13 RARS patients and did not show any mutations in any of the patients. Similarly, Steensma et al., in a recent study, found no coding mutations of ABCB7 in patients with RARS [20].

We have investigated the methylation status of the ABCB7 promoter region and the results showed >80% methylation in all samples, with no significant difference between MDS patients and healthy controls. Gene promoters can be divided into three groups according to their CpG ratio, GC content and length of CpG-rich region: high-CpG, intermediate-CpG and low-CpG promoters [21]. Based on this classification, the ABCB7 promoter region is defined as an intermediate-CpG promoter. Intermediate-CpG promoters show a high frequency of DNA methylation [21]. Our data fit with the definition of the ABCB7 promoter region as an intermediate CpG promoter, but suggest that promoter methylation is unlikely to be the mechanism underlying the reduction in expression levels of ABCB7 observed in RARS.

No genomic abnormality in the ABCB7 gene was identified in our cases, suggesting that there could be a trans-acting factor responsible for the low levels of ABCB7 in RARS (analogous to the down regulation of the alpha-globin genes in the acquired HbH associated with MDS caused by mutations in the ATRX gene) [22]. Therefore, we examined our microarray dataset for genes with expression levels correlated either strongly positively (≥0.8) or inversely (≤−0.8) with ABCB7 gene expression levels. While no other gene had a strong inverse correlation with ABCB7, interestingly two genes showed a positive correlation: ELP2 (0.82) and RTFI (0.80), both of which are associated with RNA Polymerase II [23,24]. Deregulation of these factors may cause defective gene transcription through chromatin [25].

The existence of an inherited disorder XLSA/A caused by inactivating mutations in ABCB7 is, of course, very informative of the probable consequences of reduced expression of the same gene in acquired disorders. Many of the key properties of ABCB7 have already been established by gene silencing studies and by conditional gene targeting in mice [13,14]. All of these experiments are consistent with the importance of ABCB7 in hematopoiesis and with the observed phenotype in XLSA/A. Therefore, it would be predicted that erythroblasts with low ABCB7 levels would have abnormal mitochondrial iron homeostasis. Our finding of a strong association between RARS and low gene expression levels of ABCB7 also raises the possibility of therapeutic approaches for RARS using drugs which induce the expression of ABCB7, for example, carbamazepine has been shown to induce ABCB7 in the liver of epileptic patients treated with the drug [26]. We suggest that down-regulation of the iron transporter ABCB7 plays an important role in the molecular pathogenesis of RARS, making an intriguing link between the inherited and acquired forms of sideroblastic anemia.

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Author Contributions

Conceived and designed the experiments: EH JB MC JW. Performed the experiments: AG AP MN BP CF TL LM MD MJ SK DB. Analyzed the data: JB AP HC JW. Wrote the paper: JB AP JW.
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