

09/14
0412



**MOLECULAR ANALYSIS OF
CHANGES IN ABO BLOOD GROUP
ANTIGEN EXPRESSION IN
HAEMATOLOGICAL
MALIGNANCY**

DENISE S. O'KEEFE B.SC. (HONS)

A thesis submitted in total fulfillment of the requirements for
the degree of Doctor of Philosophy

Department of Medicine

University of Adelaide,
Adelaide, South Australia

October, 1995

Copyrighted material

TABLE OF CONTENTS

Summary	xii
Declaration.....	xiii
Acknowledgments	xiv
Publications.....	xv
Conference Presentations	xvi
List of abbreviations used in this thesis	xviii

CHAPTER ONE: REVIEW OF THE LITERATURE

1.0 Introduction.....	2
1.1 Genetics and biochemistry of the ABO blood group system.....	3
1.2 Precursor structures	5
1.3 The H antigen.....	7
1.4 The Lewis system.....	9
1.5 The I system.....	11
1.6 The molecular basis of the ABO blood group.....	13
1.7 The A ² allele.....	14
1.8 The A ³ and B ³ alleles	15
1.9 The A ^x and cis-AB alleles	17
1.10 Another kind of O allele	18
1.11 Intron and exon structure of the ABO gene.....	18
1.12 Interactions between the different blood group systems	19
1.13 Loss of blood group antigens in haemopoietic malignancy.....	19
1.14 Masking of antigens by sialyl-transferases	21
1.15 The association between chemotherapy and loss of ABH antigens.....	23
1.16 Loss of the H antigen precursor	24

1.17 Loss of the I antigen.....	25
1.18 Intolerance to "self" antigens.....	27
1.19 Intrinsic factors that may modify ABH antigens	27
1.20 A genetic basis for loss of ABH antigens	28
1.21 Loss of A or B antigens - an indicator of pre-leukaemic status?	30
1.22 Family studies of patients with loss of A or B antigens.....	31
1.23 Loss of the ABH antigens in carcinoma	34
1.24 Relationship between loss of ABH antigens and tumour aggressiveness.....	35
1.25 Biochemical studies of blood group antigens in tumours.....	36
1.26 Studies using monoclonal antibodies that detect the A and B glycosyltransferases	37
1.27 Genetic analysis of urothelial cell lines with loss of blood group A antigen expression.....	40
1.28 Expression of incompatible A antigen in carcinoma.....	40
1.29 The haemopoietic malignancies	42
1.30 Disruption of the ABL proto-oncogene at 9q34.1 in CML.....	44
1.31 Disruption of other genes at 9q34 in leukaemia.....	45
1.32 Tumour suppressor genes and loss of ABO antigens	46
1.33 Aim of this project.....	49

CHAPTER TWO: GENERAL MATERIALS AND METHODS

2.0 Reagents and solutions	51
2.1 Solutions used for the analysis of red cells.....	51
Antibodies.....	51
30% solution of polyvinyl pyrrolidone (PVP).....	51
Phosphate-buffered saline (PBS).....	51

2.2 Solutions used for cell isolation and culture.....	52
Trypsin-versene.....	52
Growth Media.....	52
2.3 Solutions for molecular biology.....	52
EDTA (ethylenediamine tetra-acetic acid), 0.5M.....	52
TEN1 buffer.....	52
Phenol.....	52
TE buffer.....	53
TES1 buffer.....	53
Solution D.....	53
DEPC (diethylpyrocarbonate) treated H ₂ O.....	53
Loading buffer (6x).....	53
5x Tris-Borate (TBE) buffer.....	53
20x SSPE.....	53
10mg/ml Salmon Sperm DNA.....	53
Sephadex column.....	54
Prehybridization mix.....	54
100x Denhardt's solution.....	54
Stripping solution.....	54
40mM dNTP solution.....	54
Ligase 10x buffer.....	55
IPTG stock solution (0.1M).....	55
X-Gal (50mg/ml).....	55
Luria Broth (LB) Medium.....	55
Preparation of LB plates with ampicillin/IPTG/X-Gal.....	55

2.4 Techniques used for serological analysis of red cells.....	56
2.4.1 Preparation of red cells for long-term storage.....	56
2.4.2 Thawing frozen red cells.....	56
2.4.3 Blood grouping.....	57
2.5 Cell isolation, culture and regular maintenance.....	59
2.5.1 Isolation of the mononuclear cell fraction from patient samples.....	59
2.5.2 Cell lines.....	59
2.5.3 Freezing and thawing of cells.....	60
2.6 Molecular biology techniques.....	61
2.6.1 Preparation of genomic DNA.....	61
Phenol-Chloroform extraction.....	61
Salting out procedure for extracting DNA.....	61
2.6.2 Preparation of total RNA.....	62
2.6.2.1 Modification of the RNA isolation method for isolating RNA and DNA from limited patient samples.....	63
2.6.3 Quantitation of DNA and RNA.....	63
2.6.4 Polymerase Chain Reaction (PCR).....	64
Primer Design.....	64
Synthesis and isolation of oligonucleotides.....	65
Standard amplification conditions.....	65
2.6.5 Reverse-transcriptase polymerase chain reaction (RT-PCR).....	66
Removal of contaminating DNA from RNA prior to reverse- transcription.....	66
2.6.6 Gel electrophoresis.....	67
Agarose gel electrophoresis.....	67
Polyacrylamide gel electrophoresis.....	67

2.6.7 Southern blot analysis.....	67
Preparation of samples.....	67
Electrophoresis	68
Alkali blotting procedure.....	68
Radio-labelling of probes	69
Procedure for membrane hybridisation	69
Washing the membrane	69
Autoradiography	70
Re-use of blots	70
2.6.8 Purification of PCR products for use in cloning and sequencing experiments.....	70
2.6.9 Cloning of PCR products.....	71
Ligation of purified PCR product into the pGEM -T Vector	71
Preparation of competent bacterial cells	71
Transformation of XL1-Blue cells.....	72
Analysis of Transformants.....	72
Screening bacterial colonies for recombinant plasmids using PCR.....	72
Small scale isolation of recombinant plasmid.....	73
Larger scale isolation of recombinant plasmids	74
2.6.10 Sequencing.....	74
Automatic sequencing	75
Denaturation of plasmid template for manual sequencing.....	75
Manual sequencing	75
Sequencing gels	76

CHAPTER THREE: RED CELL SEROLOGY

3.0 Introduction.....	78
3.1 Considerations and difficulties encountered when collecting samples from patients with changes in ABO blood group status.....	79
3.1.1 Evidence that mixed-field reactions are not perceived.....	80
3.2 Patients used in this study.....	83
3.2.1 Patients with haematological malignancy and loss of A or B antigens.....	83
3.2.2 Ante-natal patients with loss of A antigen expression.....	85
3.2.3 Red cell analysis.....	86
3.2.4 Cytogenetics.....	87
3.2.5 Quality of samples.....	88
Case-histories of patients used in this study (Table 3-1).....	89
Summary.....	96

CHAPTER FOUR: GENOTYPING THE ABO LOCUS

4.0 Introduction.....	99
4.1 Development of a sensitive method for molecular ABO genotyping.....	100
4.1.1 Rationale.....	100
4.1.2 Materials and Methods.....	102
4.1.3 Results and Discussion.....	102
4.2 ABO genotyping of patients with malignancy.....	108
4.2.1 Materials and Methods.....	109
4.2.2 Results and Discussion.....	109
4.2.2.1 Patients with malignancy.....	109
4.2.2.2 Patients with haematological malignancy and loss of ABO antigens.....	112

4.2.2.3	Ante-natal patients with abnormal A antigen expression detected during routine screening.....	118
4.3	Development of a method to genotype the A ² allele.....	118
4.3.1	Rationale.....	119
4.3.2	Materials and methods.....	119
4.3.3	Results and Discussion.....	120
4.4	A ² Genotyping of patients with loss of A antigen expression.....	122
4.4.1	Results and Discussion.....	122
4.4.1.1	Patients of A ² blood group.....	122
4.4.1.2	A ² genotyping of patients with loss of A antigen expression and haematological malignancy.....	125
4.5	Confirmation of allele dosage using Southern blotting.....	126
4.5.1	Rationale.....	127
4.5.2	Materials and Methods.....	127
4.5.3	Results and Discussion.....	128
	Summary.....	130

CHAPTER FIVE: EXPRESSION OF THE ABO GENE

5.0	Introduction.....	133
5.1	Development of a sensitive method for ABO phenotyping.....	134
5.1.1	Rationale.....	134
5.2	Positive controls for the reverse-transcription reaction.....	134
5.2.1	Assessment of β -actin as a positive control for the reverse-transcription reaction.....	134
5.2.2	Materials and methods.....	135
5.2.3	Results and Discussion.....	135

5.2.4 Assessment of β -globin as a positive control for the reverse-transcription reaction	136
5.2.5 Materials and Methods	137
5.2.6 Results and Discussion	137
5.3 Expression of the ABO gene.....	138
5.3.1 Materials and methods.....	139
5.3.2 Results and Discussion	139
5.4 Sequencing of the new "O" ABO allele.....	142
5.4.1 Materials and Methods	142
5.4.2 Results and Discussion	142
5.5 Development of a PCR method to detect the O ² allele.....	147
5.5.1 Rationale.....	147
5.5.2 Materials and methods.....	148
5.5.3 Results and Discussion	149
5.6 Development of a modified method to assess the relative expression of the A/B and O alleles.	150
5.6.1 Materials and methods.....	151
5.6.2 Results and Discussion	151
5.7 Detection of a minor splice variant of the ABO gene	155
5.8 Analysis of ABO gene expression in patients with colon cancer	159
5.8.1 Expression of the ABO gene in colon tumours	159
5.9 Analysis of ABO gene expression in patients with haematological malignancy and loss of antigen expression.....	161
Patient AN.....	163
Patient GN.....	164
Patient MR.....	164
Patients NR and WM.....	165
Patient MA.....	165

Patient CG.....	165
Patient PM	166
Patient BA.....	166
Patients WD and SR	167
Patients KN and DH.....	167
5.10 Imprinting and loss of ABO antigens	170
5.10.1 Results and Discussion	171
Summary.....	174
CHAPTER SIX: ANALYSIS OF THE ADENYLATE KINASE 1 GENE	
6.0 Introduction.....	177
6.1 Rationale.....	178
6.2 Theoretical localization of the <i>BanI</i> restriction enzyme polymorphism in the AK1 gene	178
6.2.1 Materials and Methods	180
6.2.2 Results and Discussion	180
6.3 Theoretical localization of the <i>TaqI</i> restriction enzyme polymorphism in the AK1 gene	182
6.3.1 Materials and Methods	183
6.3.2 Results and Discussion	183
6.4 Actual Localization of the <i>TaqI</i> polymorphism.....	185
6.4.1 Materials and Methods	185
6.4.2 Results and Discussion	186
6.5 Expression analysis of the polymorphic <i>TaqI</i> site	187
6.5.1 Results and Discussion	187
6.6 Sequencing of the new AK1 allele	188
6.6.1 Materials and Methods	188
6.6.2 Results and Discussion	189

6.7 Analysis of patients with loss of A/B antigens for loss of heterozygosity of the AK1 gene.....	191
6.7.1 Materials and Methods	191
6.7.2 Results and Discussion	192
6.8 Analysis of AK1 gene expression in patients with haematological malignancy and loss of antigen expression.....	195
6.8.1 Materials and Methods	195
6.8.2 Results and Discussion	196
Summary.....	201

CHAPTER SEVEN: ANALYSIS OF THE ABL GENE

7.0 Introduction.....	204
7.1 Analysis of the ABL polymorphism in normal individuals.....	205
7.1.1 Rationale.....	205
7.1.2 Materials and Methods	208
7.1.3 Results and Discussion	208
7.2 Analysis of the ABL gene in patients with haematological malignancy and loss of ABO antigen expression.....	209
7.2.1 Optimization of the RT-PCR method to assess allelic expression of the ABL gene.....	211
7.2.2 Materials and Methods	211
7.2.3 Results and Discussion	212
7.3 Analysis of the ABL gene in patients with malignancy and no loss of ABO antigen expression.....	212
7.3.1 Analysis of allele dosage	212
7.3.2 Analysis of ABL expression in patients with haematological malignancy and no loss of blood group antigen expression.	214
7.3.3 Results and Discussion	218

Summary.....	220
CHAPTER EIGHT: CONCLUDING DISCUSSION	222
Future Directions	224
BIBLIOGRAPHY	226
APPENDIX A	255
Table 1. Comparison of ABO genotype with ABO blood group of patients with a wide range of haematological malignancies.	256
Table 2. Comparison of ABO genotype of tumours and blood group of patients.....	259
APPENDIX B	260
Table 1: Patients analysed at the ABL locus.....	261

Summary

Loss of expression of the A and B antigens of the ABO blood group system from the red cells of patients with haematological malignancy was first observed in the 1950s. Loss of antigen expression *per se* is probably not of direct importance in disease progression, but reflects a genetic alteration that has occurred in the malignant stem cell, as both the leukaemic cells and erythrocytes can be derived from the same precursor.

The recent cloning of the coding region of the ABO gene has made it possible to investigate loss of ABO antigens at the molecular level. This thesis describes the development of techniques to genotype and simultaneously assess allele dosage at the ABO locus using PCR and allele-specific restriction enzyme digestion. Fifteen patients with a variety of haematological malignancies and loss of A and/or B antigen expression were analysed. Loss of antigen expression in two cases was not due to changes occurring at the ABO locus. Four of the remaining thirteen patients had abnormal allele dosage of the ABO gene; one with physical loss of the A allele, one patient with two copies of the O allele and one A allele, and two patients that had lost the O allele. As the O allele of the ABO gene is non-functional, and loss of a non-functional allele can not provide a growth or other advantage to the cell, it was concluded that the changes seen at the ABO locus are an indication of other genetic events occurring in the same chromosomal region.

Analysis of the relative expression of the ABO alleles using RT-PCR and restriction enzyme digestion revealed that the A allele was transcriptionally silenced in four patients. However, these four patients, all of whom genotyped as AO, expressed the O allele. As monoallelic expression of autosomal genes usually indicates imprinting, studies of the patients and their parents were carried out to determine if there was any parental bias in the contribution of the affected allele. Including data from the literature, 11 of 12 patients with loss of A or B antigen expression had lost

expression of the maternally inherited allele of the ABO gene. Therefore, it seems highly likely that imprinting is involved in the etiology of this phenomenon.

Other reports show that some patients with loss of A or B antigens have markedly reduced levels of the enzyme adenylate kinase 1. As both the ABO and AK1 genes are located in chromosome band 9q34, it was possible that the AK1 gene was also affected by the silencing or loss of heterozygosity (LOH) event occurring at the ABO locus. A *Taq1* restriction enzyme polymorphism in exon 7 of the AK1 gene was identified. Using this polymorphism, it was determined that the loss of heterozygosity event occurring at the ABO gene in one patient, does not extend to the AK1 gene. However, analysis of the allelic expression of the AK1 gene in a patient with severely reduced AK1 enzyme activity and allelic silencing of the ABO gene, revealed decreased expression of one of the alleles, although this patient had normal allele dosage at the AK1 locus. It was therefore concluded that this patient has allelic silencing of both the linked ABO and AK1 genes.

As recurrent LOH in a given region usually indicates the presence of a tumour suppressor gene, the ABL gene, which is located in between the ABO and AK1 genes, was examined. The ABL gene is involved in regulation of the the cell cycle, and therefore it can be considered a potential tumour suppressor gene. Two patients with allelic silencing of the ABO gene and no LOH at the ABO locus were examined. Although both patients had normal allele dosage at the ABL locus, expression analysis of the ABL gene was inconclusive. Although normal controls always produced repeatable results using three different PCR-based methods for the analysis, the relative allelic expression of the ABL gene in the patients was highly variable. The reasons for this are unclear, but are probably due to low mRNA copy numbers of the ABL gene in these patients.

This is the first report of LOH events occurring at the ABO locus and of allelic silencing in haematological malignancy, and represents as yet undescribed genetic and epi-genetic mechanisms for leukemogenesis.