DNA Methylation of the ABO Promoter Underlies Loss of ABO Allelic Expression in a Significant Proportion of Leukemic Patients

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Abstract

Background: Loss of A, B and H antigens from the red blood cells of patients with myeloid malignancies is a frequent occurrence. Previously, we have reported alterations in ABH antigens on the red blood cells of 55% of patients with myeloid malignancies.

Methodology/Principal Findings: To determine the underlying molecular mechanisms of this loss, we assessed ABO allelic expression in 21 patients with ABH antigen loss previously identified by flow cytometric analysis as well as an additional 7 patients detected with ABH antigen changes by serology. When assessing ABO mRNA allelic expression, 6/12 (50%) patients with ABH antigen loss detected by flow cytometry and 5/7 (71%) of the patients with ABH antigen loss detected by serology had a corresponding ABO mRNA allelic loss of expression. We examined the ABO locus for copy number and DNA methylation alterations in 21 patients, 11 with loss of expression of one or both ABO alleles, and 10 patients with no detectable allelic loss of ABO mRNA expression. No loss of heterozygosity (LOH) at the ABO locus was observed in these patients. However in 8/11 (73%) patients with loss of ABO allelic expression, the ABO promoter was methylated compared with 2/10 (20%) of patients with no ABO allelic expression loss (P = 0.03).

Conclusions/Significance: We have found that loss of ABH antigens in patients with hematological malignancies is associated with a corresponding loss of ABO allelic expression in a significant proportion of patients. Loss of ABO allelic expression was strongly associated with DNA methylation of the ABO promoter.

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Introduction

ABH antigens are carbohydrate structures present on the surface of red blood cells (RBCs) and platelets, as well as endothelial and epithelial cells. The antigens are generated by the stepwise addition of monosaccharides to protein or lipid core structures. Two glycosyltransferase genes catalyze the final steps of the stepwise addition of monosaccharides to protein or lipid core structures. The A and B glycosyltransferases, which add different monosaccharides to the precursor H antigen, are encoded by separate alleles of the ABO gene [2,3]; the A glycosyltransferase which adds N-acetylgalactosamine to give the A antigen, and the B glycosyltransferase which adds galactose to give the B antigen. There are numerous weaker alleles of A and B coding for less active glycosyltransferases, the most common of which is A+. [4]. The O allele is a null allele which is transcribed but is enzymatically inactive [3].

Alteration of ABH antigens in hematological malignancy was first reported by van Loghem et al [5] who described very weak A antigen expression on the RBCs of an acute myeloid leukemia (AML) patient, who had previously shown normal A antigen expression. Loss of A, B, or H antigens from the surface of RBCs has since then been a recurrent observation in transfusion laboratories dealing with hematological malignancy patients [6–8].

We previously described the use of a flow cytometric method for the sensitive detection of alterations of A, B and H antigens on RBCs [8]. Fifty-five percent (16/29) of patients with myeloid malignancies of blood group A, B, or AB had a detectable population of RBCs with decreased expression of A or B antigens compared with no detectable changes in 127 normal A, B, and AB individuals. Loss of H was detected in 21% (6/28) of group O patients compared with no changes in 51 normal O individuals.

Possible mechanisms for inactivation of ABO include allelic loss (loss of heterozygosity–LOH), mutation (loss of function) and silencing by DNA methylation. Loss of ABH antigens from tumor
tissue is frequently seen in solid tumors including carcinomas of the buccal epithelium, stomach, colon, lung, ovary, prostate, bladder, and breast [9-18], and is associated with poor prognosis, high tumor grade and increased metastatic potential [9,19-23]. Previous studies have found that loss of ABH antigens in solid tumors is associated with LOH [24-26].

The ABO promoter region is rich in CpG dinucleotides [27,28] and previous analysis of this region in several human carcinoma cell lines and cancers has shown that DNA methylation of the ABO promoter region was inversely correlated with gene expression [25,26,29]. We set out to determine whether LOH and/or DNA methylation of ABO was responsible for ABH antigen alterations in patients with hematological malignancy.

Materials and Methods

Patient samples

The patients analyzed in this study presented to the Haematology-Oncology Department at The Queen Elizabeth Hospital during the period 1996–2000 with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) or myeloproliferative disorders (MPD) including chronic myeloid leukemia (CML). Twenty-one of the patient specimens analyzed were previously described in an analysis of ABH antigens by flow cytometry [8]. Seven additional patients were identified by serology as having loss of ABH antigens. Peripheral blood stem cell (PBSC) and bone marrow (BM) samples from breast cancer patients were used as controls, as well as peripheral blood mononuclear cells (PBMCN) from anonymous voluntary blood donors. For the leukemic patient samples, either bone marrow aspirates or peripheral blood, all samples were taken as part of routine clinical care and were surplus to diagnostic needs. The use of patient samples followed a protocol approved by the Human Research Ethics Committee of The Queen Elizabeth Hospital. Mononuclear cells were prepared from all patient specimens using Ficoll-Paque (Pharmacia, Uppsala, Sweden).

Cell lines and 5-aza-2'-deoxycytidine treatments

Human leukemia cell lines EM-2, HEL, HL-60, K-562, KCL-22, JURKAT and RAJI were grown in RPMI 1640 with 10% fetal bovine serum (FBS), penicillin and streptomycin. Cells were starved in medium supplemented with 0.1% FBS for 48 h prior to treatment. Following this, the medium was changed to include 10% FBS and cells were treated with 5-AZA (1 µM, 2 µM or vehicle - ultra pure water) daily for 3 days. Twenty-four hours after the final treatment, the media was removed, cells were washed with PBS and fresh media was added. Cells were allowed to recover for 24 h and then harvested at 48, 72 and 96 h post treatment. DNA and RNA were isolated as outlined below, however, if there were less than 10^6 cells after treatment due to extensive cell death by 5-AZA treatment, the cells were lysed with 0.5% Nonidet P40, 20 µM RNAsin, 0.01 M DTT [30] and the supernatant was placed in TriPure for RNA extraction while the cell nuclei were bisulfite modified.

RNA and DNA isolation

RNA was isolated with TriPure (Sigma) and genomic DNA was extracted by proteinase K/SDS treatment.[31] RNA was reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Genomic DNA was bisulfite modified as described previously [32,33].

PCR amplification

PCR reactions were performed in a volume of 50 µl and included 0.5 U HotStarTaq polymerase (Qiagen, Hilden, Germany), 2 mM MgCl2, 0.2 mM of each dNTP, 10 µM of each primer, 100 ng of bisulfite modified DNA or 2 µl of cDNA in the supplied buffer (Qiagen).

A BO allelic expression analysis

ABO genotypes were determined as described previously [8]. The relative allelic expression of the ABO alleles in heterozygous patients was determined by restriction digestion of RT-PCR products. cDNA was amplified with primers ABO x5/5F (5'-cgaaggtgctgacaccgtagga-3') and ABO x6/7R (5'-ggaagaacgcttattccttggc-3') for A0² or B0² genotypes with the following PCR conditions 5 cycles of (30 s at 96°C, 60 s at 70°C - 1°C per cycle, 60 s at 72°C) followed by 35 cycles of (30 s at 96°C, 60 s at 65°C, 60 s at 72°C). The 160 bp PCR product was then digested with BstEI and KpnI. Digestion with KpnI was indicative of O' allele expression while digestion with BstEI was indicative of A or B allelic expression (Figure 1A). For genotypes which did not include the O² allele (for example AB and A²B² genotypes), the primers ABO x6/7F (5'-ggcatacgaaaattgtcttc-3') and ABO R (5'-ctgctgctggcttcacttga-3') were used to amplify a 529 bp PCR product with the following PCR conditions: 40 cycles of (30 s at 96°C, 60 s at 68°C, 60 s at 72°C). Digestion of this PCR product with PmlI indicated A² allelic expression and digestion with Abl indicated B allelic expression. The primers and PCR conditions for the 377 bp reference gene were PBGD x1F (5'-ctttcaagggagccgcttg-3') and PBGD x6/7R (5'-cataggtctctccttgctg-3'), conditions 33–35 cycles of (30 s at 96°C, 60 s at 68°C, 60 s at 72°C).

DNA methylation analysis

Methylation independent PCR (MIP) primers for ABO were designed to amplify bisulfite modified sequences regardless of methylation status[34] and are ABO bisF (5'-ggggtggtggtaataggtagtgTT-3') and ABO bisR (5'-gcggaaactcctgcaccatccc-3'). The uppercase Ts or As in the primer sequences indicate the position of a non CpG cytosine. The 269 bp PCR product (~ABO BIS) was amplified from bisulfite modified DNA with the following PCR conditions: 10 cycles (60 s at 94°C, 45 s at 65°C - 1°C per cycle, 45 s at 72°C) followed by 35 cycles of (60 s at 94°C, 60 s at 55°C, 60 s at 72°C). The ABO BIS PCR products were analyzed by methylation sensitive - single strand conformation analysis (MS-SSCA) [33] and/or COBRA (combined bisulfite restriction analysis) [35] and/or melt curve analysis (MCA) [25,36]. For MS-SSCA, the ABO BIS products were analyzed on 0.5 x and/or 0.75 x MDE gels (FMC, Rockland, ME) [33]. For COBRA, the ABO BIS products were digested with the following restriction enzymes: BstUI, HpaII and TaqI (all New England Biolabs, Beverly, MA). The restriction enzymes only digested the PCR product if the cytosine within the restriction enzyme recognition sequence was methylated. For methylation analysis by MCA, the ABO BIS PCR reactions were performed in 20 µl reactions using a Rotorgene 3000 real time PCR machine (Corbett Research, Sydney, Australia). Each reaction consisted of 10 µl of 2× Quantitect SYBR Green real time PCR mix (Qiagen), 2 µl of each primer (5 µM stock), and 6 µl of bisulfite modified DNA. Reactions were heated to 95°C for 15 min, then subjected to 55 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s (acquisition at this step). After a final incubation at 72°C for 4 min, the melting profile was obtained by 90 s of pre-melt conditioning at 60°C then heating the reactions from 60°C to 99°C and acquiring the fluorescence at each 0.5°C increment. The controls
The different nucleotide substitutions characteristic of the major ABO alleles create allele specific restriction enzyme sites. The deleted G in the O1 allele creates a KpnI site while the A and B alleles create allele specific restriction enzyme sites. The capital Ts identify thymines that are a result of bisulfite modification of cytosines and loss of methylation status), one spanned the KpnI/BstEII site. Genotyping of patients for ABO did not reveal any allele shifts indicative of LOH (data not shown). We therefore decided to determine whether the ABO locus underwent allelic expression changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes. Thirteen of the 27 (48%) patient samples had altered allelic expression, either loss or reduced expression of an allele or changes.
expression of the A1 allele. F10 and F60, both with loss of A by flow analysis had no expression of any ABO allele but were positive for the reference gene PBGD. However, for F60, a sample taken 3 years prior was positive for ABO expression. Though there was no flow cytometry performed for that sample, it seems likely that loss of ABO occurred as the malignancy progressed from the chronic phase to the blast crisis of CML. Loss of A allele expression was also seen in the leukemic cells of four other patients, F7, F11, F39 and F53 (Figure 1B and 1C). F39 and F53 samples showed loss of A and H for the reference gene flow cytometry performed for that sample, it seems likely that loss of A allele expression was also seen in the leukemic cells of four other patients, F7, F11, F39 and F53 (Figure 1B and 1C). F39 and F53 samples showed loss of A and H for the reference gene.

| ID  | DIAGNOSIS   | GENO  | FLOW ANALYSIS | EXP     |
|-----|-------------|-------|---------------|---------|
| F7  | AML M2      | A1O1  | no loss       | loss of A1 |
| F9  | AML M2      | A1O1  | LOSS of A & H | loss of A1 |
| F10 | MDS         | A2O1  | no loss       | loss of A1 |
| F11 | AML M1      | A1O1  | no loss       | loss of A1 |
| F14 | AML M2      | A2O1  | no loss       | no loss   |
| F15 | MDS         | A1O2  | no loss       | no loss   |
| F17 | MDS         | A1O1  | no loss       | no loss   |
| F20 | CML         | A1O1  | LOSS of H     | no loss   |
| F23 | CML chronic | A2B   | no loss       | no loss   |
| F24 | AML         | B01   | LOSS of B     | no loss   |
| F25 | MDS         | A1A1  | LOSS of A     | no loss   |
| F26 | AML M3      | A1O1  | no loss       | no loss   |
| F27 | AML M4      | A1O1  | LOSS of A & H | loss of A1 |
| F30 | CML chronic | B01   | LOSS of H     | no loss   |
| F39 | AML M4      | A1O1  | LOSS of H     | loss of A1 |
| F42 | AML M3      | B01   | LOSS of B     | no loss   |
| F46 | AML M3      | A1O1  | LOSS of A     | no loss   |
| F51 | AML M4      | A1O1  | LOSS of A     | no loss   |
| F53 | AML M1      | A1O1  | LOSS of H     | loss of A1 |
| F57 | AML M7      | B01   | LOSS of H     | no loss   |
| F60 | CML blast crisis | A1A2 | LOSS of A | ‘93+’/’96+’ |

In the ID column a F prefix denotes patients analyzed by flow cytometry [8] while a S prefix denotes loss of ABH antigen patients as detected by serology. The GENO column refers to ABO genotype and ‘EXP’ to ABO allelic expression. For SEROLOGY ‘mfr’ refers to a mixed field reaction. In the EXP column, which allele is lost is shown in italics and underline. For patient F60 there were 2 samples analyzed for ABO mRNA expression, one in 1993 and one in 1996. The 1993 sample (93+) was positive for ABO expression however the 1996 sample (96−) which was when the flow analysis was performed, was negative for ABO mRNA expression. The bold indicates the samples with loss of ABH antigens. F25 was an A1A1 sample therefore determining allelic expression was not possible.

Table 1. ABO genotyping, ABH antigen status and ABO allelic expression.

| ID  | DIAGNOSIS   | GENO  | FLOW ANALYSIS | EXP     |
|-----|-------------|-------|---------------|---------|
| S1  | RAJEB       | A1O1  | weak A2 expression | loss of A1 |
| S2  | MDS         | A1O1  | LOSS of A     | loss of A1 |
| S3  | CML         | A1O1  | LOSS of A     | no loss   |
| S5  | AML M2      | A1O1  | mfr—only H antigen | loss of A1 |
| S6  | AML M3      | A1B   | mfr with anti-A | loss of A1 |
| S7  | AML M3      | B01   | mfr with anti-B | no loss   |
| S8  | AML M0      | A1O1  | LOSS of A     | loss of A1 |

Table 2. ABO genotyping, expression and methylation analysis of leukemic cell lines.

| NAME | TYPE                      | GENO  | EXP | METH | 5-AZA |
|------|---------------------------|-------|-----|------|-------|
| EM-2 | Human CML in blast crisis | A1A2  | M   | +    |       |
| HEL  | Human erythroleukemia     | O1O1  | M   | +    |       |
| JURKAT | Human T cell leukemia     | O1O2  | M   | +    |       |
| K-562 | Human CML in blast crisis | O1O1  | U   | +    |       |
| KCL-22 | Human CML in blast crisis | A1O2  | U   | +    |       |
| RAJI | Human Burkitt lymphoma    | O1O1  | M   | +    |       |

*TYPE* refers to the cell line type and origin, ‘GENO’ refers to the ABO genotype, ‘EXP’ to ABO mRNA expression and ‘METH’ to the ABO promoter CpG island methylation status as determined by MS-SSCA and COBRA (Figure 2). ‘5-AZA’ refers to ABO mRNA expression after the cell line was treated with 5-aza-2-deoxyxycytidine as outlined in the ‘Materials and Methods’. ‘+’ refers to positive ABO expression, ‘−’ refers to unmethylated and ‘M’ to methylated ABO promoter region.

To express ABO, an attempt to re-express ABO was performed by treating the cells with the demethylating agent, 5-aza-2-deoxyxycytidine (5-AZA). K-562 and HEL already expressed ABO and hence 5-AZA treatment resulted in no change (Table 2). The other cell lines EM-2, JURKAT, KCL-22 and RAJI were

ABO allele expression studies in leukemic cell lines

Since LOH of the ABO locus was not observed in patient samples, we went on to assess DNA methylation of the ABO promoter. The region we examined spans the transcription start site and the bisulfite modified methylated sequence is shown in Figure 1D. We firstly investigated ABO promoter DNA methylation in leukemic cell lines since only 2/6 cell lines expressed ABO (Table 2), although it must be recognized that JURKAT and RAJI, which derive from lymphocytic leukemia would not be expected to express ABO. We investigated ABO DNA methylation in the leukemic cell lines by two methodologies, MS-SSCA [33] and COBRA [35]. From Figure 2A it is evident that only the K-562 cell line had the same banding pattern as the unmethylated PBMC and PBSC samples. The remaining cell lines were methylated and lacked expression of ABO. The only cell line which expressed ABO but was methylated was the HEL cell line. Restriction enzyme digestion of the ABO BIS PCR products again showed that all the leukemic cell lines, except K-562 were methylated to various degrees (Figure 2B). HEL was the least methylated of the cell lines, perhaps indicative that only one of the ABO alleles was methylated and thus this cell line may still express ABO from the other allele.

Since many of the leukemic cell lines were methylated and failed to express ABO, an attempt to re-express ABO was performed by treating the cells with the demethylating agent, 5-aza-2-deoxyxycytidine (5-AZA). K-562 and HEL already expressed ABO and hence 5-AZA treatment resulted in no change (Table 2). The other cell lines EM-2, JURKAT, KCL-22 and RAJI were
methylated and did not express ABO but after treatment with 5-AZA they all were demethylated and re-expressed ABO. The re-expression of ABO after treatment with 5-AZA in the JURKAT cell line is shown in Figure 2C as well as the corresponding demethylation at the ABO promoter (ie presence of uncut band in 5Aza treated JURKAT compared to vehicle treated - Figure 2D). Since ABO DNA promoter methylation was shown to be responsible for the silencing of this locus in the leukemic cell

Figure 2. ABO promoter methylation in leukemic cell lines. (A) MS-SSCA analysis of the ABO BIS PCR products. PBMNC refers to peripheral blood mononuclear cells and PBSC to peripheral blood stem cells. These were used as unmethylated controls. It is clear from the SSCA gel that only the K-562 leukemic cell line is unmethylated as it has the same banding pattern as the PBMNC and PBSC. The other cell lines all have varying amounts of methylation as seen by the various banding patterns. The JURKAT and RAJI cell lines were hypermethylated, as seen by the dramatic shift of the bottom doublet of bands. (B) Restriction enzyme digests of the ABO BIS PCR products. Digestion with any of the restriction enzymes is indicative of methylation at that Cpg site within the restriction enzyme recognition sequence. All the products will cut with TaqI since there is a TaqI site in the reverse primer. (C) ABO re-expression in the JURKAT cell line after 24 h treatment with 5-aza-2’-deoxycytidine treatment. On the gel, the NEGATIVE was an RT control (RNA only), the VEHICLE lane was JURKAT cells treated with ultra pure water, the following lanes are JURKAT cells treated with 1 µM or 2 µM of 5-aza-2’-deoxycytidine respectively showing ABO re-expression. PBGD is the reference gene. (D) The ABO promoter is demethylated in JURKAT cells after 5-aza-2’-deoxycytidine treatment. In the VEHICLE treated JURKAT cells there is no evidence of unmethylated ABO promoter which would be a band at the same size as the UNCT sample. However, after treatment with 1 or 2 µM of 5-aza-2’-deoxycytidine the ABO promoter is unmethylated as evidenced by a band at the same size as the UNCT sample.

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lines, we assessed whether ABO methylation was responsible for the loss of ABO allelic expression in the patient specimens.

**ABO methylation analysis in patients with hematological malignancies**

The ABO promoter region was analyzed for methylation in the patient samples by three different methodologies: MS-SSCA, COBRA and MCA. Initially the samples were analyzed by MS-SSCA with 2 running conditions, [42,43] followed by COBRA as a second method [35]. The restriction enzymes used to analyze ABO promoter methylation analysis were TaqI, HinfI and BstUI. There were 34 CpGs in the ABO BIS PCR product (Figure 1D) and restriction enzyme digestion allowed analysis of 16 of these CpGs. No ABO promoter methylation was detected in the normal specimens, four PBMC and four PBSC, despite a vast preponderance of non-expressing cells (Figure 2A and 2B).

Of the 21 patients analyzed for ABO promoter DNA methylation, 11 had loss of expression of one or both of the ABO alleles while 10 had no ABO allelic loss (Table 3). The ABO promoter was methylated in 8/11 (73%) of the patients with loss of ABO allelic expression compared with 2/10 (20%) of patients with no allelic loss (P = 0.03 by Fisher’s exact test) (Table 3; Figure 3).

Five of the 7 patients that were previously ascertained by serology and RT-PCR as having loss of A or B antigens were methylated at the ABO locus when analyzed by MS-SSCA and COBRA (patients with S prefixes, Table 3). COBRA was concordant with MS-SSCA except for sample S4 which was only methylated at one restriction site but unmethylated by MS-SSCA (data not shown), thus providing support for why more than one methodology was used. Methylation for 16 patients was additionally assessed using a more sensitive assay, MCA. After MCA, 6 additional patient samples, originally classified as unmethylated by MS-SSCA and COBRA, were found to be methylated (Figure 3), again providing further support for using more than one methodology. It is not surprising that MCA classified 6 additional patients as methylated as unlike MS-SSCA it does not rely on alterations in DNA strand conformations that result in obvious gel shifts, and unlike COBRA it does not rely on the CpG being assessed residing in a restriction enzyme site. MCA uses differences in melting temperature between methylated and unmethylated sequence, is relatively independent of the location of sequence differences within the PCR product, and is therefore able to detect differences in methylation at more sites than MS-SSCA or COBRA. Further, changes in melting curve profiles can result from even a single nucleotide change, therefore MCA can even detect methylation of one CpG site [36,44].

**Table 3. ABO methylation analysis in patient specimens with ABO allelic loss of expression.**

| ID   | DIAGNOSIS | GENO | EXP | METH |
|------|-----------|------|-----|------|
| F9   | AML       | A1°  | little A1 | U    |
| F10  | MD5       | A1°  | loss of A & O | M    |
| F11  | AML M1    | A1°  | loss of A | U    |
| F15  | CML blast crisis | A1A2 | No loss | M    |
| F17  | MD5       | A0°  | No loss | U    |
| F20  | CML       | A1°  | No loss | U    |
| F23  | CML chronic | A0°  | No loss | U    |
| F24  | AML       | BO1  | No loss | U    |
| F27  | AML M4    | A0°  | loss of A | M    |
| F30  | CML chronic | BO1  | No loss | U    |
| F39  | AML M4    | A0°  | loss of A | M    |
| F51  | AML M4    | A0°  | No loss | U    |
| F53  | AML M1    | A0°  | loss of A | M    |
| F57  | AML M7    | BO1  | No loss | U    |
| F60  | CML blast crisis | A1A2 | 93-96- | M    |

In the ID column a F prefix denotes patients analyzed by flow cytometry while a S prefix denotes loss of ABO antigens patients detected by serology. ‘GENO’ refers to ABO genotype, ‘METH’ to ABO promoter CpG island methylation assessed either by MS-SSCA, COBRA and/or melt curve analysis (MCA), ‘U’ unmethylated at the ABO promoter and ‘M’ is methylated at the ABO promoter. For patient F60 two samples were available for analysis; the 1993 (93) sample was positive for ABO expression whereas the 1996 (96) sample was negative for ABO expression.

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**Discussion**

Loss of ABH antigens in a subset of RBCs derived from a malignant stem cell is likely to be indicative of genetic or epigenetic changes that have occurred in the malignant stem cell. Malignant stem cells often retain the ability to differentiate along several lineages including the erythroid lineage [45–47]. RBCs that are deficient in A or B antigens have been reported to have decreased transferrase activities, supporting the notion that loss of antigens reflects a change at the ABO locus and not at the cell surface or membrane precursors [48].

In this study, we examined whether loss of the A and B blood group antigens could be related to LOH at the ABO locus, differential expression of ABO alleles, or DNA methylation of the ABO promoter. It is surprising that no ABO LOH was observed as a substantial proportion of myeloid leukemias have deletions including the 9q34 region where ABO is located [49].

However, loss of mRNA expression of the corresponding ABO allele was seen for 11/19 (58%) of patients with loss of A and B antigens. Of these, 71% of samples shown to have loss by serology had detectable allelic loss at the mRNA expression level compared with 50% seen in patients with ABH antigen loss by flow analysis. The increased agreement in the samples with ABH antigen loss as determined by serology is not surprising since more than 50% of the cells need to have abnormal antigen expression to be readily detected by serology. The flow cytometric analysis can detect alterations in cell populations as low as 10%, and thus in these patient samples the larger normal population of cells would mask the expression changes of the smaller abnormal cell population. Therefore, it was not surprising that the mRNA analysis was more concordant with the loss of ABH antigen samples detected by serology compared with flow cytometry.

The 5-AZA treatment of the ABO negative leukemic cell lines indicated that ABO DNA methylation was associated with lack of ABO expression since demethylation of the ABO promoter resulted in re-expression of the gene. In the patients with ABO allelic loss, methylation of the ABO promoter was detected for 73% of the samples. Recently there has been a report in which ABO DNA methylation was found in leukemic patients [50] however since
only the abstract was available we are not able to compare our results with this study. However, we show that DNA methylation is significantly associated with silencing of the ABO transcript in patients with hematological malignancies and that the ABO transcript can be re-expressed in leukemic cell lines by treating with a demethylating agent. DNA methylation of the ABO promoter would explain much of the reported loss of ABH antigens in patients with hematological malignancies [6].

There may of course be multiple mechanisms underlying the loss of ABH antigens in hematopoietic malignancy. It is intriguing to consider that ABO methylation may be part of a long range epigenetic silencing mechanism [51] leading to the co-ordinate silencing of a linked tumor suppressor gene may also be important in which case the alterations in ABO may be a sign post to a recurrent oncogenic mechanism. This is supported by the observation that methylation in some cases is likely to lead to no cellular phenotype as was observed in OO individuals in which both alleles of the ABO locus are null or where the leukemic stem cells are unable to differentiate.

Additionally, since changes at the ABO locus have been associated with changes at other 9q34 loci, it is likely that ABO alterations are not the leukemia causing event but rather a marker of other events occurring at this chromosomal region. This is supported by the reported observation of individuals with decreases in both ABO and adenylyl kinase (AKT) expression [52–54] in their leukemic cells. AKT is localized at 9q34.11, not too distant from ABO at 9q34.2. Further studies are needed to determine the importance of ABO alterations in leukemia and whether these are causative or an epiphenomenon.

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Author Contributions
Conceived and designed the experiments: AD. Performed the experiments: TBM DJH TKD. Analyzed the data: TBM DJH. Contributed reagents/materials/analysis tools: DSO. Wrote the paper: TBM DJH AD.

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