DIFFERENTIAL GENE EXPRESSION ANALYSIS IN DRUG RESISTANT MULTIPLE MYELOMA CELL LINES

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ABSTRACT

DIFFERENTIAL GENE EXPRESSION ANALYSIS IN DRUG RESISTANT MULTIPLE MYELOMA CELL LINES

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The emergence of drug-resistance of tumor cells is a major complication for succesful chemotherapy. In this study, the molecular mechanisms of resistance to prednisone, vincristine and melphalan in multiple myeloma cell lines, RPMI-8226 and U-266 were investigated. Drug resistance was induced by application of the drugs by stepwise dose increments and confirmed by XTT cytotoxicity assay.

Gene expression analysis demostrated that *MDR1* gene is one of the most important factor causing the multidrug resistance phenotype in prednisone, vincristine and melphalan resistant multiple myeloma cell lines. According to microarray analysis alterations in laminin, integrin and collagen genes were detected. Additionally, upregulation of some oncogenes and growth factors (*Rho family of GTPases, YES1, ACT2, TGFBR, EPS15, PDGF*) was shown to have a role in MDR in multiple myeloma. Significant downregulation of suppressors of cytokine signalling gene expressions and upregulation of different types of interleukine and interferon gene expressions (*IL3 and interferon-gamma receptor*) which are related to JAK-STAT signalling pathay was shown. Alterations in expression levels of genes related to ceramide metabolism were shown especially for melphalan resistance in multiple myeloma.

The data from vincristine/prednisone and vincristine/melphalan drug combination studies were shown that the usage of vincristine on prednisone and melphalan resistant multiple myeloma cell lines increase the efficacy of the chemotherapy. On the other hand the cross-resistance development of prednisone and melphalan resistant sublines to irradiation was detected.

These results may help to understand the molecular mechanisms of prednisone, vincristine and melphalan resistance in multiple myeloma model cell lines RPMI-8226 and U-266.

Keywords: Multiple myeloma, multidrug resistance, microarray analysis

ÖZ

İLAÇ DİRENÇLİ MULTİPL MYELOMA HÜCRE HATLARINDA FARKLI GEN ANLATIM ANALİZLERİ

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Tümör hücrelerinde görülen ilaç dirençliliği kemoterapi başarısını olumsuz yönde etkileyen bir faktördür. Bu çalışmada, multipl myeloma hücre hatları olan RPMI-8226 ve U-266'da prednison, vinkristin ve melfalan'a karşı gelişen dirençliliğin moleküler mekanizmaları araştırılmıştır. Dirençlilik ilaçların artan dozlarda uygulanmasıyla geliştirilmiş ve XTT sitotoksisite deneyi ile gösterilmiştir.

Gen anlatım analizleri *MDR1* geninin multipl myelomada prednisone, vinkristin ve melphalan direnci açısından en önemli faktörlerden biri olduğunu göstermektedir. Mikrodizi analizlerinden elde edilen sonuçlarda laminin, integrin ve kollajen genlerinde farklılıklar tespit edilmiştir. Bunlara ek olarak, bazı onkogen ve büyüme faktör genlerindeki (*Rho ailesi GTPazları, YES1, ACT2, TGFBR, EPS15, PDGF* vb) artışın multiple myelomada çoklu ilaç dirençliliğinde rolü olduğu bulunmuştur. JAK-STAT sinyal yolağında görevli bazı interlökin ve interferonların (*(IL3, interferon-gama reseptör* vb) artışı ve sitokin sinyal genlerinin supresörlerinin düşüşü gözlenmiştir. Seramid metabolizmasıyla ilgili genlerdeki değişikliklerin multipl myelomada özellikle melfalan direnci ile bağlantılı olduğu gözlenmiştir.

Vinkristin/prednison ve vinkristin/melfalan ilaç kombinasyon çalışmalarından elde edilen sonuçlar vinkristinin prednison ve melfalana dirençli olan multipl myeloma

hücre hatlarında daha etkili olduğunu göstermiştir. Diğer taraftan prednisona ve melfalana dirençli hatların radyasyona çapraz direnç gösterdikleri görülmüştür.

Bu sonuçların multipl myeloma model hücre hatları RPMI-8226 ve U-266'da prednison, vinkristin ve melfalana karşı gelişen ilaç dirençliliğinin moleküler mekanizmalarının anlaşılmasında önemli olabileceği düşünülmektedir.

Anahtar Kelimeler: Multipl myeloma, çoklu ilaç dirençliliği, mikrodizin analizi

To my lovely children Gamze and Burak

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TABLE OF CONTENTS

ABSTRACT..iv

ÖZ...vi

ACKNOWLEDGMENTS...ix

TABLE OF CONTENTS..x

LIST OF TABLES...xiv

LIST OF FIGURES...xvii

ABBREVIATIONS..xxii

CHAPTERS

1. INTRODUCTION..1

1.1 Multiple Myeloma...1

1.2 Drug Therapy For Multiple Myeloma 4

1.2.1 Standard Chemotherapy...4

1.2.2 Combination Chemotherapy...4

1.2.3 Refractory and Relapsing Myeloma...5

1.2.4 High Dose Chemotherapy...5

1.3 Radiotherapy for Multiple Myeloma...... 6

1.4 Multidrug Resistance in Multiple Myeloma...7

1.4.1 Differential Expression Levels of ABC Transporter Protein Family Members..7

1.4.1.1 P-glycoprotein...11

1.4.1.2 Multidrug Resistance Associated Protein 1 (MRP1)..............................11

1.4.1.3 Breast Cancer Resistance Protein (BCRP)...12

1.4.1.4 Lung Resistance Protein (LRP)...12

1.4.2 Altered Expression Levels of Genes Related to Apoptosis.......................13

1.4.2.1 Apoptosis triggered by internal signals: the intrinsic or mitochondrial pathway...13

1.4.2.2 Apoptosis triggered by external signals: the extrinsic or death receptor pathway...14

1.4.3 Involvement of JAK-STAT Signaling Pathway in MDR..............................15

1.4.4 Involvement of Extracellular Matrix and Cell Adhesion in MDR.....16
1.4.5 Altered Expression Levels of Oncogenes Related to Multiple Myeloma.....17
1.4.5.1 The c-myc oncogene.....18
1.4.5.2 The bcl-2 oncogene.....18
1.4.5.3 The ras oncogene.....19
1.4.5.4 The p53 tumor suppressor gene.....20
1.4.5.5 The Rb1 tumor suppressor gene.....20
1.4.6 Involvement of Growth Factors in MDR.....20
1.4.7 Differential Expression Levels of Genes Related to Cell Cycle.....22
1.4.8 Involvement of Ceramide Metabolism in MDR.....23
1.4.9 Involvement of Cytochromes and Drug Metabolism in MDR.....25
1.5 Aim of The Study.....29
2. MATERIALS AND METHODS.....30
2.1 Cell Lines and Growth Conditions.....30
2.1.1 RPMI-8226.....30
2.1.2 U-266.....30
2.2 Anticancer Drugs.....31
2.2.1 Melphalan.....31
2.2.2 Prednisone.....31
2.2.3 Vincristine.....32
2.3 Primers.....32
2.4 Generation of Resistant Sublines.....34
2.5 Cell Viability Assay.....35
2.6 Total RNA Isolation.....36
2.7 RNA Quantification.....36
2.8 Agarose Gel Electrophoresis of RNA Samples.....37
2.9 cDNA Preperation.....37
2.10 Expression Analysis.....37
2.10.1 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).....37
2.10.2 Evaluation of the PCR Results.....38

2.10.3 Protein Analysis....38
2.11 Microarray Analysis....39
2.11.1 Target Preparation....39
2.11.2 Target Hybridization and Scanning....40
2.11.3 Data Analysis....40
2.11.3.1 Normalization....40
2.11.3.2 Statistical Analysis....41
2.12 Proliferation Assay For Cross-Resistance To Vincristine on Prednisone and Melphalan Resistant Cell Lines....41
2.13 Proliferation Assay For Cross-Resistance To Cobalt 60 Gamma (γ) Radiation....42
3. RESULTS AND DISCUSSION....43
3.1 Cell Culture....43
3.2 Application of Drugs....44
3.3 Cell Viability Assay....45
3.4 Gel Electrophoresis of RT-PCR Products....50
3.5 Densitometric Analysis of PCR Products on Gels....57
3.6 Protein analysis....66
3.7 Microarray Analysis....69
3.7.1 RNA Isolation and Target Preparation....69
3.7.2 Normalization....70
3.7.3 Data Analysis....71
3.7.4 Data Grouping....78
3.7.4.1 Extracellular Matrix Related Gene Expression....78
3.7.4.2 Oncogene Related Gene Expressions....91
3.7.4.3 Growth Factor Related Gene Expressions....109
3.7.4.4 JAK-STAT Signaling Pathway and Interleukin and Interferon Encoding Gene Expressions....120
3.7.4.5 Cell Cycle Related Gene Expressions....131
3.7.4.6 Ceramide Metabolism Related Gene Expressions....141
3.7.4.7 Apoptosis and Death Associated Gene Expressions....149
3.7.4.8 Cytochrome Related Gene Expressions....160

3.7.4.9 ABC Transporter Gene Expressions............................171
3.8 Proliferation Assay For Cross-Resistance To Vincristine On Prednisone and Melphalan Resistant Cell Lines..181
3.9 Proliferation Assay For Cross-Resistance To Cobalt-60 Gamma (γ) Radiation...184
4. CONCLUSIONS..188
REFERENCES..192
APPENDICES...209
A. BUFFERS AND SOLUTIONS..209
B. RT-PCR REACTION MIXTURES AND AMPLIFICATION CONDITIONS..215
C. PROTEIN CONCENTRATION DETERMINATION BY BRADFORD...220
CURRICULUM VITAE..222

LIST OF TABLES

TABLES

Table 1.1 List of ABC transporter family genes, chromosomal locations and functions........9

Table 1.2 Genetic alterations described in multiple myeloma........17

Table 1.3 Different types of CYP families........27

Table 3.1a Dose and period of application of vincristine on multiple myeloma cell lines during resistance development........44

Table 3.1b Dose and period of application of prednisone on multiple myeloma cell lines during resistance development........45

Table 3.1c Dose and period of application of melphalan on multiple myeloma cell lines during resistance development........45

Table 3.2 Expression levels of extracellular matrix related genes in RPMI-8226/Pred and U-266/Pred cell lines with respect to control........82

Table 3.3 Expression levels of extracellular matrix related genes in RPMI-8226/Vinc and U-266/Vinc cell lines with respect to control........85

Table 3.4 Expression levels of extracellular matrix related genes in RPMI-8226/Melp and U-266/Melp cell lines with respect to control........89

Table 3.5 Expression levels of oncogene related genes in RPMI-8226/Pred and U-266/Pred cell lines with respect to control........97

Table 3.6 Expression levels of oncogene related genes in RPMI-8226/Vinc and U-266/Vinc cell lines with respect to control........102

Table 3.7 Expression levels of oncogene related genes in RPMI-8226/Melp and U-266/Melp cell lines with respect to control........106

Table 3.8 Expression levels of growth factor related genes in RPMI-8226/Pred and U-266/Pred cell lines with respect to control........112

Table 3.9 Expression levels of growth factor related genes in RPMI-8226/Vinc and U-266/Vinc cell lines with respect to control........115

Table 3.10 Expression levels of growth factor related genes in RPMI-8226/Melp and U-266/Melp cell lines with respect to control........118

Table 3.11 Expression levels of JAK-STAT signaling pathway and interleukin and interferon encoding genes in RPMI-8226/Pred and U-266/Pred cell lines with respect to control 123
Table 3.12 Expression levels of JAK-STAT signaling pathway and interleukin and interferon encoding genes in RPMI-8226/Vinc and U-266/Vinc cell lines with respect to control...126
Table 3.13 Expression levels of JAK-STAT signaling pathway and interleukin and interferon encoding genes in RPMI-8226/Melp and U-266/Melp cell lines with respect to control...129
Table 3.14 Expression levels of cell cycle related genes in RPMI-8226/Pred and U-266/Pred cell lines with respect to control...134
Table 3.15 Expression levels of cell cycle related genes in RPMI-8226/Vinc and U-266/Vinc cell lines with respect to control...137
Table 3.16 Expression levels of cell cycle related genes in RPMI-8226/Melp and U-266/Melp cell lines with respect to control...140
Table 3.17 Expression levels of ceramide metabolism related genes in RPMI-8226/Pred and U-266/Pred cell lines with respect to control............................144
Table 3.18 Expression levels of ceramide metabolism related genes in RPMI-8226/Vinc and U-266/Vinc cell lines with respect to control............................146
Table 3.19 Expression levels of ceramide metabolism related genes in RPMI-8226/Melp and U-266/Melp cell lines with respect to control............................148
Table 3.20 Expression levels of apoptosis and death associated genes in RPMI-8226/Pred and U-266/Pred cell lines with respect to control............................153
Table 3.21 Expression levels of apoptosis and death associated genes in RPMI-8226/Vinc and U-266/Vinc cell lines with respect to control............................156
Table 3.22 Expression levels of apoptosis and death associated genes in RPMI-8226/Melp and U-266/Melp cell lines with respect to control............................159
Table 3.23 Expression levels of cytochrom related genes in RPMI-8226/Pred and U-266/Pred cell lines with respect to control...163
Table 3.24 Expression levels of cytochrom related genes in RPMI-8226/Vinc and U-266/Vinc cell lines with respect to control...166

Table 3.25 Expression levels of cytochrom related genes in RPMI-8226/Melp and U-266/Melp cell lines with respect to control...169
Table 3.26 Expression levels of ABC transporter related genes in RPMI-8226/Pred and U-266/Pred cell lines with respect to control.......................................175
Table 3.27 Expression levels of ABC transporter related genes in RPMI-8226/Vinc and U-266/Vinc cell lines with respect to control.......................................178
Table 3.28 Expression levels of ABC transporter related genes in RPMI-8226/Melp and U-266/Melp cell lines with respect to control.......................................180
Table C.1 Total protein concentrations..221

LIST OF FIGURES

FIGURES

Figure 1.1 The general scheme of mechanisms that are related with apoptosis......14
Figure 1.2 IL-6 signal transduction activates STAT3 and increases anti-apoptotic gene expression...16
Figure 1.3 Regulation of cell cycle...23
Figure 1.4 Major synthetic and metabolic pathways for ceramide.....................24
Figure 2.1 Chemical structure of melphalan...31
Figure 2.2 Chemical structure of prednisone..31
Figure 2.3 Chemical structure of vincristine...32
Figure 3.1a RPMI-8226 cells under inverted microscope with 40X magnification...43
Figure 3.1b U-266 cells under inverted microscope with 40X magnification.........43
Figure 3.2 Percent viability of RPMI-8226 and RPMI-8226/50nM vincristine cells at increasing doses of vincristine...46
Figure 3.3 Percent viability of U-266 and U-266/2nM vincristine cells at increasing doses of vincristine...47
Figure 3.4 Percent viability of RPMI-8226 and RPMI-8226/500μM prednisone cells at increasing doses of prednisone...48
Figure 3.5 Percent viability of U-266 and U-266/300μM prednisone cells at increasing doses of prednisone..49
Figure 3.6 Percent viability of RPMI-8226 and RPMI-8226/1μM melphalan cells at increasing doses of melphalan. 49
Figure 3.7 Percent viability of U-266 and U-266/1μM melphalan cells at increasing doses of melphalan. 50
Figure 3.8 Expression levels of *GCS* (532 bp), *SK-1* (551 bp) and *LASS-1* (555 bp) genes in RPMI-8226 sublines (2% w/v agarose gel); Ladder: 50 bp ladder, A) *GCS*, B) *SK-1*, C) *LASS-1* products...51
Figure 3.9 Expression levels of *Bcl-2* (232 bp), $Bcl\text{-}X_L$ (422 bp) and *LRP* (483 bp) genes in RPMI-8226 sublines (2% w/v agarose gel); Ladder: 50 bp ladder, A) *Bcl-2*, B) $Bcl\text{-}X_L$, C) *LRP* products..52

Figure 3.10 Expression levels of *MDR1* (258 bp), *MRP1* (256 bp) and *BCRP* (342 bp) genes in RPMI-8226 sublines (2% w/v agarose gel); Ladder: 50 bp ladder, A) *MDR1*, B) *MRP1*, C) *BCRP* products....53
Figure 3.11 Expression levels of *β-actin* gene (209 bp) in RPMI-8226 sublines (2% w/v agarose gel); Ladder: 50 bp ladder....53
Figure 3.12 Expression levels of *GCS* (532 bp), *SK-1* (551 bp) and *LASS-1* (555 bp) genes in U-266 sublines (2% w/v agarose gel); Ladder: 50 bp ladder, A) *GCS*, B) *SK-1*, C) *LASS-1* products....54
Figure 3.13 Expression levels of *Bcl-2* (232 bp), *Bcl-X_L* (422 bp) and *LRP* (483 bp) genes in U-266 sublines (2% w/v agarose gel); Ladder: 50 bp ladder, A) *Bcl-2*, B) *Bcl-X_L*, C) *LRP* products....55
Figure 3.14 Expression levels of *MDR1* (258 bp), *MRP1* (256 bp) and *BCRP* (342 bp) genes in RPMI-8226 sublines (2% w/v agarose gel); Ladder: 50 bp ladder, A) *MDR1*, B) *MRP1*, C) *BCRP* products....56
Figure 3.15 Expression levels of *β-actin* gene (209 bp) in U-266 sublines (2% w/v agarose gel); Ladder: 50 bp ladder....56
Figure 3.16 a *MDR1* gene expression levels of RPMI-8226 cell lines....57
Figure 3.16 b *MDR1* gene expression levels of U-266 cell lines....58
Figure 3.17 a *MRP1* gene expression levels of RPMI-8226 cell lines....58
Figure 3.17 b *MRP1* gene expression levels of U-266 cell lines....59
Figure 3.18 a *BCRP* gene expression levels of RPMI-8226 cell lines....59
Figure 3.18 b *BCRP* gene expression levels of U-266 cell lines....60
Figure 3.19 a *LRP* gene expression levels of RPMI-8226 cell lines....60
Figure 3.19 b *LRP* gene expression levels of U-266 cell lines....61
Figure 3.20 a *Bcl-2* gene expression levels of RPMI-8226 cell lines....61
Figure 3.20 b *Bcl-2* gene expression levels of U-266 cell lines....62
Figure 3.21 a *Bcl-X_L* gene expression levels of RPMI-8226 cell lines....62
Figure 3.21 b *Bcl-X_L* gene expression levels of U-266 cell lines....63
Figure 3.22 a *GCS* gene expression levels of RPMI-8226 cell lines....63
Figure 3.22 b *GCS* gene expression levels of U-266 cell lines....64
Figure 3.23 a *SK-1* gene expression levels of RPMI-8226 cell lines....64
Figure 3.23 b *SK-1* gene expression levels of U-266 cell lines....65

Figure 3.24 a *LASS-1* gene expression levels of RPMI-8226 cell lines.....................65

Figure 3.24 b *LASS-1* gene expression levels of U-266 cell lines.........................66

Figure 3.25 Western blot analysis for Bcl-2 protein (25kDa; 50µg/ml)................67

Figure 3.26 Western blot analysis for GAPDH protein (37kDa; 10µg/ml)..............67

Figure 3.27 a Bcl-2 protein levels of RPMI-8226 cell lines..............................68

Figure 3.27 b Bcl-2 protein levels of U-266 cell lines....................................68

Figure 3.28 Electropherograms for a. RPMI-8226/50nM Vinc b. U-266/1µM Melp total RNA...69

Figure 3.29 Denaturing agarose gel electrophoresis of RNA samples..................70

Figure 30 Heatmap for original and 500µM Pred resistant RPMI-8226 cell lines (data dublicate)...72

Figure 3.31 Heatmap for original and 50nM Vinc resistant RPMI-8226 cell lines (data dublicate)...73

Figure 3.32 Heatmap for original and 1µM Melp resistant RPMI-8226 cell lines (data dublicate)..74

Figure 3.33 Heatmap for original and 300µM Pred resistant U-266 cell lines (data dublicate)..75

Figure 3.34 Heatmap for original and 2nM Vinc resistant U-266 cell lines (data dublicate)..76

Figure 3.35 Heatmap for original and 1µM Melp resistant U-266 cell lines (data dublicate)..77

Figure 3.36 Expression levels of extracellular matrix related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines......................................81

Figure 3.37 Expression levels of extracellular matrix related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines...84

Figure 3.38 Expression levels of extracellular matrix related genes in RPMI-8226/1µMMelp and U-266/1µMMelp cell lines...88

Figure 3.39 Expression levels of oncogene related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines...96

Figure 3.40 Expression levels of oncogene related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines...101

Figure 3.41 Expression levels of oncogene related genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines..105
Figure 3.42 Expression levels of growth factor related genes in RPMI-8226/500μMPred and U-266/300μMPred cell lines..................................111
Figure 3.43 Expression levels of growth factor related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines..114
Figure 3.44 Expression levels of growth factor related genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines..117
Figure 3.45 Expression levels of JAK-STAT signaling pathway and interleukin and interferon encoding genes in RPMI-8226/500μMPred and U-266/300μMPred cell lines...122
Figure 3.46 Expression levels of JAK-STAT signaling pathway and interleukin and interferon encoding genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines...125
Figure 3.47 Expression levels of JAK-STAT signaling pathway and interleukin and interferon encoding genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines 128
Figure 3.48 Expression levels of cell cycle related genes in RPMI-8226/500μMPred and U-266/300μMPred cell lines...133
Figure 3.49 Expression levels of cell cycle related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines..136
Figure 3.50 Expression levels of cell cycle related genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines..139
Figure 3.51 Expression levels of ceramide metabolism genes in RPMI-8226/500μMPred and U-266/300μMPred cell lines..................................143
Figure 3.52 Expression levels of ceramide metabolism related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines..145
Figure 3.53 Expression levels of ceramide metabolism related genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines..147
Figure 3.54 Expression levels of apoptosis and death associated genes in RPMI-8226/500μMPred and U-266/300μMPred cell lines..................................152

Figure 3.55 Expression levels of apoptosis and death associated genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines..155
Figure 3.56 Expression levels of apoptosis and death associated genes in RPMI-8226/1µMMelp and U-266/1µMMelp cell lines..158
Figure 3.57 Expression levels of cytochrome related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines....................................162
Figure 3.58 Expression levels of cytochrome related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines..165
Figure 3.59 Expression levels of cytochrome related genes in RPMI-8226/1µMMelp and U-266/1µMMelp cell lines..168
Figure 3.60 Expression levels of ABC transporter genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines..174
Figure 3.61 Expression levels of ABC transporter genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines..177
Figure 3.62 Expression levels of ABC transporter genes in RPMI-8226/1µMMelp and U-266/1µMMelp cell lines..179
Figure 3.63. Effect of vincristine on RPMI-8226 and RPMI-8226/500µM Pred cell lines..181
Figure 3.64 Effect of vincristine on RPMI-8226 and RPMI-8226/1µM Melp cell lines..182
Figure 3.65 Effect of vincristine on U-266 and U-266/300µM Pred cell lines......183
Figure 3.66 Effect of vincristine on U-266 and U-266/1µM Melp cell lines.........183
Figure 3.67 Effect of 200 and 800cGy γ radiation on viability of RPMI-8226 sensitive and drug resistant sublines..185
Figure 3.68 Effect of 200 and 800cGy γ radiation on viability of U-266 sensitive and drug resistant sublines..185
Figure C.1 Bradford standard curve..220

ABBREVIATIONS

Ag	: Antigen
Ab	: Antobody
BCNU	: Nitrosourea carmustine
BCRP	: Breast cancer resistance protein
BSA	: Bovine serum albumin
CAM-DR	: Cell adhesion mediated drug resistance
Cy-E	: Cyclophosohamide, etoposide
DC-IE	: Dexamethasone, cyclophosphamide, idorubicin, etoposide
DNA-PK	: DNA-dependent protein kinase
ECOG	: The Eastern Cooperative Oncology Group
GAPDH	: Glyceraldehayde 3-phosphate dehydrogenase
GCS	: Glycosylceramide synthase
GC	: Glucocorticoid
GR	: Glucocorticoid receptor
GRE	: Glucocorticoid-responsive element
4-HPR	: Fenretinide
HDMP	: Melphalan, methylprednisone
IgA	: Immunoglobulin-A
IgG	: Immunoglobulin-G
IL-6	: Interleukin-6
IgH	: Immunoglobulin heavy chain
LASS-1	: Longevity assurance gene 1
LRP	: Lung resistance protein
MDR	: Multidrug resistance
Melp	: Melphalan
MM	: Multiple myeloma
MRP	: Multidrug resistant associated protein
OAF	: Osteoclast activating factor
PCR	: Polymerase chain reaction

P-gp : P-glycoprotein

Pred : Prednisone

RT-PCR : Reverse transcriptase polymerase chain reaction

SK-1 : Sphingosine kinase-1

VAD : Vincristine, doxorubicin, dexamethasone

VAMP : Vincristine, doxorubicin, dexamethasone, methylprednisone

VBMCP : Vincristine, BCNU, melphalan, cyclophosphamide, prednisone

Vin : Vincristine

Xrcc : X-ray repair cross-complementing protein

XTT : 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H- tetrozolium hydroxide

CHAPTER 1

INTRODUCTION

1.1 MULTIPLE MYELOMA

Multiple myeloma (MM) fits in the group of plasma cell disorders characterized by neoplastic proliferation of single clone of plasma cells engaged in the production of monoclonal immunoglobulins, usually monoclonal IgG or IgA (MacLennan, 1991).

Plasma cells are the end-effector cells of the B-lymphocyte lineage that produce and secrete antigen (Ag)-specific antibodies (Ab). After the activated B cells enter the bone marrow, they stop proliferation and differentiate into plasma cells, under the influence of adhesion molecules and factors such as interleukin-6. They start to produce immunoglobulin and die by apoptosis after several weeks or months. The prototype plasma cell malignancy is multiple myeloma, a neoplasm characterized by the accumulation of monoclonal plasma cells which show three major features (Bargolie *et al.,* 1989).

1. The secreted monoclonal immunoglobulins are usually of the IgG and IgA isotype
2. In contrast with the distribution of normal plasma cells, MM plasma cells localize uniquely within the bone marrow
3. MM plasma cells produce a number of cytokines, some formally identified as osteoclast activating factors (OAF)

Multiple myeloma patients may show various clinical alterations. Bone lesions are very common, bone pain being the most prevalent symptom. These lytic lesions caused by tumor expansion and activation of osteoclasts by myeloma cells secretion of osteoclasts activating factor (OAF). Interleukin-6 may have an important role in osteoclast activation (Klein *et al.,* 1989). Because of the great bone reabsorbtion,

another common problem is hypercalcemia that may lead to renal alterations and neurologic symptoms if not treated (Perillie *et al.,* 1958).

The diagnosis of MM is confirmed when bone marrow plasmocytosis (>10 %), lytic bone lesions and monoclonal immunoglobulin in serum or urine is found. There is a complex staging system in multiple myeloma that is actually a functional system which serves to evaluate the prognosis using various types of clinical and laboratory tests. This differs from the anatomic staging systems for solid tumors (Durine *et al,* 1975).

Stage I: Low tumor mass ($<0.6 \times 10^{12}/m^2$) number of neoplastic plasma cells

All of the following must be present.

A. Hemoglobin > 10.5 g/dl

B. Serum calcium level normal

C. Low serum myeloma protein production rates

- IgG peak < 5g/dl
- IgA peak < 3g/dl
- Bence Jones protein < 4g/24hr

D. No bone lesions or osteoporosis

Stage II: Intermediate tumor mass (0.6 to $1.2 \times 10^{12}/m^2$) number of neoplastic plasma cells

All patients who do not qualify for high or low tumor mass categories are considered to have intermediate tumor mass

Stage III: High tumor mass ($>1.2 \times 10^{12}/m^2$) of neoplastic plasma cell number

One of the following abnormalities must be present

A. Hemoglobin < 8.5 g/dl

B. Serum calcium > 12 mg/dl

C. Very high serum or urine myeloma protein products rate

- IgG peak > 7g/dl
- IgA peak >5g/dl
- Bence Jones protein >12g/24hr

D. >3 lytic bone lesion on bone survey

Multiple myeloma accounts for 1 % of all malignant disease and slightly more than 10 % of hematologic malignancies. The median age of patients at the time of diagnosis is 61 years. It is more common in men and black population (Herrinton *et al.*, 1993).

The development of MM may proceed via a multistep transformation process which involves a series of molecular events, including gene mutation, chromosomal abnormalities, oncogene activation and growth factor dysregulation.

The myeloma cells are aneuploid and their chromosomes have many numerical and structural abnormalities that seems to prevent the differentiation and death of these cells, which continue to proliferate and accumulate in the bone marrow (Bottura, 1963).

The translocation t(11;14) (q13;q32) is the single most common chromosomal abnormality in myeloma which involves the *Bcl-1* oncogene and is found in other lymphoproliferative disorders (Durine BGM *et al.*, 1990).

Several infrequent structural abnormalities have also been specifically reported. Some of them, such as t(8;14), t(14;18) (Gould *et al.*, 1988) and Philadelphia chromosomes (van den Berghe, 1979) are well known to be involved in other hematopoietic malignancies and associated to specific oncogenes and whereas others such as t(1;16) (p11;p11) (Facon, 1993) , inv (7)(p11;122) (Kokkinou, 1990), 17p+ (Manolova *et al.*, 1979) , t(1;20)(q12.3;p13) and del(3)(p12), (Weh *et al.*, 1993) t(6;14)(p21.1;q32.3) (Nishida *et al.*, 1989) are as yet identified in few studies only .

Aneuploidy is a common finding, with both hypodiploidy and hyperdiploidy. Hyperdiploidy mostly involves chromosomes 3, 5, 7, 9, 11, 19 and 21, whereas hypodiploidy commonly affects chromosomes 13, 8 and X in MM. Abnormal karyotypes indicate poor survival, but hyperdiploidy is suggested to indicate good prognosis (Anday *et al.*, 1974).

1.2 DRUG THERAPY FOR MULTIPLE MYELOMA

1.2.1 Standard Chemotherapy

Oral administration of melphalan and prednisone (corticosteroid, glucocorticoid) has remained a standard form of therapy in the treatment of MM for almost 30 years (Huang *et al.*, 1999). Numerous prospective trials have demonstrated that treatment with this regimen yields a response rate of about 50 % with few complete remissions (CR, <5 %), an average remission duration of approximately 18 months and a median survival of 24 to 30 months (Oken, 1984). The reported 5-year survival rate is less than 20 % (Bergsagel *et al.*, 1988). Most regimens used in the treatment of MM include corticosteroids, which are beneficial in increasing response rates when combined with cytotoxic drugs and may prolong survival (Salmon *et al.*, 1994).

1.2.2 Combination Chemotherapy

Because of the modest success attained using standard chemotherapy, a variety of regimens using multiple chemotherapeutic agents have been studied. One of the best known of these regimens is the M-2 protocol (VBMCP), devised at Memorial Sloan-Kettering Cancer Center in 1977. This regimen consisted of vincristine, the nitrosourea carmustine (BCNU), melphalan, cyclophosphamide and prednisone, and was associated with a response rate of 78 % and a median survival of 38 months (Case *et al.*, 1977). The Eastern Cooperative Oncology Group (ECOG) conducted a large randomised study in which VBMCP was compared with treatment of melphalan, the results of which demonstrated that VBMCP induced a higher response rate (72 % vs 51 %), a larger response duration (median 24 vs 18 months) and a slightly higher 5-year survival rate (26 % vs 19 %), but no significant difference in overall survival (29 vs 27 months). The regimen consisting of vincristine, doxorubicin and dexamethasone (VAD), and a similar combination containing high dose methylprednisone (VAMP) were shown to produce less bone marrow toxicity than other regimens containing alkylating agents, and were particularly notable for their rapid induction of remission (Alexanian *et al.*, 1992). These combinations have not been found to be superior to standard melphalan therapy when used as first line agents, but may have role in the treatment of patients

in whom autologous transplantation is anticipated and in patients with refractory myeloma (Oken, 1997). Although a proper combination therapy has increased the cross-resistance between these drugs has been and still is a major rationale for combination chemotherapy (Dalton, 1997). Drug interactions may result in antagonism, as well as synergistic, additive or indifferent effects so drug combination studies are important when using combination chemotherapy.

1.2.3 Refractory and Relapsing Myeloma

Patients who relapse later than 6 months after stopping initial therapy have 60 to 70 % chance of responding to re-initiation of the previously used induction therapy. If no response ensues, then treatment with VAD or other regimens may be attempted. Both VAD and treatment with high dose dexamethasone have induced remissions in approximately 25 % of patients with disease resistant to initial treatment, and have prolonged survival by 1 year in patients who responded (Alexanian *et al.*, 1986). Several salvage regimens are available for petients with VAD resistance. One such regimen utilises etoposide, cisplatin, cytarabine and dexamethasone. Other second line chemotherapeutic regimens include Cy-E (cyclophosphamide and etoposide) and HDMP (melphalan and methylprednisone) (Ballester *et al.*, 1997).

1.2.4 High Dose Chemotherapy

With conventional dose chemotherapy, complete remissions are rare. The first attempt to induce complete remission with high-dose therapy was with the use of high-dose melphalan, pioneered by McElwain and colleagues at the Royal Marsden Hospital. However with longer follow-up it became evident that these remissions were not durable and the median duration of remission was only 18 months. Although high-dose melphalan ultimately proved disappointing as the first-line treatment, this work neverthless provided an impetus for the subsequent development of even more intensive therapy combined with bone marrow or peripheral blood stem cell rescue. High-dose melphalan and other high-dose therapies such as high-dose cyclophosphamide or high-dose cyclophosphamide and etoposide with growth factor support are also being increasingly used in relapsed and refractory patients (Huang *et al.*, 1999).

1.3 RADIOTHERAPY FOR MULTIPLE MYELOMA

Radiotherapy is also used in the treatment of multiple myeloma. Patients who have multiple myeloma sometimes receive radiation therapy in addition to chemotherapy. Interventions with radiation can provide significant clinical benefit for myeloma patients. Radiation therapy may be used in several ways in myeloma (www.multiplemyeloma.org/treatments/3.03.01.php)

- Local radiation therapy at higher doses (with chemotherapy in some cases) is used in the treatment of solitary tumors in bone or soft tissue (plasmacytomas)
- High-dose radiation to a larger part of the body may be used to reduce tumor burden or as salvage therapy
- Local low-dose radiation therapy is sometimes used as palliative treatment to relieve uncontrolled pain and is also used to help prevent or treat bone fractures or spinal-cord compression
- Total body irradiation is used in conjunction with high-dose chemotherapy prior to stem cell transplantation in order to help kill myeloma cells in the bone marrow

Cobalt 59 is a naturally occuring, stable (non-radioactive) metal found in various minerals. After being placed in an intense radiation field (neutron field), cobalt 59 is transformed into cobalt 60, which is radioactive. During the transformation from cobalt 60 back to a stable state, gamma radiation is emitted and the cobalt atoms are reduced to stable nickel atoms. The radioactive substance cobalt 60 is extensively used in medical and industrial applications as well as in the treatment of malignant tumors. (de Laeter *et al.*, 2003).

1.4 MULTIDRUG RESISTANCE IN MULTIPLE MYELOMA

The emergence of drug-resistance of tumor cells is a major complication for successful anti-cancer chemotherapy. At relapse, tumor cells often exhibit cross-resistance to a broad spectrum of chemically non-related cytotoxic drugs, called multidrug resistance (MDR) (Klappe *et al.*, 2004). The more critical mechanism of cytotoxic drug resistance in MM is considered to reside at the cellular level. Malignant cells may exhibit genetic instability allowing spontaneous generation of variant forms that may result in drug resistance. Altered gene products may result in the development of resistance at the cellular level by causing reduced intracellular drug accumulation, altered drug distribution within the cell, modification of the drug target or an enhanced ability to repair drug damage and decrease apoptosis. Any single one or combination of these alterations may lead to clinically significant cellular drug resistance (Ueda *et al.*, 1987). Unfortunately, when one mechanism of resistance is eliminated, the cancer cell adapts and becomes resistant by another mechanism. Therefore, addressing a single mechanism of resistance at the cellular level is insufficient. Multiple mechanisms must be addressed to eliminate the maximum number of myeloma cells (Dalton *et al.*, 2002).

1.4.1 Differential Expression Levels of ABC Transporter Protein Family Members

There are several mechanisms by which tumor cells develop resistance to cytotoxic agents. One mechanism is mediated by ABC drug transporter proteins. Hydrophobic drugs enter the cell by diffusion across plasma membrane. Energy dependent transport systems are needed to expel these drugs out of the cell. These pumps namely ATP binding cassette (ABC) transporters work by coupling the hydrolysis of ATP to substrate transport across the cell membrane (Liu *et al.*, 2005). ATP-binding cassette superfamily is a very large family including over 50 members which are divided into seven subfamilies (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG). Although members of this family share little sequence homology outside the nucleotide binding sites, their general topologic organization is strikingly conserved. A list of all known human ABC genes is displayed in Table 1.1 (Dean, 2002). ABC transporters are responsible for the transport of a wide variety of substrates including

ions, phospholipids, steroids, polysaccharides, amino acids and peptides across biological membranes (Ambudkar *et al.*, 1999). Among these transporters P-glycoprotein (P-gp) or multidrug resistance protein (MDR1), multidrug resistance associated protein (MRP1), breast cancer resistance protein (BCRP) and lung resistance-related protein (LRP) are most important ones for the multidrug resistance phenotype in most of the cancer types including hematological malignancies.

Table 1.1 List of ABC transporter family genes, chromosomal locations and functions (Dean *et al.*, 2002)

Symbol	Alias	Location	Function
ABCA1	*ABC1*	9q31.1	Cholesterol efflux onto HDL
ABCA2	*ABC2*	9q34.3	Drug resistance
ABCA3	*ABC3, ABCC*	16p13.3	Surfactant secretion
ABCA4	*ABCR*	1p21.3	N-Retinylidiene-PE efflux
ABCA5		17q24.3	
ABCA6		17q24.3	
ABCA7		19p13.3	
ABCA8		17q24.3	
ABCA9		17q24.3	
ABCA10		17q24.3	
ABCA12		2q34	
ABCA13		7p12.3	
ABCB1	*PGY1, MDR*	7q21.12	Multidrug resistance
ABCB2	*TAP1*	6p21.3	Peptide transport
ABCB3	*TAP2*	6p21.3	Peptide transport
ABCB4	*PGY3*	7q21.12	PC transport
ABCB5		7p21.1	
ABCB6	*MTABC3*	2q35	Iron transport
ABCB7	*ABC7*	Xq21-q2	Fe/S cluster transport
ABCB8	*MABC1*	7q36.1	
ABCB9		12q24.3	
ABCB10	*MTABC2*	1q42.13	
ABCB11	*SPGP*	2q24.3	Bile salt transport
ABCC1	*MRP1*	16p13.1	Drug resistance
ABCC2	*MRP2*	10q24.2	Organic anion efflux
ABCC3	*MRP3*	17q21.3	Drug resistance
ABCC4	*MRP4*	13q32.1	Nucleoside transport

Table 1.1 continued

ABCC5	*MRP5*	3q27.1	Nucleoside transport
ABCC6	*MRP6*	16p13.1	
CFTR	*ABCC7*	7q31.31	Chloride ion channel
ABCC8	*SUR*	11p15.1	Sulfonylurea receptor
ABCC9	*SUR2*	12p12.1	K(ATP) channel regulation
ABCC10	*MRP7*	6p21.1	
ABCC11		16q12.1	
ABCC12		16q12.1	
ABCD1	*ALD*	Xq28	VLCFA transport regulation
ABCD2	*ALDL1, ALDR*	12q11	
ABCD3	*PXMP1, PMP70*	1p22.1	
ABCD4	*PMP69, P70R*	14q24.3	
ABCE1	*OABP, RNS4I*	4q31.31	Oligoadenylate binding protein
ABCF1	*ABC50*	6p21.1	
ABCF2		7q36.1	
ABCF3		3q27.1	
ABCG1		21q22.3	Cholesterol transport
ABCG2		4q22	Toxin efflux, drug resistance
ABCG4		11q23	
ABCG5		2p21	Sterol transport
ABCG8		2p21	Sterol transport

1.4.1.1 P-glycoprotein

P-glycoprotein is the first protein to be described in the multidrug resistance. It encodes a 170 kDa transmembrane glycoprotein is capable of extruding a wide variety of large heterocyclic compounds, including anthracyclines, vinca alkaloids and epipodophyllotoxins that are frequently used for treating hematologic malignancies. P-glycoprotein is encoded by *MDR1 (ABCB1)* gene. The gene is located on chromosome 7q21.1 with 28 exons (Safa, 2004; Germann, 1996). The protein contains 12 transmembrane segments, 6 domains and 2 nucleotide binding domains (Germann *et al.*, 1996). Immunohistochemical analyses of normal human tissues show the localization of P-glycoprotein to the adrenal gland, specialized endothelial cells in brain and testis and to the lumenal surface of specific epithelial cell types in pancreas, kidney, colon, jejunum and liver (Thiebaut *et al.*, 1987). These localizations suggest a possible function of P-glycoprotein as a transporter of toxic exogenous materials and endogenous metabolites (Thorgiersson *et al.*, 1991). On the other hand during chemotherapy, P-gp functions as a pump extruding drug molecules through the plasma membrane. Uncharged drug molecules entering the cell by passive diffusion are removed from membrane before they can enter the cell (Mechetner *et al.*, 1998, Filipits *et al.*, 1996). P-gp does not seem to be expressed *de novo* in myeloma cells obtained from patients before they receive chemotherapy. The expression of P-gp has not been shown to be elevated in patients treated with melphalan either. However, P-gp expression does increase in approximately 75 % of patients treated with vincristine, doxorubicin and dexamethasone (Grogan *et al.*, 1993).

1.4.1.2 Multidrug Resistance Associated Protein 1 (MRP1)

The *MRP1 (ABCC1)* gene is a member of the superfamily of membrane drug transporters and is located on chromosome 16p13.1. It encodes a full transporter (190 kDa) and transports glutathione-linked organic compounds from cells (Cole, 1996). The glutathione S-transferase (GST) family of enzymes catalyse the conjugation of reduced glutathione via the sulfhydryl group, to electrophilic centers on a wide variety of substrates (Douglas, 1987). The MRP family includes seven members; MRP1 and MRP2 can lead to the extrusion of anthracyclines and vinca alkaloids.

MRP expression has been reported in a variety of untreated and refractory hematologic malignancies, including AML and chronic leukemias (Sonneveld, 2000).

1.4.1.3 Breast Cancer Resistance Protein (BCRP)

BCRP protein (72.6 kDa) was isolated from multidrug resistant breast cancer cells. It is an ATP-dependent transporter but evolutinary distinct from the families that contain P-gp and MRP. It has motifs characteristic of an ABC half transporter (Rabindran, 2000). BCRP is encoded by the *ABCG2* gene which is mapped on chromosome 4 (4q22) (Kawabata *et al.,* 2001). Overexpression of BCRP results in cross-resistance to mitoxantrone, daunorubicin and topotecan but not to microtubular inhibitors such as paclitaxel and vinblastine (Yang *et al.,* 2003). BCRP was found to be expressed in the mitoxantrone-selected human multiple myeloma cell line 8226; however, its clinical relevance in patients with multiple myeloma remains to be validated (Ross *et al.,* 1999).

1.4.1.4 Lung Resistance Protein (LRP)

The lung resistance protein was initially identified in an anthracycline-resistant, non-small cell lung cancer cell line that lacked P-gp overexpression (Scheper *et al.,* 1993). The *LRP* gene is located on chromosome 16 (16p11.2), close to the *MRP1* and encodes a 110 kDa protein (Slovak *et al.,* 1995). LRP is a major vault protein that assembles as a barrel-shaped structure. It forms central plugs of the nuclear pore complexes and functions to block the transport of drugs from the cytoplasm to the nucleus. The spectrum of cross-resistance of LRP is wide covering the classical MDR phenotype as well as the platinol- and melphalan-resistant phenotype (Yang *et al.,* 2003). LRP is found to be expressed in approximately half of patients with multiple myeloma. LRP was found to be expressed more frequently in patients with p53 deletion and P-gp overexpression. LRP and P-gp might share a similar regulatory mechanism mediated by p53 (Yang *et al.,* 2003).

1.4.2 Altered Expression Levels of Genes Related to Apoptosis

Another mechanism which is related to resistance of tumor cells to cytotoxic agents and radiation is mediated by the alterations in apoptotic pathways. Apoptosis is the process of programmed cell death that occur in multicellular organisms. The process of apoptosis is controlled by a diverse range of cell signals, which may originate either extracellularly or intracellularly. It is triggered by either stimulation of cell surface receptors like Fas or mitochondrial response to stress (Hu *et al.*, 2003). The cellular molecules involved in this mechanism include overexpression of antiapoptotic proteins, such as B-cell leukemia protein 2 (Bcl-2), Bcl-X_L, A1/Bfl1, and mutations in the p53 protein (Yang *et al.*, 2003). Figure 1.1 demonstrates the extracellular and intracellular mechanisms of apoptosis.

1.4.2.1 Apoptosis triggered by internal signals: the intrinsic or mitochondrial pathway

In a healthy cell, the outer membranes of its mitochondria display the protein Bcl-2 on their surface. Bcl-2 inhibits apoptosis. Internal damage to the cell (e.g., from reactive oxygen species) causes a related protein, Bax, to migrate to the surface of the mitochondrion where it inhibits the protective effect of Bcl-2 and inserts itself into the outer mitochondrial membrane punching holes in it and causing cytochrome c to leak out. The released cytochrome c binds to the protein Apaf-1 ("apoptotic protease activating factor-1"). Using the energy provided by ATP, these complexes aggregate to form apoptosomes. The apoptosomes bind to and activate caspase-9. Caspase-9 is one of a family of over a dozen caspases. They are all proteases. They get their name because they cleave proteins — mostly each other — at aspartic acid (Asp) residues. Caspase-9 cleaves and, in so doing, activates other caspases (caspase-3 and -7). The activation of these "executioner" caspases creates an expanding cascade of proteolytic activity (rather like that in blood clotting and complement activation) which leads to digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA, and phagocytosis of the cell (Dejean *et al.*, 2006).

1.4.2.2 Apoptosis triggered by external signals: the extrinsic or death receptor pathway

Fas and the TNF receptor are integral membrane proteins with their receptor domains exposed at the surface of the cell. Binding of the complementary death activator (FasL and TNF respectively) transmits a signal to the cytoplasm that leads to activation of caspase 8. Caspase 8, like caspase 9, initiates a cascade of caspase activation leading to phagocytosis of the cell (Wajant, 2002).

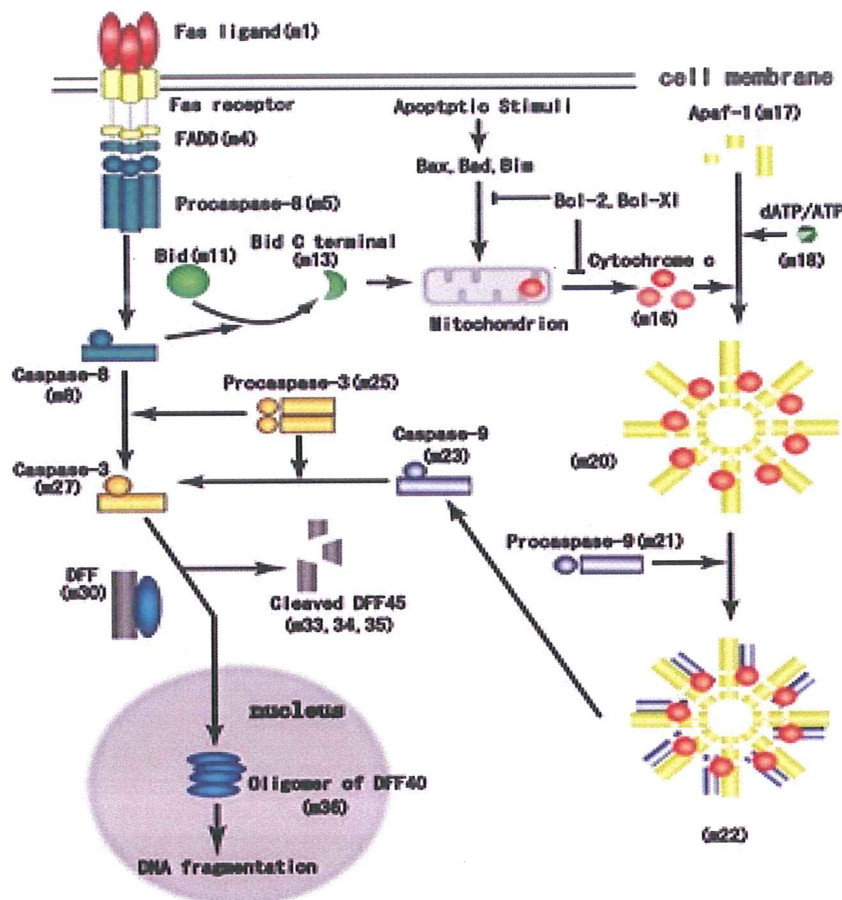


Figure 1.1 The general scheme of mechanisms that are related with apoptosis (www.csml.org/.../fas-induced-apoptosis-pathway/)

1.4.3 Involvement of JAK-STAT Signaling Pathway in MDR

Interleukins are group of cytokines that were first seen to be expressed by white blood cells as means of communication. The majority of interleukins are synthesized by helper CD4+ T lymphocytes, as well as through monocytes, macrophages and endothelial cells. They promote the development and differentiation of T, B and hematopoietic cells (Alaverdi *et al.*, 2007). Some cytokines are involved in the pathogenesis of multiple myeloma. There is some evidence that interleukin-6 is produced by bone cells and stromal cells after stimulation by myeloma cells. The interleukin-6 system (interleukin-6, soluble interleukin-6 receptor a, interleukin-1-b) play an important role in the pathogenesis of bone lesions because it activates osteoclasts in the vicinity of myeloma cells and thus provoke bone reabsorbtion. The first evidence of an interaction that prevents cell death upon exposure to toxic stresses is IL-6, the major growth factor of MM. It is a major stimulant of cell growth, and the associated cell signalling principally involves the mitogen-activated protein kinase pathway (Figure 1.2). IL-6 promotes cell survival by the upregulation of antiapoptotic genes, in particular Bcl-X_L. This is accomplished by JAK2/STAT3 pathway. Bcl-X_L is the primary anti-apoptotic gene that confers resistance to drugs, dexamethasone and Fas-mediated apoptosis (Dalton *et al.*, 2002).

Interferons belong to the large class of glycoproteins known as cytokines. They assist the immune response by inhibiting viral replication within host cells, activating natural killer cells and macrophages, increasing antigen presentation to lymphocytes, and inducing the resistance of host cells to viral infection. Interleukins and interferons are important cytokines in the JAK-STAT signaling pathway (Lin *et al.*, 2004)

The JAK-STAT signalling pathway takes part in the regulation of cellular responses to cytokines such as interleukins and interferons and also to growth factors. Employing Janus Kinases (JAKs) and Signaling Transducers and Activators of Transcription (STATs), the pathway transduces the signal carried by these extracellular polypeptides to the nucleus, where activated STAT proteins modify gene expression. The pathway plays a central role in principle cell fate decision,

regulating the process of cell proliferation, differentiation and apoptosis. It is particularly important in hematopoiesis (Hebenstreit *et al.*, 2005).

The pathway is negatively regulated on multiple levels. Protein tyrosine phosphatases remove phosphates from cytokine receptors as well as activated STATs. More recently identified suppressors of cytokine signaling (SOCS) inhibit STAT phosphorylation by binding and inhibiting JAKs or competing with STATs for phosphotyrosine binding sites on cytokine receptors (Krebs *et al.*, 2001).

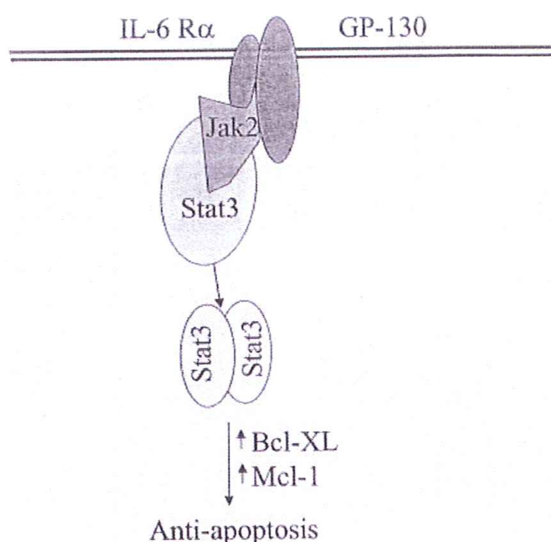


Figure 1.2 IL-6 signal transduction activates STAT3 and increases anti-apoptotic gene expression (Dalton *et al.*, 2002)

1.4.4 Involvement of Extracellular Matrix and Cell Adhesion in MDR

The extracellular matrix (ECM) is the extracellular part of animal tissue that usually provides structural support to the animal cells in addition to performing various other important functions. Many cells bind to components of the extracellular matrix. The cell-to-ECM adhesion is regulated by specific cell surface cellular adhesion molecules (CAM) known as integrins. Integrins are cell surface proteins that bind cells to ECM structures, such as fibronectin and laminin and also to integrin proteins

on the surface of other cells which initiates many intracellular signalling pathways (Alberts *et al.*, 2004)

Cell adhesion also has been shown to prevent cell death through a number of mechanisms. This is called cell adhesion mediated drug resistance (CAM-DR). IL-6 has been shown to promote cell survival by enhancing adhesion of myeloma cells to bone marrow and fibronectin, through macrophage inflammatory protein-1α. Macrophage inflammatory protein-1α is secreted by myeloma cells and has been found to be elevated in the bone marrow plasma of the majority of patients with advanced MM. When myeloma cells and other hematopoietic cancer cells adhere to fibronectin, they become resistant to multiple drugs, including doxorubicin, melphalan and even target-based therapy such as imatinib mesylate (Dalton *et al.*, 2002).

1.4.5 Altered Expression Levels of Oncogenes Related to Multiple Myeloma

The molecular lesions of lymphoid neoplasia can be divided into broad categories according to their mechanisms, which include activation of proto-oncogenes by chromosomal translocation or point mutation, proto-oncogene amplification and tumor suppressor gene inactivation (Table 1.2).

Table 1.2 Genetic alterations described in multiple myeloma

Involved oncogene	**Chromosome location**	**Frequency (%)**	**Mechanism of activation**
c-myc	8q24	0-5	Rearrangement
bcl-2	18q21	0-5	Rearrangement
N-*ras*	1p11-13	9-27	Point mutation
K-*ras*	12p11-12	0-7	Point mutation
p53	17p13	6-13	Point mutation/ deletion
Rb1	13q14	3	Deletion

Proto-oncogene activation by chromosomal translocations is the common result of a subset of molecular lesions typical of lymphoid neoplasia, very frequently associated with a specific subtype of lymphoproliferation. The most frequent translocation in adult lymphoid tumors involves proto-oncogene (*Bcl-2*), which does not directly influence cell proliferation, but prevents cell death. Activation of proto-oncogenes by mutation in lymphoid neoplasia is restricted to the *ras* gene family. Gene amplification as a mechanism of proto-oncogene activation seems of little importance in lymphoid neoplasia, though it is a relatively common mechanism in the molecular pathogenesis of solid tumors. Distruption of tumor suppressor genes by chromosomal deletion of one allele and inactivation of the other is an additional general mechanism of pathogenesis in lymphoid tumors, best exemplified by the case of p53 (Gaidano, 1992).

1.4.5.1 The *c-myc* oncogene

The *c-myc* proto-oncogene codes for a protein which acts as a transcription factor and regulates the activity of other genes by binding to specific DNA sequences. Its stimulation of B-cell proliferation and repression of B-cell differentiation are important steps in the pathogenesis of human B-cell malignancies (Gaidano *et al.*, 1992). *C-myc* rearrengement, especially through chromosomal translocation that juxtaposes the *c-myc* proto-oncogene and the gene encoding the immunoglobulin heavy chain (IgH). The main molecular consequence of this translocation is nonregulated c-myc expression throughout the cell cycle, leading to cellular proliferation. Rearrangements of *c-myc* have been observed in some cases of MM. Most of these rearrangements are t(8;14) (Avet-Loiseau *et al.*, 2001).

1.4.5.2 The *bcl-2* oncogene

The *bcl-2* oncogene belongs to a class of proto-oncogenes that apparently control cell survival by blocking programmed cell death (Gaidano *et al.*, 1992). The t(14;18) translocation found in follicular B cell lymphomas is the most common translocation associated with human lymphoid malignancies. This translocation creates Bcl-2-Ig fusion gene and results in overexpression of wild-type Bcl-2 mRNA and protein in the malignant cells (Bloem *et al.*, 1999)

The *bcl-2* is a member of a large gene family; the best studied counterpart of bcl-2 is bax. The bcl-2 and bax proteins can interact and form heterodimers which appears to be important for the ability of bcl-2 to block apoptosis (Oltvai *et al.*, 1993). Other members of the bcl-2 family, like mcl-1 and bcl-x_L also participate in completing dimerizations (Bloem *et al.*, 1999). It was shown that mcl-1 and bcl-x_L expressions were related in myeloma cells and are tightly regulated by IL-6 (Puthier *et al.*, 1999).

1.4.5.3 The *ras* oncogene

Signals transmitted from the cell surface to the nucleus are essential components of cell viability. A complex network of signal transduction pathways link cell surface receptors to the control of cellular proliferation, diffrentiation and survival. The ras family, comprised of H-ras, N-ras and K-ras, are critical components of many of these signal transduction pathways. H-, N-, and K-ras are small monomeric G-proteins that act as molecular switches. Ras cycles between the GDP-bound inactive state and the GTP-bound active state. Point mutations at codons 12, 13 or 61 lead to a constitutively active, GTP-bound Ras protein (Rowley *et al.*, 2002).

Activated *ras* genes have been identified in several human cancers, including both myeloid and lymphoid malignancies. Mutations, however, have been described in only two B-cell disorders, namely acute lymphoblastic leukemia and multiple myeloma (Ahuja *et al.*, 1990). *Ras* mutations have been shown to protect myeloma cell lines from apoptosis induced by dexamethasone, doxorubicin or melphalan (Rowley *et al.*, 2002). This demonstrates the importance of ras dependent signal transduction in myeloma cells.

1.4.5.4 The *p53* tumor suppressor gene

The *p53* gene encodes a 53 kDa nuclear phosphoprotein that controls the normal cell cycle by regulating transcription and possibly DNA replication. Loss of this growth inhibitor activity is usually the result of point mutations of one allele associated with the loss of the other. The majority of mutations occur between codons 110 and 307 encompassing exon 5-9 and are clustered in four regions that are highly conserved among several different species (Soussi *et al.*, 1990; Hollstein *et al.*, 1991).

The incidence of *p53* mutations in bone marrow samples of patients with plasma cell dyscrasis ranges from 10 to 20 per cent. They are typically associated with advanced and clinically aggressive forms of multiple myeloma (Portier *et al.*, 1992).

1.4.5.5 The *Rb1* (Retinoblastoma) tumor suppressor gene

The *Rb1* tumor suppressor gene encodes a 110 kDa phosphoprotein which accumulates in the nucleus and is associated with DNA binding activiy. Preliminary findings suggest that Rb1 protein is involved in the control of transition of normal cells from G0/G1 into S-phase of the cell cycle (Goodrich *et al.*, 1991).

Various mechanisms of *Rb1* inactivation have been described in human tumors: point mutations, gross rearrangements, large intragenic deletions and complete deletions. Immunohistochemical analysis has shown complete absence of the protein in 17 % of multiple myeloma patients and 18 % of pasma cell leukemia patients. Also U-266 cell line (MM cell line) showed lack of protein expression. Sequencing analysis of the Rb1 cDNA revealed two abnormal mRNA species carrying the deletion of both exon 13 and exon 14, respectively (Corradini *et al.*, 1994).

1.4.6 Involvement of Growth Factors in MDR

The term growth factor refers to a naturally occuring protein capable of stimulating cellular growth, proliferation and cellular differentiation. Growth factors typically act as signaling molecules between cells. They bind to receptors on the cell surface, with the result of activating cellular proliferation and/or differentiation. Growth factors

function as growth stimulators (mitogens) and/or growth inhibitors, stimulate cell migration, act as chemotactic agents, inhibit cell migration, inhibit invasion of tumor cells, modulate differentiated functions of cells, involved in apoptosis, involved in angiogenesis and promote survival of cells without influencing growth and differentiation. Individual growth factor proteins tend to occur as members of larger families of structurally and evolutionarily related proteins. There are many families which are listed below (www.mercksource.com).

- Bone morphogenetic proteins (BMPs)
- Epidermal growth factor (EGF)
- Erythropoietin (EPO)
- Fibroblast growth factor (FGF)
- Granulocyte-colony stimulating factor (G-CSF)
- Granulocyte-macrophage colony stimulating factor (GM-CSF)
- Growth differentiation factor-9 (GDF9)
- Hepatocyte growth factor (HGF)
- Insulin-like growth factor (IGF)
- Myostatin (GDF-8)
- Nerve growth factor (NGF) and other neurotrophins
- Platelet-derived growth factor (PDGF)
- Thrombopoietin (TPO)
- Transforming growth factor alpha(TGF-α)
- Transforming growth factor beta (TGF-β)
- Vascular endothelial growth factor (VEGF)

1.4.7 Differential Expression Levels of Genes Related to Cell Cycle

The cell cycle, is the series of events that take place in a cell leading to its division. Regulation of the cell cycle also involves processes crucial to the survival of a cell, including the detection and repair of genetic damage as well as the prevention of uncontrolled cell division. Two key classes of regulatory molecules, cyclins and cyclin-dependent kinases determine a cell's progress through the cell cycle (Nigg, 1995).

Cyclin D is the first cyclin produced in the cell cycle, in response to extracellular signals (growth factors). Cyclin D binds to existing CDK4, forming the active cyclin D-CDK4 complex. Cyclin D-CDK4 complex in turn phosphorylates the retinoblastoma susceptibility protein (RB). The hyperphosphorylated RB dissociates from the E2F/DP1/RB comlex, activating E2F. Activation of E2F results in transcription of various genes like cyclin E, cyclin A, DNA polymerase, thymidine kinase etc. Cyclin E thus produced binds to CDK2, forming the cyclin E-CDK2 complex, which pushes the cell from G1 to S phase. Cyclin B/CDK1 complex initiates the G2/M transition (Norbury, 1995).

A disregulation of the cell cycle components may lead to tumor formation. Some genes like the cell cycle inhibitors *(p15, p16, p18, p19, p21, p27), RB,* and *p53*, when they mutate, may cause the cell to multiply uncontrollably, forming a tumor. The cells which are actively undergoing cell cycle are targeted in cancer therapy as the DNA is relatively exposed during cell division and hence susceptible to damage by drugs or radiation (Nigg *et al.*, 1995). Figure 1.3 shows a schematic representation of cell cycle regulation.

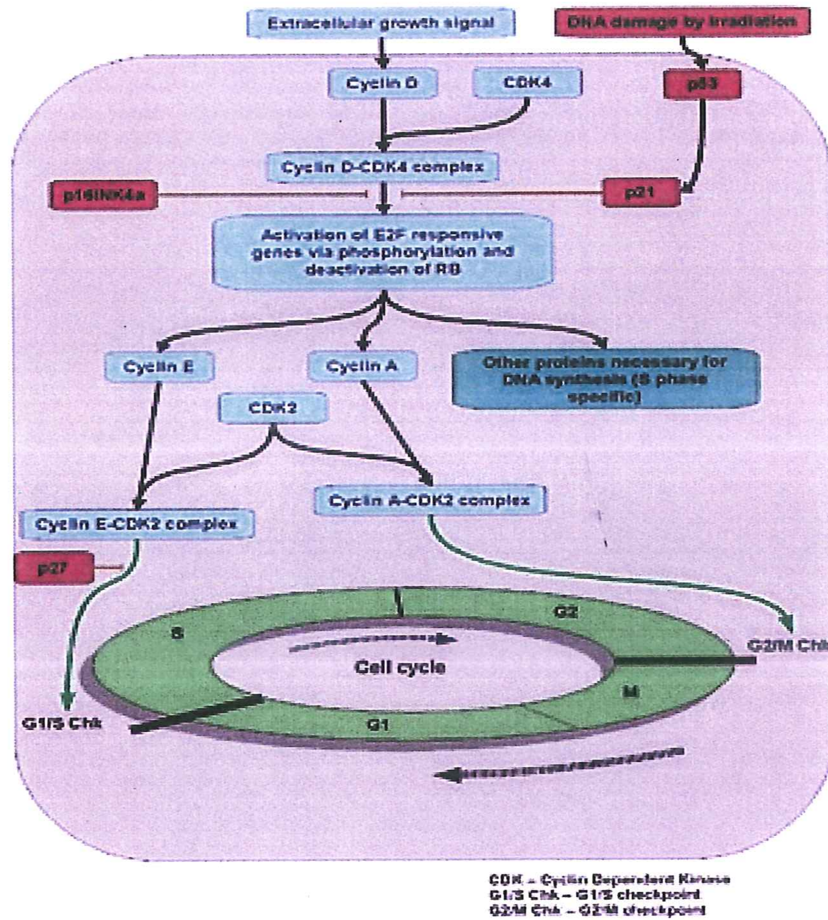


Figure 1.3 Regulation of cell cycle (Made from scratch in OpenOffice.org Draw)

1.4.8 Involvement of Ceramide Metabolism in MDR

In addition to changes in the expression level of particular proteins, multidrug-resistant cells exhibit major alterations in their sphingolipid composition. A 2- to 3-fold overexpression of glucosylceramide appears to be a rather general aspect of P-gp expressing MDR cells. Consequently it has been proposed that increased levels of glucosylceramide serve as a marker for MDR cancers. Sphingolipids might be directly involved in the regulation of MDR protein activity. On the other hand, an increased turnover of ceramide to glucosylceramide may confer the ability of MDR cells to escape from apoptosis, since ceramide plays a major role in the regulation of

apoptosis. As such, this mechanism may represent a novel strategy for MDR cells to increase their resistance to environmental stress-inducers (Klappe *et al.*, 2004)

Sphingolipids, which include ceramides and sphingosine, are essential structural components of cell membranes that also have messenger functions that regulate the proliferation, survival and death of cells (Reynolds *et al.*, 2004).

Intracellular ceramide can be formed either by *de novo* synthesis, or through the sphingomyelinase-dependent catabolism of sphingomyelin, in various seperate cellular compartments. The generation of ceramide from both catabolism of sphingomyelin and by *de novo* synthesis have been implicated in the response to cytotoxic agents, and it is possible that cell death signalling by ceramide may be enhanced by the summation of cellular ceramide levels that are derived from both pathways (Fig 1.4) (Reynolds *et al.*, 2004)

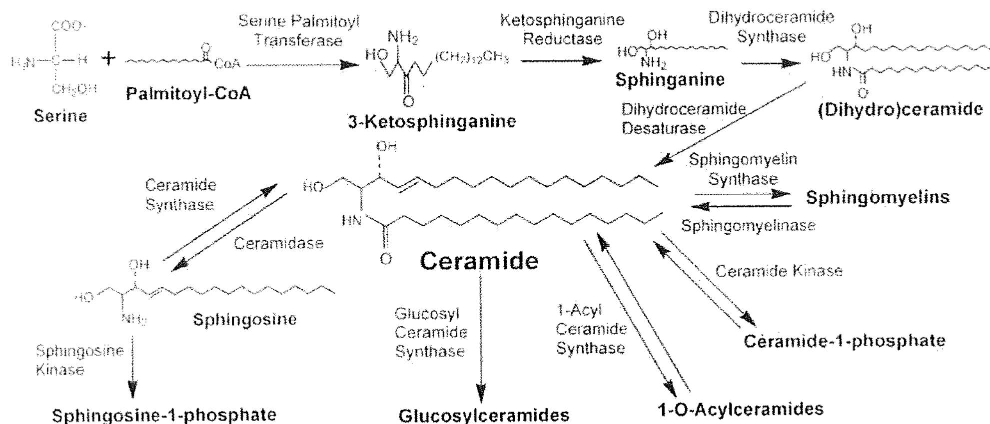


Fig 1.4 Major synthetic and metabolic pathways for ceramide (Reynolds *et al.*, 2004)

Ceramide can be metabolized to less toxic forms by glycosylation, acylation or by catabolism to sphingosine, which is then phosphorylated to the anti-apoptotic sphingosine 1-phosphate. Glycosylceramide synthase overexpression has been shown to enhance resistance to doxorubicin, suggesting that inhibition of ceramide metabolism or catabolism might enhance cancer chemotherapy (Reynolds *et al.*, 2004).

Several anticancer agents, including the cytotoxic retinoid, fenretinide (4-HPR), have been shown to act, at least in part, by increasing tumor cell ceramide via de novo synthesis. Phase I trials of ceramide metabolism inhibitors in combination with 4-HPR and with other cytotoxic agents are in development. Thus, pharmacological manipulation of sphingolipid metabolism to enhance tumor cell ceramide is being realized and offers a novel approach to cancer chemotherapy (Reynolds *et al.*, 2004).

1.4.9 Involvement of Cytochromes and Drug Metabolism in MDR

All drugs are detoxified and eventually excreted from the body, and many require bioactivation to form the active compound. Cytochromes are the major enzymes involved in drug metabolism and bioactivation. Human CYPs are primarily membrane-associated proteins, located either in the inner membrane of mitochondria or in the endoplasmic reticulum of cells. Table 1.3 shows different types of human CYP families. CYPs metabolize thousands of endogenous and exogenous compounds. Most CYPs can metabolize multiple substrates, and many can catalyze multiple reactions, which accounts for their central importance in metabolizing the extremely large number of endogenous and exogenous molecules. In the liver, these substrates include drugs and toxic compounds as well as metabolic products such as bilirubin (a breakdown product of hemoglobin). Cytochrome P450 enzymes are present in most other tissues of the body, and play important roles in hormone synthesis and breakdown (including estrogen and testosterone synthesis and metabolism), cholesterol synthesis, and vitamin D metabolism (Nelson, 2003).

Many drugs may increase or decrease the activity of various CYP isozymes by either inducing the biosynthesis of an isozyme (enzyme induction) or by directly inhibiting the activity of the CYP (enzyme inhibition). This is a major source of adverse drug interactions, since changes in CYP enzyme activity may affect the metabolism and clearance of various drugs. For example, if one drug inhibits the CYP-mediated metabolism of another drug, the second drug may accumulate within the body to toxic levels, possibly causing an overdose. Hence, these drug interactions may necessitate dosage adjustments or choosing drugs which do not interact with the CYP system. Such drug interactions are especially important to take into account when using drugs of vital importance to the patient, drugs with important side effects and

drugs with small therapeutic windows, but any drug may be subject to an altered plasma concentration due to altered drug metabolism (Guengerich *et al.*, 2008).

Table 1.3 Different types of CYP families (http://drnelson.utmem.edu/human.P450.table.html)

Family	Function	Members	Names
CYP1	drug and steroid (especially estrogen) metabolism	3 subfamilies, 3 genes, 1 pseudogene	*CYP1A1, CYP1A2, CYP1B1*
CYP2	drug and steroid metabolism	13 subfamilies, 16 genes, 16 pseudogenes	*CYP2A6, CYP2A7, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2F1, CYP2J2, CYP2R1, CYP2S1, CYP2U1, CYP2W1*
CYP3	drug and steroid (including testosterone) metabolism	1 subfamily, 4 genes, 2 pseudogenes	*CYP3A4, CYP3A5, CYP3A7, CYP3A43*
CYP4	arachidonic acid or fatty acid metabolism	6 subfamilies, 11 genes, 10 pseudogenes	*CYP4A11, CYP4A22, CYP4B1, CYP4F2, CYP4F3, CYP4F8, CYP4F11, CYP4F12, CYP4F22, CYP4V2, CYP4X1, CYP4Z1*
CYP5	thromboxane A_2 synthase	1 subfamily, 1 gene	*CYP5A1*
CYP7	bile acid biosynthesis 7-alpha hydroxylase of steroid nucleus	2 subfamilies, 2 genes	*CYP7A1, CYP7B1*
CYP8	*varied*	2 subfamilies, 2 genes	*CYP8A1* (prostacyclin synthase), *CYP8B1* (bile acid biosynthesis)

Table 1.1 continued

CYP11	steroid biosynthesis	2 subfamilies, 3 genes	*CYP11A1*, *CYP11B1*, *CYP11B2*
CYP17	steroid biosynthesis, 17-alpha hydroxylase	1 subfamily, 1 gene	*CYP17A1*
CYP19	steroid biosynthesis: aromatase synthesizes estrogen	1 subfamily, 1 gene	*CYP19A1*
CYP20	unknown function	1 subfamily, 1 gene	*CYP20A1*
CYP21	steroid biosynthesis	2 subfamilies, 2 genes, 1 pseudogene	*CYP21A2*
CYP24	vitamin D degradation	1 subfamily, 1 gene	*CYP24A1*
CYP26	retinoic acid hydroxylase	3 subfamilies, 3 genes	*CYP26A1*, *CYP26B1*, *CYP26C1*
CYP27	*varied*	3 subfamilies, 3 genes	*CYP27A1* (bile acid biosynthesis), *CYP27B1* (vitamin D3 1-alpha hydroxylase, activates vitamin D3), *CYP27C1* (unknown function)
CYP39	7-alpha hydroxylation of 24-hydroxycholesterol	1 subfamily, 1 gene	*CYP39A1*
CYP46	cholesterol 24-hydroxylase	1 subfamily, 1 gene	*CYP46A1*
CYP51	cholesterol biosynthesis	1 subfamily, 1 gene, 3 pseudogenes	*CYP51A1* (lanosterol 14-alpha demethylase)

1.5 AIM OF THE STUDY

Multidrug resistance is described as a complex phenotype of tumor cells which is characterized by the resistance to wide range of structurally unrelated anticancer agents used in cancer chemotherapy The emergence of drug-resistant tumor cells is a major complication for succesful anti-cancer chemotherapy in many cancer types including multiple myeloma. There are various mechanisms that are responsible for this phenotype. Identification of these mechanisms clearly will help to develop new treatment strategies for multiple myeloma. For this purpose the objectives in this study were:

- To develop prednisone, melphalan and vincristine resistant RPMI-8226 and U-266 multiple myeloma sublines by applying the drugs in dose increments.
- To investigate gene expressions of *P-gp, MRP1, BCRP, LRP, Bcl-2, Bcl-*X_L*, GCS, SK-1* and *LASS-1* at transcriptional level in original and drug resistant sublines by RT-PCR.
- To investigate genome wide gene expression analysis at transcriptional level of drug resistant sublines by microarray analysis.
- To evaluate cross-resistance of vincristine on prednisone and melphalan resistant sublines.
- To investigate development of cross-resistance to gamma radiation.

As a result by combining these data we aimed to understand the molecular mechanisms of prednisone, vincristine and melphalan resistance in multiple myeloma model cell lines RPMI-8226 and U-266.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL LINES AND GROWTH CONDITIONS

2.1.1 RPMI-8226

RPMI-8226 is a human multiple myeloma cell line (donated by Gülhane Military Medical Academy). It was established from the peripheral blood of a 61-year-old man with multiple myeloma (IgG lambda-type) at diagnosis in 1966. Cells grow mostly as single cells in suspension in a medium containing 90 % RPMI 1640 (Biochrom AG, Berlin) medium supplemented with 10 % fetal bovine serum (FBS) (Biochrom AG, Berlin), 1.5 % L-glutamine and 0.1 mg/ml gentamicin (Biological Industries, Israel) at 37^0C with 5 % CO_2. Doubling time of cells is 60-70 hours. (DSMZ Cell Culture Data).

2.1.2 U-266

U-266 is also a human multiple myeloma cell line (donated by Gülhane Military Medical Academy). It was established from the peripheral blood of a 53-year-old man with IgE-secreting myeloma (refractory, terminal) in 1968. Cells express mRNA for *bcl-2* gene. Their morphology is round to polygonal, single or clustered cells in suspension, some loosely adherent and they grow in the same medium and growth conditions as in the case of RPMI-8226 cell line with 20 % FBS instead. Doubling time of cells is 55 hours. (DSMZ Cell Culture Data).

2.2 ANTICANCER DRUGS

2.2.1 Melphalan

It is one of a group of anti-cancer drugs called alkylating agents which kills cancer cells by damaging DNA and stops them from dividing. Figure 2.1 shows the chemical structure of melphalan.

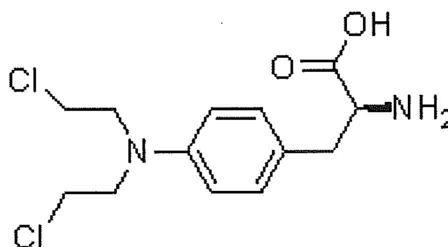


Figure 2.1 Chemical structure of melphalan

Molecular formula of melphalan is $C_{13}H_{18}Cl_2N_2O_2$ with molecular weight 305.20.

2.2.2 Prednisone

Prednisone is a member of the glucocorticoid class of hormones. The chemical structure of prednisone is seen in Figure 2.2.

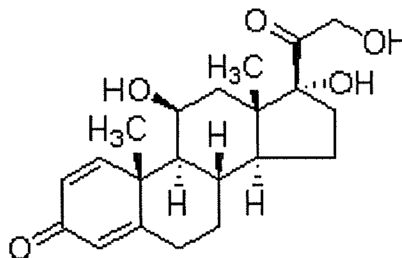


Figure 2.2 Chemical structure of prednisone

Molecular formula of prednisone is $C_{21}H_{28}O_5$ and its molecular weight is 360.4.

2.2.3 Vincristine

Vincristine belongs to a group of anticancer drugs called vinca alkaloids. It prevents the formation of chromosome spindles necessary for cell duplication at the mitosis. Figure 2.3 shows the chemical structure of vincristine.

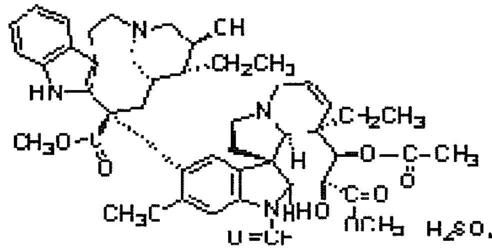


Figure 2.3 Chemical structure of vincristine

Vincristine's molecular weight is 923.04 having molecular formula of $C_{46}H_{56}N_4O_{10}H_2SO_4$.

2.3 PRIMERS

The PCR primers used to amplify *MDR1* gene were 21/20 oligonucleotides. (Dong *et al.,* 2005)

Sense primer : 5'-TACAGTGGAATTGGTGCTGGG-3'
Antisense primer : 5'-CCCAGTGAAAAAATGTTGCCCA-3'

The PCR primers used to amplify *MRP1* gene were 20/20 oligonucleotides. (Miao *et al,* 2003)

Sense primer : 5'-TAGAGGACTTCGTGTCAGCC-3'
Antisense primer : 5'-GTCCATGATGGTGTTGAGCC-3'

The PCR primers used to amplify *BCRP* gene were 22/23 oligonucleotides. (Pallissot *et al.,* 2005)

Sense primer : 5'-TACAGTTCTCAGCAGCTCTTCG-3'

Antisense primer : 5'-CAACTTGAAGATGGAATATCGAG-3'

The PCR primers used to amplify *LRP* gene were 20/20 oligonucleotides. (Schaich *et al.*, 2005)

Sense primer : 5'-CGCTGCTTGATTTTGAGGAT-3'

Antisense primer : 5'-CGAGAATCACGCAGTAGTTG-3'

The PCR primers used to amplify *Bcl-2* gene were 21/21 oligonucleotides. (Douarre *et al*, 2005)

Sense primer : 5'-GGTGAACTGGGGGAGGATTGT-3'

Antisense primer : 5'-CTTCAGAGACAGCCAGGAGAA-3'

The PCR primers used to amplify $Bcl\text{-}X_L$ gene were 20/20 oligonucleotides. (Assaf *et al*, 2004)

Sense primer : 5'-AGGATACAGCTGGAGTCAGT-3'

Antisense primer : 5'-ACCTGCATCTCCTTGTCTAC-3'

The PCR primers used to amplify *GCS* gene were 19/18 oligonucleotides. (Baran *et al.*, 2007)

Sense primer : 5'-ATGACAGAAAAAGTAGGCT-3'

Antisense primer : 5'-GGACACCCCTGAGTGGAA-3'

The PCR primers used to amplify *SK-1* gene were 22/22 oligonucleotides. (Baran *et al.*, 2007)

Sense primer : 5'-CCGACGAGGACTTTGTGCTAAT-3'

Antisense primer : 5'-GCCTGTCCCCCCAAAGCATAAC-3'

The PCR primers used to amplify *LASS-1* gene were 23/23 oligonucleotides. (Baran *et al.*, 2007)

Sense primer : 5'-CTATACATGGACACCTGGCGCAA-3'
Antisense primer : 5'-TCAGAAGCGCTTGTCCTTCACCA-3'

The PCR primers used to amplify *β-actin* gene were 20/20 oligonucleotides. (Baran *et al.*, 2007)

Sense primer : 5'-CAGAGCAAGAGAGGCATCCT-3'
Antisense primer : 5'-TTGAAGGTCTCAAACATGAT-3'

Primers were selected as to anneal on different exons to prevent amplification of contaminating DNA.

2.4 GENERATION OF RESISTANT SUBLINES

Before application of drugs, both cell lines (RPMI-8226 and U-266) were grown for 4 weeks in media without any drug to optimize growth conditions. Melphalan, prednisone and vincristine were applied seperately in dose increments to RPMI-8226 and U-266 cell lines for generation of resistant sublines. The resistant sublines to melphalan (RPMI-8226/1μMMelp, U-266/1μMMelp), prednisone (RPMI-8226/500μMPred, U-266/300μMPred) and vincristine (RPMI-8226/50nMVinc, U-266/2nMVinc) were developed by increasing the doses starting from 5nM melphalan, 5μM prednisone and 0.5nM vincristine. At these concentrations the cells were grown for 4 weeks. Before increasing the dose of drugs, viable cell counts were performed. The cells were counted by tryphan blue assay on hematocytometer under a light microscope and at least 90 % viability was seen before passing to a higher dose. Melphalan was purchased from Sigma (St. Louis, MO, USA). Prednisone and vincristine was denoted by Gülhane Military Medical Academy, School of Medicine as injection form and the drugs were diluted with deionized water.

2.5 CELL VIABILITY ASSAY

The XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) (Biological Industries, Israel) assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazen dye by metabolic active cells and mitochondrial reduction of the XTT reagent in nonviable cells. The formazan dye formed is soluble in aqueous solutions and is directly quantified using a scanning multiwell spectrophotometer (ELISA reader). Cells, grown in a 96 well tissue culture plate in the presence of drug, are incubated with the yellow XTT solution for 4 h. After this incubation period, orange formazan solution is formed, which is spectrophotometrically quantified using an ELISA plate reader. An increase in number of living cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This increase directly correlates to the amount of orange formazan formed. By this assay it is possible to determine the inhibitory concentrations (IC_{50} or IC_{80}) of the drugs on original and resistant RPMI-8226 and U-266 cell lines. Two different IC values were used for comparison because in some of the cell lines IC_{50} values could not calculated. The resistance indices 'R' were evaluated according to the following equations:

$R = IC_{50}$ resistant cell line/ IC_{50} original cell line [Equation 1]

$R = IC_{80}$ resistant cell line/ IC_{80} original cell line [Equation 2]

2.6 TOTAL RNA ISOLATION

Prior to RNA isolation all the plastic and glassware were treated with DEPC-treated dH_2O and were autoclaved. Total RNA was isolated from original and drug resistant RPMI-8226 and U-266 cell lines. Cells (nearly 10^8) were centrifuged at 800 rpm for 5 minutes. After the supernatant was discarded 1ml of TRIzol (Sigma, St. Louis, MO, USA) was added to the cell pellet. It was incubated for 5 minutes at room temperature while shaking by hand. Then 0.4 ml of chloroform was added. The tube was shaked vigorously by hand for 15 seconds and incubated at room temperature for 2-3 minutes. Then a centrifugation was carried out at 13000 rpm for 15 minutes. After this centrifugation, three distinct layers were formed. The upper clear RNA containing phase was taken into a sterile eppendorf tube. Then mixing with 0.5ml isopropyl alcohol, RNA was precipated by incubating at room temperature for 10 minutes. After incubation the tube was centrifuged at 13000 rpm for 10 minutes and supernatant was removed before the pellet was dried near the Bunsen burner. Pellet was washed with 1ml of 75 % ethanol and centrifuged at 6500 rpm for 10 minutes. After the pellet was dried it was dissolved in RNase free water and finally incubated at 55-60 °C for 10 minutes.

2.7 RNA QUANTIFICATION

In order to determine the RNA yield spectrophotometric analysis was performed. 10µl of RNA was diluted with 990µl RNase free water (1/100 dilution) and its absorbance values were measured both at 260nm and 280nm by Shimadzu (Columbia, USA) spectrophotometer. Then concentration of RNA was calculated by the formula;

$$260\text{nm OD} \times \text{dilution factor } (100) \times 40 = \mu\text{g/ml RNA} \qquad \text{[Equation 3]}$$

2.8 AGAROSE GEL ELECTROPHORESIS OF RNA SAMPLES

For the detection of the intactness of RNA molecules 1.2 % w/v agarose gel electrophoresis with ethidium bromide staining was performed (70V for 1 hour). The solutions and buffers used in agarose jel was given in Appendix A.

2.9 cDNA PREPARATION

cDNA synthesis (reverse transcription) was performed by using both oligodT's and random hexamers. cDNAs which were preapared by random hexamers were used for the PCR products that are greater than 350 bp in size. In a sterile Eppendorf tube 5μg total RNA and 0.5μg oligo dT (or 1.25μg random hexamer) were added. The mixture was incubated at 70 °C for 5 minutes and chilled on ice for 1-2 minutes. Then 4μl 5x reaction buffer, 2μl dNTP (2.5mM of each dNTP) and 20U Moloney Murine Reverse Transcriptase (Fermentas, Vilnius, Lithuania) was added onto the mixture and incubated at 42 °C for 60 minutes. After this incubation period, reaction was stopped by heating to 70 °C for 10 minutes. Tube was chilled on ice for 2-3 minutes. cDNAs were stored at -20 °C.

2.10 EXPRESSION ANALYSIS

2.10.1 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

cDNAs that was reverse transcribed from isolated RNA was used as template for polymerase chain reaction in order to optimize *MDR1, MRP1, BCRP, LRP, Bcl-2,* *Bcl-*X_L*, GCS, SK-1, LASS-1* and *β-actin* gene expressions. The product of *β-actin* gene was used as constitutively expressed common internal control. The optimum PCR reaction mixtures and conditions were given in Appendix B. The PCR products were examined on 2 % w/v agarose gel electrophoresis (90V for 1 hour) stained with ethidium bromide. RT-PCR experiments were performed twice independently.

2.10.2 Evaluation of the PCR Results

Using a computer program named as Scion Image Software (Scion Corporation, USA), densitometric analysis was performed to quantify the expression levels of the *MDR1, MRP1, BCRP, LRP, Bcl-2,* $Bcl\text{-}X_L$*, GCS, SK-1* and *LASS-1* genes. The densitometric values obtained for genes were divided by densitometric values of *β-actin* gene for normlization of the data. Then the band intensities of resistant sublines were compared with that of the original cell lines. The results were subjected to student t-test (SPSS Inc.,USA). $p<0.05$ was considered as statistically significant.

2.10.3 Protein Analysis

Total cell lysate extraction was done as described previously (Han *et al.,* 2000) and protein concentrations were calculated by Bradford assay (Bradford, 1976). Bovine serum albumin (BSA) was used as standard and 6 dilutions (1/100, 1/50, 1/25, 1/16.6, 1/10 and 1/6.25) were done to draw a standard curve. Standard curve was the absorbances at OD_{595}. Protein determination by using standard curve was given in Appendix C. Then the concentrations of unknown samples to be used in SDS-PAGE were calculated from the standard curve. The reagents and buffers used in protein isolation, Bradford assay and Western Blot were given in Appendix A.

The calculated concentrations of cell lysates were run in 7.5 % polyacrylamide gel SDS-PAGE (40mA-60mA for 5 hours). They were transferred to a 0.45 μm nitrocellulose membrane (Protan BA 85, Schleicher & Schuell) at 25 V for overnight by using BioRad ElectroBlot System. The membrane was washed with TBST buffer and the nonspecific binding sites were blocked with 5 % non-fat dry milk. After that membrane was incubated with monoclonal antibodies GAPDH (glyceraldehydes-3-phosphate dehydrogenase) (Chemicon, 10μg/ml) which is an internal control and Bcl-2 (Biovision, 50μg/ml) for 2 hours and peroxidase conjugated goat anti mouse IgG secondary antibody (Chemicon, 1:5000) for 1 hour. Finally the bands were seen by applying diaminobenzidine (DAB) solution (1:20). The samples were washed in PBS for 2x2 minutes. Densitometric analysis was done using Scion Image program.

2.11 MICROARRAY ANALYSIS

Microarray studies was done by using Affymetrix GeneChip® Human Genome kit (Human Genome U133 Plus 2.0 Array) . The studies were done at Ankara University Biotechnology Institude. The solutions and buffers used in microarray analysis were given in Appendix A.

2.11.1 Target Preparation

High quality total RNA was isolated from original and drug resistant RPMI-8226 and U-266 cell lines by using TRIzol reagent (Sigma St. Louis, Mo, USA) as previously described. RNA isolation was done dublicate from all of the cell lines in order to do statistical analysis. The quantification of RNA yield was determined by spectrophotometric analysis. The A260/A280 ratio should be close to 2.0 for pure RNAs. Integrity of total RNA samples were also be assessed qualitatively on an Agilent 2100 Bioanalyzer.

The double-stranded cDNA was synthesized from the RNAs that were isolated from original and drug resistant multiple myeloma cell lines. Since all of the isolated RNAs were greater than 10-100 ng, One-Cycle Target Labeling Assay (Affymetrix, Santa Clara, CA, USA) was performed according to manufacturer's instructions.
The RNAs were reverse transcribed using a T7-oligo(dT) promoter primer in the first-strand cDNA synthesis reaction and after that RNase H-mediated second strand cDNA synthesis was performed. The double-stranded cDNA is purified and serves as a template in the subsequent *in vitro* transcription (IVT) reaction. IVT reaction is carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analog/ribonucleotide mix for (cRNA) amplification and biotin labeling. The cleanup of cRNAs is essential to remove unincorporated NTPs, so that the concentration and purity of cRNA can be accurately determined by 260nm absorbances. After that the cRNAs were fragmented before hybridization.

2.11.2 Target Hybridization and Scanning

A hybridization cocktail was prepared, including the fragmented target and probe array controls. *E.coli* (*bioB, bioC, bioD*) and P1 Bacteriophage (*cre*) antisense biotinylated cRNAs are used as hybridization controls. The cocktail was then hybridized to the probe array during a 16-hour incubation. After 16 hours of hybridization in Affymetrix GeneChip® Hybridization Oven 640, the arrays were removed from the hybridization oven and the hybridization cocktail was extracted with a pipettor through the septum of the arrays. The probe arrays were refilled completely with the appropriate volume of wash buffer. After washing step the arrays were stained using hybridization stain kit which contains streptavidin-phycoerythrin biotinylated anti-streptavidin antibody. Once the probe arrays had been hybridized, washed, and stained, they were scanned in Affymetrix GeneChip® Scanner 3000 device. The software defines the probe cells and computes an intensity for each cell.

2.11.3 Data Analysis

Totally 16 microarray chips with HGU13Plus2 platform and labelled as RPMI-8226, RPMI-8226/500µM Pred, RPMI-8226/50nM Vinc, RPMI-8226/1µM Melp, U-266, U-266/300µM Pred, U-266/2nM Vinc, U-266/1µM Melp (each dublicate) were used at data analysis. Each chip contains 604258 oligonucleotide (probe) within 54674 oligonucleotide set (probe set). The chips were single chanelled Affymetrix chips.

2.11.3.1 Normalization

In the normalization studies RMA (Robust Multichip Analysis) method which is the most widely used method in the literature was performed. In the RMA method only specific binding data are used, the non-specific binding data are eliminated. This method is based on three main steps. These are background improvement, quantile normalization and summarization of the final data at the level of oligonucleotide sets by " median polish" method (Irizarry *et al.*, 2003).

In the normalization studies first of all by using specific binding data RMA background improvements were done. Then the datas were quantile-normalized seperately. Finally, by using RMA method the gene expression levels were calculated at the level of oligonucleotide sets, median polish method was performed and log2 equivalents were calculated. The gene expressions that are 2 times greater or lower than the controls (original RPMI-8226 and U-266 cell lines) were selected for discussion.

2.11.3.2 Statistical Analysis

At the data analysis the gene expression profiles of the RPMI-8226 and U-266 cell lines were compared seperately. In each cell line the original RPMI-8226 and U-266 lines were used as control and the drug applied lines were compared with their own control. For statistical analysis the t-test was used and p-values were calculated. The statistically significant ($p < 0.05$) gene expressions were selected.

2.12 PROLIFERATION ASSAY FOR CROSS-RESISTANCE TO VINCRISTINE ON PREDNISONE AND MELPHALAN RESISTANT CELL LINES

The effect of vincristine on original and resistant RPMI-8226 (RPMI-8226/500μMPred and RPMI-8226/1μMMelp) and U-266 (U-266/300μMPred and U-266/1μMMelp) cell lines were tested by performing cell viability assay as previously described in section 2.5. The resistance indices of each cell line to vincristine were calculated to determine the cross-resistance of each cell line to vincristine. The resistance indices were calculated according to Equation 1. Student t-test was used for statistical analysis (SPSS Inc.,USA). $p<0.05$ was considered as statistically significant.

2.13 PROLIFERATION ASSAY FOR CROSS-RESISTANCE TO COBALT-60 GAMMA (γ) RADIATION

Original and drug resistant sublines of RPMI-8226 and U-266 cell lines were placed into 96-well microtiter plates ($20x10^3$ cells/well) and incubated in fresh medium for 24 hours at standard culture conditions. Then plates were exposed to 200cGy and 800cGy cobalt-60 γ radiation by a Theratron 780 Cobalt 60 Teletherapy Unit (AECL Medical, Ontario, Canada) and incubated for additional 24 hours at 37°C in a 5 % CO_2 athmosphere. Finally cell proliferation assay was evaluated using XTT Cell Proliferation Kit (Biological Industries, Israel). The viability ratio of radiated cell line to its non-radiated control represents the degree of cross-resistance of each cell line to cobalt-60 gamma radiation. The data tested with student t-test considering $p<0.05$ as statistically significant.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 CELL CULTURE

RPMI-8226 and U-266 cell lines were examined daily, observing the morphology, the color of the medium and the density of the cells. Figure 3.1 shows the RPMI-8226 and U-266 cells that are not treated with any chemotherapeutic under inverted microscope with 40X magnification. The medium were refreshed once a week. Before application of drugs, both cell lines were grown for 4 weeks to adapt the cells to laboratory conditions and to optimize the growth conditions.

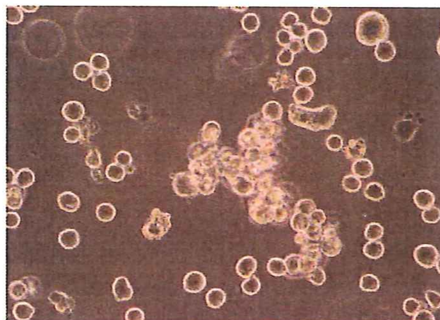


Figure 3.1a. RPMI-8226 cells under inverted microscope with 40X magnification.

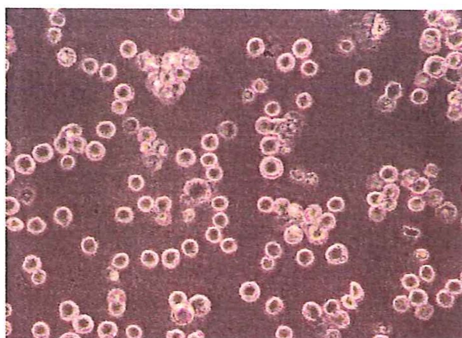


Figure 3.1b. U-266 cells under inverted microscope with 40X magnification.

3.2 APPLICATION OF DRUGS

Drug applications was done in dose increments. Before increasing each dose, cells were incubated at least 4 weeks for adaptation. Table 3.1 was shown the starting and final concentrations and also the application periods of the drugs. Finally RPMI-8226/50nMVin, U-266/2nMVin, RPMI-8226/500μMPred, U-266/300μMPred, RPMI-8226/1μMMelp and U-266/1μMMelp resistant sublines were generated. However the final concentrations of vincristine and prednisone were not same in RPMI-8226 and U-266 cell lines. These cell lines had different origin since RPMI-8226 was isolated from a patient at diagnosis whereas U-266 was from another patient at refractory phase. Because of the individual differences, different resistance mechanisms are active in these cell lines. For example U-266 cell line lacks *Rb1* tumor suppressor gene expression (Corradini *et al.*, 1994) whereas it express mRNA for *Bcl-2* gene (DSMZ Cell Culture Data). It is known that Rb1 protein is involved in the control of transition of normal cells from G0/G1 into S-phase of the cell cycle and bcl-2 is an antiapoptotic protein. Vincristine, being an alkylating agent that acts during mitosis, it can be more effective on U-266 cell line than RPMI-8226. This can be an explanation of the different final vincristine concentrations.

Table 3.1a. Dose and period of application of vincristine on multiple myeloma cells during resistance development

Vincristine Concentration (nM)	0.5nM	0.7nM	1 nM	2nM	5nM	10nM	20nM	50nM
RPMI-8226	4 weeks	8 weeks	4 weeks	4 weeks	8 weeks	4 weeks	4 weeks	4 weeks
U-266	4 weeks	8 weeks	4 weeks	4 weeks	-	-	-	-

Table 3.1b. Dose and period of application of prednisone on multiple myeloma cells during resistance development

Prednisone Concentration (µM)	5µM	10µM	20µM	40µM	80µM	100µM	300µM	500µM
RPMI-8226	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks	8 weeks	8 weeks
U-266	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks	8 weeks	-

Table 3.1c. Dose and period of application of melphalan on multiple myeloma cells during resistance development

Melphalan Concentration (nM)	5nM	10nM	20nM	50nM	100nM	200nM	500nM	1µM
RPMI-8226	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks	8 weeks	8 weeks
U-266	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks	4 weeks	8 weeks	8 weeks

3.3 CELL VIABILITY ASSAY

In order to quantify the acquired resistance by the RPMI-8226 and U-266 cells, after drug (melphalan, vincristine, prednisone) application, XTT assay was performed both for original and resistant cell lines. The aim is to obtain the inhibitory concentration 50 (IC_{50}) or 80 (IC_{80}) values of the drugs, which is the toxic dose of the drug that has the capacity to kill 50 % and 20 % of the cells respectively. The assay performed for original cells for vincristine, prednisone and melphalan; also for resistant sublines RPMI-8226/500µM prednisone, RPMI-8226/50nM vincristine, RPMI-8226/1µM melphalan, U-266/300µM prednisone, U-266/2nM vincristine and U-266/1µM melphalan.

Figure 3.2 and Figure 3.3 shows the viabilities of RPMI-8226, RPMI-8226/50nMVinc and U-266, U-266/2nMVinc cell lines with respect to applied vincristine concentrations (0-400nM; 0-200nM).

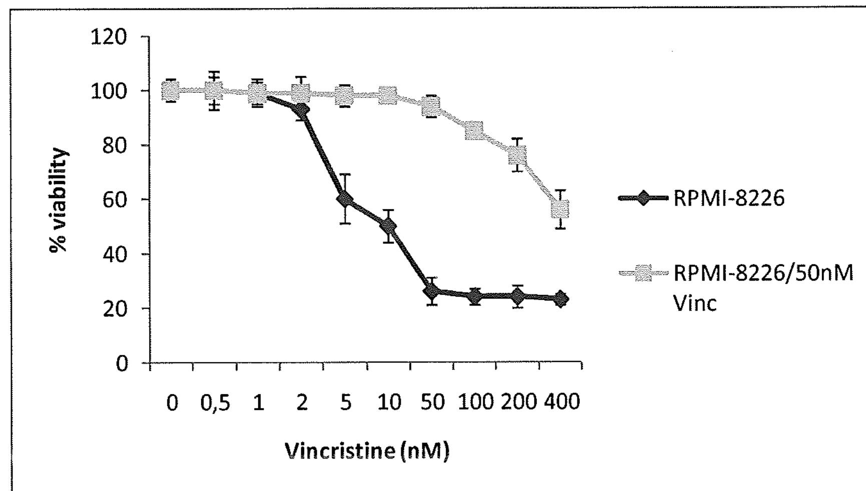


Figure 3.2 Percent viability of RPMI-8226 and RPMI-8226/50nM vincristine cells at increasing doses of vincristine.

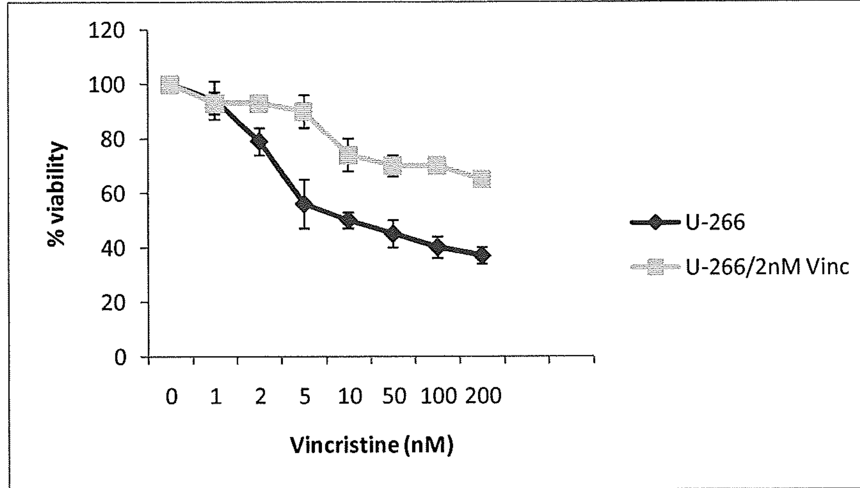


Figure 3.3 Percent viability of U-266 and U-266/2nM vincristine cells at increasing doses of vincristine.

IC_{50} value of vincristine on RPMI-8226 cells is 10nM while IC_{50} value for RPMI-8226/50nM vincristine is 400nM. On the other hand IC_{80} value of vincristine on sensitive U-266 cells is 2nM and IC_{80} value for U-266/2nM vincristine is 8.1nM. From these results it was seen that original RPMI-8226 cell line gained 40 fold and U-266 cell line gained 4 fold resistance to vincristine.

Figure 3.4 shows the viabilities of RPMI-8226 and RPMI-8226/500μM prednisone cell lines with respect to applied prednisone concentrations (0-5000μM) whereas Figure 3.5 shows the same data for U-266 and U-266/300μM prednisone with the prednisone concentrations ranging from 0-3000μm.

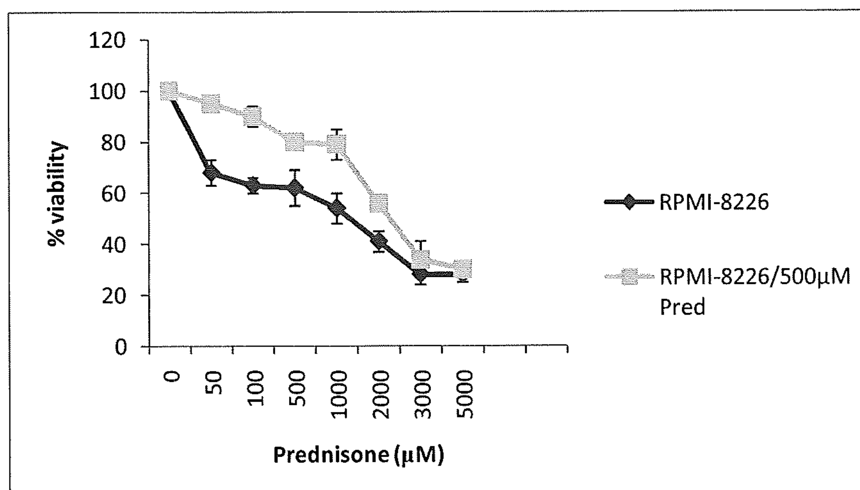


Figure 3.4 Percent viability of RPMI-8226 and RPMI-8226/500μM prednisone cells at increasing doses of prednisone

IC_{50} value of prednisone on RPMI-8226 cells is 1308μM while IC_{50} value for RPMI-8226/500μM prednisone is 2270μM. The original RPMI-8226 cell line gained 2 fold resistance to prednisone. However IC_{80} values of prednisone on U-266 and U-266/300μM prednisone cells are 43.4μM and 1624μM respectively which indicates 37 fold drug resistance development.

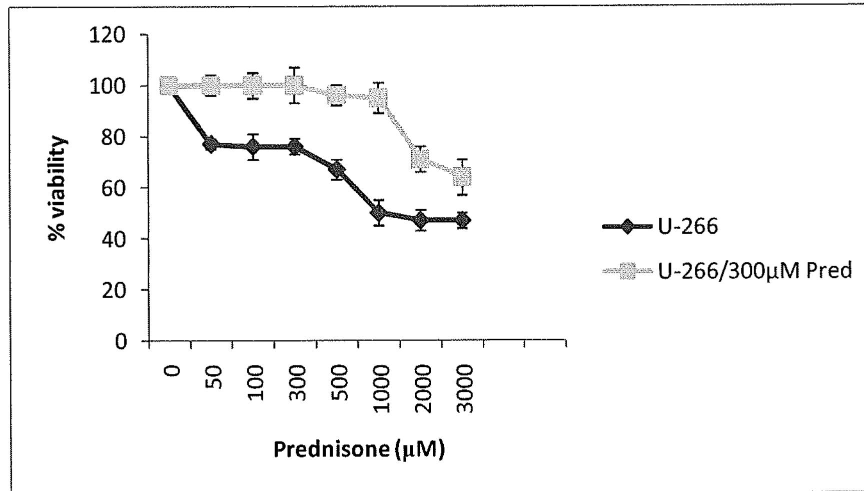


Figure 3.5 Percent viability of U-266 and U-266/300μM prednisone cells at increasing doses of prednisone.

Viabilities of RPMI-8226, RPMI-8226/1μM melphalan and U-266, U-266/1μM melphalan cell lines with respect to applied melphalan concentrations (0-5000μM) are seen in Figures 3.6 and 3.7.

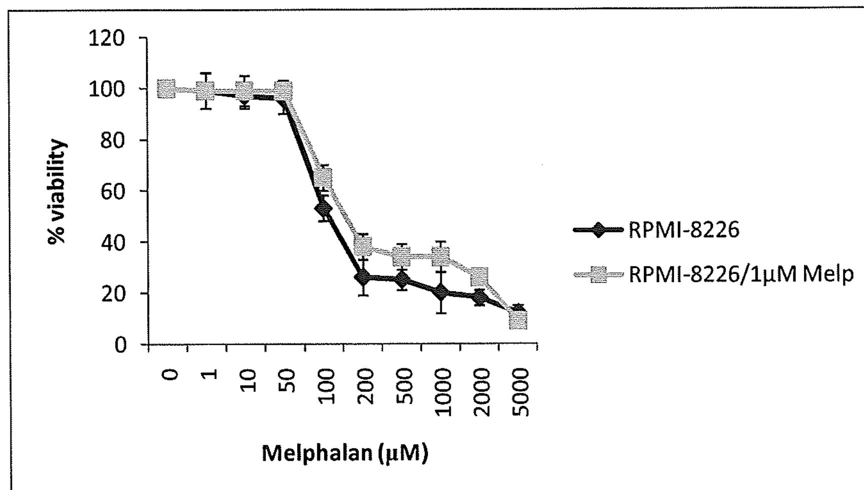


Figure 3.6 Percent viability of RPMI-8226 and RPMI-8226/1μM melphalan cells at increasing doses of melphalan.

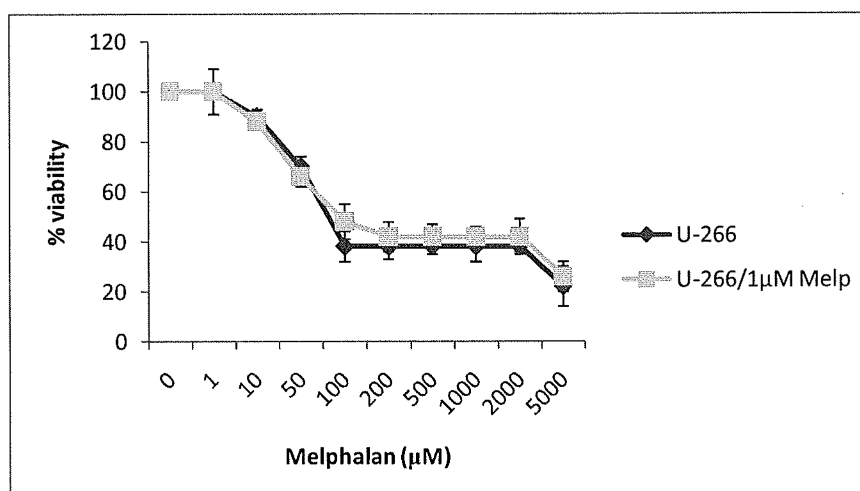


Figure 3.7 Percent viability of U-266 and U-266/1µM melphalan cells at increasing doses of melphalan.

RPMI-8226 and U-266 cells gained 1.5 and 2 fold resistance to melphalan respectively. IC_{50} value of melphalan on RPMI-8226 111.1µM while IC_{50} value for is RPMI-8226/1µM melphalan 155.5µM. On the other hand IC_{50} value of melphalan on sensitive cells is U-266 81.2µM while IC_{50} value for is U-266/1µM melphalan 188µM.

From these results it was seen that all of the sublines gained resistance to the applied drugs seperately (RPMI-8226/50nMVin, U-266/2nMVin, RPMI-8226/500µMPred, U-266/300µMPred, RPMI-8226/1µMMelp and U-266/1µMMelp) and the resistant sublines were investigated for the drug resistance mechanisms.

3.4 GEL ELECTROPHORESIS OF RT-PCR PRODUCTS

The expression levels of the target genes (*MDR1, MRP1, BCRP, LRP, Bcl-2, Bcl-X$_L$, GCS, SK-1* and *LASS-1*) were firstly optimized for original RPMI-8226 and U-266 cell lines by RT-PCR. *B-actin*, a constitutively expressed gene (housekeeping gene) was chosen as the reference gene. After drug application, the RNAs from drug resistant sublines were isolated and RT-PCR was performed for these lines in order to see the expression profile of the same genes.

Figures 3.8 - 3.11 show the RT-PCR results for *β-actin, MDR1, MRP1, BCRP, LRP, Bcl-2, Bcl-X_L, GCS, SK-1* and *LASS-1* genes for original and drug resistant sublines of RPMI-8226. Data from RPMI-8226/100μM Pred and RPMI-8226/20nM Vinc were not further considered for resistance analysis. Negative controls indicating there was no contaminating DNA were checked (data not shown).

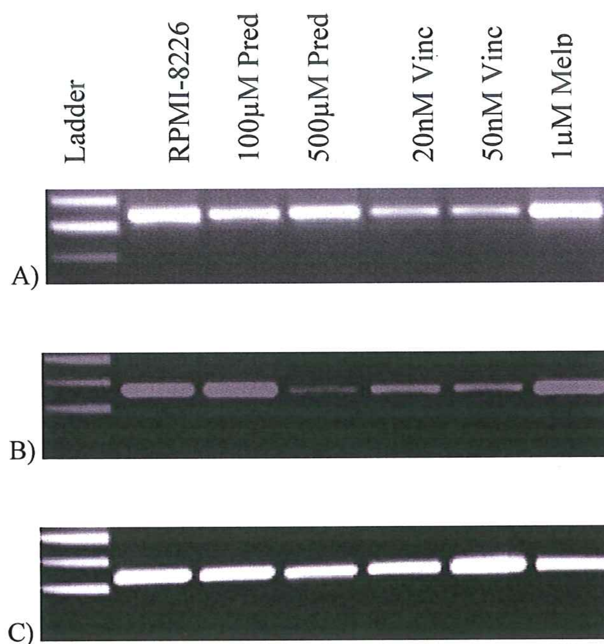


Figure 3.8 Expression levels of *GCS* (532 bp), *SK-1* (551 bp) and *LASS-1* (555 bp) genes in RPMI-8226 sublines (2% w/v agarose gel); Ladder: 50 bp ladder, A) *GCS*, B) *SK-1,* C) *LASS-1* products

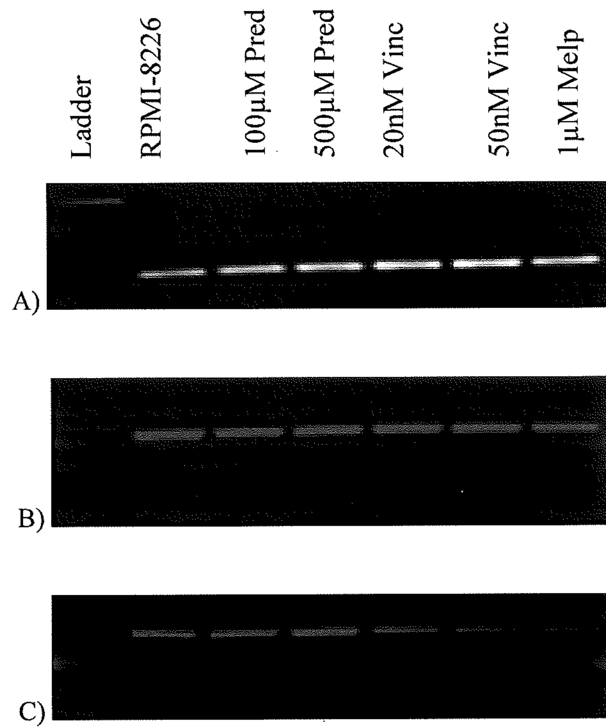


Figure 3.9 Expression levels of *Bcl-2* (232 bp), *Bcl-X$_L$* (422 bp) and *LRP* (483 bp) genes in RPMI-8226 sublines (2% w/v agarose gel); Ladder: 50 bp ladder, A) *Bcl-2*, B) *Bcl-X$_L$*, C) *LRP* products

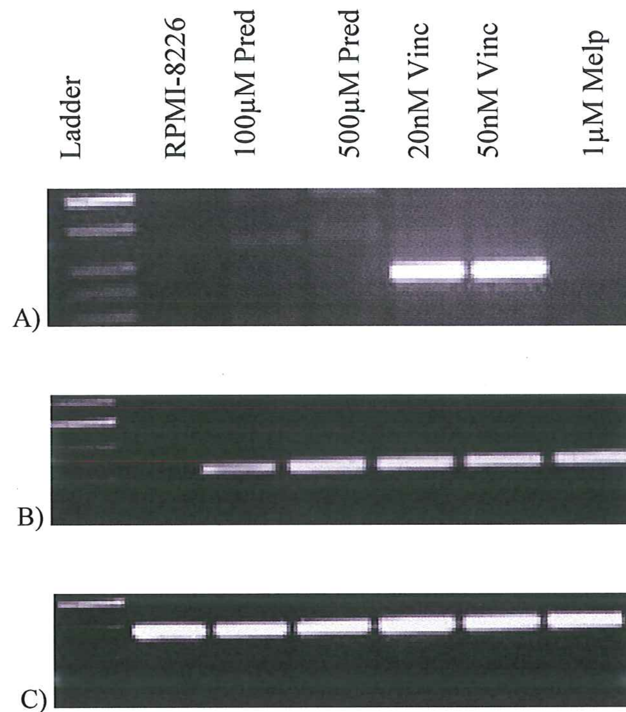


Figure 3.10 Expression levels of *MDR1* (258 bp), *MRP1* (256 bp) and *BCRP* (342 bp) genes in RPMI-8226 sublines (2% w/v agarose gel); Ladder: 50 bp ladder, A) *MDR1*, B) *MRP1*, C) *BCRP* products

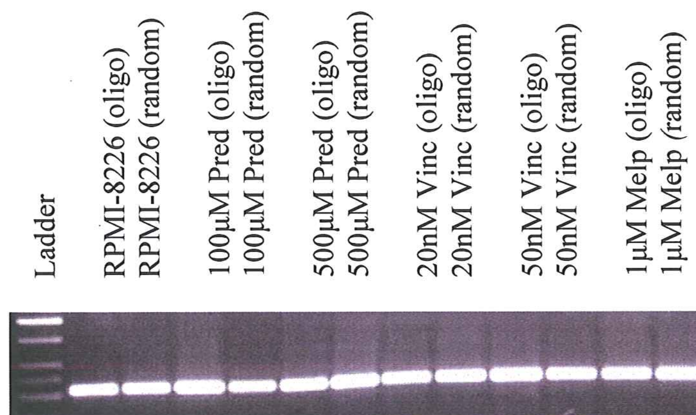


Figure 3.11 Expression levels of *β-actin* gene (209 bp) in RPMI-8226 sublines (2% w/v agarose gel); Ladder: 50 bp ladder

Figures 3.12 - 3.15 show the RT-PCR results for *β-actin, MDR1, MRP1, BCRP, LRP, Bcl-2, Bcl-X_L, GCS, SK-1* and *LASS-1* genes for original and drug resistant U-266 cell lines. Datum from U-266/100μM Pred was not considered in this study. Negative controls indicating there was no contaminating DNA were checked (data not shown).

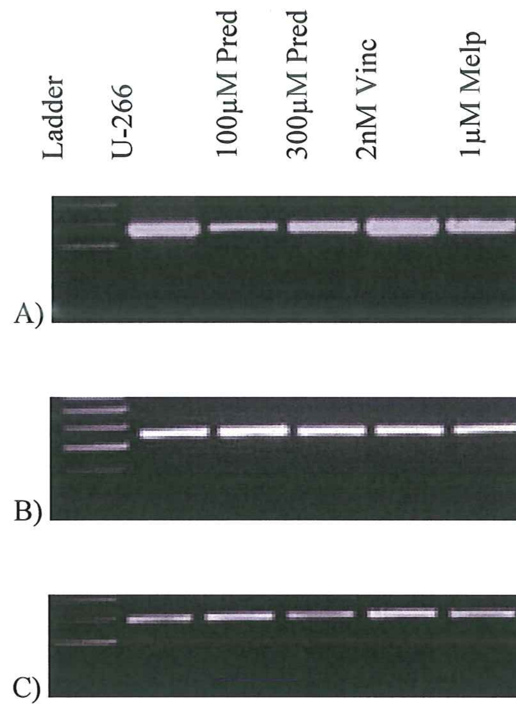


Figure 3.12 Expression levels of *GCS* (532 bp), *SK-1* (551 bp) and *LASS-1* (555 bp) genes in U-266 sublines (2% w/v agarose gel); Ladder: 50 bp ladder, A) *GCS*, B) *SK-1,* C) *LASS-1* products

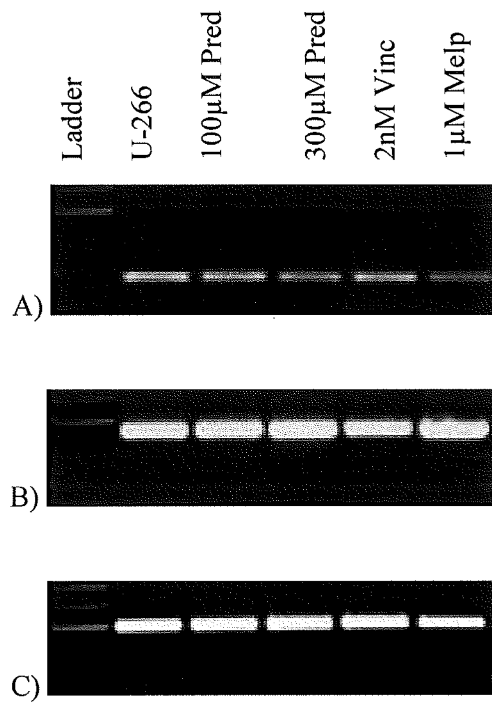


Figure 3.13 Expression levels of *Bcl-2* (232 bp), *Bcl-X$_L$* (422 bp) and *LRP* (483 bp) genes in U-266 sublines (2% w/v agarose gel); Ladder: 50 bp ladder, A) *Bcl-2*, B) *Bcl-X$_L$*, C) *LRP* products

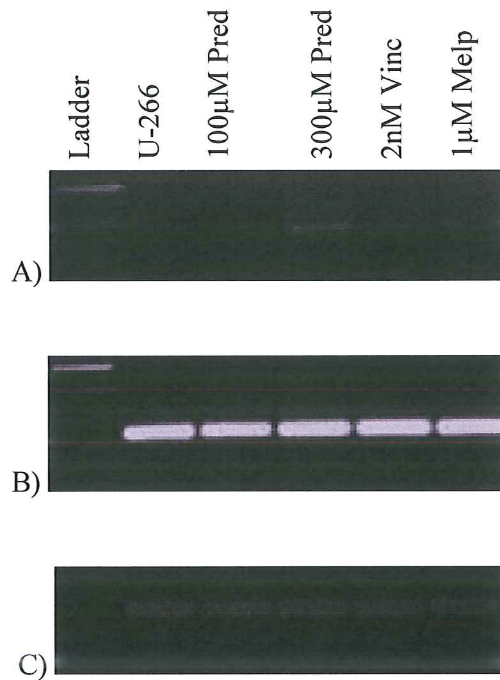


Figure 3.14 Expression levels of *MDR1* (258 bp), *MRP1* (256 bp) and *BCRP* (342 bp) genes in RPMI-8226 sublines (2% w/v agarose gel); Ladder: 50 bp ladder, A) *MDR1*, B) *MRP1*, C) *BCRP* products

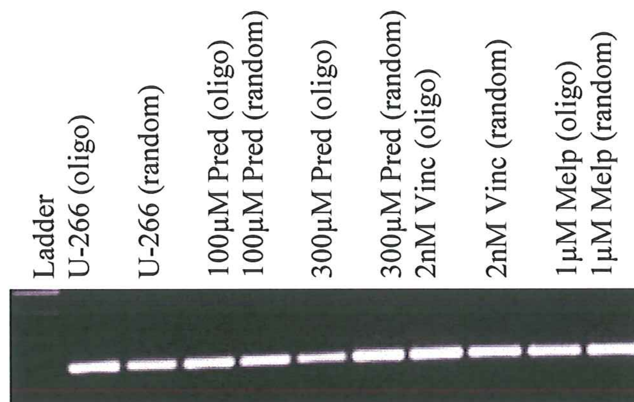


Figure 3.15 Expression levels of *β-actin* gene (209 bp) in U-266 sublines (2% w/v agarose gel); Ladder: 50 bp ladder

3.5 DENSITOMETRIC ANALYSIS OF PCR PRODUCTS ON GELS

In order to quantify the amounts of the PCR products, a software programme of Scion Image was used. The intensity of each band was measured and normalized with its control band (β-actin). The densitometric analyses were done twice and Student t-test was used for statistical analysis.

In Figures 3.16-3.24 the gene expression levels of *MDR1, MRP1, BCRP, LRP, Bcl-2,* $Bcl\text{-}X_L$, *GCS, SK-1* and *LASS-1* genes were shown.

Figure 3.16a and Figure 3.16b shows the expression levels of *MDR1* in RPMI-8226 and U-266 cell lines. *MDR1* expression increased at prednisone (2.6 fold, $p<0.05$), vincristine (31.6 fold, $p<0.05$) and melphalan (2.5 fold, $p<0.05$) resistant cell lines of RPMI-8226. For U-266 cells the expression levels of *MDR1* were also increased 8.5 fold ($p<0.05$) in prednisone, 5 fold ($p<0.05$) in vincristine and 6.5 ($p<0.05$) fold in melphalan resistant sublines respectively.

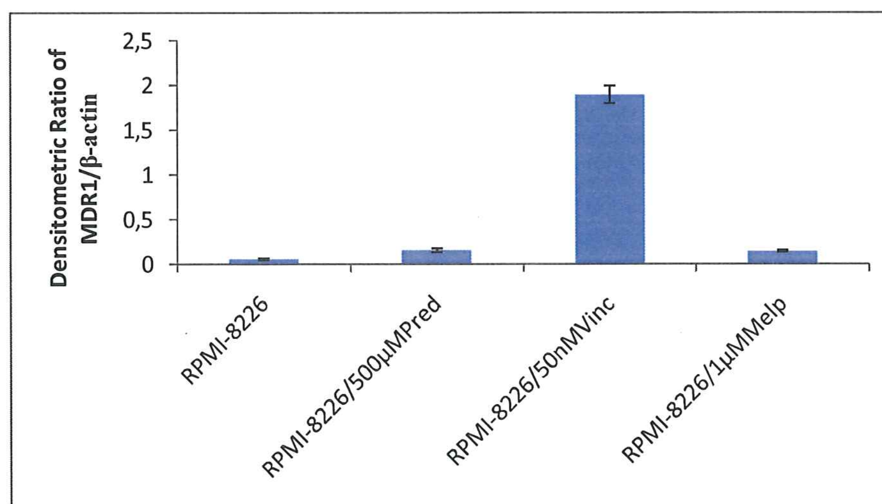


Figure 3.16 a. *MDR1* gene expression levels of RPMI-8226 cell lines

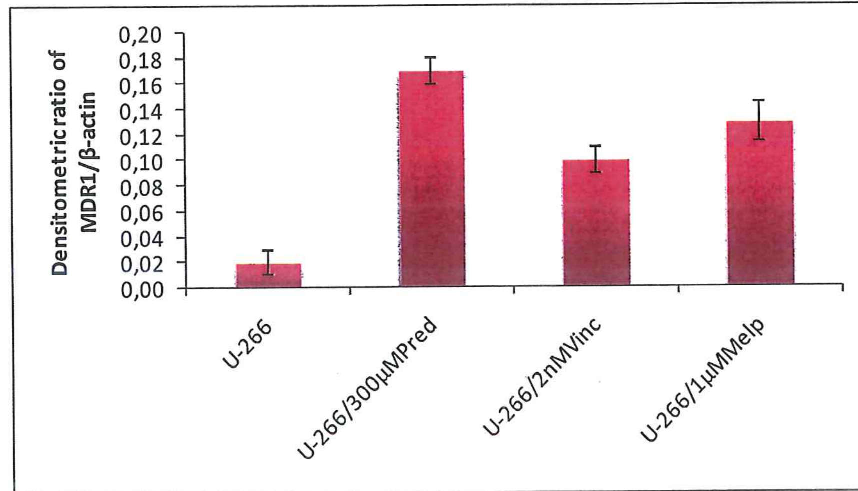


Figure 3.16 b. *MDR1* gene expression levels of U-266 cell lines

As it is seen from Figure 3.17 *MRP1* levels increased at prednisone (9.9 fold, $p<0.05$), vincristine (9.7 fold, $p<0.05$) and melphalan (11 fold, $p<0.05$) resistant RPMI-8226 cell lines. However, gene expression levels did not change significantly at drug applied U-266 cell lines. It can be because of the *MRP1* gene expression level is much higher initially at sensitive U-266 cell line compared to RPMI-8226.

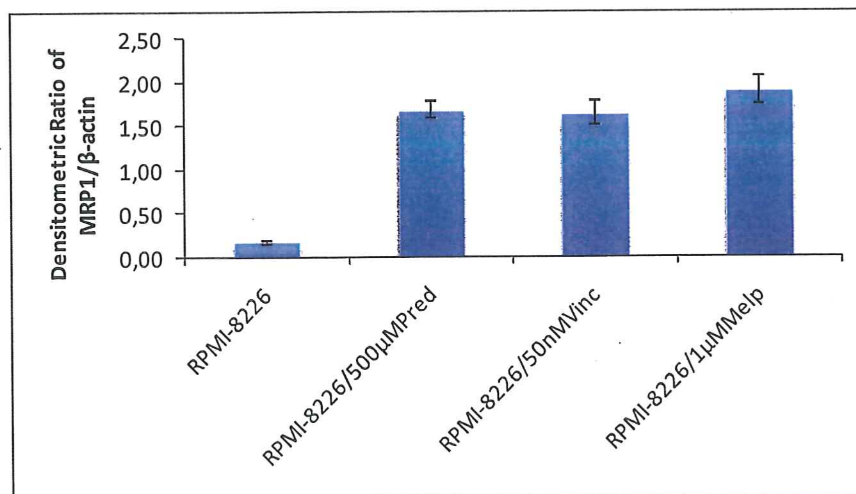


Figure 3.17 a. *MRP1* gene expression levels of RPMI-8226 cell lines

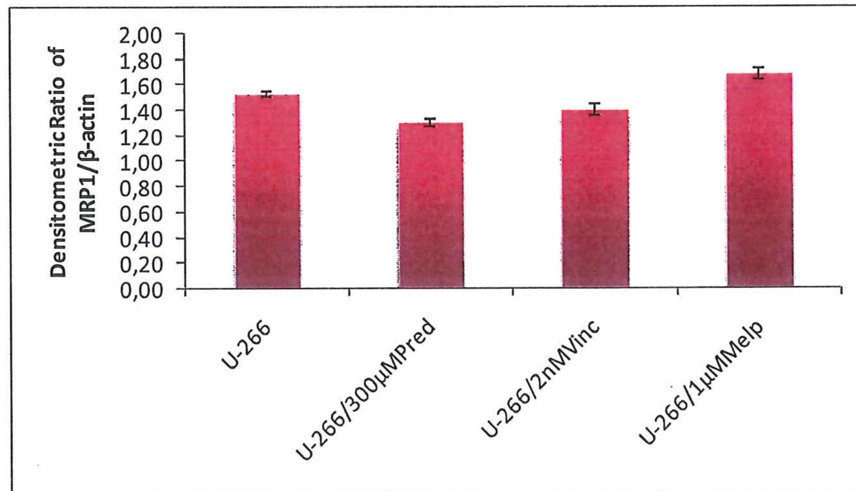


Figure 3.17 b. *MRP1* gene expression levels of U-266 cell lines

BCRP gene expression did not change significantly at non of the resistant sublines of RPMI-8226 and U-266 as it is seen from Figure 3.18.

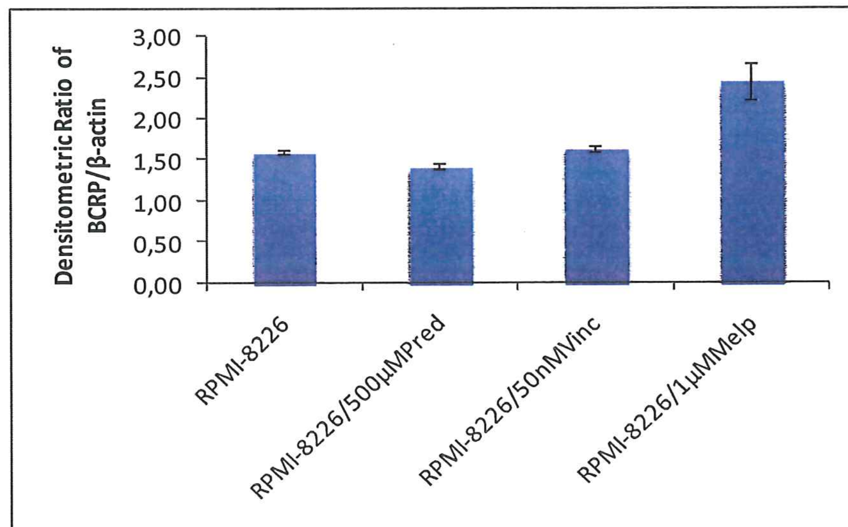


Figure 3.18 a. *BCRP* gene expression levels of RPMI-8226 cell lines

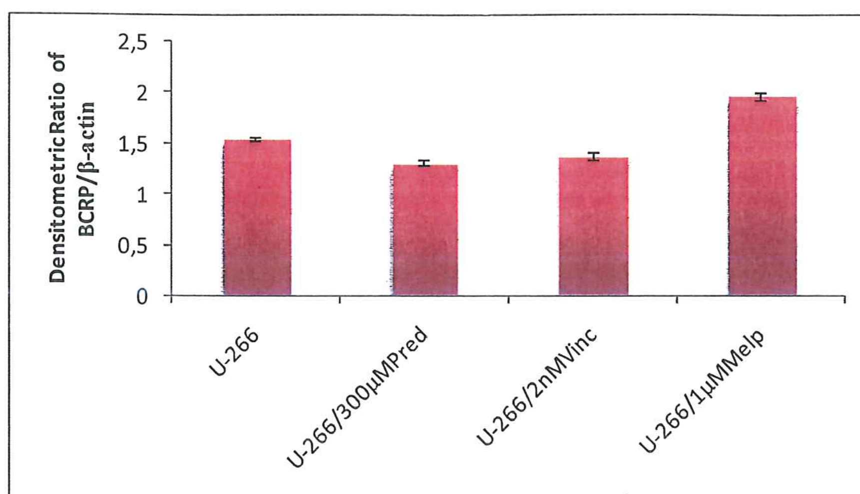


Figure 3.18 b. *BCRP* gene expression levels of U-266 cell lines

Figure 3.19a and 19b indicates *LRP* gene expression levels in the original cells and the sublines. The levels decreased at vincristine 1.4 fold and melphalan 1.6 fold resistant RPMI-8226 cell lines. However these were not statistically significant as in the case of other sublines.

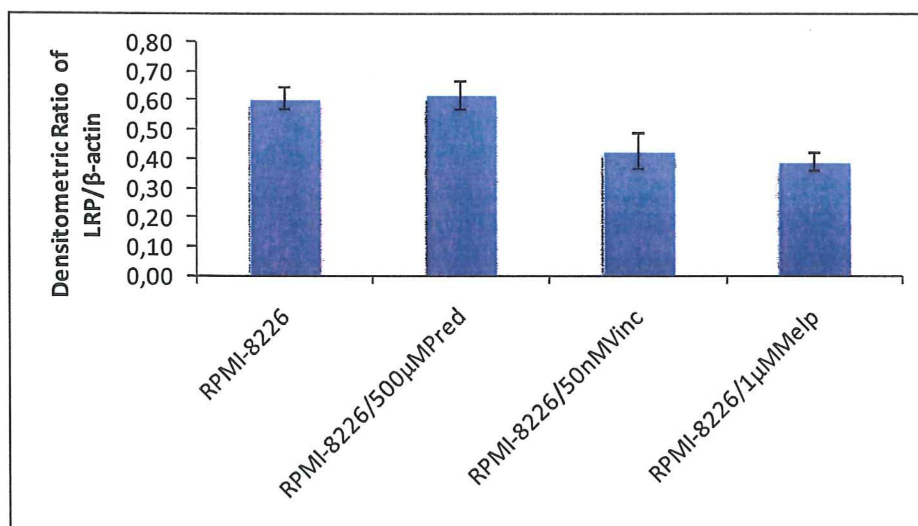


Figure 3.19 a. *LRP* gene expression levels of RPMI-8226 cell lines

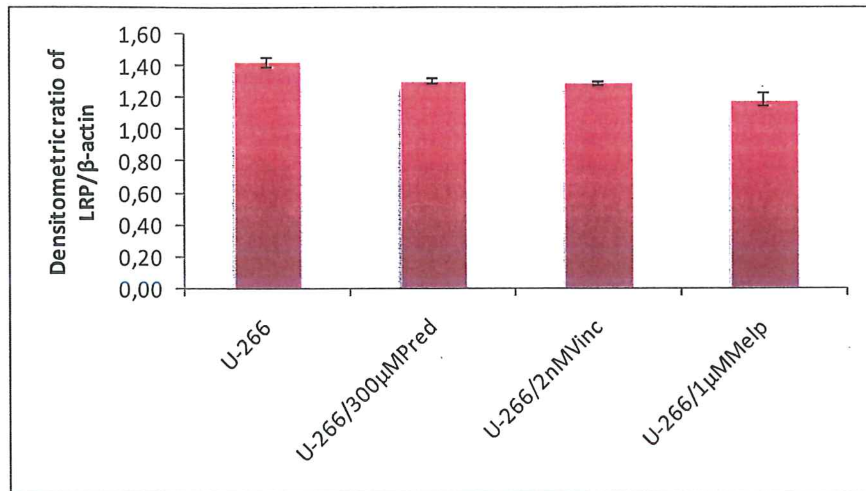


Figure 3.19 b. *LRP* gene expression levels of U-266 cell lines

Bcl-2 gene expression did not chance significantly at vincristine, prednisone and melphalan resistant RPMI-8226 and U-266 cell lines as it is seen from Figure 3.20a.

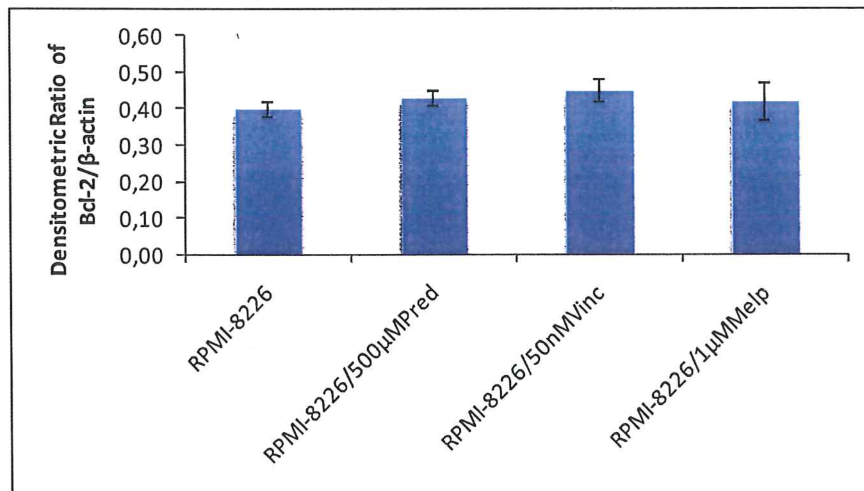


Figure 3.20 a. *Bcl-2* gene expression levels of RPMI-8226 cell lines

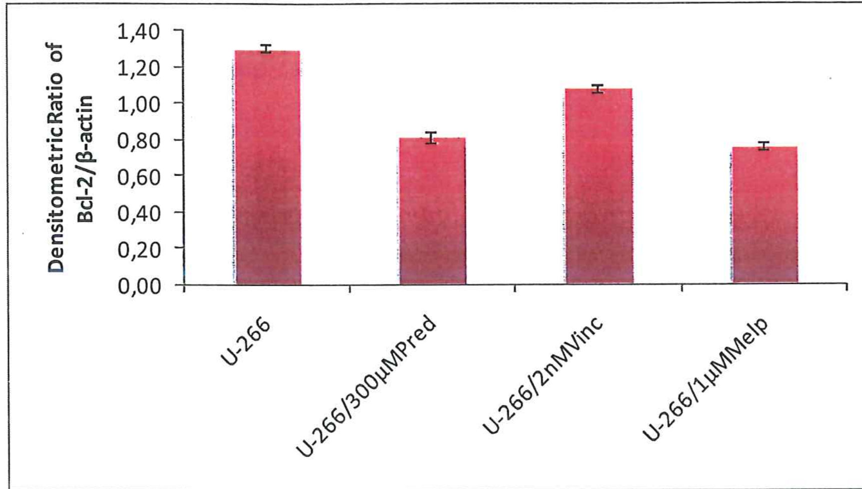


Figure 3.20 b. *Bcl-2* gene expression levels of U-266 cell lines

Antiapoptotic *Bcl-*X_L gene expression did not change significantly at vincristine, prednisone and melphalan applied RPMI-8226 and U-266 cell lines as it is observed from the Figures 3.21a and 3.21b.

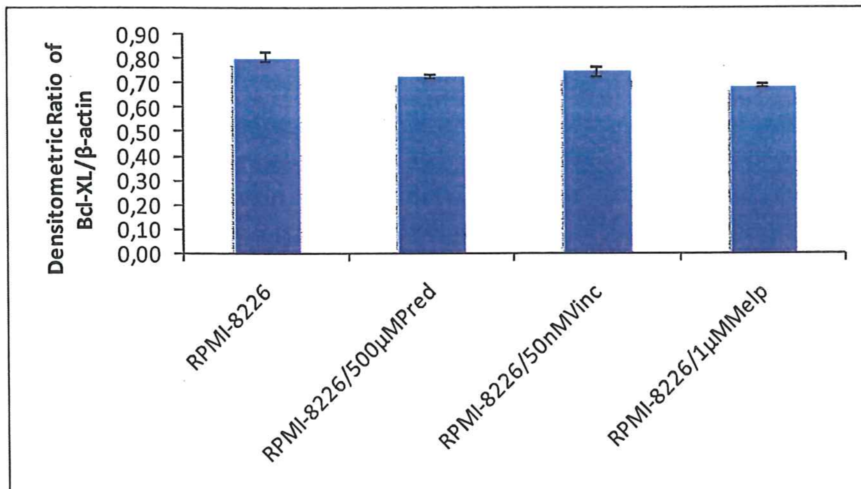


Figure 3.21 a. *Bcl-*X_L gene expression levels of RPMI-8226 cell lines

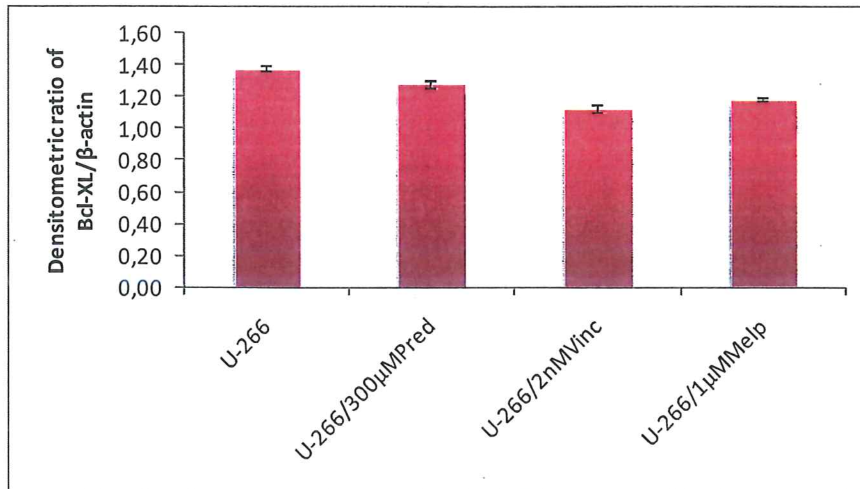


Figure 3.21 b. *Bcl-X_L* gene expression levels of U-266 cell lines

GCS gene expression decreased at vincristine applied RPMI-8226 cell lines (2 fold, $p<0.05$) while increased at melphalan applied RPMI-8226 cell line (2 fold, $p<0.05$). On the other hand it was only decrease at prednisone applied U-266 cell line (2 fold, $p<0.05$) and did not change more at vincristine and melphalan applied ones. The results are seen in Figure 3.22.

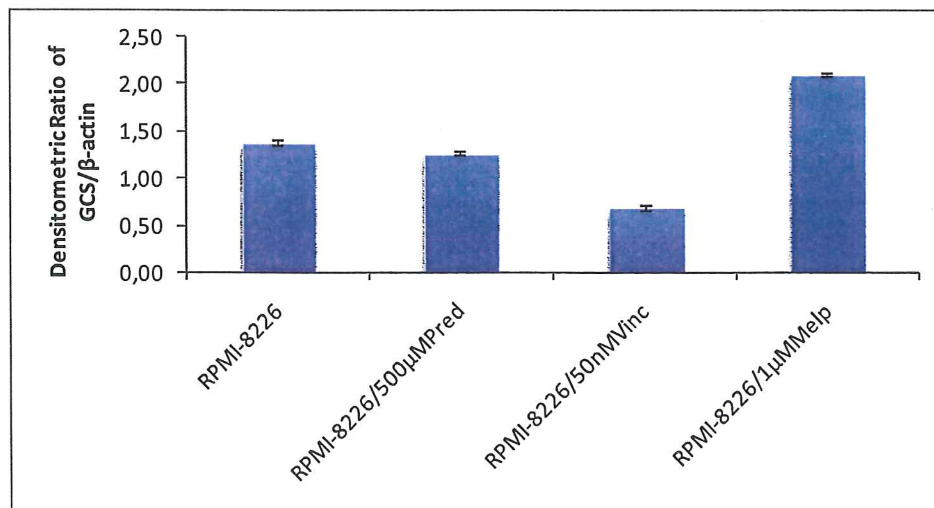


Figure 3.22 a. *GCS* gene expression levels of RPMI-8226 cell lines

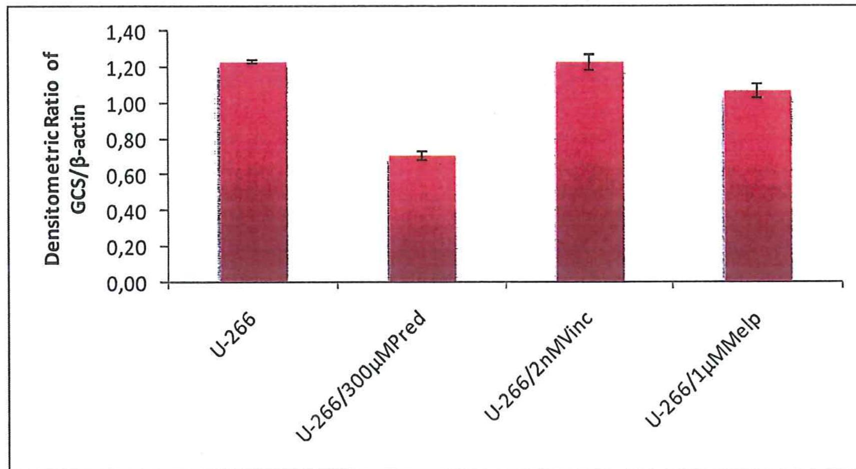


Figure 3.22 b. *GCS* gene expression levels of U-266 cell lines

Figure 3.23 indicates the mRNA expression levels for *SK-1* gene. *SK-1* gene expression levels decreased at prednisone (3 fold, $p<0.05$) and increased at melphalan (2 fold, $p<0.05$) resistant RPMI-8226 cells. The expression levels did not change for the rest.

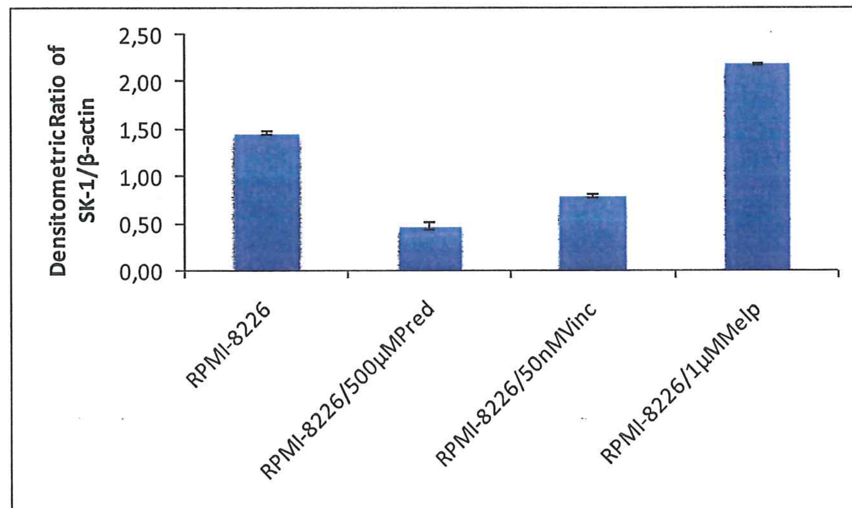


Figure 3.23 a. *SK-1* gene expression levels of RPMI-8226 cell lines

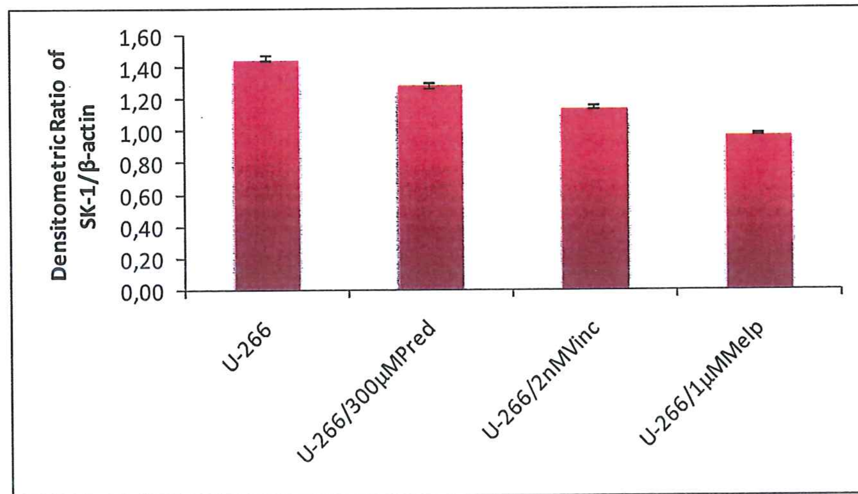


Figure 3.23 b. *SK-1* gene expression levels of U-266 cell lines

LASS-1 gene expression did not change significantly in resistant sublines of RPMI-8226 and U-266 (Figure 3.24a and Figure 3.24b)

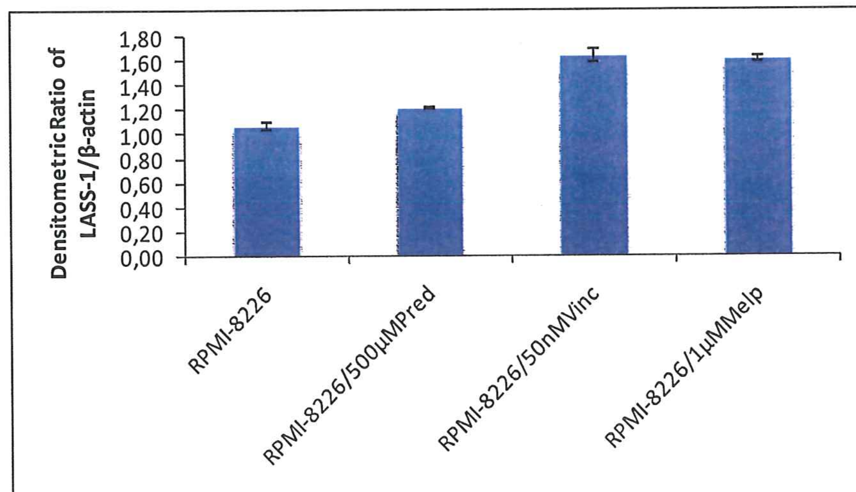


Figure 3.24 a. *LASS-1* gene expression levels of RPMI-8226 cell lines

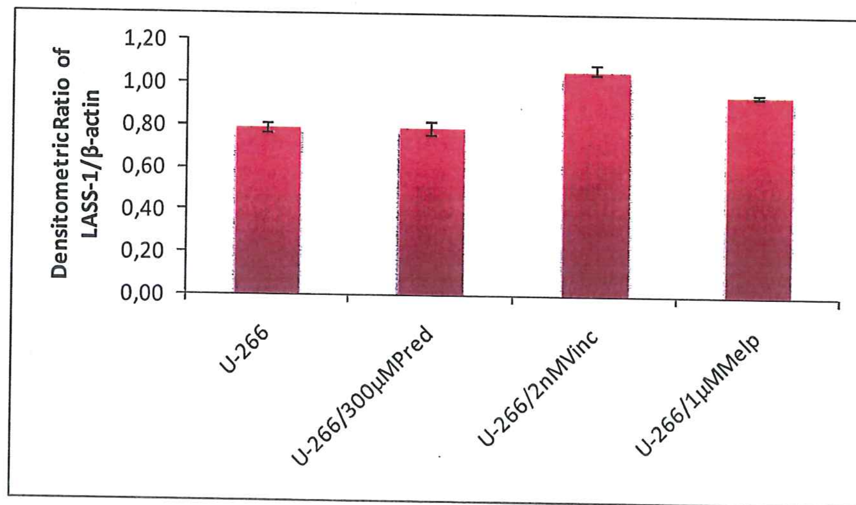


Figure 3.24 b. *LASS-1* gene expression levels of U-266 cell lines

3.6 PROTEIN ANALYSIS

Protein analysis was performed for determination of level of protein encoded by *Bcl-2* gene only in order to make a relation between RT-PCR results with protein level.

Western Blot analysis was optimized for Bcl-2 (50µg/ml) and GAPDH (10µg/ml) proteins. GAPDH was used as control. Figure 3.25 and 3.26 show the results of Western Blot analysis for Bcl-2 and GAPDH respectively. Densitometric analysis was done using Scion Image program. Figures 3.27 shows the shematic representation of the protein levels of Bcl-2 protein which are normalized with GAPDH. The densitometric analyses were done twice and Student t-test was used for statistical analysis.

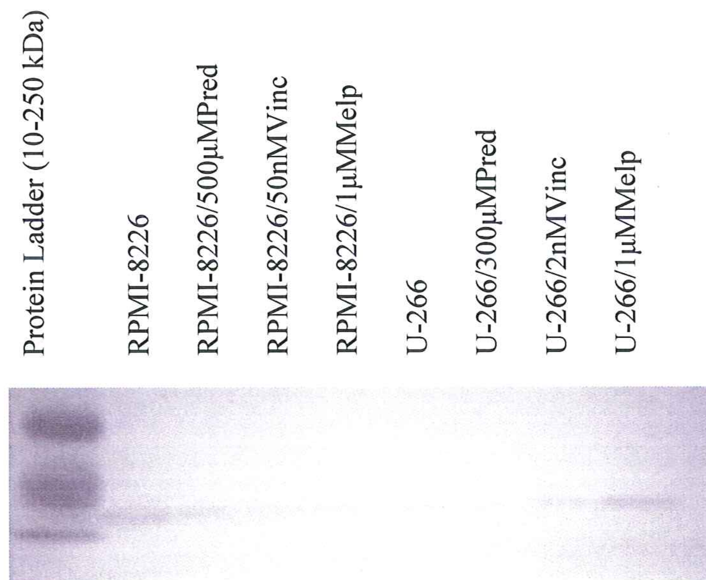


Figure 3.25 Western blot analysis for Bcl-2 protein (25kDa; 50µg/ml).

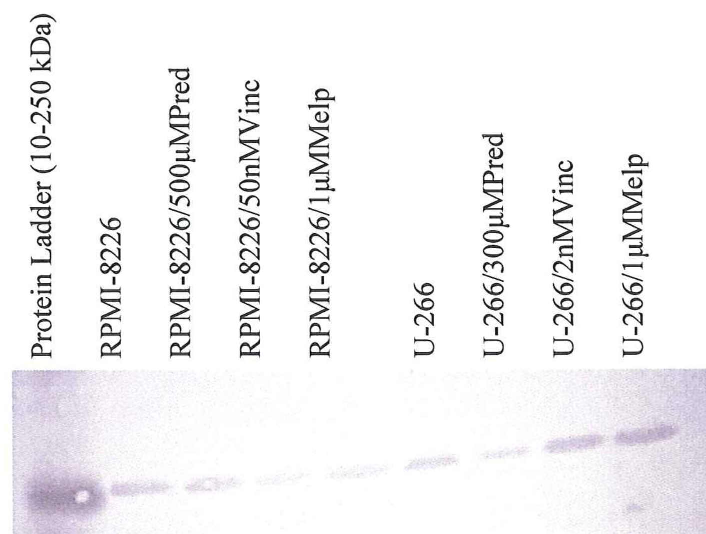


Figure 3.26 Western blot analysis for GAPDH protein (37kDa; 10µg/ml).

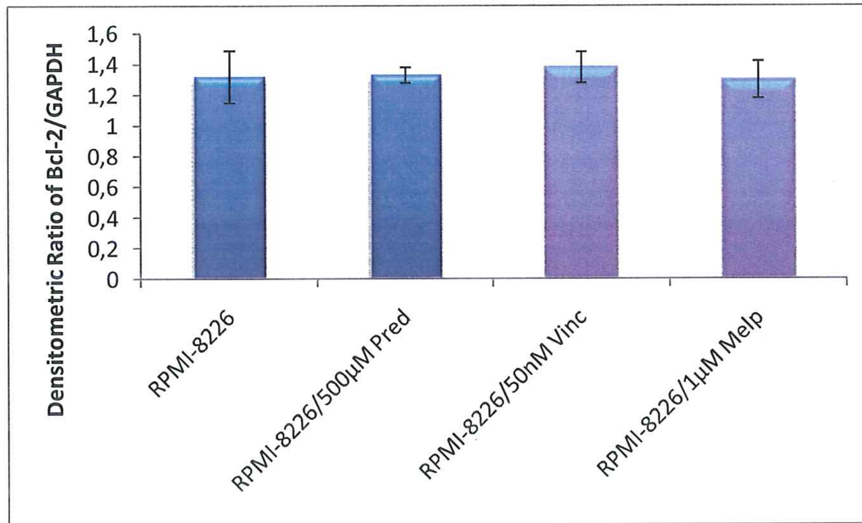


Figure 3.27 a. Bcl-2 protein levels of RPMI-8226 cell lines

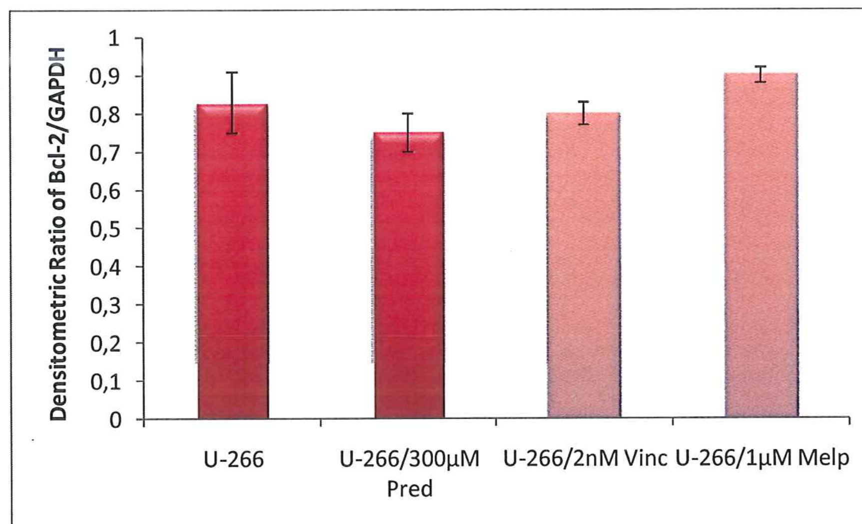


Figure 3.27 b. Bcl-2 protein levels of U-266 cell lines

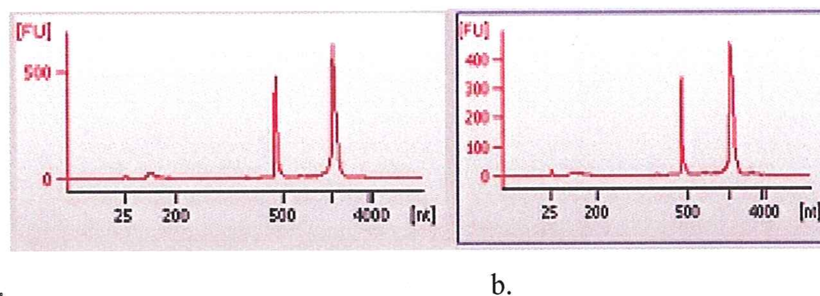
The results of the Western blot analyses confirm gene expression analyses. Bcl-2 protein levels did not change much between drug resistant and original RPMI-8226 and U-266 cell lines.

3.7 MICROARRAY ANALYSIS

3.7.1 RNA Isolation and Target Preparation

For an efficient microarray analysis purity (A_{260}/A_{280} between 1.80-2.00) and concentration (>2500µg/ml) of the RNAs are very important. RNAs having high purity and concentration was isolated from all of the cell lines (each dublicate).

Integrity of total RNA samples were assessed qualitatively on an Agilent 2100 Bioanalyzer. Figure 3.28 shows the electropherogram of the isolated RNAs. For a high-quality total RNA sample, two well-defined peaks corresponding to the 18S and 28S ribosomal RNAs should be observed, with ratios approaching 2:1 for the 28S to 18S bands. Figure 3.29 shows the results of the same samples on the denaturing agarose gel. For both of the samples 18S and 28S bands were seen without smear. Only the electropherogram and gel electrophoresis results of the RNAs that were isolated from RPMI-8226/50nM Vinc (5) and U-266/1µM Melp (15) were given. Similar results were obtained for all of the isolated RNAs.



a. b.

Figure 3.28. Electropherograms for a. RPMI-8226/50nM Vinc b. U-266/1µM Melp total RNA

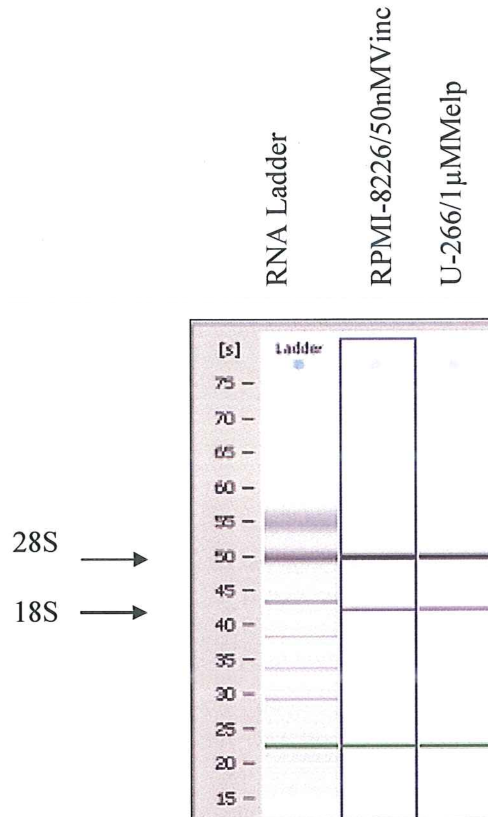


Figure 3.29 Denaturing agarose gel electrophoresis of RNA samples

3.7.2 Normalization

Normalization was done by using RMA method. In the RMA method, maximum non-specific binding data were eliminated with minimum change of data characteristics.

3.7.3 Data Analysis

The gene expression profiles of the drug resistant (prednisone, vincristine, melphalan) RPMI-8226 and U-266 cell lines were compared with their own controls. Dublicate data from same cell line were used for statistical analysis. The statistically significant ($p<0.05$) gene expressions were selected and these genes were discussed in order to find out which drug triggers which pathway. There were approximately 7000 statistically significant ($p<0.05$) different gene expression data between RPMI-8226/500µMPred resistant cell line and its control. This number was 4500 for RPMI-8226/50nMVinc and 3500 for RPMI-8226/1µMMelp resistant cell lines. Expressions of 6000 genes ($p<0.05$) were changed in U-266/300µM Pred resistant subline. This number was 9500 for U-266/2nM Vinc and 3500 for U-266/1µM Melp resistant cell lines. Significant fold change values greater than 2-fold and less than 2-fold were considered and by using KEGG pathway, data grouping which can contribute to drug resistance was performed. The gene expression levels between 2-fold and -2-fold were considered as not significant (NS).

Figure 30-35 demostrate the heatmaps for the genes which expression level changes were greater than 10-fold and less than 10-fold. Expression levels increase from light blue to dark red.

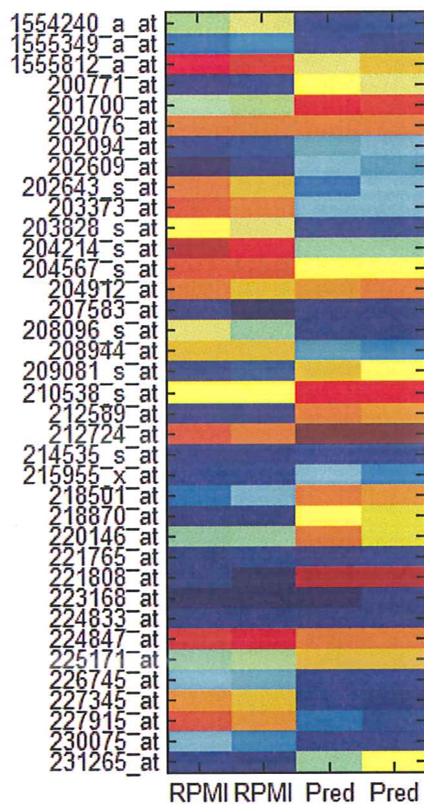


Figure 3.30 Heatmap for original and 500μM Pred resistant RPMI-8226 cell lines (data dublicate)

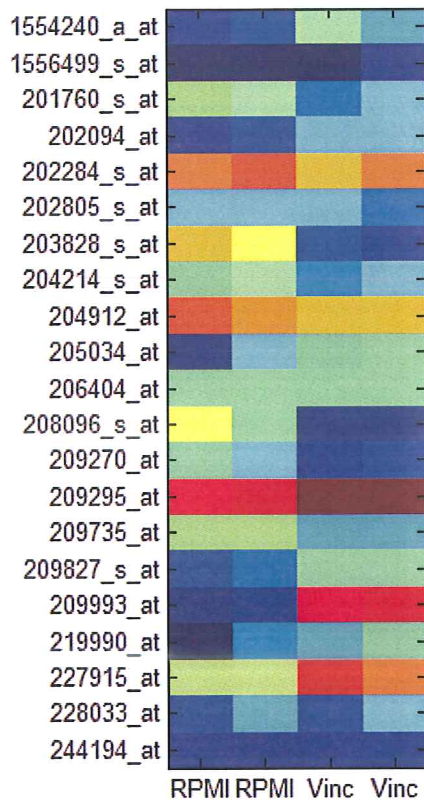


Figure 3.31 Heatmap for original and 50nM Vinc resistant RPMI-8226 cell lines (data dublicate)

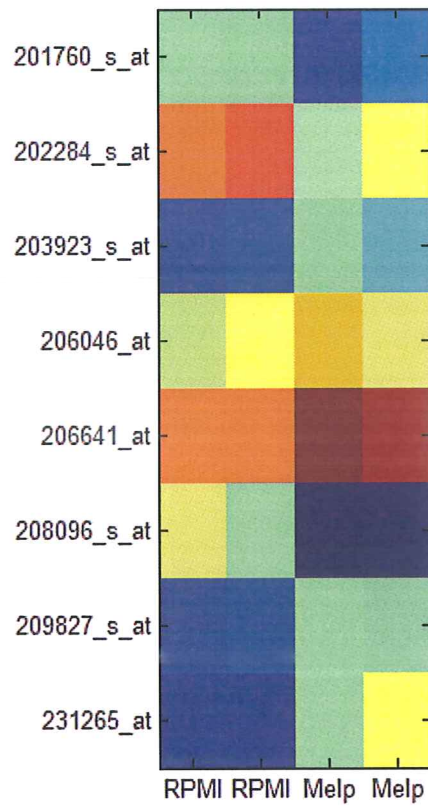


Figure 3.32 Heatmap for original and 1μM Melp resistant RPMI-8226 cell lines (data dublicate)

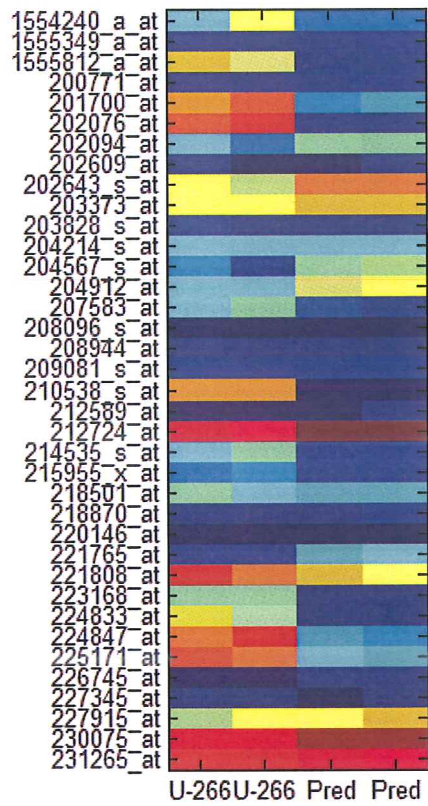


Figure 3.33 Heatmap for original and 300μM Pred resistant U-266 cell lines (data dublicate)

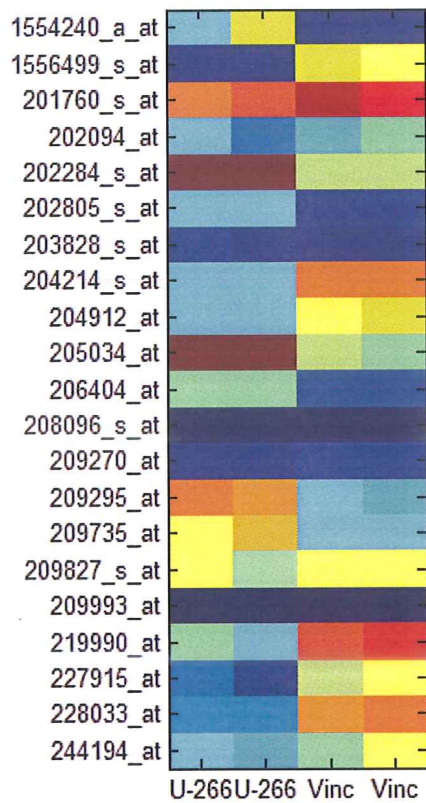


Figure 3.34 Heatmap for original and 2nM Vinc resistant U-266 cell lines (data dublicate)

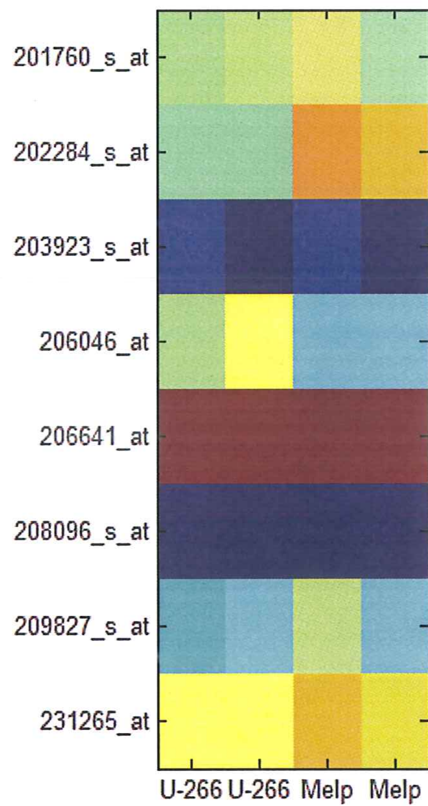


Figure 3.35 Heatmap for original and 1μM Melp resistant U-266 cell lines (data dublicate)

3.7.4 Data Grouping

3.7.4.1 Extracellular Matrix Related Gene Expressions

Alterations in gene expression levels of extracellular matrix related genes were shown in Figure 3.36-3.38. Integrins are cell surface receptors that interact with the extracellular matrix and mediate various intracellular signals. They define cellular shape, mobility and regulate the cell cycle. At least 15 different α subunits and 8 different β subunits of integrins have been identified. Theoretically, over a hundred possible pairings of α and β subunits could occur as heterodimers. Specific integrins (beta 1, alpha 5, alpha v, alpha IIb) were expressed and allowed the cells to bind preferentially to ECM proteins, fibronectin. Cell surface integrin expression (beta 1, alpha 5 and alpha v) on platinum resistant ovarian cancer cell lines were shown in the literature (Jun, 2003). Also beta 1 integrin adhesion was shown to enhance IL-6-mediated STAT3 signaling in myeloma cells (Shain *et al.*, 2009). ICAM-1 which is intercellular adhesion molecule 1 is a ligand for ITGAL (integrin alpha L). When activated, leukocytes bind to endothelial cells via ICAM-1/ITGAL and then transmigrate into tissues (Yang *et al.*, 2005).

Among the genes encoding the integrins, αv integrin (RPMI-8226/500µMPred, U-266/2nMVinc, RPMI-8226/1µMMelp), αIIb integrin (U-266/300µMPred) and β1 integrin (RPMI-8226/50nMVinc and RPMI-8226/1µMMelp) was overexpressed (Table 3.2-3.4). Other fibronectin encoding genes such as FN1, FLRT2 were also overexpressed in RPMI-8226/500µMPred (Table 3.2) and U-266/2nMVinc (Table 3.3) sublines. This shows that except the cell line U-266/1µMMelp, all of the drug resistant MM cell lines show increased interaction with fibronectin. ICAM-1 ligand ITGAL was only overexpressed in vincristine resistant RPMI-8226 cell line (Table 3.3) which can be an indicator to invasiveness.

Another glycoprotein of the ECM is laminin which act in a regulatory capacity, influencing a cell's potential for growth and differentiation. The presence of receptors capable of binding to laminin is correlated with metastatic behavior in human breast cancer and melanoma cells (Benton *et al.*, 2009). LAMC1 was

drastically overexpressed in RPMI-8226/500μMPred subline (145 fold) whereas LAMB1 and LAMA1 were overexpressed in U-266/300μMPred subline (Table 3.2).

Collagen is the main protein of connective tissue in animals and the most abundant protein in mammals. Tough bundles of collagen called collagen fibers are a major component of the extracellular matrix that supports most tissues and gives cells structure from the outside. Collagen is also found inside certain cells. The relation of collagen and invasive behavior of tumor cells was reported previously. In one of the studies, it was shown that there is a significant decrease in the amount of collagen in breast cancer tissue. Alteration in collagen metabolism in breast cancer tissue reflect tissue remodelling, characteristic for invasive phenotype of cancer cells (Cechowska-Pasko *et al.*, 2006). In another study, it was shown that for cancer to metastasize from a primary tumor to distinct sites in the body, physiological barriers whose primary structural constituent is collagen must be degraded to allow the passege of tumor cells (Nerenberg *et al.*, 2007). Also increased penetration of human multidrug-resistant overian carcinoma cells into *in vitro* collagen fibroblasts matrix was reported in another study (Sedlak *et al.*, 1996). Tancred *et al.*, showed that expression levels of fibronectin and collagen I were reduced in MM patients with high-level plasmacytosis (Tancred *et al.*, 2008).

According to our results; different types of collagen encoding genes were upregulated in all of the drug resistant MM cell lines. However, it is interesting that only COL21A1 was drastically downregulated in prednisone, vincristine and melphalan resistant RPMI-8226 cell lines (128-, 157- and 167-fold) (Table 3.2). Type XXI collagen is an extracellular matrix component of the blood vessel walls. Platelet-derived growth factor (PDGF) has a pronounced effect on the stimulation of COL21A1 expression in cultured aortic smooth-muscle cells, suggesting that XXI collagen may contribute to the extracellular matrix assembly of the vascular network during blood vessel formation (Chou *et al.*, 2002). The downregulation of COL21A1 gene in drug resistant RPMI-8226 cell lines can be an indicator of more invasive behavior of RPMI-8226 cells.

The ADAMs (a disintegrin and metalloproteinase) are a fascinating family of transmembrane and secreted proteins with important roles in regulating cell

phenotype via their effects on cell adhesion, migration, proteolysis and signaling. The functional ADAM metalloproteinases are involved in ectodomain shedding of diverse growth factors, cytokines, receptors and adhesion molecules (Edwards *et al.*, 2008).

The archetypal activity is shown by ADAM-17 (tumor necrosis factor-α convertase, TACE), which is the principle protease involved in the activation of pro-TNF-α, but whose sheddase functions cover a broad range of cell surface molecules. In particular, ADAM-17 is required for generation of the active forms of epidermal growth factor receptor (EGFR) ligands, and its function is essential for the development of epithelial tissues. Another major family member, ADAM-10, is a principal player in signaling via the Notch and Eph/ephrin pathways. For a growing number of substrates, foremost among them being Notch, cleavage by ADAM sheddases is essential for their subsequent "regulated intramembrane proteolysis" (RIP), which generates cleaved intracellular domains that translocate to the nucleus and regulate gene transcription (Edwards *et al.*, 2008).

Different types of genes which belong to ADAMs family were downregulated in prednisone and vincristine resistant RPMI-8226 and U-266 cell lines. However, ADAM17 was upregulated in RPMI-8226/1μMMelp and ADAM10 and ADAM23 were upregulated in U-266/1μMMelp cell lines (Table 3.4). Also LICAM was upregulated in U-266/1μMMelp cell line (Table 3.4). LICAM is a substrate for ADAM10 and their relation was reported in many reports. In one of the reports it was shown that ectodomain shedding of L1 adhesion molecule promotes cell migration by autocrine binding to integrins (Mechtersheimer *et al.*, 2001). In another study, it was shown that expression of L1-CAM and ADAM10 in human colon cancer cells induces metastasis (Gavert *et al.*, 2007). Upregulation of both ADAM10 and L1CAM genes in U-266/1μMMelp cell line can be an indicator of invasiveness.

Figure 3.36 Expression levels of extracellular matrix related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines

Table 3.2 Expression levels of extracellular matrix related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines with respect to control

Gene Name	**Gene Symbol**	**Description**	**RPMI-8226/500µMPred Fold Change**	**U-266/300µMPred Fold Change**
1554240_a_at	ITGAL	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	-95.2	-3.58
1555349_a_at	ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	-62.2	-2.44
202351_at	ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	+2.80	NS
200771_at	LAMC1	laminin, gamma 1 (formerly LAMB2)	+145.3	NS
201505_at	LAMB1	laminin, beta 1	NS	+2.15
227048_at	LAMA1	laminin, alpha 1	NS	+2.10
211719_x_at	FN1	fibronectin 1	+5.8	+2.05
204358_s_at	FLRT2	fibronectin leucine rich transmembrane protein 2	NS	+2.12
208096_s_at	COL21A1	collagen, type XXI, alpha 1	-164.1	NS
209081_s_at	COL18A1	collagen, type XVIII, alpha 1	+128.9	NS

Table 3.2 continued

221900_at	COL8A2	collagen, type VIII, alpha 2	+2.14	NS
234387_at	COL4A5	collagen, type IV, alpha 5 (Alport syndrome)	+2.11	NS
225292_at	COL27A1	collagen, type XXVII, alpha 1	NS	+2.49
204345_at	COL16A1	collagen, type XVI, alpha 1	NS	+2.36
204724_s_at	COL9A3	collagen, type IX, alpha 3	NS	+3.18
211809_x_at	COL13A1	collagen, type XIII, alpha 1	NS	+2.09
211239_s_at	ADAM7	ADAM metallopeptidase domain 7	-2.09	NS
207422_at	ADAM20	ADAM metallopeptidase domain 20	NS	-2.23
214535_s_at	ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	NS	-22.62
217007_s_at	ADAM15	ADAM metallopeptidase domain 15 (metargidin)	NS	-2.15

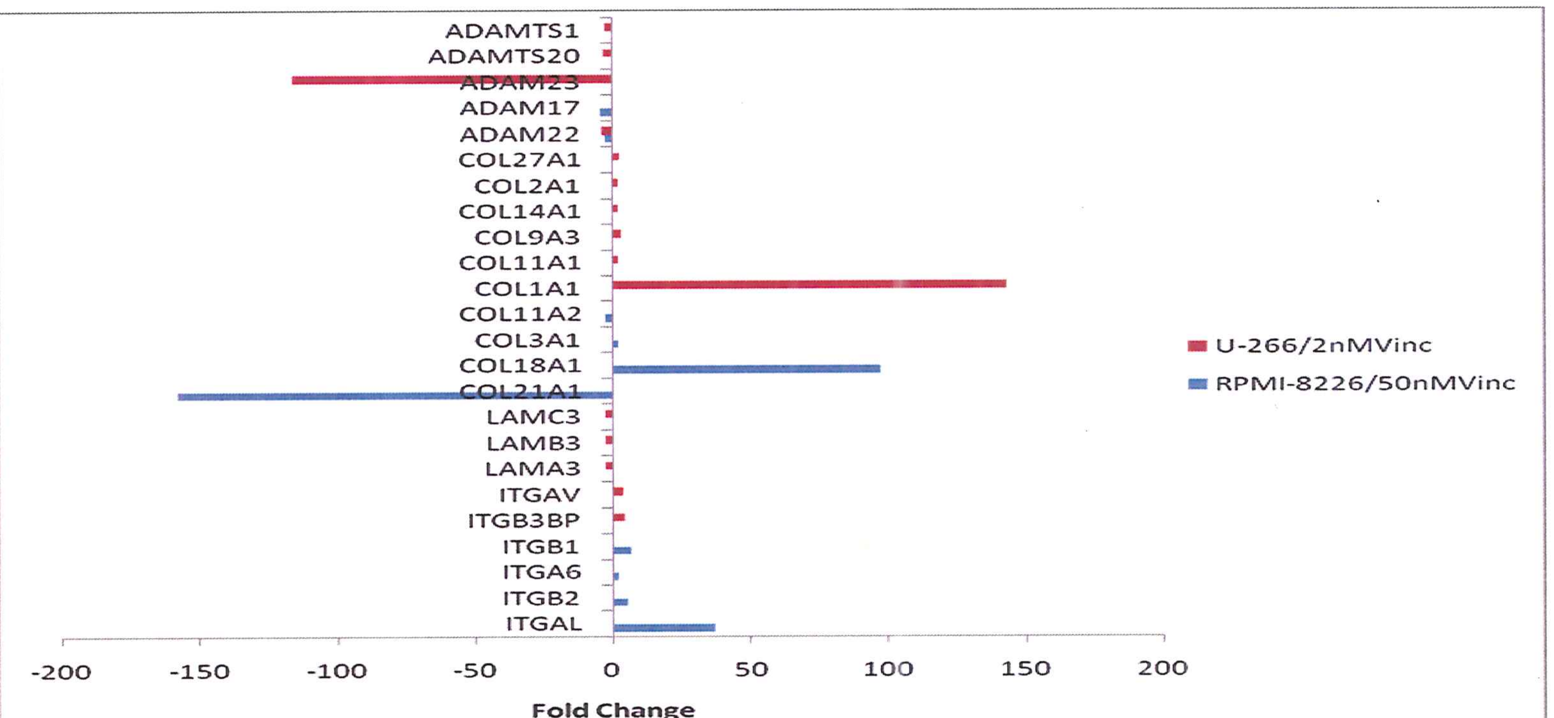


Figure 3.37 Expression levels of extracellular matrix related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines

Table 3.3 Expression levels of extracellular matrix related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/50nMVinc Fold Change	U-266/2nMVinc Fold Change
1554240_a_at	ITGAL	Integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	+37.53	NS
202803_s_at	ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	+5.93	NS
201656_at	ITGA6	integrin, alpha 6	+2.07	NS
211945_s_at	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	+6.63	NS
205176_s_at	ITGB3BP	integrin beta 3 binding protein (beta3-endonexin)	NS	+4.69
202351_at	ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	NS	+3.94

Tabl e 3.3 continued

1560078_at	LAMA3	laminin, alpha 3	NS	-2.25
209270_at	LAMB3	laminin, beta 3	NS	-2.44
232558_at	LAMC3	laminin, gamma 3	NS	-2.14
208096_s_at	COL21A1	collagen, type XXI, alpha 1	-157.4	NS
209082_s_at	COL18A1	collagen, type XVIII, alpha 1	+97.3	NS
215076_s_at	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	+2.34	NS
216993_s_at	COL11A2	collagen, type XI, alpha 2	-2.25	NS
1556499_s_at	COL1A1	collagen, type I, alpha 1	NS	+143.1
204320_at	COL11A1	collagen, type XI, alpha 1	NS	+2.09
204724_s_at	COL9A3	collagen, type IX, alpha 3	NS	+3.43
212865_s_at	COL14A1	collagen, type XIV, alpha 1 (undulin)	NS	+2.18
213492_at	COL2A1	collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)	NS	+2.01
225288_at	COL27A1	collagen, type XXVII, alpha 1	NS	+2.90

Table 3.3 continued

244194_at	ADAM22	ADAM metallopeptidase domain 22	-2.26	-3.45
213532_at	ADAM17	ADAM metallopeptidase domain 17 (tumor necrosis factor, alpha, converting enzyme)	-3.78	NS
244463_at	ADAM23	ADAM metallopeptidase domain 23	NS	-116
220717_at	ADAMTS20	ADAM metallopeptidase with thrombospondin type 1 motif, 20	NS	-2.94
222162_s_at	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	NS	-2.11
237411_at	ADAMTS6	ADAM metallopeptidase with thrombospondin type 1 motif, 6	NS	-2.04

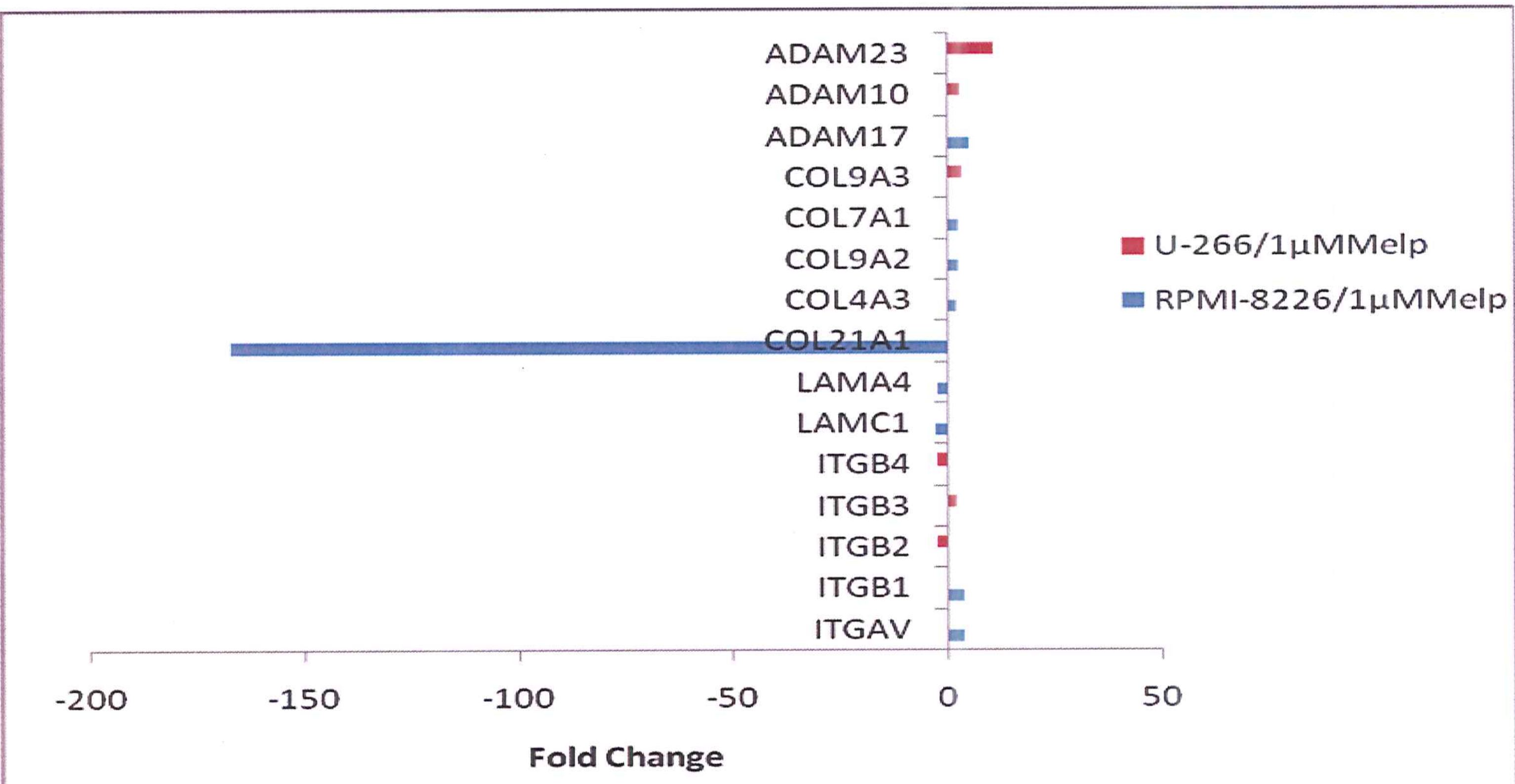


Figure 3.38 Expression levels of extracellular matrix related genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines

Table 3.4 Expression levels of extracellular matrix related genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/1μMMelp Fold Change	U-266/1μMMelp Fold Change
202351_at	ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	+3.75	NS
211945_s_at	ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	+3.83	NS
1555349_a_at	ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	NS	-2.37
204626_s_at	ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	NS	+2.25
204990_s_at	ITGB4	integrin, beta 4	NS	-2.09
200770_s_at	LAMC1	laminin, gamma 1 (formerly LAMB2)	-2.60	NS
210990_s_at	LAMA4	laminin, alpha 4	-2.25	NS

Table 3.4 continued

208096_s_at	COL21A1	collagen, type XXI, alpha 1	-167	NS
216893_s_at	COL4A3	collagen, type IV, alpha 3 (Goodpasture antigen)	+2.12	NS
232542_at	COL9A2	collagen, type IX, alpha 2	+2.49	NS
204136_at	COL7A1	collagen, type VII, alpha 1 (epidermolysis bullosa, dystrophic, dominant and recessive)	+2.34	NS
204724_s_at	COL9A3	collagen, type IX, alpha 3	NS	+3.45
213532_at	ADAM17	ADAM metallopeptidase domain 17 (tumor necrosis factor, alpha, converting enzyme)	+5,27	NS
202603_at	ADAM10	ADAM metallopeptidase domain 10	NS	+2.80
206046_at	ADAM23	ADAM metallopeptidase domain 23	NS	+11
204585_s_at	L1CAM	L1 cell adhesion molecule	NS	+2.15

3.7.4.2 Oncogene Related Gene Expressions

Changes in the expression levels of the oncogenes were shown schematically in Figures 3.39-3.41. The Ras family, comprised of H-ras, N-ras and K-ras are critical components of many of the transduction pathways which are essential components of cell viability (Rowley *et al.*, 2002). Activated ras genes have been identified in several human cancers, including both myeloid and lymphoid malignancies. Ras mutations have been shown to protect myeloma cell lines from apoptosis induced by dexamethasone, doxorubicin or melphalan (Rowley *et al.*,2002). The Ras superfamily of small GTPases includes the Ras, Rho, Arf, Rab and Ran families. Ras-regulated signal pathways control such processes as actin cytoskeletal integrity, proliferation, differentiation, cell adhesion, apoptosis and cell migration. Ras and ras-related proteins are often deregulated in cancers, leading to increased invasion and metastasis, and decreased apoptosis. Ras activates a number of pathways but an especially important one seems to be the mitogen-activated protein (MAP) kinases, which themselves transmit signals downstream to other protein kinases and gene regulatory proteins (Lodish *et al.*, 2000).

According to Table 3.5 prednisone resistant MM cell lines the oncogenes which belong to Ras superfamily were upregulated, especially Rho family of GTPases. Rho proteins are involved in a wide variety of cellular functions such as cell polarity, vesicular trafficking, the cell cycle and transcriptional dynamics (Ellenbroek *et al.*, 2007). Especially in prednisone resistant U-266 cell line Rho GTPase activating proteins such as ARHGAP18 (67 fold) and ARHGAP25 (9 fold) were upregulated while RhoGDP dissociation inhibitor ARHGDIB was downregulated (212 fold) (Table 3.5). It is important that RPMI-8226/500µMPred cell line was drastically overexpressed RAB9 gene (870 fold) (Table 3.5). In one of the studies it was shown that when recombinant VP22-Rab9 was transduced into NPC1 fibroblasts, nearly all cells showed significant reduction in cellular free cholesterol levels (Narita, 2005). Prednisone structure is similar to cholesterol and overexpression of RAB9 can be important for the reduction in cellular level of prednisone in RPMI-8226 cells and can be related to prednisone resistance.

ETS family is one of the largest families of transcription factors. The ETS family is present throughout the body and is involved in a wide variety of functions including the regulation of cellular differentiation, cell cycle control, cell migration, cell proliferation, apoptosis and angiogenesis. ETS2 was 10 fold and ETS1 was 162 fold downregulated in RPMI-8226/500μMPred and U-266/300μMPred cell lines respectively (Table 3.5).

NF-κB is a protein complex that acts as a transcription factor. NF-κB family members share structural homology with the retroviral oncogene v-Rel, resulting in their classification as NF-κB/Rel proteins. There are five proteins in the mammalian NF-κB family. These are grouped as Class I (NF-κB1 and NF-κB2) and Class II (RelA, RelB and c-Rel) (Nabel *et al*, 1993).

In tumor cells, NF-κB is active either due to mutations in genes encoding the NF-κB transcription factors themselves or in genes that control NF-κB activity (such as IκB genes). In addition, some tumor cells secrete factors that cause NF-κB to became active. Blocking NF-κB can cause tumor cells to stop proliferating, to die, or to become more sensitive to the action of anti-tumor agents. Thus NF-κB is the subject of much active research among pharmaceutical companies as a target for anti-cancer therapy (Escarcega *et al.*, 2007).

According to Table 3.5 NF-κB2 was downregulated in both of the prednisone resistant MM cell lines while its inhibitors NFKBIB and NKIRAS1 was upregulated. This result is meaningful since prednisone is an inhibitor of NF-κB. However, in RPMI-8226/500μMPred subline RELA and RELB were much more upregulated with respect to NFκB2 which they may overcome the effect of NFκB2. On the other hand also RELA and RELB genes were downregulated in U-266/300μMPred subline (Table 3.5). This result can be an explanation of why RPMI-8226 cell line became more resistant to prednisone with respect to U-266.

The ubiquitin-specific proteases (Ubps) are a family of largely dissimilar enzymes. Unp is tumorigenic when overexpressed in mice, leading to the suggestion that Unp play a role in the regulation of ubiquitin-dependent protein degradation as yet

undescribed substrates (Gilchrist *et al.*, 2000). USP4 and USP6 were upregulated in only vincristine resistant MM sublines (Table 3.6).

The Ras superfamily gene expressions seems not to be correlated with vincristine resistance. Since most of the Ras related genes were either downregulated or not much more upregulated as in prednisone resistant sublines of MM. However NFκB and RELA genes were upregulated in both of the vincristine resistant MM sublines (Table 3.6).

ETS1 and ETS2 genes were both downregulated in vincristine resistant RPMI-8226 subline but not in U-266/2nMVinc (Table 3.6). On the other hand YES1 and FYN genes were upregulated in vincristine resistant U-266 subline (Table 3.6). FYN oncogene related to SRC, FGR, YES is a human gene. This gene is a member of the protein-tyrosine kinase oncogene family. FYN has been shown for the signaling pathways such as T and B cell receptor signaling, integrin-mediated signaling growth factor and cytokine receptor signaling, platelet activation, ion channel function, cell adhesion, axon guidance, fertilization, entry into mitosis (Zamoyska *et al.*, 2003; Palacios *et al.*, 2004).

According to Table 3.7 similar to the case of vincristine the Ras superfamily gene expressions seems not to be correlated with melphalan resistance. On the other hand RELA and RELB genes were downregulated in RPMI-8226/1μMMelp subline whereas they were upregulated in U-266/1μMMelp (Table 3.7). NFκB genes were both upregulated in melphalan resistant MM cell lines (Table 3.7).

YES1 and ACT2 genes were both upregulated in both of the melphalan resistant MM cell lines which can be related to melphalan resistane (Table 3.7). In humans, there are three genes in the "Akt family"; Akt1, Akt2, Akt3. These genes code for enzymes that are members of the serine/threonine specific protein kinase family. Akt regulates cellular survival and metabolism by binding and regulating many downstream effectors, e.g. NFκB, Bcl-2 family proteins (Song *et al.*, 2005). Akt is also known to play a role in the cell cycle. Under various circumstances, activation of Akt was shown to overcome cell cycle arrest in G1 and G2 phases (Ramaswamy *et al.*, 1999; Kandel *et al.*, 2002). Akt2 is required for the insulin-induced translocation of glucose

transport 4 (GLUT4) to the plasma membrane. Glycogen synthase kinase 3 (GSK-3) could be inhibited upon phosphorylation by Akt, which results in promotion of glycogen synthesis.

TPR (translocated promoter region), encodes a large coiled-coil protein that forms intranuclear filaments attached to the inner surface of nuclear pore complexes (NPCs). The protein directly interacts with several components of the NPC. It is required for the nuclear export of mRNAs and some proteins (Hansson *et al.*, 2005). The gene was only 5 fold upregulated in U-266/1µMMelp subline (Table 3.7). Although it was not upregulated in RPMI-8226/1µMMelp cell line still it can be related to melphalan resistance since melphalan is carried into nucleus and interacts with nuclear pore complexes (Table 3.7).

Toll-like receptor family members play fundamental role in pathogen recognition and activation of innate immunity. They recognize pathogen associated molecular patterns that are expressed an infectious agents and mediate the production of cytokines necessary for the development of effective immunity. TLR 1/2/4/5/7/9 activates NFκB. Active NFκB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis.

The TLR pathways, which play a critical role in tissue repair are also key regulators in cancer progression as well as chemoresistance. TLRs serve as cell surface sensors that can initiate pathways leading to proliferation and chemoresistance; as well as mediators that are able to regulate the infiltrating immune cells to provide further support for cancer progression (Chen *et al.*, 2008). The reation between toll-like receptors and proliferation and survival of multiple myeloma cells was shown. TLR-specific ligands induce increased proliferation and survival of the MM cells, partially due to an autocrine interleukin-6 production (Bohnhorst *et al.*, 2006).

TLR-4 signaling was shown to promote tumor growth and paclitaxel chemoresistance in overian cancer (Kelly *et al.*, 2006). Down-regulated expression of TLR-7 mRNA was found to be more sensitive chronic lymphocytic leukemia cells to cytotoxic chemotherapeutic agents in another study (Shi *et al.*, 2007). Mature B-cell

neoplasias such as Burkitt lymphoma, follicular lymphoma and multiple myeloma was shown to express TLR9 and/or TLR10, whereas pre-B cell lines were negative (Bourke *et al.*, 2003).

According to our results in Tables 3.5-3.7 different types of toll-like receptors (TLR 2-4-7 and10) were overexpressed in drug resistant MM cell lines except U-266/300μMPred and RPMI-8226/50nMVinc. However, only in prednisone resistant MM cell lines NFκB expressions were downregulated. This result is meaningful since prednisone is an inhibitor of NFκB. For melphalan resistant MM cell lines, in both of them, both toll-like receptor and NFκB expressions were upregulated. This can be an indicator of melphalan resistance in multiple myeloma.

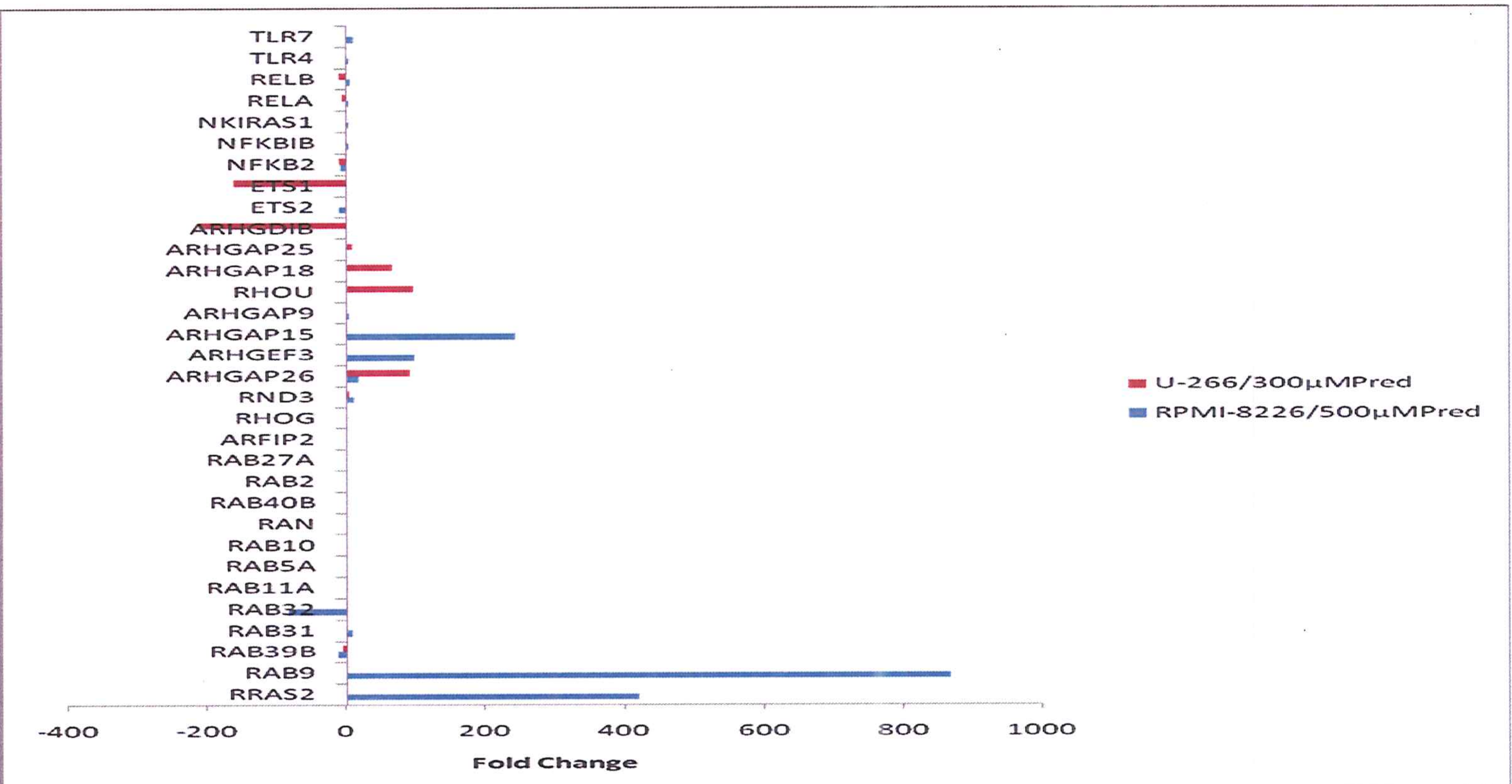


Figure 3.39 Expression levels of oncogene related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines

Table 3.5 Expression levels of oncogene related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/500µMPred Fold change	U-266/300µMPred Fold Change
212589_at	RRAS2	related RAS viral (r-ras) oncogene homolog 2	+420	NS
221808_at	RAB9	RAB9, member RAS oncogene family	+870	NS
230075_at	RAB39B	RAB39B, member RAS oncogene family	-11.31	-4.08
217762_s_at	RAB31	RAB31, member RAS oncogene family	+8.33	+3.29
204214_s_at	RAB32	RAB32, member RAS oncogene family	-83.9	NS
200863_s_at	RAB11A	RAB11A, member RAS oncogene family	+2.37	+2.20
209089_at	RAB5A	RAB5A, member RAS oncogene family	+2.47	NS
222980_at	RAB10	RAB10, member RAS oncogene family	+2.37	
200749_at	RAN	RAN, member RAS oncogene family	NS	+2.88
204547_at	RAB40B	RAB40B, member RAS oncogene family	NS	+3.01

Table 3.5 continued

208731_at	RAB2	RAB2, member RAS oncogene family	NS	+2.54
222294_s_at	RAB27A	RAB27A, member RAS oncogene family	NS	+3.30
202109_at	ARFIP2	ADP-ribosylation factor interacting protein 2 (arfaptin 2)	+3.03	+2.28
203175_at	RHOG	ras homolog gene family, member G (rho G)	+2.36	+3.18
212724_at	RND3	Rho family GTPase 3	+11.63	+4.37
215955_x_at	ARHGAP26	Rho GTPase activating protein 26	+18.89	+91.4
218501_at	ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3	+98.6	NS
218870_at	ARHGAP15	Rho GTPase activating protein 15	+242.6	NS
224451_x_at	ARHGAP9	Rho GTPase activating protein 9	+4.03	NS
223168_at	RHOU	ras homolog gene family, member U	NS	+95.9
225171_at	ARHGAP18	Rho GTPase activating protein 18	NS	+66.8
38149_at	ARHGAP25	Rho GTPase activating protein 25	NS	+8.63

Table 3.5 continued

1555812_a_at	ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	NS	-212
201328_at	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	-9.64	NS
224833_at	ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	NS	-162
209636_at	NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	-5.89	-8.16
214062_x_at	NFKBIB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	+3.60	+3.11
225930_at	NKIRAS1	NFKB inhibitor interacting Ras-like 1	+4.59	+3.11

Table 3.5 continued

201783_s_at	RELA	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	+4.46	-4.72
205205_at	RELB	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	+6.14	-8.69
224341_x_at	TLR4	toll-like receptor 4	+3.53	NS
220146_at	TLR7	toll-like receptor 7	+10.77	NS

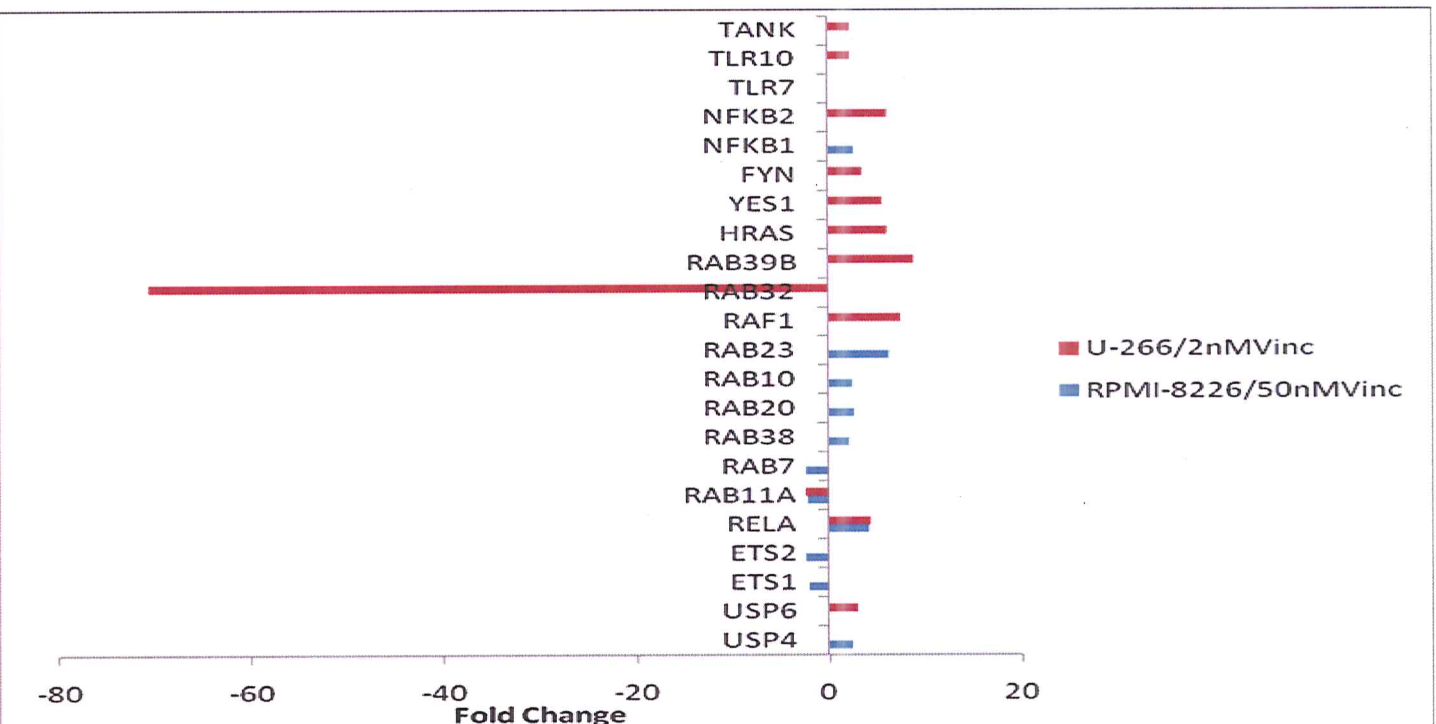


Figure 3.40 Expression levels of oncogene related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines

Table 3.6 Expression levels of oncogene related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/50nMVinc Fold Change	U-266/2nMVinc Fold Change
202682_s_at	USP4	ubiquitin specific peptidase 4 (proto-oncogene)	+2.42	NS
206405_x_at	USP6	ubiquitin specific peptidase 6 (Tre-2 oncogene)	NS	+2.86
1555355_a_at	ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	-2.08	NS
201329_s_at	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	-2.37	NS
201783_s_at	RELA	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	+4.22	+4.25
200863_s_at	RAB11A	RAB11A, member RAS oncogene family	-2.25	-2.47

Table 3.6 continued

211961_s_at	RAB7	RAB7, member RAS oncogene family	-2.42	NS
219412_at	RAB38	RAB38, member RAS oncogene family	+2.09	NS
219622_at	RAB20	RAB20, member RAS oncogene family	+2.67	NS
222980_at	RAB10	RAB10, member RAS oncogene family	+2.47	NS
223463_at	RAB23	RAB23, member RAS oncogene family	+6.19	NS
201244_s_at	RAF1	v-raf-1 murine leukemia viral oncogene homolog 1	NS	+7.46
204214_s_at	RAB32	RAB32, member RAS oncogene family	NS	-70.7
230075_at	RAB39B	RAB39B, member RAS oncogene family	NS	+8.87
212983_at	HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	NS	+6.10
202933_s_at	YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	NS	+5.46
210105_s_at	FYN	FYN oncogene related to SRC, FGR, YES	NS	+3.43

Table 3.6 continued

209239_at	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	+2.65	NS
209636_at	NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	NS	+6.10
222952_s_at	TLR7	toll-like receptor 7	NS	+2.21
223750_s_at	TLR10	toll-like receptor 10	NS	+2.25
207616_s_at	TANK	TRAF family member-associated NFKB activator	NS	-2.88

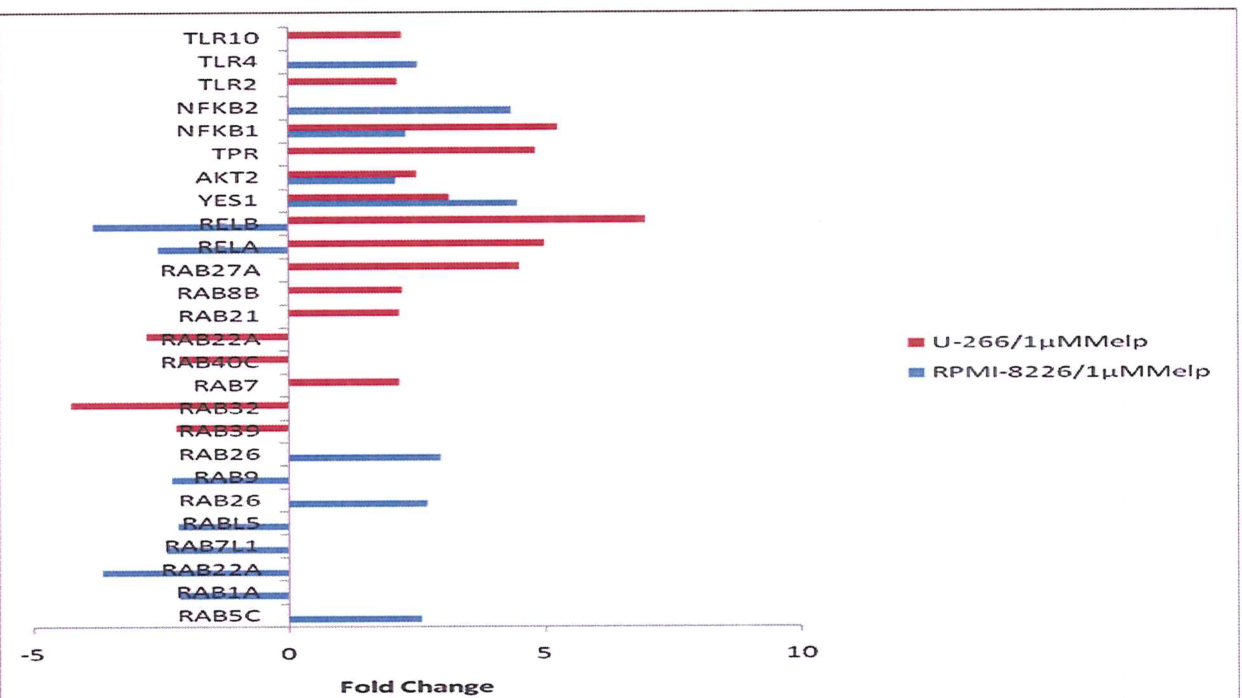


Figure 3.41 Expression levels of oncogene related genes in RPMI-8226/1µMMelp and U-266/1µMMelp cell lines

Table 3.7 Expression levels of oncogene related genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/1μMMelp Fold Change	U-266/1μMMelp Fold Change
201140_s_at	RAB5C	RAB5C, member RAS oncogene family	+2.60	NS
213440_at	RAB1A	RAB1A, member RAS oncogene family	-2.12	NS
218360_at	RAB22A	RAB22A, member RAS oncogene family	-3.65	NS
218699_at	RAB7L1	RAB7, member RAS oncogene family-like 1	-2.39	NS
218785_s_at	RABL5	RAB, member RAS oncogene family-like 5	-2.17	NS
219562_at	RAB26	RAB26, member RAS oncogene family	+2.69	NS
221808_at	RAB9	RAB9, member RAS oncogene family	-2.29	NS
50965_at	RAB26	RAB26, member RAS oncogene family	+2.96	NS
1554800_at	RAB39	RAB39, member RAS oncogene family	NS	-2.20
204214_s_at	RAB32	RAB32, member RAS oncogene family	NS	-4.25

Table 3.7 continued

211961_s_at	RAB7	RAB7, member RAS oncogene family	NS	+2.15
213466_at	RAB40C	RAB40C, member RAS oncogene family	NS	-2.12
218360_at	RAB22A	RAB22A, member RAS oncogene family	NS	-2.78
226268_at	RAB21	RAB21, member RAS oncogene family	NS	+2.15
226633_at	RAB8B	RAB8B, member RAS oncogene family	NS	+2.21
235766_x_at	RAB27A	RAB27A, member RAS oncogene family	NS	+4.50
201783_s_at	RELA	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	-2.53	+4.99
205205_at	RELB	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	-3.81	+6.96

Table 3.6 continued

202932_at	YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	+4.46	+3.13
236664_at	AKT2	v-akt murine thymoma viral oncogene homolog 2	+2.09	+2.51
1557227_s_at	TPR	translocated promoter region (to activated MET oncogene)	NS	+4.82
209239_at	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	+2.31	+5.24
209636_at	NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	+4.34	NS
204924_at	TLR2	toll-like receptor 2	NS	+2.12
224341_x_at	TLR4	toll-like receptor 4	+2.53	NS
223750_s_at	TLR10	toll-like receptor 10	NS	+2.20

3.7.4.3 Growth Factor Related Gene Expressions

Figures 3.42-3.43 represents the differential expression of growth factor related genes in drug resistant MM sublines. Transforming growth factor beta controls proliferation, cellular differentiation and other functions in most cells. It acts as an antiproliferative factor in normal epithelial cells and at early stages of oncogenesis (Khalil, 1999). Transforming growth factor, beta receptor is a tumor suppressor gene. This gene encodes a member of Ser/Thr protein kinase family and binds TGF-beta. This receptor/ligand complex phosphorylates proteins, which then enter the nucleus and regulate the transcription of a subset of genes related to cell proliferation (Dore *et al.*, 1998). TGFB-receptor genes were downregulated in both of the prednisone resistant MM cell lines (Table 3.8) and only in melphalan resistant RPMI-8226 cell line (Table 3.10) indicating cell proliferation. However, TGFBR1 was upregulated in both of the vincristine resistant MM cell lines (Table 3.9).

Fibroblast growth factors (FGFs) are family of growth factors involved in angiogenesis, wound healing and embryonic development. The FGFs are heparin-binding proteins and interactions with cell-surface associated heparan sulfate proteoglycans have been shown to be essential for FGF-signal transduction. FGFs are key players in the process of proliferation and differentiation of wide variety of cells and tissues (Olsen *et al.*, 2003). Fibroblast growth factor related genes were downregulated in all of the drug resistant MM sublines except RPMI-8226/50nMVinc subline (Table 3.8-3.10).

Platelet-derived growth factor plays a role in embryonic development, cell proliferation, cell migration and angiogenesis. In essence, the PDGFs allow a cell to skip the G1 checkpoints in order to divide (Barres *et al.*, 1992). Increased angiogenic potential has been identified in multiple myeloma and it was shown that high levels of platelet-derived growth factor and hepatocyte growth factor (HGF) were associated with advanced disease stage; in addition, HGF played a significant role in disease processing and was related to disease severity (Kara *et al.*, 2006). PDGF related genes were overexpressed in all of the drug applied RPMI-8226 cell lines and also in melphalan resistant U-266 cell line indicating the importance of this growth

factor in melphalan resistance (Table 3.8-3.10). HGF gene was upregulated only in prednisone and vincristine resistant RPMI-8226 cell lines (Table 3.8 and Table 3.9).

Epidermal growth factor plays an important role in the regulation of cell growth, proliferation and differentiation by binding to its receptor EGFR. The EPS8 protein functions as part of the EGFR pathway, though its exact role has not been determined. Highly similar proteins in other organisms are involved in the transduction signals from Ras to Rac and growth factor-mediated actin remodeling. It is only overexpressed in RPMI-8226/500μMPred subline (31 fold). In one of the studies it was shown that expression of the eps8 cDNA in fibroblastic or hematopoietic target cells expressing the EGFR resulted in increased mitogenic response to EGF, implicating the eps8 gene product in the control of mitogenic signals (Fazioli *et al.*, 1993). EPS15 gene also encodes a protein that is part of the EGFR pathway. The protein is present at clatherin-coated pits and is involved in receptor-mediated endocytosis of EGF. Notably, this gene is rearranged with the HRX/ALL/MLL gene in acute myelogenous leukemias (Salcini *et al.*, 1999). In one of the studies it was suggested that EPS15 and EPS15R mediated internalization of activated EGF receptor to result in receptor recycling as to reinforce the proliferative response to EGF (Niehof *et al.*, 2008). EPS15 was upregulated in both of the prednisone resistant MM cell lines indicating its importance in prednisone resistance (Table 3.8). On the other hand it was overexpressed in U-266/2nMVinc cell line while it was downregulated in RPMI-8226/50nMVinc (Table 3.9). This can be because of the individual differences with respect to the same drug and also can be an explanation of why U-266 cell line could not became more resistant to vincristine with respect to RPMI-8226.

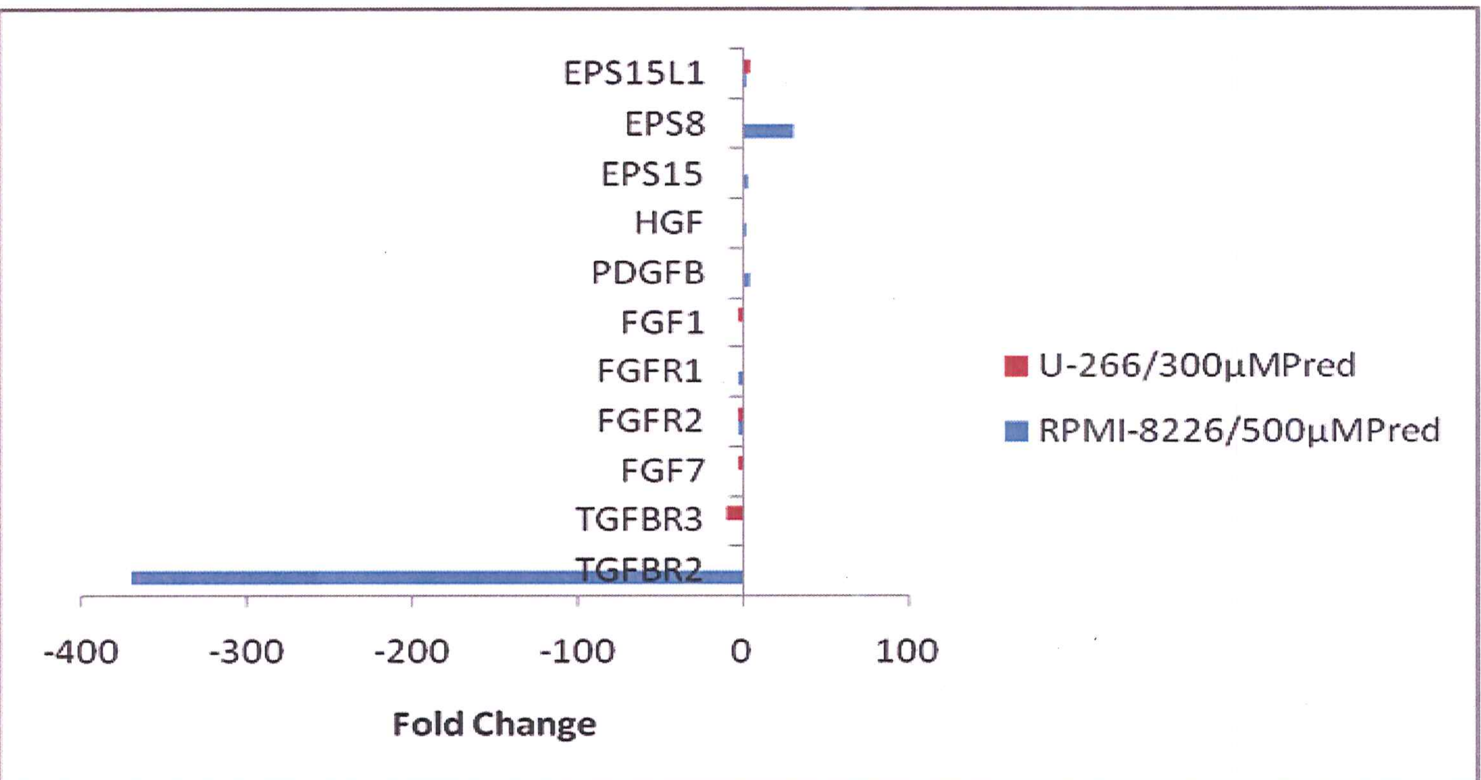


Figure 3.42 Expression levels of growth factor related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines

Table 3.8 Expression levels of growth factor related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/500µMPred Fold Change	U-266/300µMPred Fold Change
208944_at	TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	-369.64	NS
226625_at	TGFBR3	transforming growth factor, beta receptor III (betaglycan, 300kDa)	NS	-9.51
231031_at	FGF7	fibroblast growth factor 7 (keratinocyte growth factor)	NS	-2.05
208234_x_at	FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)	-2.44	-2.37

Table 3.8 continued

211535_s_at	FGFR1	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	-2.28	NS
205117_at	FGF1	fibroblast growth factor 1 (acidic)	NS	-2.63
216061_x_at	PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	+4.78	NS
210997_at	HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	+2.49	NS
217886_at	EPS15	epidermal growth factor receptor pathway substrate 15	+3.29	NS
202609_at	EPS8	epidermal growth factor receptor pathway substrate 8	+30.90	NS
221056_x_at	EPS15L1	epidermal growth factor receptor pathway substrate 15-like 1	+2.60	+4.28

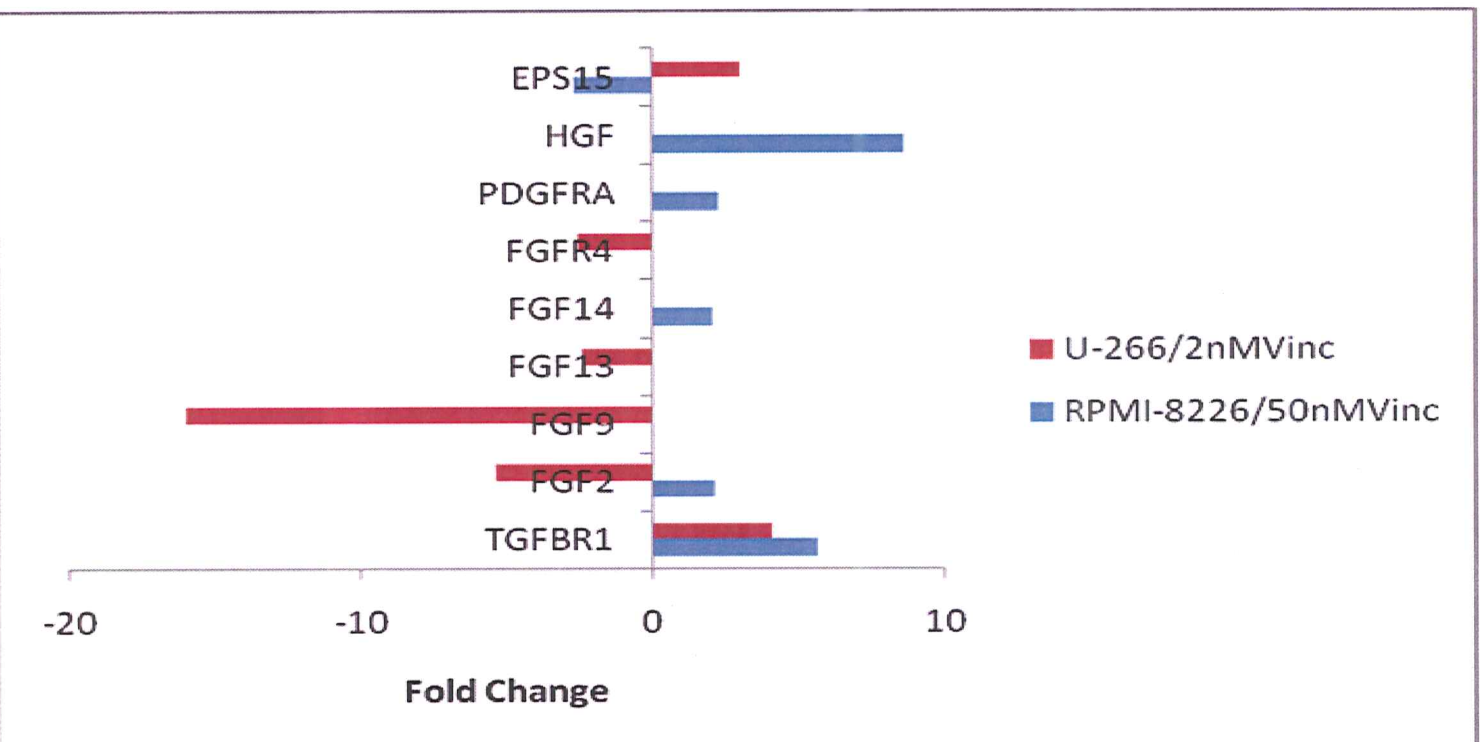


Figure 3.43 Expression levels of growth factor related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines

Table 3.9 Expression levels of growth factor related genes in RPMI-8226/2nMVinc and U-266/50nMVinc cell lines with respect to control

Gene Name	**Gene Symbol**	**Description**	**RPMI-8226/50nMVinc Fold Change**	**U-266/2nMVinc Fold Change**
224793_s_at	TGFBR1	transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kDa)	+5.61	+4.05
204422_s_at	FGF2	fibroblast growth factor 2 (basic)	+2.08	-5.42
206404_at	FGF9	fibroblast growth factor 9 (glia-activating factor)	NS	-16
205110_s_at	FGF13	fibroblast growth factor 13	NS	-2.46
230231_at	FGF14	fibroblast growth factor 14	+2.04	NS
1554962_a_at	FGFR4	fibroblast growth factor receptor 4	NS	-2.54
203131_at	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	+2.21	NS
209960_at	HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	+8.57	NS

Table 3.9 continued

217887_s_at	EPS15	epidermal growth factor receptor pathway substrate 15	-2.71	+2.94

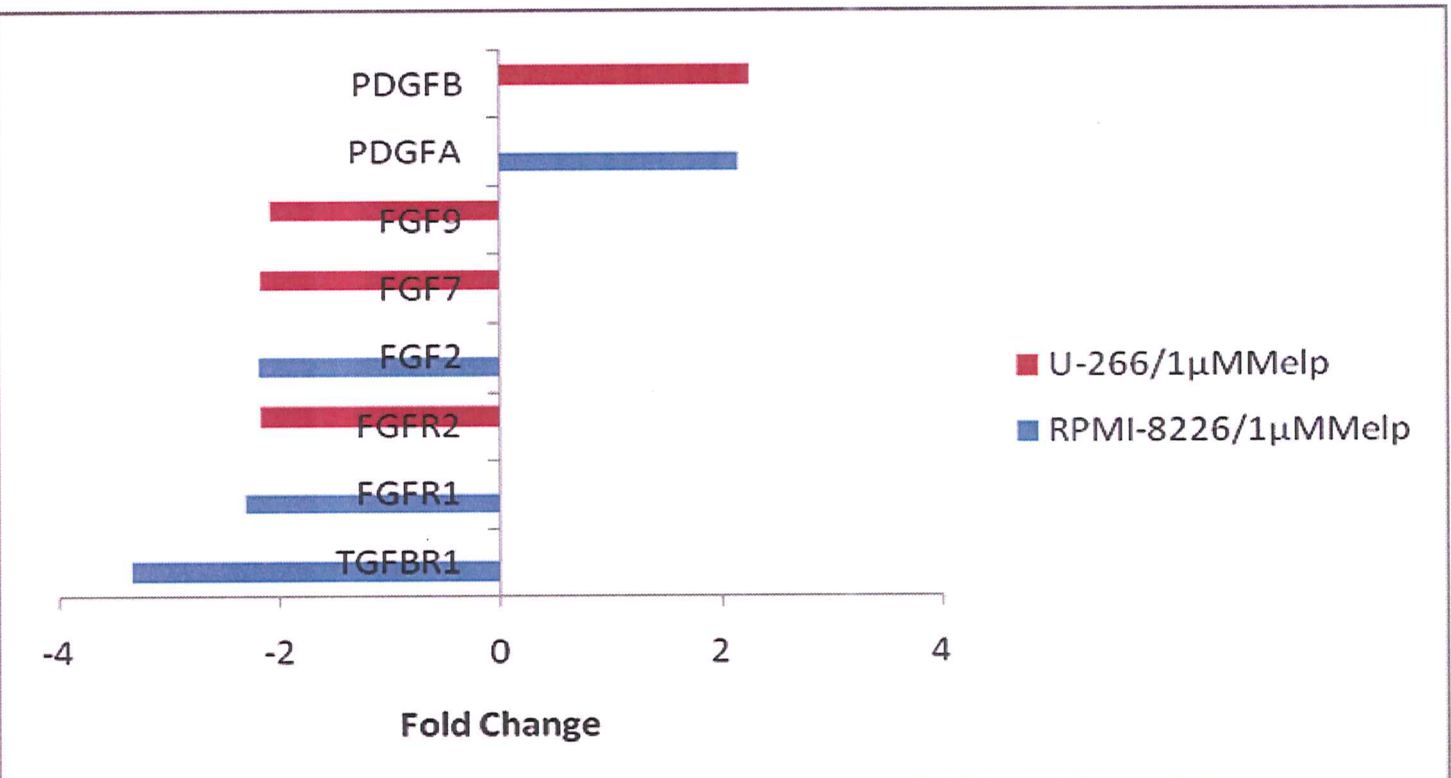


Figure 3.44 Expression levels of growth factor related genes in RPMI-8226/1µMMelp and U-266/1µMMelp cell lines

Table 3.10 Expression levels of growth factor related genes in RPMI-8226/1µmMelp and U-266/1µMMelp cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/1µMMelp Fold Change	U-266/1µMMelp Fold Change
224793_s_at	TGFBR1	transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kDa)	-3.34	NS
226705_at	FGFR1	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	-2.31	NS
203638_s_at	FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)	NS	-2.17
204421_s_at	FGF2	fibroblast growth factor 2 (basic)	-2.18	NS

Table 3.10 continued

231031_at	FGF7	fibroblast growth factor 7 (keratinocyte growth factor)	NS	-2.17
239178_at	FGF9	fibroblast growth factor 9 (glia-activating factor)	NS	-2.08
216867_s_at	PDGFA	platelet-derived growth factor alpha polypeptide	+2.17	NS
204200_s_at	PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	NS	+2.26

3.7.4.4 JAK-STAT Signaling Pathway and Interleukin and Interferon Encoding Gene Expressions

Different types of interleukin and interferon coding gene expressions were changed in drug resistant MM sublines (Figure 3.45-3.47). Among them IL3 upregulation was common in prednisone resistant MM cell lines (Table 3.11). In the literature it was shown that IL3 upregulates the expression of the antiapoptotic proteins cIAP2, Mcl-1 and Bcl-X(L) and induces a Pim1-dependent antiapoptotic pathway in primary human basophils (Didichenko *et al.*, 2008). Also IL19 upregulation was common in vincristine resistant sublines (Table 3.12). IL19 upregulation is related with induced proliferation of the squamous cell carcinoma cell lines (Hsing *et al.*, 2008). It is interesting that IL32 was significantly downregulated in RPMI-8226/500µMPred and RPMI-8226/50nMVinc cell lines (Table 3.11-3.12). IL32 is known to provoke tumor necrosis factor-alpha (TNF-α) (Kim *et al.*, 2005). Its significant downregulation can be related to proliferation of these cells. IL16 is a cytokine that released by a variety of cells including lymphocytes and some epithelial cells that has been characterized as a chemoattractant for certain immune cells expressing the cell surface molecule CD4 (Wilson *et al.*, 2005). IL16, 20 and 17 fold overexpressed in RPMI-8826/50nMVinc (Table 3.12) and RPMI-8226/1µMMelp (Table 3.13) sublines respectively. In one of the studies it was shown that interleukin 16 was significantly increase in high-grade astrocytic brain tumors (Liebrich *et al.*, 2007). Also in another study it was reported that serum level of interleukin-16 in multiple myeloma patients was significantly increased with increasing stage of the disease (Alexandrakis *et al.*, 2004). Interleukin 10 receptor alpha subunit is a subunit for the interleukin-10 receptor. The protein encoded by this gene is a receptor for interleukin 10. This protein is structurally related to interferon receptors. It has been shown to mediate the immunosuppressive signal of interleukin 10, and thus inhibits the synthesis of proinflammatory cytokines. This receptor is reported to promote survival of progenitor myeloid cells through the insulin receptor substrate-2/PI 3-kinase/AKT pathway. Activation of this receptor leads to tyrosine phosphorylation of JAK1 and TYK2 kinases (Ho, 1993). IL10RA was significantly overexpressed in prednisone

(14 fold) (Table 3.11) and vincristine (16 fold) (Table 3.12) resistant U-266 cell lines.

On the other hand different types of suppressors of cytokine signaling genes such as ASB2, SOCS2, SOCS4 and WSB2 were downregulated in drug resistant MM cell lines (Table 3.11-3.13).

IFI16 gene encodes a member of hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats family of cytokines. The protein localizes to the nucleoplasm and nucleoli, and interacts with p53 and retinoblastoma-1. It modulates p53 function and inhibits cell growth in the Ras/Raf signaling pathway. IFI16 gene was downregulated in both of the vincristine resistant MM cell lines (Table 3.12). In the melanoma M14 cell line IFI16 was shown to reduced the development of tumors (Eqistelli *et al.*, 2009). Downregulation of IFI16 gene in vincristine resistant cell lines may be associated with proliferation of these cell lines.

Interferon-gamma receptor gene 2 was both overexpressed in prednisone resistant MM cell lines (Table 3.11). In one of the studies, it was shown that prednisolone treatment in asthma is associated with an increase in numbers of cells expressing mRNA for interferon gamma (Robinson *et al.*, 1993). However, it is interesting that interferon-gamma receptor gene 1 was upregulated in melphalan resistant RPMI-8226 cell line whereas it was downregulated in U-266/1µMMelp (Table 3.13). This can be because of the individual differences.

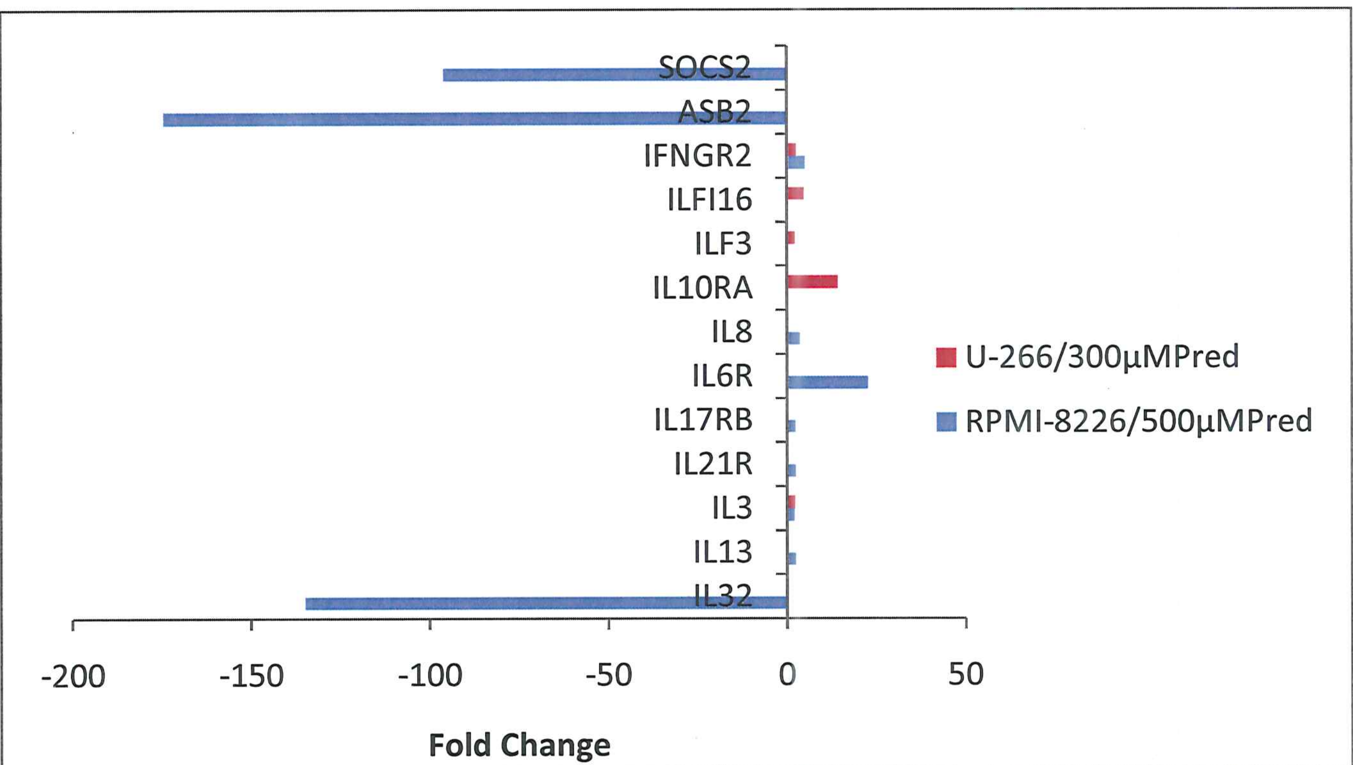


Figure 3.45 Expression levels of JAK-STAT signaling pathway and interleukin and interferon encoding genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines

Table 3.11 Expression levels of JAK-STAT signaling pathway and interleukin and interferon encoding genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines with respect to control

Gene Name	**Gene Symbol**	**Description**	**RPMI-8226/500µMPred Fold Change**	**U-266/300µMpred Fold Change**
203828_s_at	IL32	interleukin 32	-134.8	NS
207844_at	IL13	interleukin 13	+2.42	NS
207906_at	IL3	interleukin 3 (colony-stimulating factor, multiple)	+2.02	+2.14
219971_at	IL21R	interleukin 21 receptor	+2.42	NS
224361_s_at	IL17RB	interleukin 17 receptor B	+2.31	NS
226333_at	IL6R	interleukin 6 receptor	+22.6	NS
211506_s_at	IL8	interleukin 8	+3.43	NS
204912_at	IL10RA	interleukin 10 receptor, alpha	NS	+14.22
217804_s_at	ILF3	interleukin enhancer binding factor 3, 90kDa	NS	+2.17
208966_x_at	ILFI16	interferon, gamma-inducible protein 16	NS	+4.69
201642_at	IFNGR2	interferon gamma receptor 2 (interferon gamma transducer 1)	+4.92	+2.47
227915_at	ASB2	ankyrin repeat and SOCS box-containing 2	-174.4	NS

Table 3.11 continued

203373_at	SOCS2	suppressor of cytokine signaling 2	-96	NS

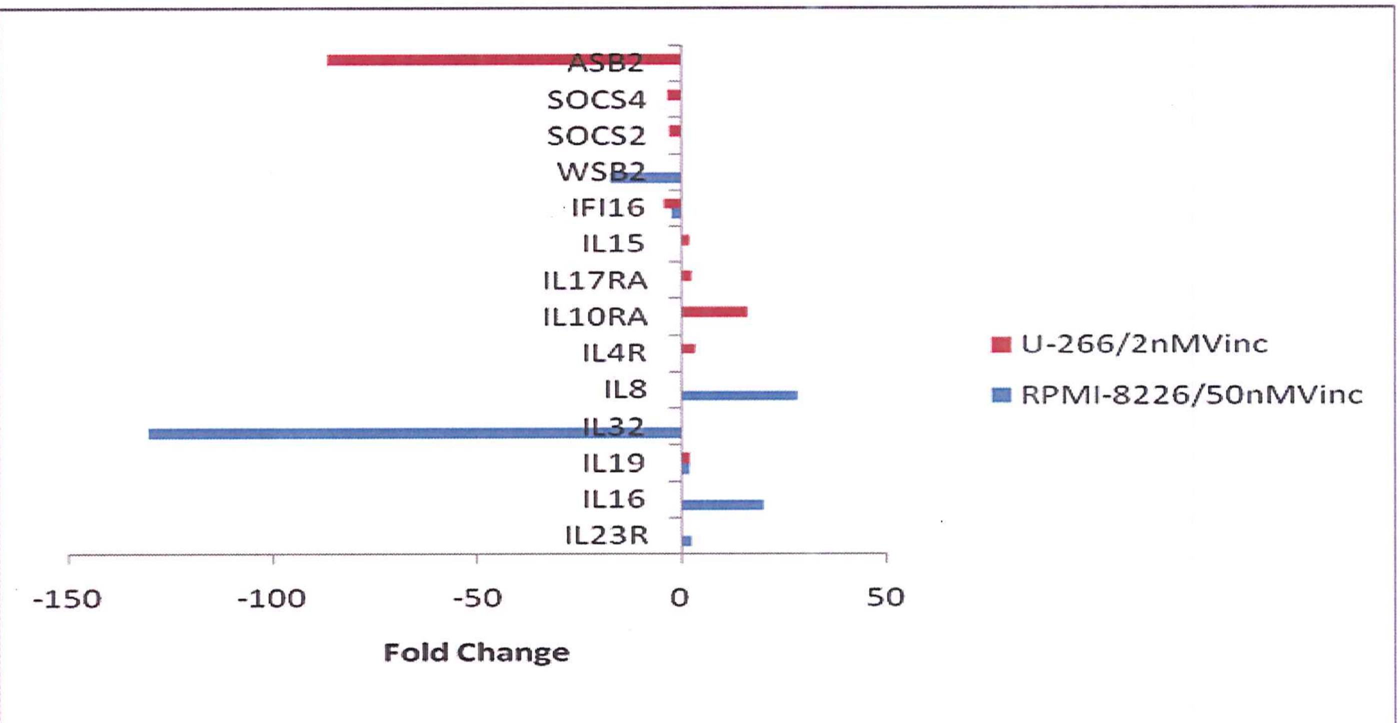


Figure 3.46 Expression levels of JAK-STAT signaling pathway and interleukin and interferon encoding genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines

Table 3.12 Expression levels of JAK-STAT signaling pathway and interleukin and interferon encoding genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/50nMVinc Fold Change	U-266/2nMVinc Fold Change
1552912_a_at	IL23R	interleukin 23 receptor	+2.67	NS
209827_s_at	IL16	interleukin16 (lymphocyte chemoattractant factor)	+19.97	NS
220745_at	IL19	interleukin 19	+2.15	+2.28
203828_s_at	IL32	interleukin 32	-130	NS
202859_x_at	IL8	interleukin 8	+28.44	NS
203233_at	IL4R	interleukin 4 receptor	NS	+3.60
204912_at	IL10RA	interleukin 10 receptor, alpha	NS	+16.11
205707_at	IL17RA	interleukin 17 receptor A	NS	+2.39
217371_s_at	IL15	interleukin 15	NS	+2.21
208966_x_at	IFI16	interferon, gamma-inducible protein 16	-2.14	-4.16
201760_s_at	WSB2	WD repeat and SOCS box-containing 2	-17.38	NS
203373_at	SOCS2	suppressor of cytokine signaling 2	NS	-2.69

Table 3.12 continued

226178_at	SOCS4	suppressor of cytokine signaling 4	NS	-3.09
227915_at	ASB2	ankyrin repeat and SOCS box-containing 2	NS	-86.22

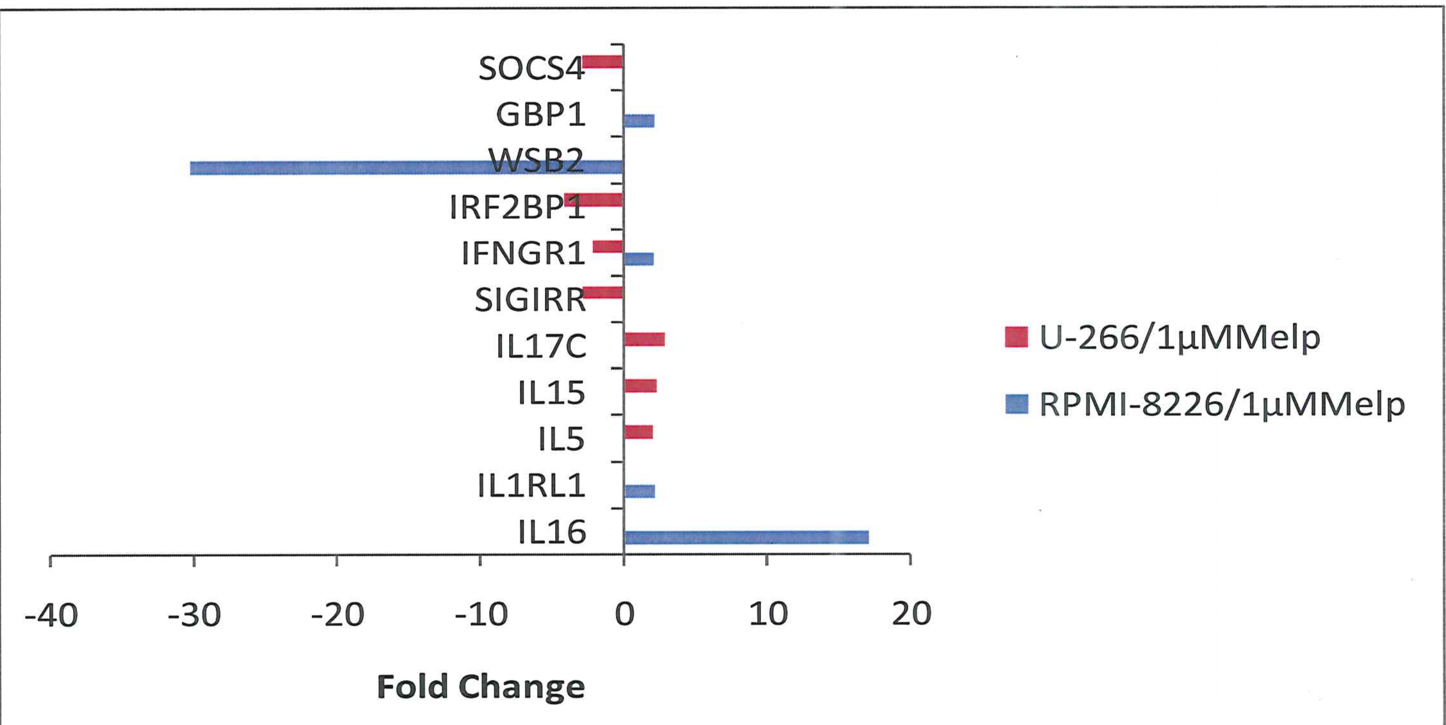


Figure 3.47 Expression levels of JAK-STAT signaling pathway and interleukin and interferon encoding genes in RPMI-8226/1µMMelp and U-266/1µMMelp cell lines

Table 3.13 Expression levels of JAK-STAT signaling pathway and interleukin and interferon encoding genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/1μMMelp Fold Change	U-266/1μMMelp Fold Change
209827_s_at	IL16	interleukin 16 (lymphocyte chemoattractant factor)	+17.02	NS
242809_at	IL1RL1	interleukin 1 receptor-like 1	+2.15	NS
207952_at	IL5	interleukin 5 (colony-stimulating factor, eosinophil)	NS	+2.04
217371_s_at	IL15	interleukin 15	NS	+2.28
224079_at	IL17C	interleukin 17C	NS	+2.84
52940_at	SIGIRR	single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	NS	-2.88
202727_s_at	IFNGR1	interferon gamma receptor 1	+2.11	-2.17
213771_at	IRF2BP1	interferon regulatory factor 2 binding protein 1	NS	-4.19
201760_s_at	WSB2	WD repeat and SOCS box-containing 2	-30.27	NS

Table 3.13 continued

202270_at	GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	+2.18	NS
226178_at	SOCS4	suppressor of cytokine signaling 4	NS	-2.88

3.7.4.5 Cell Cycle Related Gene Expressions

Alterations in cell cycle related genes in drug resistant MM sublines were shown in Figure 3.48-3.50. Different types of cell cycle regulatory cyclin encoding genes and cyclin dependent kinases were downregulated and also cyclin dependent kinase inhibitor encoding genes were upregulated in both of the prednisone resistant MM cell lines (Table 3.14) and as well as in vincristine resistant RPMI-8226 cell line (Table 3.15). These results may be correlated with the slow growth and increase in doubling time of the resistant cells. However these results were just opposite for U-266/2nMVinc subline. U-266/2nMVinc subline was actively duplicated with respect to RPMI-8226/50nMVinc subline.It can be because of the cyclin E2 gene that was 487 fold overexpressed in U-266/2nMVinc subline (Table 3.15). Cyclin E is a member of the cyclin family which is required for the transition from G1 to S phase. Since vincristine effects as a mitotic inhibitor drug it is meaningful why we can not apply the higher dose of vincristine to U-266 cell line. On the other hand, it is interesting that two recently identified E2F subunits, E2F7 and E2F8, were highly overexpressed only in U-266/2nM Vinc cell line (Table 3.15). E2F7 and E2F8, are induced in cells treated with DNA-damaging agents where they have an important role in dictating the outcome of the DNA-damage response. Depletion of either E2F7 or E2F8 prevents the cell-cycle effects that occur in response to DNA damage. Thus E2F7 and E2F8 act upstream of E2F1, and influence the ability of cells to undergo a DNA-damage response (Zalmas *et al.*, 2008). The overexpression of these genes in U-266/2nMVinc cell line shows a balance between high demand of active cell cycle progression and effect of vincristine on vincristine applied U-266 cell line.

BRCA2 and CDKN1A interacting protein is also known as BCCIP. This gene product was isolated on the basis of its interaction with BRCA2 and p21 proteins. Functional studies indicate that this protein may be an important cofactor for BRCA2 in tumor suppression, and a modulator of CDK2 kinase activity via p21 (Coleman *et al.*, 1992). BCCIP gene expression was downregulated in both of the vincristine and prednisone resistant MM cell lines (Table 3.14 and Table 3.15).

For melphalan resistant RPMI-8226 and U-266 cell lines only cyclin dependent kinase inhibitor encoding genes were downregulated with respect to their sensitive controls, which may be correlated cell proliferation and drug resistance (Table 3.16).

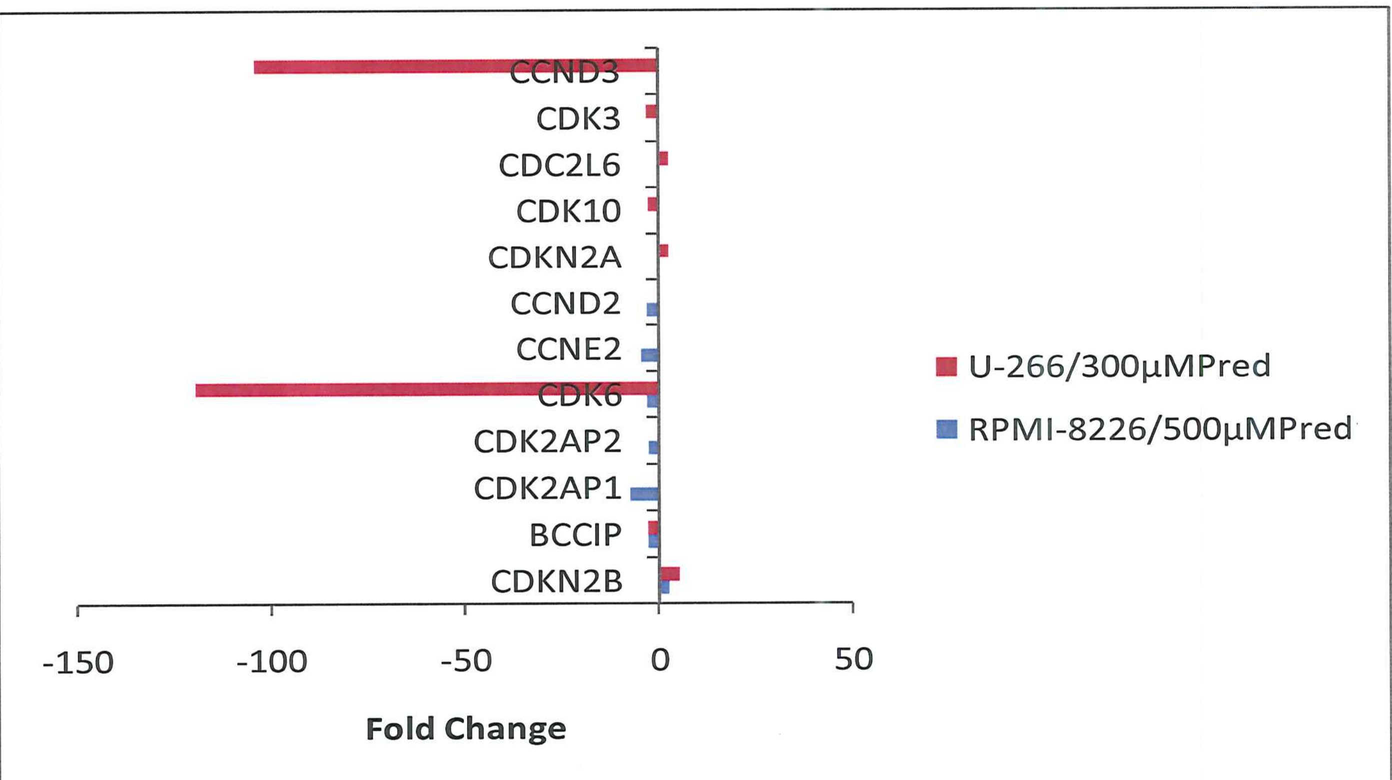


Figure 3.48 Expression levels of cell cycle related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines

Table 3.14 Expression levels of cell cycle related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/500µMPred Fold Change	U-266/300µMPred Fold Change
236313_at	CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	+2.49	+5.31
218264_at	BCCIP	BRCA2 and CDKN1A interacting protein	-2.90	-2.86
201938_at	CDK2AP1	CDK2-associated protein 1	-7.41	NS
203252_at	CDK2AP2	CDK2-associated protein 2	-2.49	NS
224847_at	CDK6	cyclin-dependent kinase 6	-3.07	-119.5
211814_s_at	CCNE2	cyclin E2	-4.50	NS
200953_s_at	CCND2	cyclin D2	-2.98	NS
209644_x_at	CDKN2A	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	NS	+2.56
203469_s_at	CDK10	yclin-dependent kinase (CDC2-like) 10	NS	-2.60
212897_at	CDC2L6	cell division cycle 2-like 6 (CDK8-like)	NS	+2.53

Table 3.14 continued

226396_at	CDK3	cyclin-dependent kinase 3	NS	-3.13
201700_at	CCND3	cyclin D3	NS	-104

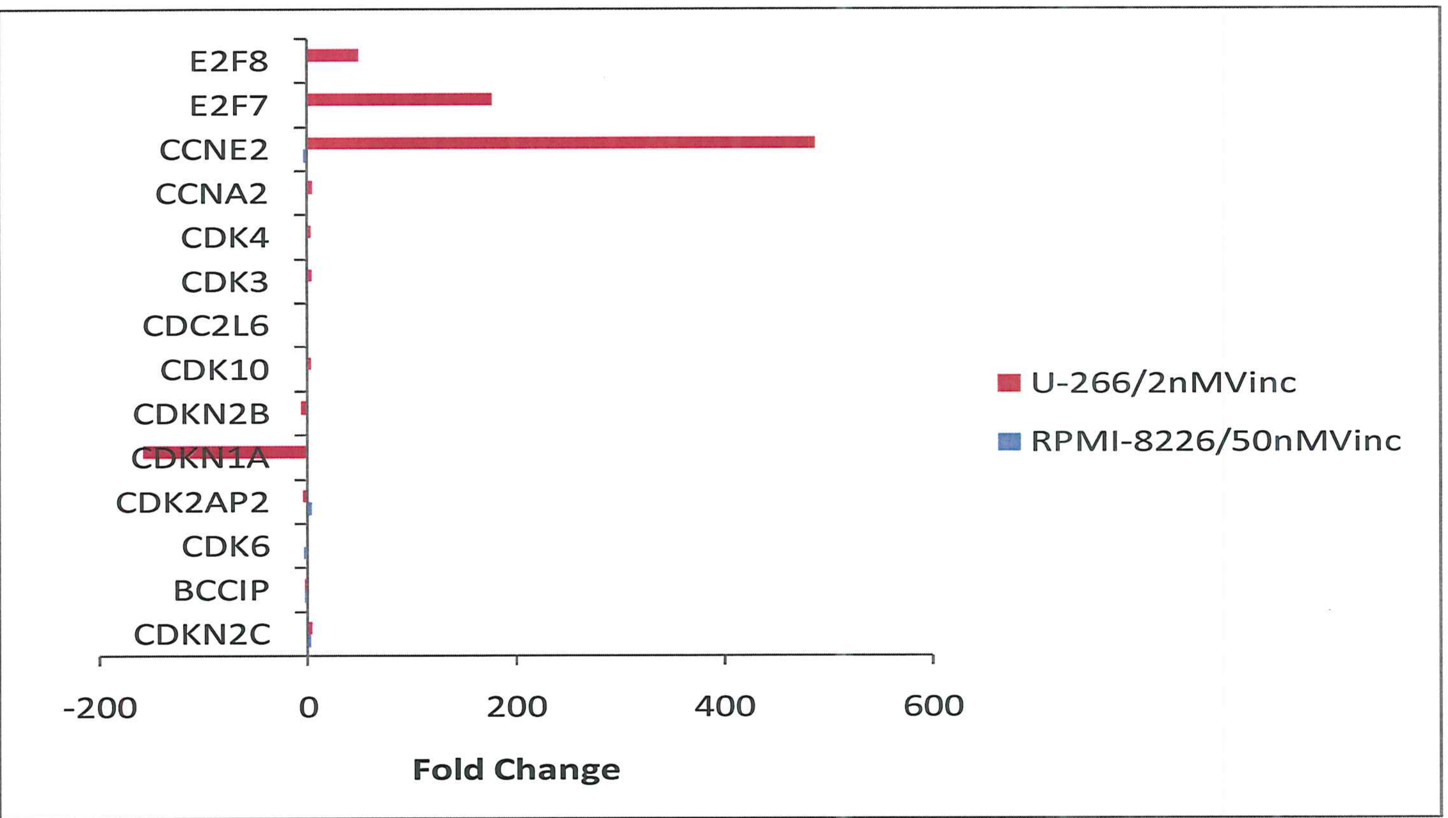


Figure 3.49 Expression levels of cell cycle related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines

Table 3.15 Expression levels of cell cycle related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines with respect to control

Gene Name	**Gene Symbol**	**Description**	**RPMI-8226/50nMVinc Fold Change**	**U-266/2nMVinc Fold Change**
211792_s_at	CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	+2.41	+3.81
218264_at	BCCIP	BRCA2 and CDKN1A interacting protein	-2.86	-3.16
243000_at	CDK6	cyclin-dependent kinase 6	-4.22	NS
203252_at	CDK2AP2	CDK2-associated protein 2	+4.19	-5.16
202284_s_at	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NS	-158.6
236313_at	CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	NS	-6.40
210622_x_at	CDK10	cyclin-dependent kinase (CDC2-like) 10	NS	+2.36
212897_at	CDC2L6	cell division cycle 2-like 6 (CDK8-like)	NS	-2.12
226396_at	CDK3	cyclin-dependent kinase 3	NS	+3.86
202246_s_at	CDK4	cyclin-dependent kinase 4	NS	+2.84
203418_at	CCNA2	cyclin A2	NS	+4.25
205034_at	CCNE2	cyclin E2	-3.89	+487.75

Table 3.15 continued

228033_at	E2F7	E2F transcription factor 7	NS	177.29
219990_at	E2F8	E2F transcription factor 8	NS	+49.18

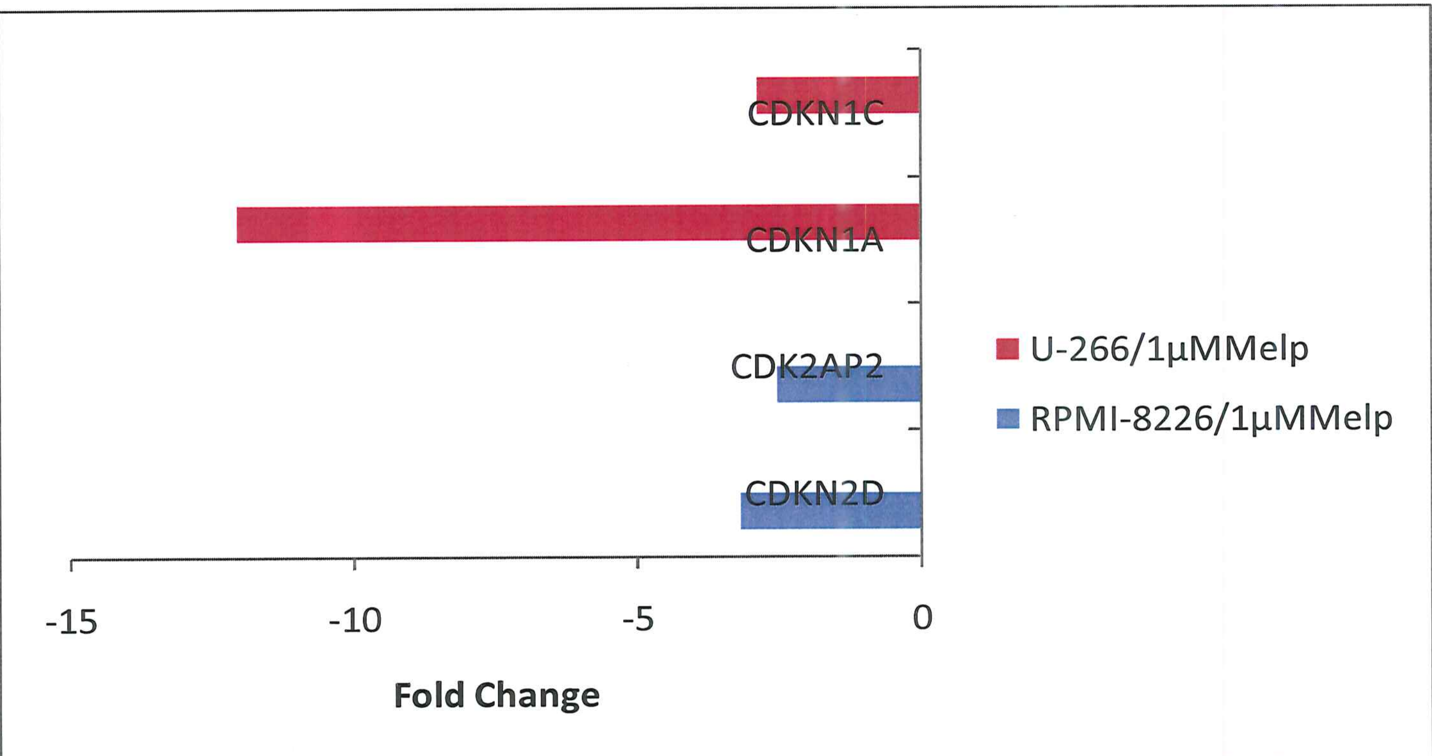


Figure 3.50 Expression levels of cell cycle related genes in RPMI-8226/1µMMelp and U-266/1µMMelp cell lines

Table 3.16 Expression levels of cell cycle related genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/1μMMelp Fold Change	U-266/1μMMelp Fold Change
210240_s_at	CDKN2D	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	-3.20	NS
203252_at	CDK2AP2	CDK2-associated protein 2	-2.54	NS
202284_s_at	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NS	-12.04
216894_x_at	CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	NS	-2.88

3.7.4.6 Ceramide Metabolism Related Gene Expressions

Figures 3.51-3.53 show the altered genes in ceramide metabolism. Ceramides are a family of lipid molecules. The most well-known functions of ceramides as cellular signals include regulating the differentiation, proliferation, programmed cell death and apoptosis of cells. (Haimovitz-Friedman *et al.*, 1994).

Sphingomyelinase was downregulated in both of the melphalan resistant MM cell lines (Table 3.19) whereas it was only downregulated in vincristine resistant RPMI-8226 subline (Table 3.18) and upregulated in prednisone resistant U-266 subline (Table 3.17). Upregulation of dihydroceramide desaturase was shown to increase the ceramide levels in the cell (Hannun *et al.*, 2008). DEGS1 gene is only upregulated in prednisone resistant MM cell lines (Table 3.17) while it is downregulated in vincristine resistant U-266 cell line (Table 3.18).

Constitutive degradation of sphingolipids and glycosphingolipids takes place in the acidic subcellular compartments, the late endosomes and the lysosomes. Ceramide can be further hydrolyzed by acid ceramidase to form sphingosine and a free fatty acid, both of which are able to leave the lysosome in contrast to ceramide. The long chain sphingoid bases released from the lysosome may then re-enter pathways for synthesis of ceramide by ceramide synthase and/or sphingosine-1-phosphate by sphingosine kinase (Kitatani *et al.*, 2008). UDP-glucose ceramide glucosyltransferase catalyzes the first glycosylation step in glycosphingolipid biosynthesis. The product, glycosylceramide is the core structure of more than 300 GSLs. UGCG is widely expressed and transcription is upregulated during keratinocyte differentiation (Kohyama-Koganaya *et al.*, 2002).

The upregulation of ceramidase, sphingosine kinase, glucosylceramide synthase and the downregulation of ceramide synthase genes can be important for drug resistance since they lower the ceramide levels within the cell. Acid ceramidase gene was not upregulated in non of the cell lines whereas ceramide synthase gene was upregulated in prednisone and vincristine resistant MM cell lines (Table 3.17 and Table 3.18) and downregulated in melphalan resistant MM (Table 3.19) cell lines. Sphingosine kinase was overexpressed in melphalan resistant RPMI-8226 cell line (Table 3.19)

and also in prednisone and vincristine resistant U-266 cell line (Table 3.17 and Table 3.18). UGCG was only overexpressed in melphalan resistant RPMI-8226 and vincristine resistant U-266 cell lines (Table 3.19).

In the literature, it was shown that cellular ceramide levels were related to drug resistance. Glycosylceramide synthase overexpression has been shown to enhance resistance to doxorubicin (Reynolds *et al.*,2004). Alteration of ceramide/ sphingosine 1-phosphate was shown to be involved in the regulation of resistance to imatinib in K562 cell lines (Baran *et al*, 2007).

Several anticancer agents, including the cytotoxic retinoid, fenretinide (4-HPR), have been shown to act by increasing tumor cell ceramide via *de novo* synthesis (Reynolds *et al.* 2004). Expression of glucosylceramide synthase mRNA in vincristine-resistant KBV200 cell line was shown in association with multidrug resistance (Yang *et al.*, 2004). However there is not much known about the relation of glucocorticoids and melphalan with ceramide metabolism. According to our results, it seems melphalan resistance is related with cellular ceramide levels because melphalan resistant MM cell lines tend to decrease ceramide level via downregulating sphingomyelinase and ceramide synthase genes while upregulating sphingosine kinase and UGCG genes (Table 3.19). On the other hand prednisone and vincristine resistance seems not to be associated with ceramide metabolism.

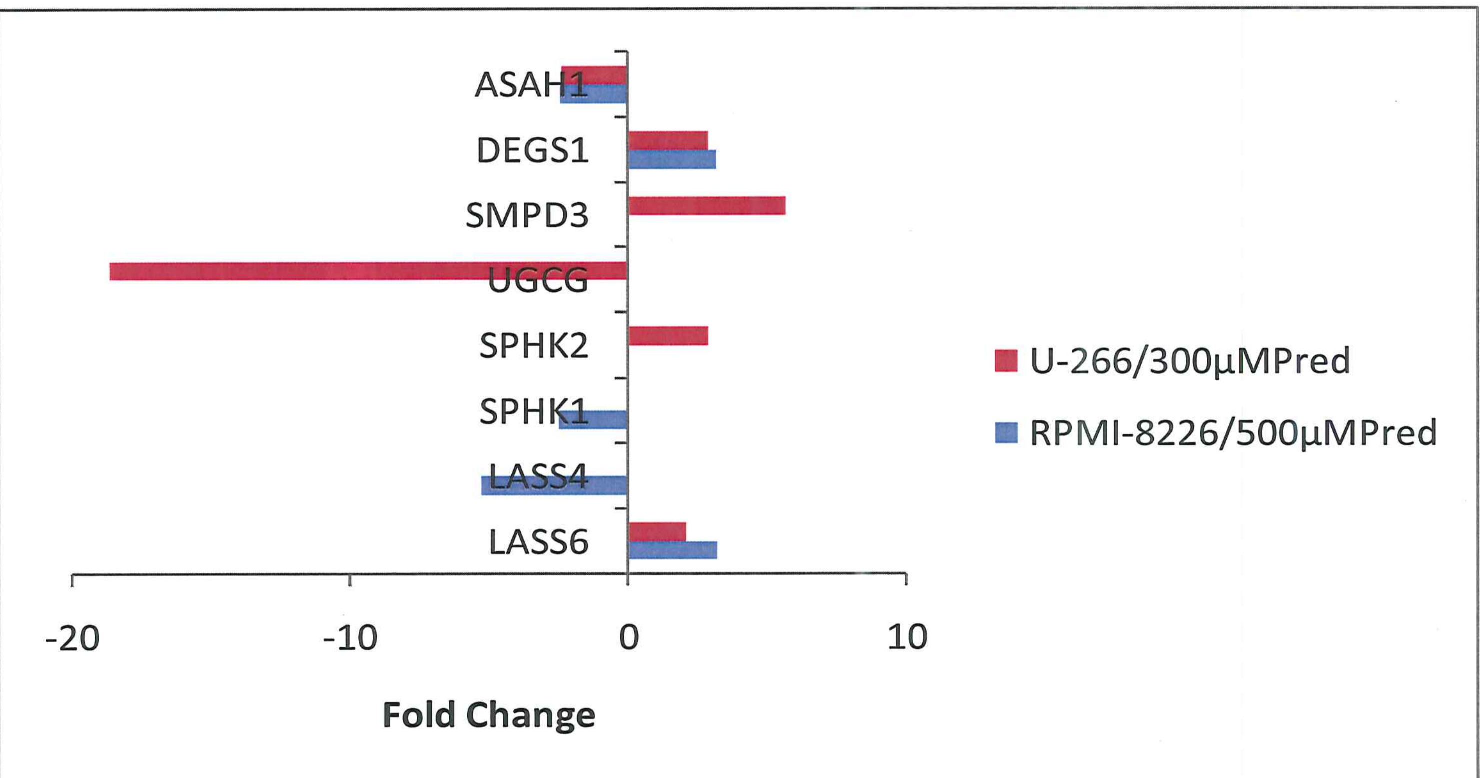


Figure 3.51 Expression levels of ceramide metabolism related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines

Table 3.17 Expression levels of ceramide metabolism related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/500µMPred Fold Change	U-266/300µMPred Fold Change
212442_s_at	LASS6	LAG1 homolog, ceramide synthase 6 (S. cerevisiae)	+3.18	+2.08
218922_s_at	LASS4	LAG1 homolog, ceramide synthase 4 (S. cerevisiae)	-5.27	NS
219257_s_at	SPHK1	sphingosine kinase 1	-2.49	NS
209857_s_at	SPHK2	sphingosine kinase 2	NS	+2.88
221765_at	UGCG	UDP-glucose ceramide glucosyltransferase	NS	-18.63
219695_at	SMPD3	sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)	NS	+5.65
207431_s_at	DEGS1	degenerative spermatocyte homolog 1, lipid desaturase (Drosophila)	+3.16	+2.88
213702_x_at	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	-2.46	-2.39

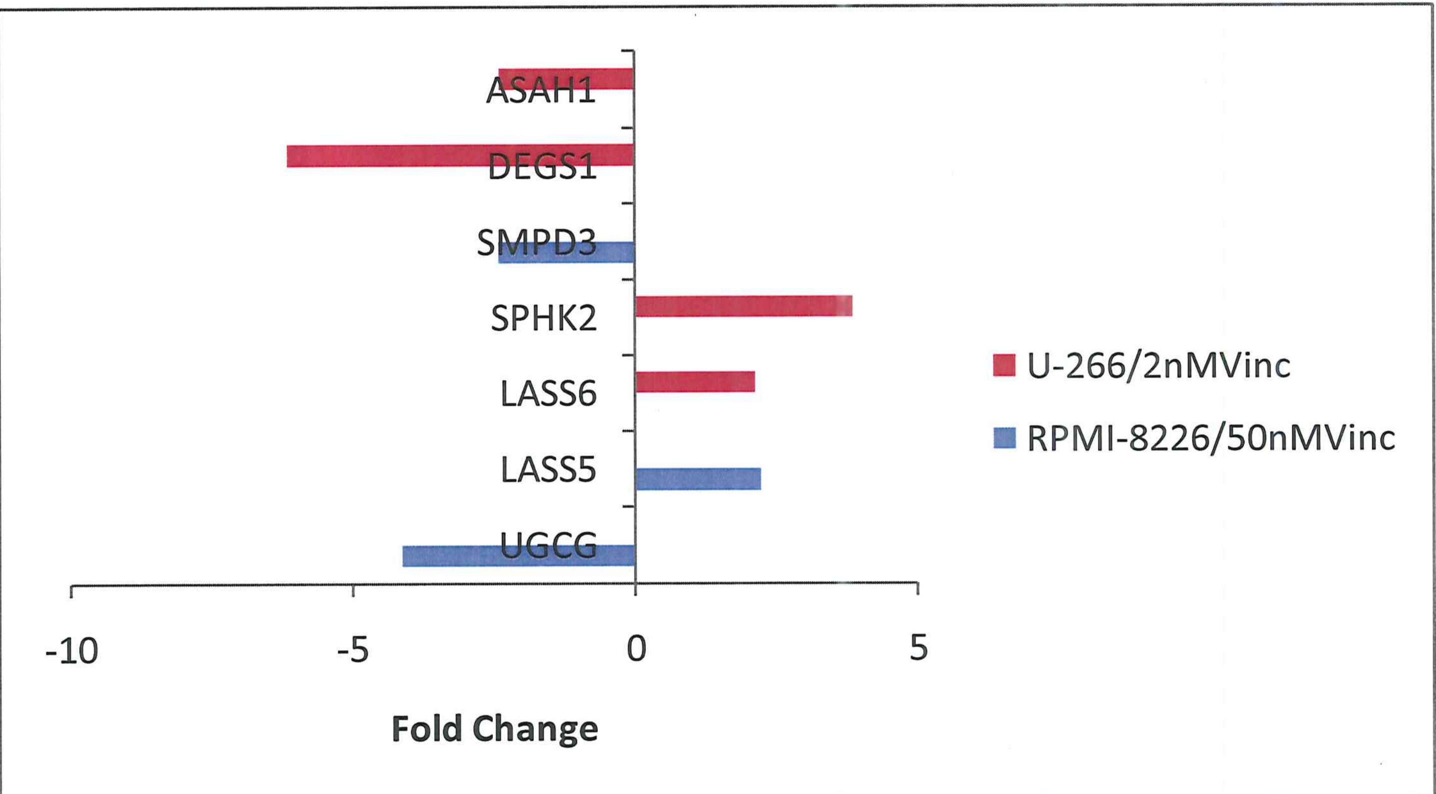


Figure 3.52 Expression levels of ceramide related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines

Table 3.18 Expression levels of ceramide metabolism related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/50nMVinc Fold Change	U-266/2nMVinc Fold Change
204881_s_at	UGCG	UDP-glucose ceramide glucosyltransferase	-4.11	NS
224951_at	LASS5	LAG1 homolog, ceramide synthase 5 (S. cerevisiae)	+2.23	NS
212442_s_at	LASS6	LAG1 homolog, ceramide synthase 6 (S. cerevisiae)	NS	+2.12
209857_s_at	SPHK2	sphingosine kinase 2	NS	+3.86
219695_at	SMPD3	sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)	-2.41	NS
207431_s_at	DEGS1	degenerative spermatocyte homolog 1, lipid desaturase (Drosophila)	NS	-6.14
213702_x_at	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	NS	-2.39

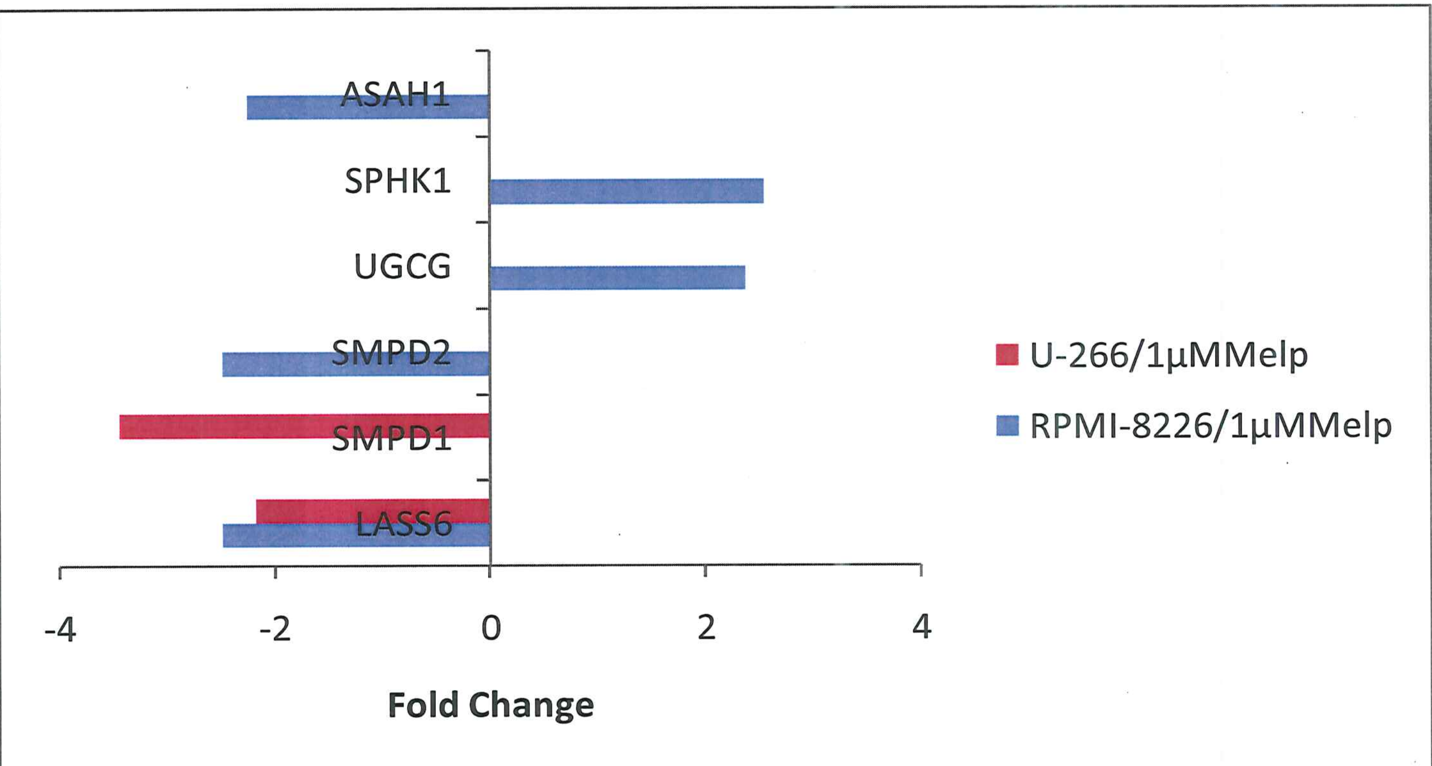


Figure 3.53 Expression levels of ceramide related genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines

Table 3.19 Expression levels of ceramide metabolism related genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/1μMMelp Fold Change	U-266/1μMMelp Fold Change
212442_s_at	LASS6	LAG1 homolog, ceramide synthase 6 (S. cerevisiae)	-2.49	-2.18
209420_s_at	SMPD1	sphingomyelin phosphodiesterase 1, acid lysosomal (acid sphingomyelinase)	NS	-3.45
205622_at	SMPD2	sphingomyelin phosphodiesterase 2, neutral membrane (neutral sphingomyelinase)	-2.49	NS
224967_at	UGCG	UDP-glucose ceramide glucosyltransferase	+2.37	NS
219257_s_at	SPHK1	sphingosine kinase 1	+2.54	NS
210980_s_at	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	-2.25	NS

3.7.4.7 Apoptosis and Death Associated Gene Expressions

Differential gene expression levels of apoptosis and death associated genes were shown in Figures 3.54-3.56. Tumor necrosis factor (TNF) acts via the TNF Receptor (TNF-R) and is part of the extrinsic pathway for trigerring apoptosis. TNF-R is associated with procaspases through adapter proteins (FADD, TRADD etc) that can cleave other inactive procaspases and trigger the caspase cascade, irreversibly commiting the cell to apoptosis.

TNFAIP3 protein has been shown to inhibit TNF-mediated apoptosis. This gene was significantly downregulated in prednisone resistant MM cell lines. TNFRSF10 which is a proapoptotic gene was shown to upregulated in melanoma cells destined to undergo apoptosis (Kokkinakis, 2006). This gene was downregulated in both of the prednisone resistant MM cell line which indicate cell survival (Table 3.20). TNFRSF10B can be activated by TNFSF10 and transduces apoptosis signal was upregulated in both of the vincristine resistant MM cell lines (Table 3.21). On the other hand TNFAIP6 is known to be involved in extracellular matrix stability and cell migration and TNFAIP8 is known as an antiapoptotic molecule (Ewing, 2007) both were upregulated in vincristine resistant MM cell lines (Table 3.21). Different types of tumor necrosis factor genes were expressed in melphalan resistant cell lines which indicates individual differences (Table 3.22). TNFRSF13C and TNFRSF17 which are both related with cell survival and proliferation were overexpressed in RPMI-8226/1µMMelp cell line. On the other hand TNFSF10 was overexpressed in U-266/1µMMelp cell line indicating cell death and TNFRSF19 which activates JNK signaling pathway and capable of inducing apoptosis by a caspase-independent mechanism was downregulated in this cell line. So both upregulation and downregulation of TNF family members in drug resistant multiple myeloma cell lines indicates that these cells may have been trying to balance the components in paralel.

Caspase-3,-6 and-7 are called effector caspases which are downstream caspases in turn cleave other protein substrates within the cell, to trigger the apoptotic processes. The initiation of this cascade reaction is regulated by caspase inhibitors. Caspase-3

gene was downregulated in both of the prednisone resistant MM cell lines (Table 3.20) as well as only in melphalan resistant U-266 cell line (Table 3.22). On the other hand caspase-6 was only downregulated in U-266/300μMPred cell line (Table 3.20) and caspase-7 was downregulated in U-266/2nMVinc cell line (Table 3.21).

The baculoviral IAP repeat-containing (BIRC) group of genes belongs to a family that inhibits apoptosis by binding to tumor necrosis factor receptor-associated factors TRAF-1 and TRAF-2, probably by interfering with activation of ICE-like proteases. BIRC related genes were significantly overexpressed in prednisone and vincristine resistant sublines but not in the melphalan resistant sublines (Tables 3.20-3.22).

BCL11A, BAG and BNIP are Bcl related genes. BCL11A gene is a common site of retroviral integration in myeloid leukemia. During hematopoietic cell differentiation, this gene is downregulated. It is probably involved in lymphoma pathogenesis since translocations associated with B-cell malignancies also deregulates its expression. BAG which is a Bcl-2 associated athanogene is a multifunctional pro-survival molecule that binds to Hsp70/Hsc70 proteins. BNIP proteins may play role in apoptosis through regulating the expression of genes associated with cell apoptosis, growth inhibition and cell proliferation (Xie *et al.*, 2004). These Bcl related genes were upregulated in RPMI-8226/500μMPred, RPMI-8226/1μMMelp and both of the vincristine resistant MM cell lines (Tables 3.20-3.22). However, they were downregulated in prednisone resistant U-266 cell line (Table 3.20). Bcl-2 and Bcl-X_L genes were not significantly altered in our sublines which is a confirmation of the results we obtained by RT-PCR and Western Blot.

Programmed cell-death is death of a cell mediated by an intracellular program. PDCD11 is a NFκB binding protein that colocalizes in the nucleus. PDCD4 gene encodes a protein localized to the nucleus in proliferating cells. The gene product is thought to play a role in apoptosis but the specific role has not yet been determined. PDCD2 gene encodes a nuclear protein expressed in a variety of tissues. Expression of the human gene has been shown to be repressed by Bcl-6, suggesting that Bcl-6 regulates apoptosis by its effects on PDCD2. These genes overexpressed in RPMI-8226/500μMPred, U-266/2nMVinc sublines, but only downregulated in U-266/300μMPred subline (Table 3.20 and Table 3.21). This shows that when one

mechanism which is related to resistance is downregulated the cells adapt themselves to survive by activating another mechanism.

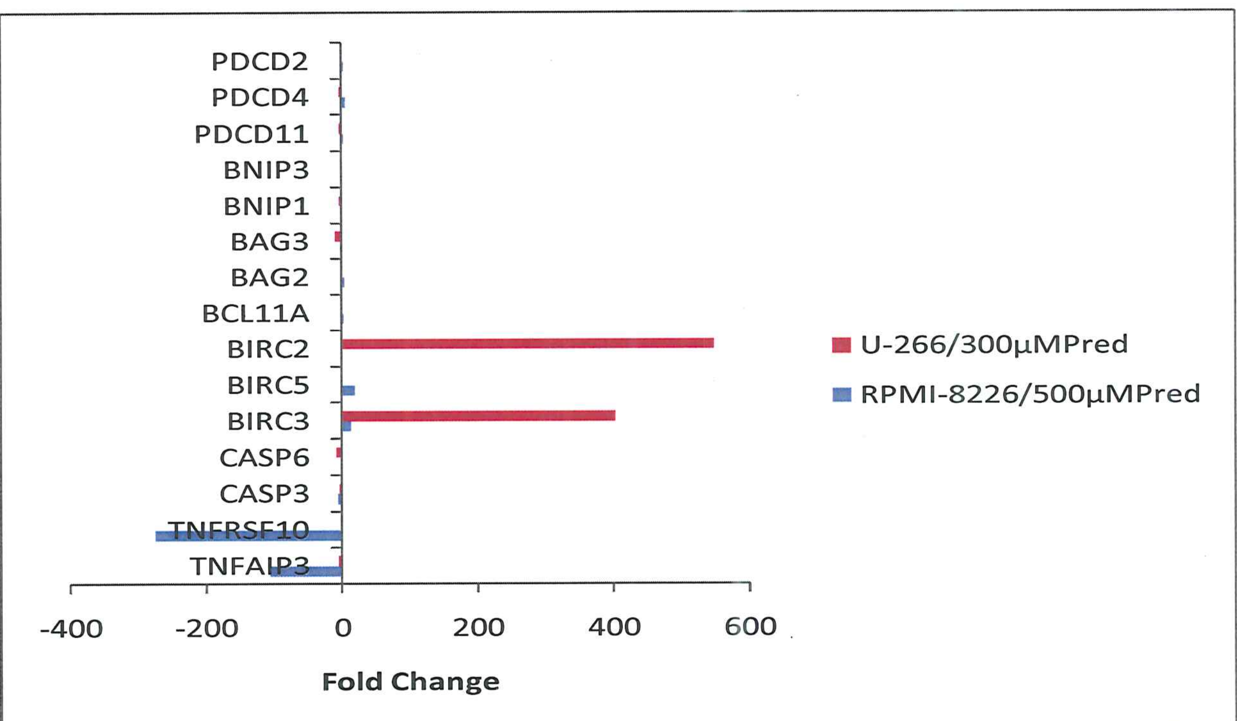


Figure 3.54 Expression levels of apoptosis and death associated genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines

Table 3.20 Expression levels of apoptosis and death associated genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/500µMPred Fold Change	U-266/300µMPred Fold Change
202643_s_at	TNFAIP3	tumor necrosis factor, alpha-induced protein 3	-106.5	-4.50
227345_at	TNFRSF10	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	-274.7	-2.02
202763_at	CASP3	caspase 3, apoptosis-related cysteine peptidase	-5.81	-3.38
209790_s_at	CASP6	caspase 6, apoptosis-related cysteine peptidase	NS	-8.33
210538_s_at	BIRC3	baculoviral IAP repeat-containing 3	+13.92	+403.5
202094_at	BIRC5	baculoviral IAP repeat-containing 5 (survivin)	+19.29	NS
202076_at	BIRC2	baculoviral IAP repeat-containing 2	NS	+547.9
219497_s_at	BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	+2.90	NS

Table 3.20 continued

209406_at	BAG2	BCL2-associated athanogene 2	+4	NS
217911_s_at	BAG3	BCL2-associated athanogene 3	NS	-9.31
37226_at	BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1	NS	-2.98
201849_at	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	NS	-2.69
212422_at	PDCD11	programmed cell death 11	+2.67	-3.48
212593_s_at	PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)	+4.59	-3.50
228420_at	PDCD2	programmed cell death 2	+3.13	NS

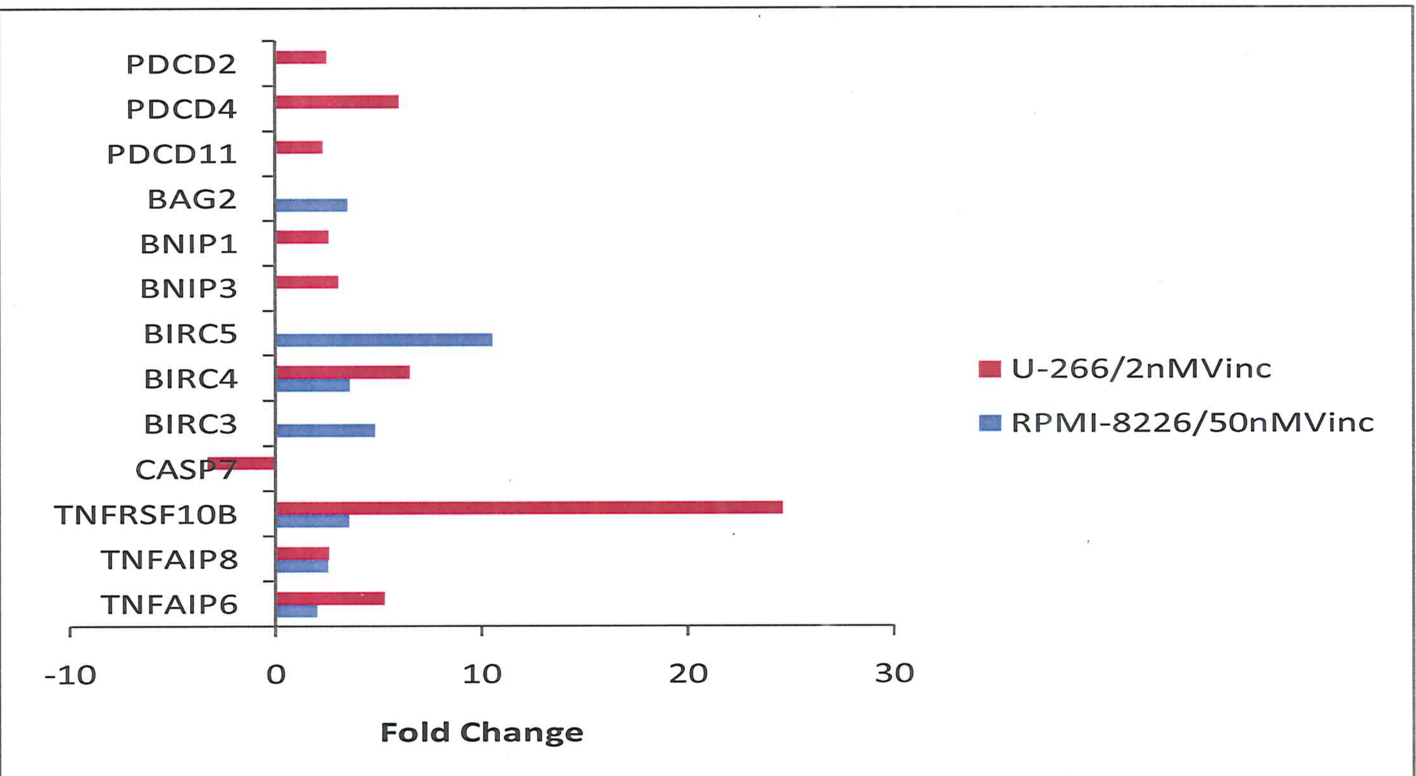


Figure 3.55 Expression levels of apoptosis and death associated genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines

Table 3.21 Expression levels of apoptosis and death associated genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/50nMVinc Fold Change	U-266/2nMVinc Fold Change
206026_s_at	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	+2.05	+5.31
208296_x_at	TNFAIP8	tumor necrosis factor, alpha-induced protein 8	+2.54	+2.62
209295_at	TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	+3.58	+24.59
207181_s_at	CASP7	caspase 7, apoptosis-related cysteine peptidase	NS	-3.27
210538_s_at	BIRC3	baculoviral IAP repeat-containing 3	+4.85	NS
225858_s_at	BIRC4	baculoviral IAP repeat-containing 4	+3.63	+6.54
202094_at	BIRC5	baculoviral IAP repeat-containing 5 (survivin)	+10.55	NS
201848_s_at	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	NS	+3.05
207829_s_at	BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1	NS	+2.60

Table 3.21 continued

209406_at	BAG2	BCL2-associated athanogene 2	+3.55	NS
212422_at	PDCD11	programmed cell death 11	NS	+2.31
212593_s_at	PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)	NS	+6.02
228420_at	PDCD2	programmed cell death 2	NS	+2.51

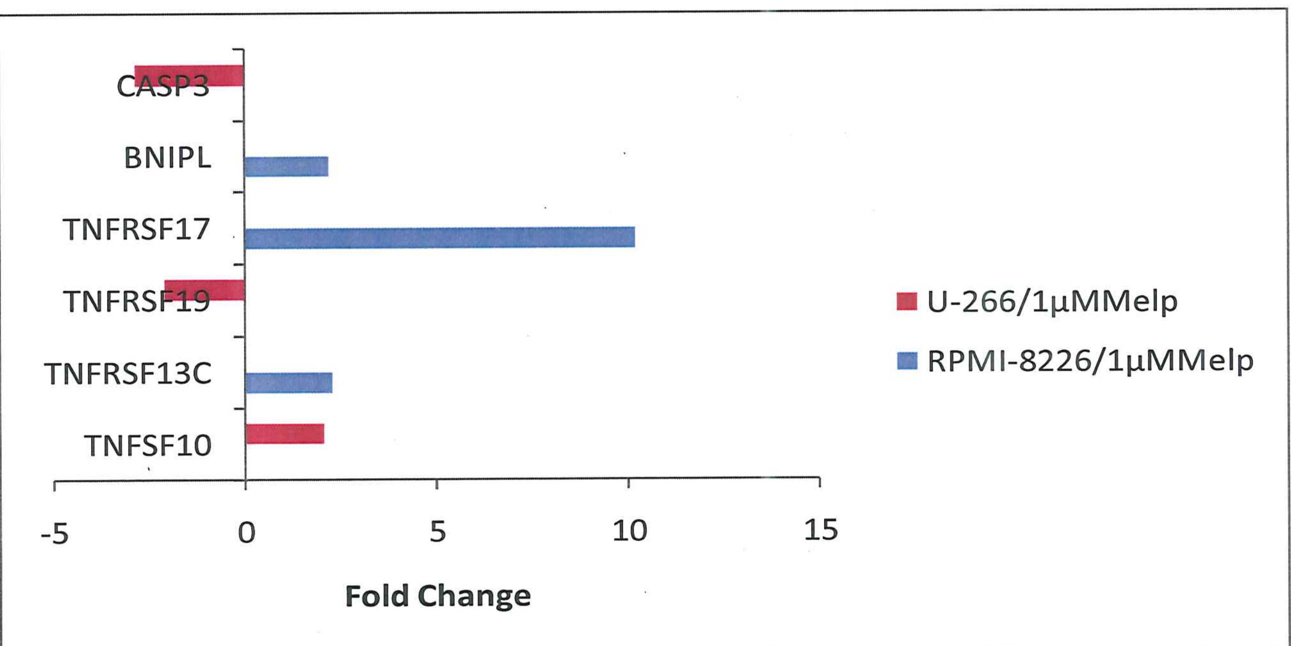


Figure 3.56 Expression levels of apoptosis and death associated genes in RPMI-8226/1µMMelp and U-266/1µMMelp cell lines

Table 3.22 Expression levels of apoptosis and death associated genes in RPMI-8226/1µMMelp and U-266/1µMMelp cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/1µMelp Fold Change	U-266/Melp Fold Change
214329_x_at	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	NS	+2.04
1552892_at	TNFRSF13C	tumor necrosis factor receptor superfamily, member 13C	+2.28	NS
224090_s_at	TNFRSF19	tumor necrosis factor receptor superfamily, member 19	NS	-2.12
206641_at	TNFRSF17	tumor necrosis factor receptor superfamily, member 17	+10.19	NS
236534_at	BNIPL	BCL2/adenovirus E1B 19kD interacting protein like	+2.18	NS
202763_at	CASP3	caspase 3, apoptosis-related cysteine peptidase	NS	-2.86

3.7.4.8 Cytochrome Related Gene Expressions

Figures 3.57-3.59 show the altered gene expression levels of cytochrome related genes. Cytochrome b/b6 is the main subunit of transmembrane cytochrome bc1 and b6f complexes. In the mitochondrion of eukaryotes and in aerobic prokaryotes, cytochrome b is a component of respiratory chain complex III- also known as the bc1 complex or ubiquinal-cytochrome c reductase which are involved in electron transport and generation of ATP (Blakely *et al.*, 2005). CYB5D2 gene was 3 fold downregulated in both of the prednisone resistant MM cell lines where as CYB5R4 gene was 5 and 2 fold upregulated in RPMI-8226/500μMPred and U-266/300μMPred respectively (Table 3.23). CYB561 gene was 3 fold upregulated in both of the vincristine resistant MM cell lines whereas CYB5A was downregulated in both (Table 3.24). On the other hand, CYBB which is the beta chain of cytochrome b and has a role in the microbicidal oxidase system of phagocytes was 13 fold upregulated in melphalan resistant RPMI-8226 subline whereas CYB5D1 was only 2 fold upregulated in U-266/1μMMelp subline (Table 3.25). Overexpression of CYB5R4, CYB561, CYBB and CYB5D1 genes may have been a consequence of increase in ATP requirement to survive under drug stress.

Cytochrome P450 oxidases (CYP) encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids (Danielson, 2002). Many drugs may either inducing the biosynthesis of an isoenzyme (enzyme induction) or by directly inhibiting the CYP (enzyme inhibition). According to Table 3.23 CYP1A2 and CYP27A1 were only upregulated in prednisone resistant RPMI-8226 cell line whereas CYP51A1, CYP4V2, CYP11B2 and CYP17A1 were downregulated. CYP1A2 is related with drug and steroid metabolism and CYP27A1 is an enzyme involved in biosynthesis of bile acids (Coli *et al.*, 1991). The structure of prednisone is similar to bile acids (cholic acid, chenodeoxycholic acid, glycocholic acid, taurocholic acid and deoxycholic acid) which may explain its upregulation in prednisone resistant RPMI-8226 cell line. In contrast; CYP51A1, CYP26A1 and CYP4V2 were significantly upregulated in prednisone resistant

U-266 cell line (Table 3.23). CYP51A1 converts lanosterol to cholesterol. It is a target for antifungal drugs, inhibiting the production of ergosterol. Overexpression of CYP51A1 can lead to resistance to antifungal agents (Vanden *et al.*, 1998). CYP26A1 regulates the cellular level of retinoic acid which is involved in regulation of gene expression in both embryonic and adult tissues. It has been shown to be implicated in clinically acquired resistance to ATRA (Idres *et al.*, 2005). CYP4V2 is implicated in the metabolism of fatty acid precursors into n-3 polyunsaturated fatty acids. In one of the studies it was shown that CYP4V2 expression was detected in lymph node metastasis in colorectal cancer (Kumarakulasingham *et al.*, 2005). These results show that non of the CYP gene expression patterns were common for prednisone resistant RPMI-8226 and U-266 MM cell lines. These results were similar for vincristine and melphalan resistances as seen from Table 3.24 and Table 3.25. Different CYP gene expressions were exist for vincristine and melphalan resistant RPMI-8226 and U-266 cell lines. This can be because of the individual differences in drug metabolism.

Cyclooxygenase (COX), also known as prostoglandin-endoperoxide synthase (PTGS), is a key regulatory enzyme in the synthetic pathway of eicosanoid production. Eicosanoids are responsible for multiple inflammatory, mitogenic and angiogenic activities in various tissue and organ systems (Khanapure *et al.*, 2007). COX expression has been implicated in colon carcinoma. Initially Tsujii and DuBois studied the effects of COX. COX-2 overexpression was increased adhesion and inhibited apoptosis, enhancing the tumorigenic potential (Tsujii *et al.*, 1995). Different types of COX genes were upregulated in all of the drug resistant multiple myeloma cell lines which may be correlated with resistance to apoptosis and MDR phenotype (Tables 3.23-3.25).

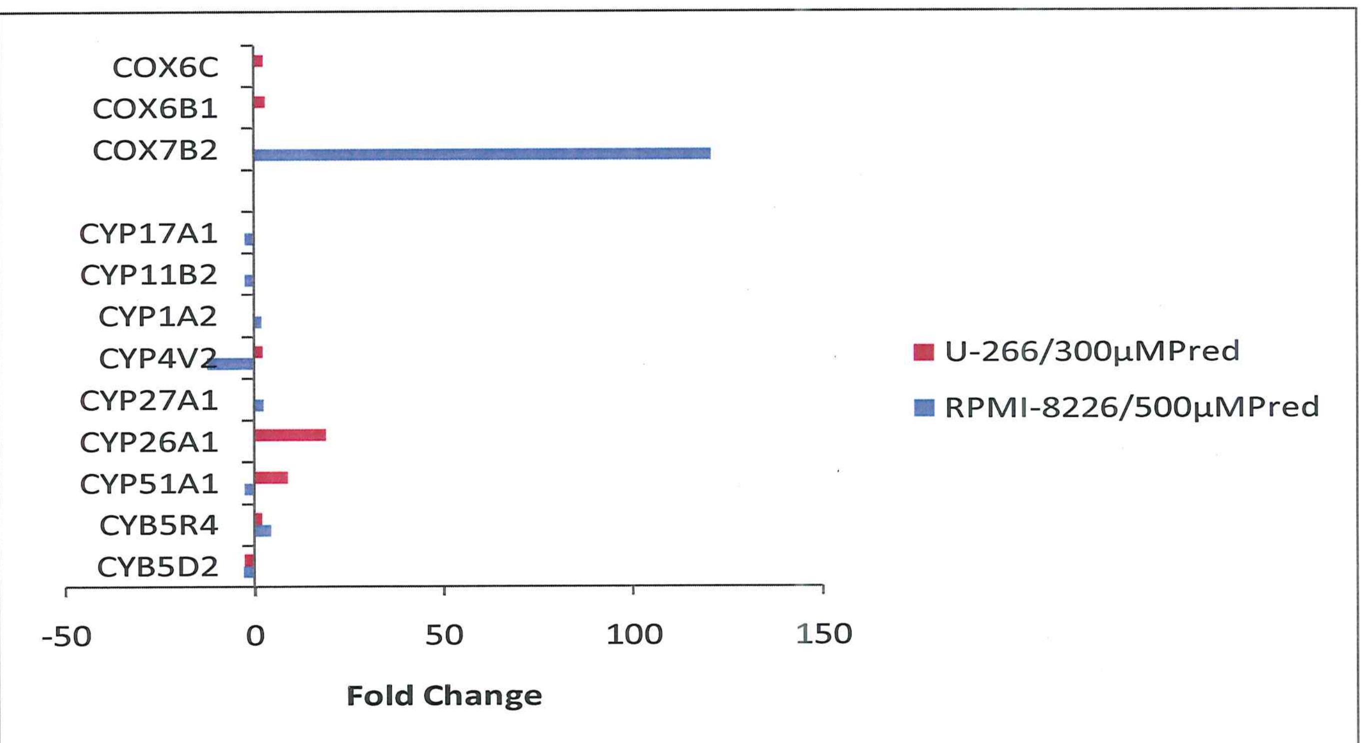


Figure 3.57 Expression levels of cytochrome related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines

Table 3.23 Expression levels of cytochrom related genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/500µMPred Fold Change	U-266/300µMPred Fold Change
225804_at	CYB5D2	cytochrome b5 domain containing 2	-2.69	-2.50
219079_at	CYB5R4	cytochrome b5 reductase 4	+4.50	+2.14
202314_at	CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	-2.54	+9.00
206424_at	CYP26A1	cytochrome P450, family 26, subfamily A, polypeptide 1	NS	+19
203979_at	CYP27A1	cytochrome P450, family 27, subfamily A, polypeptide 1	+2.60	NS
226745_at	CYP4V2	cytochrome P450, family 4, subfamily V, polypeptide 2	-12.29	+2.26
207609_s_at	CYP1A2	cytochrome P450, family 1, subfamily A, polypeptide 2	+2.18	NS

Table 3.23 continued

214630_at	CYP11B2	cytochrome P450, family 11, subfamily B, polypeptide 2	-2.25	NS
205502_at	CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide 1	-2.39	NS
231265_at	COX7B2	cytochrome c oxidase subunit VIIb2	+120.7	NS
201441_at	COX6B1	cytochrome c oxidase subunit Vib polypeptide 1 (ubiquitous)	NS	+3.11
201754_at	COX6C	cytochrome c oxidase subunit VIc	NS	+2.49

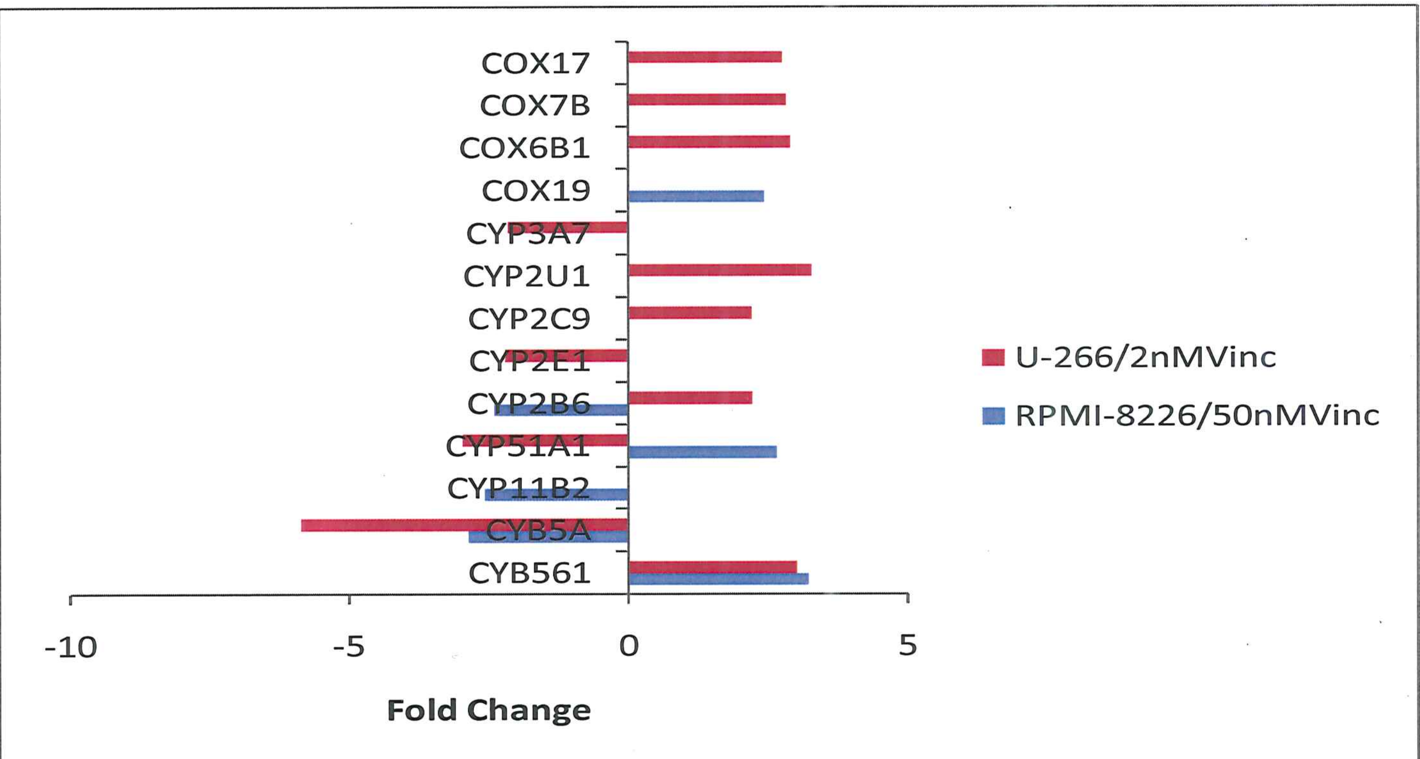


Figure 3.58 Expression levels of cytochrome related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines

Table 3.24 Expression levels of cytochrom related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/50nMVinc Fold Change	U-266/2nMVinc Fold Change
209164_s_at	CYB561	cytochrome b-561	+3.24	+3.03
209366_x_at	CYB5A	cytochrome b5 type A (microsomal)	-2.86	-5.85
214630_at	CYP11B2	cytochrome P450, family 11, subfamily B, polypeptide 2	-2.56	NS
202314_at	CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	+2.67	-2.96
206754_s_at	CYP2B6	cytochrome P450, family 2, subfamily B, polypeptide 6	-2.39	+2.23
209975_at	CYP2E1	cytochrome P450, family 2, subfamily E, polypeptide 1	NS	-2.20
220017_x_at	CYP2C9	cytochrome P450, family 2, subfamily C, polypeptide 9	NS	+2.21
226393_at	CYP2U1	cytochrome P450, family 2, subfamily U, polypeptide 1	NS	+3.29

Table 3.24 continued

205939_at	CYP3A7	cytochrome P450, family 3, subfamily A, polypeptide 7	NS	-2.15
235533_at	COX19	COX19 cytochrome c oxidase assembly homolog (S. cerevisiae)	+2.44	NS
201441_at	COX6B1	cytochrome c oxidase subunit Vib polypeptide 1 (ubiquitous)	NS	+2.90
202110_at	COX7B	cytochrome c oxidase subunit VIIb	NS	+2.84
203880_at	COX17	COX17 cytochrome c oxidase assembly homolog (S. cerevisiae)	NS	+2.77

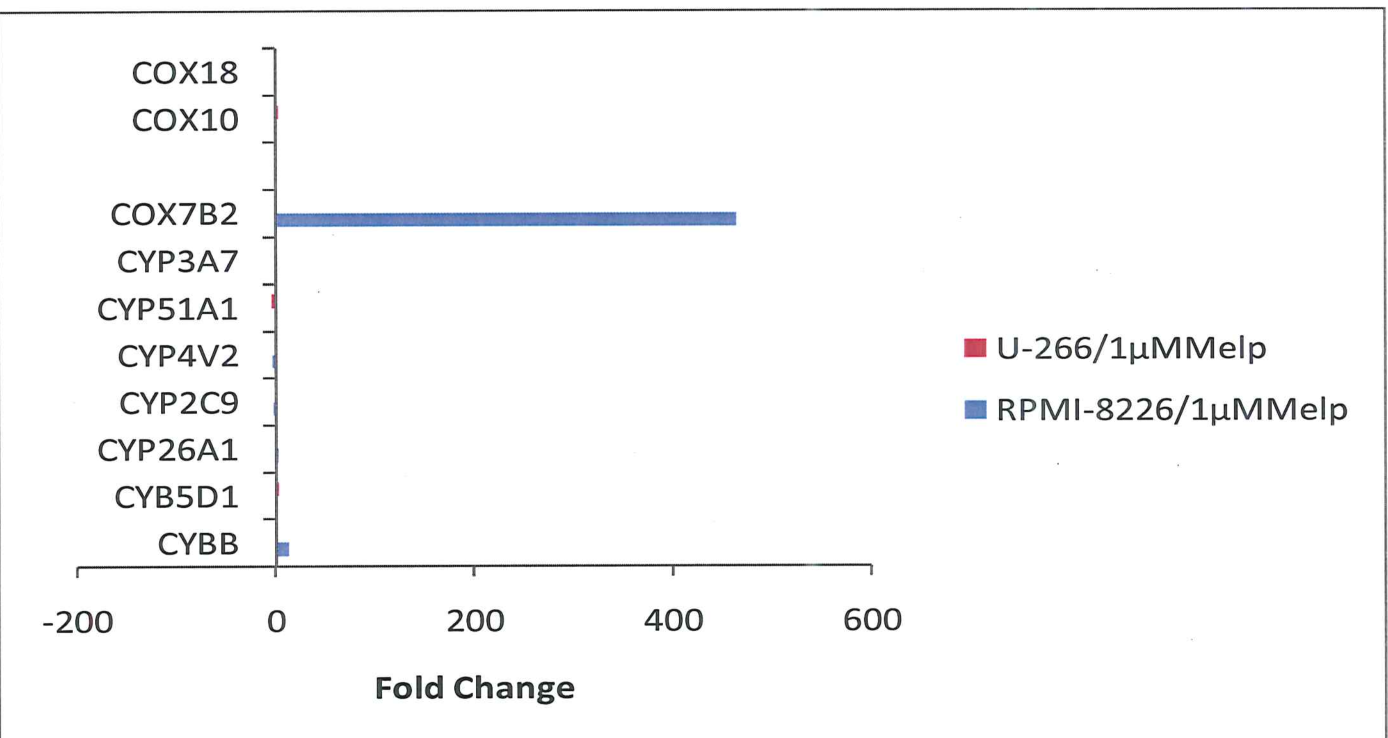


Figure 3.59 Expression levels of cytochrome related genes in RPMI-8226/1µMMelp and U-266/1µMMelp cell lines

Table 3.25 Expression levels of cytochrom related genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/1μMMelp Fold Change	U-266/1μMMelp Fold Change
203923_s_at	CYBB	cytochrome b-245, beta polypeptide (chronic granulomatous disease)	+13.17	NS
226833_at	CYB5D1	cytochrome b5 domain containing 1	NS	+2.46
206424_at	CYP26A1	cytochrome P450, family 26, subfamily A, polypeptide 1	+2.41	NS
216661_x_at	CYP2C9	cytochrome P450, family 2, subfamily C, polypeptide 9	-2.26	NS
226745_at	CYP4V2	cytochrome P450, family 4, subfamily V, polypeptide 2	-2.46	NS
202314_at	CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	NS	-3.78
211843_x_at	CYP3A7	cytochrome P450, family 3, subfamily A, polypeptide 7	NS	+2.17
231265_at	COX7B2	cytochrome c oxidase subunit VIIb2	+464.64	NS

Table 3.25 continued

203858_s_at	COX10	COX10 homolog, cytochrome c oxidase assembly protein, heme A: farnesyltransferase (yeast)	NS	+2.94
227442_at	COX18	COX18 cytochrome c oxidase assembly homolog (S. cerevisiae)	NS	+2.29

3.7.4.9 ABC Transporter Gene Expressions

Altered gene expressions of ABC transporter family members were given schematically in Figures 3.60-3.62. ABCA2 is a transporter highly expressed in the cells of the nervous and hematopoetic systems, is associated with lipid transport and drug resistance in cancer cells, including tumor stem cells (Mack *et al.*, 2008). ABCA9 involves in monocyte differentiation and macrophage lipid homeostasis. According to expression profile in Table 3.26, ABCA2 gene was 3 fold overexpressed in RPMI-8226/500μMPred subline while ABCA9 gene was 2 fold overexpressed in U-266/300μMPred subline. Since prednisone belongs to corticosteroid group of compounds its transport is meaningful by ABCA group of transporters.

MDR1 (ABCB1) was overexpressed in both of the prednisone resistant MM cell lines (Table 3.26). In the literature, decreased cytoplasmic glucocorticoid concentration secondary to increased p-glycoprotein-mediated efflux of glucocorticoid from target cells due to overexpression of the MDR1 gene was shown (Farrel *et al.*, 2003).

ABCB5 participate in ATP-dependent transmembrane transport of structurally diverse molecules ranging from small ions, sugars and peptides to more complex organic molecules and it is related to doxorubicin resistance in human malignant melanoma (Frank *et al.*, 2005). ABCB10 is a mitochondrial inner membrane erythroid transporter involved in heme biosynthesis (Graf *et al.*, 2004). ABCB5 gene was 2 fold downregulated in prednisone resistant RPMI-8226 subline while ABCB10 was 4 fold downregulated in U-266/300μMPred (Table 3.26).

Overexpression of MRP1 (ABCC1) gene was observed for the prednisone resistant RPMI-8226 cell line (Table 3.26).

ABCG1 is involved in macrophage cholesterol and phospholipid transport. It is linked to both obesity and atherosclerosis but not linked to MDR phenotype (Van Veen *et al.*, 1997). ABCG1 was downregulated in both of the prednisone resistant

multiple myeloma cell lines (Table 3.26). In one of the studies it was shown that some of the drug resistant acute myeloblastic leukemia patients had high level of cholesterol (Pallis *et al.*, 2004).

ABCD2 is a member of the ALD subfamily, which is involved in peroxisomal import of fatty acids and/or fatty acyl-CoAs in the organelle. ABCD2 gene is overexpressed 10 fold only in prednisone resistant U-266 cell line (Table 3.26). ABCD2 gene is not related with drug resistance but the loss of abcd2 results in oxidative stress providing a clue to its cellular function (Lu *et al.*, 2007).

According to Table 3.27, it is seen that MDR1 was highly overexpressed in vincristine resistant RPMI-8226 and U-266 cell lines. ABCC1 gene was only overexpressed in RPMI-8226/50nMVinc cell line and ABCG2 gene which is involved in resistance to mitoxantrone and anthracycline resistance, was downregulated in both of the vincristine resistant multiple myeloma cell lines.

ABCB9 gene was 3 fold overexpressed in vincristine resistant U-266 cell line (Table 3.27). The function of this half-transporter has not yet been determined; however, this protein may play a role in lysosomes. Alternative splicing of this gene results in distinct isoforms which are likely to have different substrate specifications. Also ABCD2 gene was 6 fold overexpressed in this subline. On the other hand, ABCA1 which is a major regulator of cellular cholesterol and phospholipid homeostasis was drastically downregulated (43 fold) in U-266/2nMVinc cell line (Table 3.27).

ABCA6 gene was 2 fold downregulated in melphalan resistant RPMI-8226 cell line whereas 2 fold overexpressed in melphalan resistant U-266 cell line (Table 3.28). It plays a role in macrophage lipid homeostasis and this gene is clustered among 4 other ABC1 family members on 17q24 (ABCA5, ABCA8, ABCA9 and ABCA10). The difference of this gene expression among two multiple myeloma cell lines can be due to this clustered or because of the individual differences since these two cell lines were isolated from two different multiple myeloma patients. ABCB1 gene was overexpressed in both of the melphalan resistant MM cell lines (Table 3.28).

ABCC12 and ABCC8 genes were 2 fold overexpressed in U-266/1μMMelp and RPMI-8226/1μMMelp sublines respectively (Table 3.28). ABCC12 gene is a member of the MRP subfamily which is involved in multi-drug resistance. This gene is arranged head-to-tail with ABCC11 on chromosome 16q12.1 (Kruh *et al.*, 2007). ABCC8 functions as a modulator of ATP-sensitive potassium channels and insulin release. Mutations and deficiencies of this protein have been observed in patients with hyperinsulinemic hypoglycemia of infancy (Klupa *et al.*, 2008). ABCC1 gene was only 4 fold overexpressed in RPMI-8226/1μMMelp subline (Table 3.28).

ABCG4 gene was also 2 fold overexpressed in melphalan resistant U-266 subline (Table 3.28). This protein is a member of the white subfamily and is expressed predominantly in liver tissue. The function has not yet been determined but may involve in cholesterol transport.

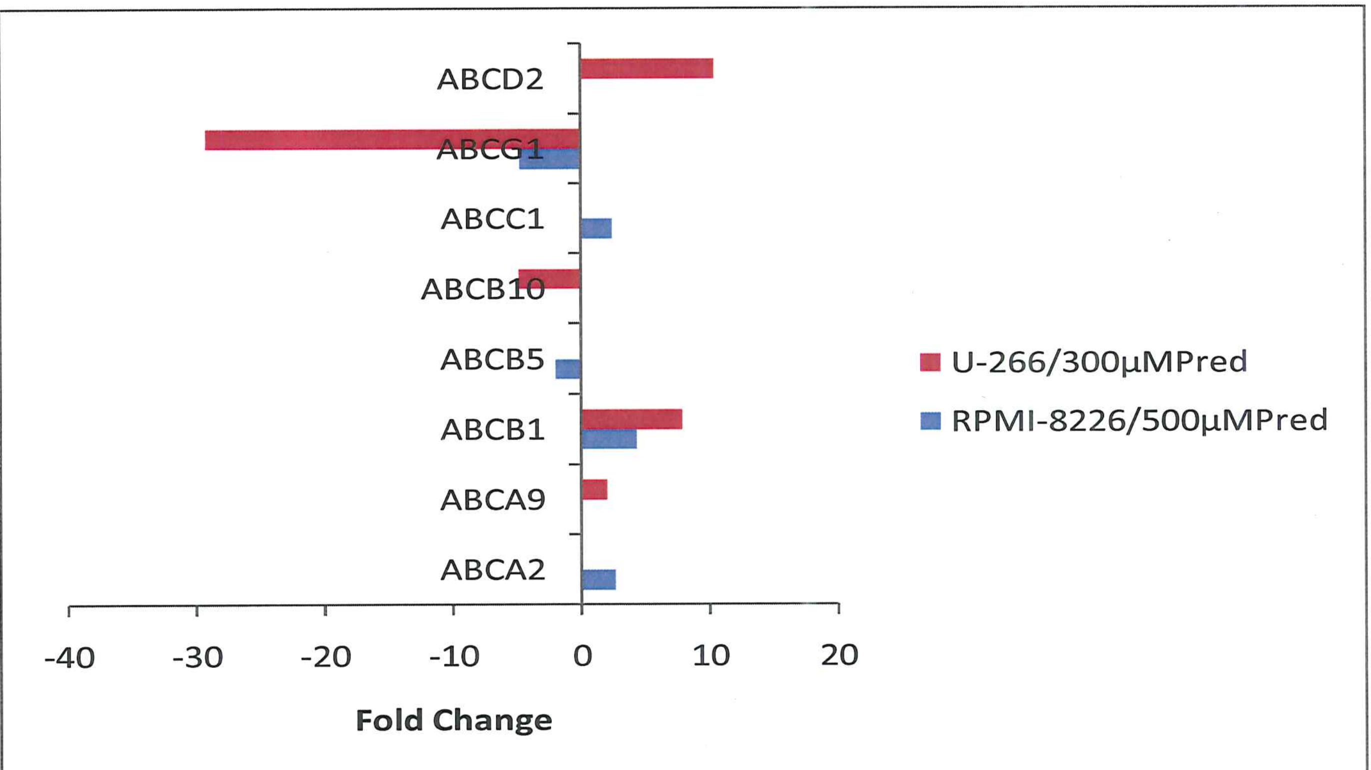


Figure 3.60 Expression levels of ABC transporter genes in RPMI-8226/500µMPred and U-266/300µMPred cell lines

Table 3.26 Expression levels of ABC transporter related genes in RPMI-8226/500μMPred and U-266/300μMPred cell lines with respect to control

Gene Name	**Gene Symbol**	**Description**	**RPMI-8226/500μMPred Fold Change**	**U-266/300μMPred Fold Change**
210100_s_at	ABCA2	ATP-binding cassette, sub-family A (ABC1), member 2	+2.6	NS
242541_at	ABCA9	ATP-binding cassette, sub-family A (ABC1), member 9	NS	+2
209993_at	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	+4.27	+7.87
243162_at	ABCB5	ATP-binding cassette, sub-family B (MDR/TAP), member 5	-2	NS
223320_s_at	ABCB10	ATP-binding cassette, sub-family B (MDR/TAP), member 10	NS	-4.82
202804_at	ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	+2.4	NS
204567_s_at	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	-4.72	-29.24

Table 3.26 continued

207583_at	ABCD2	ATP-binding cassette, sub-family D (ALD), member 2	NS	+10.41

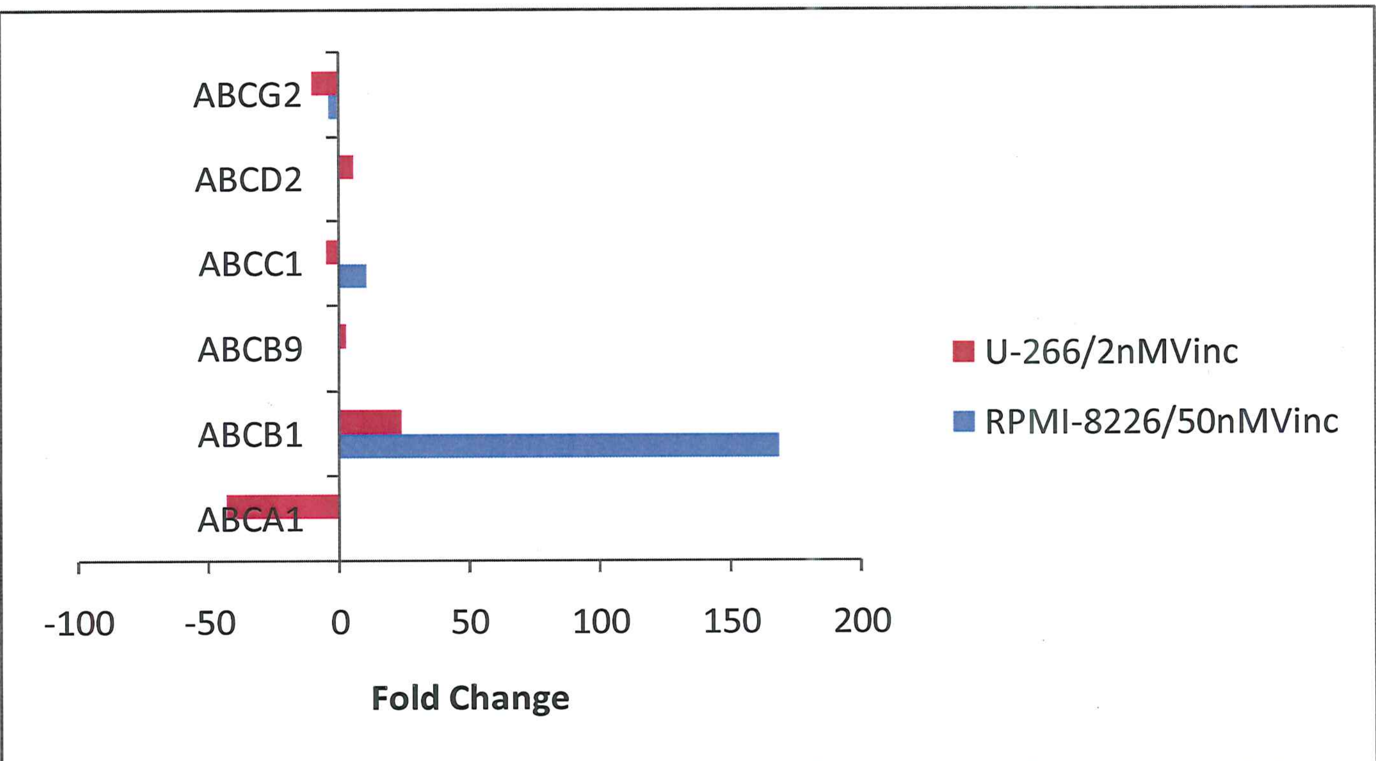


Figure 3.61 Expression levels of ABC transporter genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines

Table 3.27 Expression levels of ABC transporter related genes in RPMI-8226/50nMVinc and U-266/2nMVinc cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/50nMVinc Fold Change	U-266/2nMVinc Fold Change
203504_s_at	ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	NS	-43.11
209993_at	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	+168.89	+9.41
214209_s_at	ABCB9	ATP-binding cassette, sub-family B (MDR/TAP), member 9	NS	+2.77
202805_s_at	ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	+9.79	-4.75
207583_at	ABCD2	ATP-binding cassette, sub-family D (ALD), member 2	NS	+5.61
209735_at	ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	-3.73	-10.33

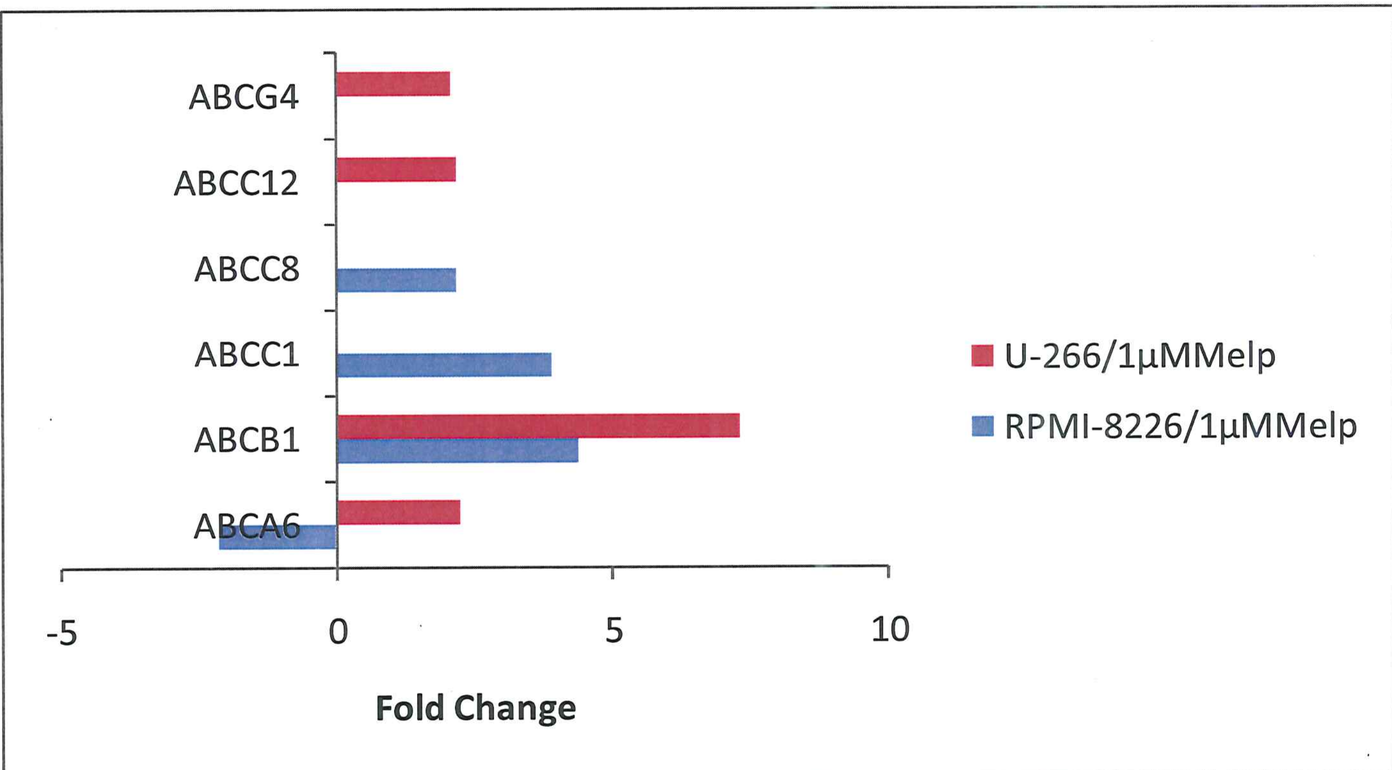


Figure 3.62 Expression levels of ABC transporter genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines

Table 3.28 Expression levels of ABC transporter related genes in RPMI-8226/1μMMelp and U-266/1μMMelp cell lines with respect to control

Gene Name	Gene Symbol	Description	RPMI-8226/1μmMelp Fold Change	U-266/1μMMelp Fold Change
217504_at	ABCA6	ATP-binding cassette, sub-family A (ABC1), member 6	-2.14	+2.23
209993_at	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	+4.38	+7.30
202805_s_at	ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	+3.89	NS
210246_s_at	ABCC8	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	+2.18	NS
1552590_a_at	ABCC12	ATP-binding cassette, sub-family C (CFTR/MRP), member 12	NS	+2.18
207593_at	ABCG4	ATP-binding cassette, sub-family G (WHITE), member 4	NS	+2.07

3.8 PROLIFERATION ASSAY FOR CROSS-RESISTANCE TO VINCRISTINE ON PREDNISONE AND MELPHALAN RESISTANT CELL LINES

Vincristine/prednisone and vincristine/melphalan drug combinations tested for cross-resistance at RPMI-8226 and U-266 cell lines. Figure 3.63 shows the effect of vincristine on original and prednisone resistant RPMI-8226 cell lines whereas Figure 3.64 shows the effect of vincristine on original and melphalan resistant RPMI-8226 cells.

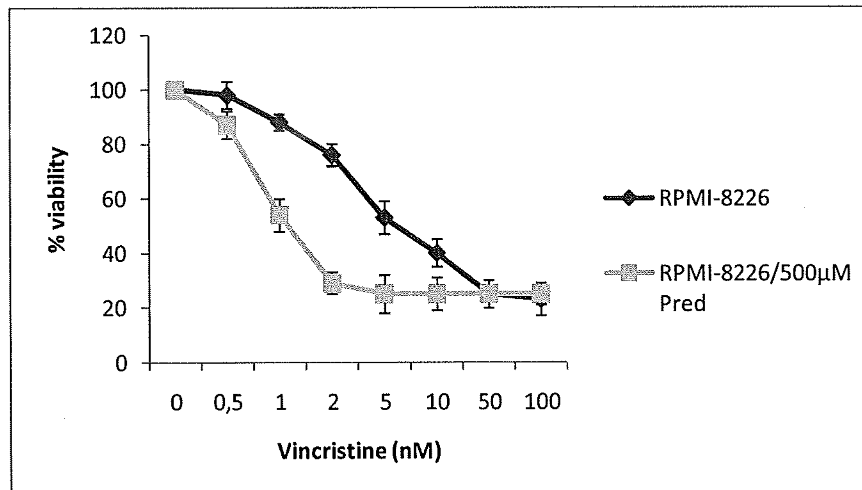


Figure 3.63 Effect of vincristine on RPMI-8226 and RPMI-8226/500µMPred cell lines.

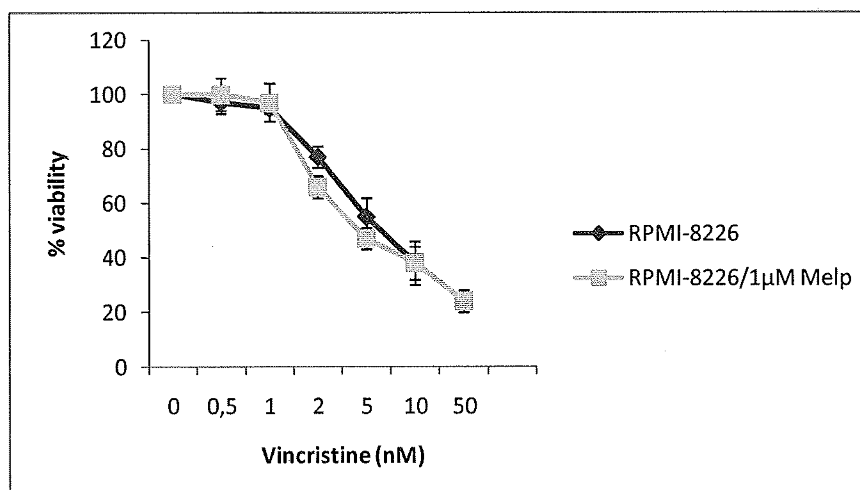


Figure 3.64 Effect of vincristine on RPMI-8226 and RPMI-8226/1μMMelp cell lines.

IC_{50} value of vincristine on original RPMI-8226 cells is 6.14nM while IC_{50} value for RPMI-8226/500μMPred is 1.16nM (Figure 3.63) and IC_{50} value of vincristine on original and melphalan resistant RPMI-8226 cells is 6.45nM and 4.06 respectively (Figure 3.64).

The effect of vincristine on original, prednisone resistant and original, melphalan resistant U-266 cell lines were shown in Figure 3.65 and Figure 3.66.

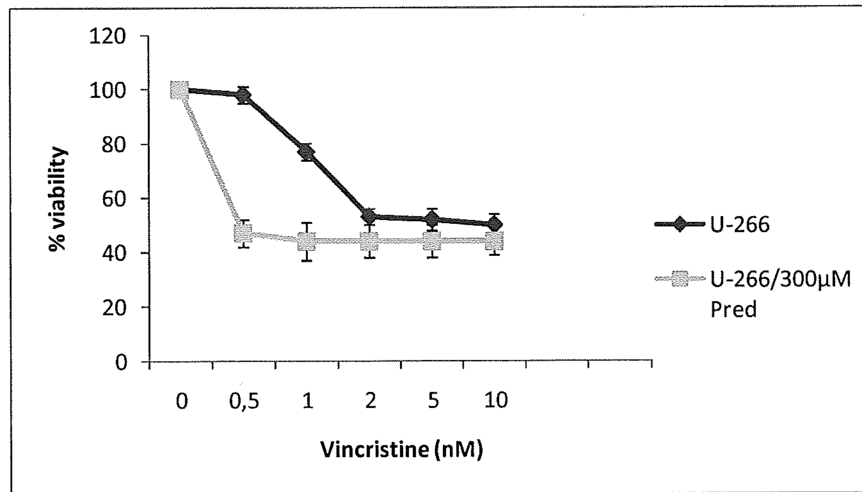


Figure 3.65 Effect of vincristine on U-266 and U-266/300µMPred cell lines.

As it is calculated from Figure 3.65 IC_{50} value of vincristine on original U-266 cells is 10nM while IC_{50} value for U-266/300µMPred is 0.5nM. On the other hand IC_{50} value of vincristine on original U-266 cells is 10nM and IC_{50} value for U-266/1µMMelp is 2nM (Figure 3.66).

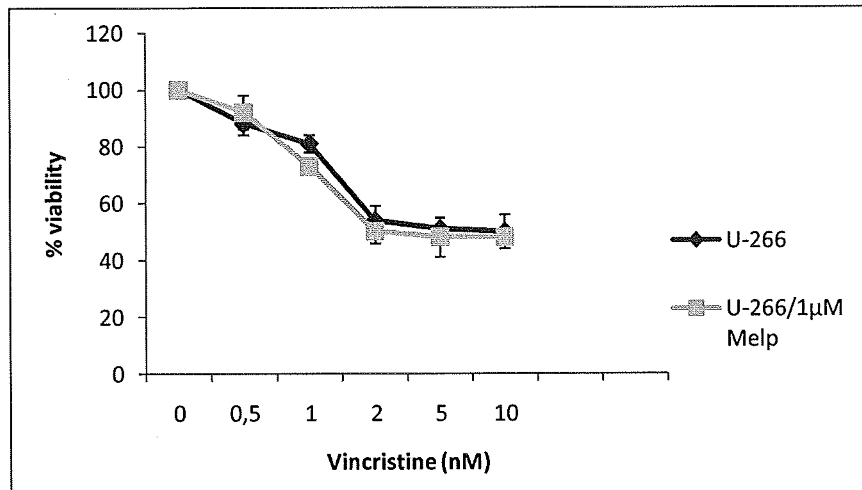


Figure 3.66 Effect of vincristine on U-266 and U-266/1µMMelp cell lines.

According to our XTT cell viability assay results in the presence of vincristine, both of the prednisone resistant RPMI-8226 and U-266 cell lines were 5 fold ($p<0.05$)

and 20 fold ($p<0.05$) more sensitive to vincristine with respect to their control cell lines. On the other hand also melphalan resistant RPMI-8226 and U-266 cell lines were 2 fold ($p<0.05$) and 5 fold ($p<0.05$) more sensitive to vincristine with respect to their controls. ACT2 gene was upregulated in both of the melphalan resistant MM cell lines. Platelet growth factor genes were overexpressed in prednisone and melphalan resistant RPMI-8226 cell lines as well as in melphalan resistant U-266 cell line. Both Akt and platelet-derived growth factor was shown to allow a cell to skip the G1 checkpoints in order to divide. Vincristine is an alkylating agent that arrests the cells at G_2M phase, so prednisone and melphalan resistant MM cell lines that were overexpressed ACT2 and PDGF related genes were became more sensitive to vincristine with respect to their original cells.

These results show that for both of the cell lines there is no cross-resistance between vincristine/prednisone and vincristine/melphalan combinations. The usage of vincristine on prednisone and melphalan resistant multiple myeloma cell lines was increase the efficacy of chemotherapy.

3.9 PROLIFERATION ASSAY FOR CROSS-RESISTANCE TO COBALT-60 GAMMA (γ) RADIATION

Original and resistant RPMI-8226 and U-266 cell lines exposed to 200 and 800cGy gamma radiation and tested for their cross-resistance to radiation. Figure 3.67 and 3.68 show the effect of radiation on RPMI-8226 and U-266 group cells respectively. The figures was determined by using the results of XTT assay for each cell line in 2 independent experiments and each point is repeated twice.

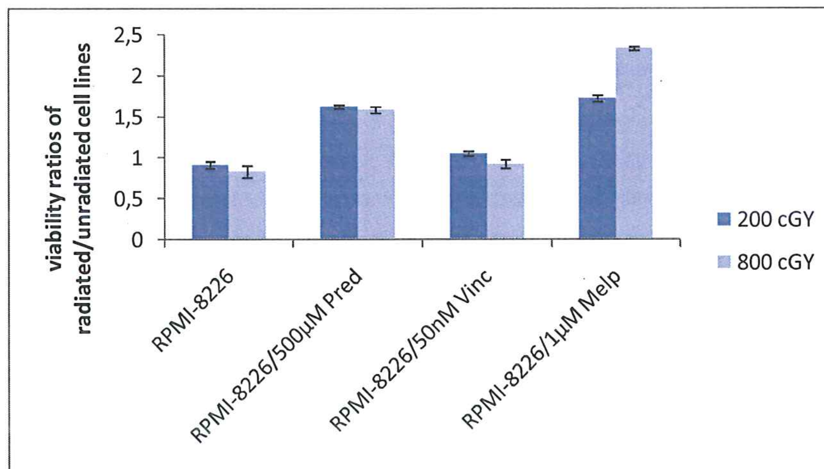


Figure 3.67 Effect of 200 and 800cGy γ radiation on viability of RPMI-8226 original and drug resistant sublines.

RPMI-8226 subline which is resistant to 500μM prednisone show 2 fold ($p<0.05$) resistance to both 200 and 800 cGy γ radiation with respect to its original control whereas RPMI-8226/50nMVinc subline is sensitive to radiation. On the other hand RPMI-8226/1μMMelp resistant cell line is 2 fold ($p<0.05$) and 2.5 fold ($p<0.05$) resistant to 200cGy and 800cGy γ radiation respectively.

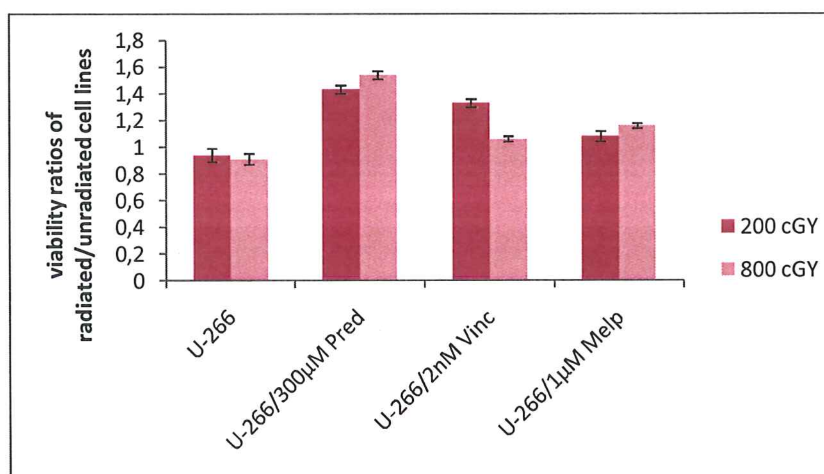


Figure 3.68 Effect of 200 and 800cGy γ radiation on viability of U-266 sensitive and drug resistant sublines.

Radiation effect is different for U-266/1μMMelp subline than RPMI-8226/1μMMelp cells. U-266/1μMMelp cells only show 1.3 fold ($p<0.05$) resistance to 800cGy γ-radiation. However, the results are similar for vincristine and prednisone resistant sublines. U-266/300μMPred cells are 1.7 fold ($p<0.05$) resistant to γ radiation. On the other hand U-266/2nMVinc subline only show 1.4 fold ($p<0.05$) resistance to 200cGy radiation whereas it is sensitive to 800cGy.

The results from these experiments show that vincristine resistant RPMI-8226/50nMVinc cell line do not show any cross-resistance with radiation similar to U-266/2nMVinc cell line. These results are correlated the other studies in the literature using paclitaxel which has the similar effect of vincristine (Milas *et al.*, 1994; Liebmann *et al.*, 1994; Raitanen *et al.*, 2002; Gupta *et al.*, 1997; Jaakkola *et al.*, 1996; Choy *et al.*, 1993; Milross *et al.*, 1997). Their results show that paclitaxel enhanced the tumor radioresponse since it is a chemotherapeutic agent with potent microtubule stabilizing activity that arrests cells in G_2M phase. Because G_2 and M are the most radiosensitive phases of the cell cycle, paclitaxel has potential as a cell cycle-specific radiosensitizer (Milas *et al.*, 1994). Vincristine is also a chemotherapeutic agent that stabilize microtubule activity like paclitaxel. In our study, vincristine resistant multiple myeloma cell lines when exposed to gamma radiation do not show any cross-resistance and vincristine resistant sublines are radiosensitive as their non-drug applied RPMI-8226 and U-266 versions.

The mechanisms responsible for the cross-resistance between radiation and melphalan have been examined in human ovarian cancer cell lines (Ozols *et al.*, 1988). Cell lines with resistance induced *in vitro* to melphalan have increased cellular levels of glutathione (GSH) compared with drug sensitive cell line and were cross-resistant to radiation. In addition depletion of GSH levels in cell lines with acquired resistance to melphalan led to a marked sensitization of these cells to irradiation (Ozols *et al.*, 1988). The results from our experiment show that RPMI-8226/1μMMelp resistant cell line is 2 fold ($p<0.05$) and 2.5 ($p<0.05$) fold resistant to 200cGy and 800cGy γ radiation respectively. However, these results were not

similar with that of U-266/1μMMelp subline since it is only 1.3 fold ($p<0.05$) resistant to 800cGy γ radiation. This can be due to different GSH levels of these two cell lines.

In the literature, there are not many studies about the effect of radiation and glucocorticoids. However in our study, we found that both prednisone resistant RPMI-8226/500μMPred and U-266/300μMPred multiple myeloma cell lines are cross-resistant to 2 fold ($p<0.05$) and 1.7 fold ($p<0.05$) resistant to applied 800cGy γ radiation.

CHAPTER 4

CONCLUSIONS

Multidrug resistance describes a complex phenotype whose predominant feature is resistance to a wide range of structurally unrelated cytotoxic compounds after exposure to a single cytotoxic agent. This phenotype is a major obstacle to successful chemotherapy.

In this study, prednisone (RPMI-8226/500µMPred, U-266/300µMPred), vincristine (RPMI-8226/50nMVinc, U-266/2nMVinc) and melphalan (RPMI-8226/1µMMelp, U-266/1µMMelp) resistant sublines of multiple myeloma were generated from the original RPMI-8226 and U-266 cell lines. Meanwhile, prednisone and melphalan resistant MM cell lines developed cross-resistance to γ irradiation whereas vincristine resistant ones did not. However, prednisone and melphalan resistant MM cell lines did not show cross-resistance to vincristine. The usage of vincristine on prednisone and melphalan resistant multiple myeloma cell lines increased the efficacy of chemotherapy.

There are multiple mechanisms of drug resistance. Therefore, adressing a single mechanism at the cellular level is insufficient. So microarray analysis, in combination with RT-PCR was performed in this study to answer the question what the molecular mechanisms at prednisone, vincristine and melphalan resistance in multiple myeloma are.

According to our results, MDR1 gene seems one of the most important mechanism causing the multidrug resistance phenotype in prednisone, vincristine and melphalan resistant RPMI-8226 and U-266 cell lines. However, upregulation of MRP1 gene seems one of the mechanisms of drug resistance for prednisone, vincristine and melphalan resistant RPMI-8226 cell line only.

Cell adhesion also has been shown to prevent cell death through a number of mechanisms so extracellular matrix related gene expressions are important. However there are not much *in vitro* studies related to cell adhesion mediated drug resistance on hematological malignancies. According to our *in vitro* studies, the genes encoding the integrins were overexpressed in all of the drug resistant MM cell lines except U-266/1µMMelp which shows increased interaction with fibronectin. Laminin related genes drastically increased in prednisone resistant RPMI-8226 and U-266 cell lines which indicates invasive property. Collagen21A1 was only downregulated in prednisone, vincristine and melphalan resistant RPMI-8226 cell line.

Activation of proto-oncogenes and inactivation of tumor suppressor genes are important in cancer and multidrug resistance development. According to our results, the oncogenes which belong to ras superfamily, especially rho family GTPases were upregulated in prednisone resistant MM cell lines. However the ras superfamily gene expression seems not to be correlated with vincristine and melphalan resistance. However, NF-κB overexpressed only in vincristine and melphalan resistant MM cell lines. YES1 and ACT2 genes which play role in cell cycle were both upregulated in both of the melphalan resistant MM cell lines which can be an indicator to melphalan resistance.

Transforming growth factor beta receptor which is a tumor suppressor gene was downregulated in both of the prednisone resistant MM cell lines and epidermal growth factor, EPS15 which has a role in cell proliferation was overexpressed. These growth factors can be important for prednisone resistance. On the other hand, platelet-derived growth factor, having important role in cell proliferation, cell migration and angiogenesis was overexpressed in all of the drug applied RPMI-8226 cell lines and also in melphalan resistant U-266 cell line indicating the importance of this growth factor in melphalan resistance.

An additional mechanism which is important for drug resistance in multiple myeloma is related to JAK-STAT signaling pathway. Different types of interleukin

and interferon coding genes which are important cytokines in JAK-STAT signaling pathway were upregulated in drug resistant MM sublines. Among them IL3 and interferon-gamma receptor gene upregulation were common in prednisone resistance. On the other hand, different types of suppressors of cytokine signaling genes such as ASB2, SOCS2, SOCS4 and WSB2 were downregulated in all of the drug resistant MM cell lines.

According to our results different types of cell cycle regulatory cyclin encoding genes and cyclin dependent kinases were downregulated and cyclin dependent kinase inhibitor encoding genes were upregulated in both of the prednisone resistant MM cell lines and as well as in vincristine resistant RPMI-8226 cell line. These results may be correlated with the slow growth and increase in doubling time of the resistant cells. BCCIP gene which is important in tumor suppression was downregulated in both of the vincristine and prednisone resistant MM cell lines.

Among the genes that are involved in ceramide metabolism it seems melphalan resistance is related with cellular ceramide levels because melphalan resistant MM cell lines tend to decrease ceramide level via downregulating sphingomyelinase and ceramide synthase genes while upregulating sphingosine kinase and UGCG genes. However, prednisone and vincristine resistance seems not to be associated with ceramide metabolism.

Different types of apoptosis and death associated genes were expressed in drug resistant multiple myeloma cell lines. Also different types of cyclooxygenase and cytochrome related genes were upregulated in all of the drug resistant multiple myeloma cell lines which may be correlated with resistance to apoptosis and MDR phenotype.

As a conclusion; prednisone, vincristine and melphalan resistant RPMI-8226 and U-266 cell lines show both similar and different gene expression profiles. Similarities can be related to common action mechanisms of the drugs whereas differences can be because of the individual differences such as polymorphisms since these cell lines were isolated from two different multiple myeloma patients at

different disease stage. Overall these results elucidated some molecular mechanisms of prednisone, vincristine and melphalan resistance in multiple myeloma model cell lines RPMI-8226 and U-266. These may be used to predict the development of new treatment strategies in multiple myeloma with keeping in mind the individualize therapy.

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APPENDIX A

BUFFERS AND SOLUTIONS

Agarose Gel Electrophoresis

TAE (Tris-Acetate-EDTA) Buffer (50X, 1L)

Tris-base (MW: 121.4)	242g
Glacial acetic acid	57.1 mL
EDTA disodium dihydrate (MW: 372.24) (0.5 M)	100 mL

Volume was adjusted to 1 L with dH_2O. pH was adjusted to 8.5. Solution was autoclaved and stored at 4°C.

Ethidium Bromide (EtBr) Solution

EtBr (MW: 394.31)	10 mg
dH_2O	1 mL

Solution was stored at 4°C in dark

Protein Isolation

Tris HCI (1M, 10 mL)

Tris-base (MW: 121.14)	1.21 g

Volume was adjusted to 10 mL with dH_2O and pH was adjusted to 7.5. Solution was autoclaved and stored at 4°C.

EDTA (0.125M, 10 mL)

EDTA (MW: 292.3)

Volume was completed to 10 mL with dH_2O and with a few drops of NaOH to enhance solubility. Solution was stored at RT.

CHAPS Buffer (5% w/v, 5 mL)

CHAPS (MW: 614.9)	0.25 g

Volume was adjusted to 5 mL with dH_2O. Solution was stored at 4°C.

PMSF (100nM, 1 mL)

PMSF	0.018 g
EtOH	1 mL

Solution was stored at 4°C.

Aprotinin (0.1 mg/mL, 1 mL)

Aprotinin	0.01 g
Physiologic saline	1 mL

10 mg/mL aprotinin was diluted 1:100 by physiologic saline to achieve 0.1 mg/ml stock solution and the solution was kept at -20°C.

Pepstatin A (0.2 mg/mL)

Pepstatin	0.002 g
DMSO	1 mL

2 mg/mL pepstatin solution was diluted to 1:10 by DMSO to achieve 0.2 mg/mL stock solution and the solution was kept at -20°C.

β-mercaptoethanol (0.5M, 5 mL)

β-mercaptoethanol (14.3M)	175 μL
dH_2O	4825 μL

Lysis Buffer (300μL)

TrisHCL (1M)	3 μL
$MgCl_2$ (25mM)	12 μL
EDTA (0.125M)	2.4 μL
CHAPS (5% w/v)	30 μL
Glycerol (87% v/v)	34.5 μL
PMSF (100mM)	3 μL
Aprotinin (0.1 mg/mL)	6 μL

Pepstatin A (0.2 mg/mL)	1.5 µL
β-mercaptoethanol (0.5M)	3 µL
dH_2O	204.6 µL

Solution was freshly prepared prior to use.

Bradford Reagent (5X, 1L)

Coomassie Brillant Blue G250	500 mg
EtOH (95% v/v)	250 mL
Phosphoric acid (85% v/v)	500 mL

Solution was completed to 1L with dH_2O, filtered from coarse fitler and stored at 4°C.

SDS-PAGE

Gel Solution (50 mL)

Acrylamide	14.6 g
Bis acrylamide	0.4 g

Solution was completed to 50 mL with dH_2O, filtered from coarse fitler and stored at dark at 4°C.

Separating Gel Buffer (1.5M TrisHCl, 100 mL)

Tris-base (MW: 121.14)	18.15 g
dH_2O	60 mL

Solution was comleted to 100 mL, pH was adjusted to 8.8 and stored at 4°C.

Stacking Gel Buffer (0.5M TrisHCI, 100 mL)

Tris-base (MW: 121.14)	6 g
dH_2O	60 mg

Solution was comleted to 100 mL, pH was adjusted to 6.8 and stored at 4°C.

SDS (10% w/v, 10 mL)

SDS (MW: 288.38)	1 g

Solution was completed to 10 mL with dH_2O and stored at RT.

APS (10% w/v, 1 mL)

APS (MW: 228.2)	100 mg
dH_2O	1 mL

Solution was freshly prepared prior to use.

Sample Dilution (SDS Reducing) Buffer (4X, 10 mL)

TrisHCI (1M, pH 6.8)	2.5 mL
Glycerol	4 mL
β-mercaptoethanol	2 mL
SDS (MW: 288.38)	0.8 g
Bromophenol blue	0.001 g

Solution was completed to 10 mL with dH_2O and stored at dark at 4°C.

Running Buffer (10X, 500 mL)

Tris-base (MW: 121.14)	15 g
Glycine (MW: 75.07)	72 g

Solution was completed to 500 mL with dH_2O and stored at 4°C.

Western Blotting

Transfer (Blotting) Buffer (4L)

Tris-base (MW: 121.14)	12.114 g
Glycine (MW: 75.07)	57.65 g
MetOH	800 mL

Solution was completed to 4 L with dH_2O and stored at 4°C.

TBST Buffer (1 L)

NaCL (MW: 58.44)	29.25 g
TrisHCI (MW: 157.6)	3.16 g
Tween 20 (MW: 1227.7)	500.5 µL

Solution was completed to 1 L with dH_2O and stored at 4°C.

Blocking Buffer (300 mL)

Non fat dry milk	15 g

Volume was completed to 300 mL with TBST buffer. Solution was freshly prepared prior to use.

Microarray

MES Stock (12X, 100 mL)

MES free-acid monohydrate	70.4 g
MES sodium salt	193.3 g

Volume was completed to 100 mL with DEPC-treated dH_2O, fitler sterilized and stored at 4°C.

MES Hybridization Buffer (2X, 500 mL)

NaCL (5M)	200 mL
12X MES stock	82 mL

Volume was completed to 500 mL with DEPC-treated dH_2O, fitler sterilized and stored at RT.

MES Stain Buffer (2X, 250 mL)

12X MES stock	41.7 mL
NaCL (5M)	92.5 mL
Tween 20 (10% v/v)	2.5 mL

Volume was completed to 250 mL with DEPC-treated dH_2O, fitler sterilized and stored at RT.

Hybridization Coctail

Fragmented cRNA	30 µL
Control Oligo B2	5 µL
20X Hybridization Control	15 µL
Herring Sperm DNA	3 µL
BSA (50 mg/mL) (freshly prepared)	3 µL

2X MES Hybridization Buffer	150 µL
DMSO	30 µL
dH_2O	64 µL

The solution was freshly prepared prior to use.

SAPE (Streptavidin Phycoerythrin) Solution

2X MES Stain Buffer	600 µL
BSA (50 mg/mL)	48 µL
SAPE (50 mg/mL)	12 µL
DI H_2O	540 µL

The solution was freshly prepared prior to use and kept in dark.

Antibody Solution

2X MES Stain Buffer	300 µL
BSA (50 mg/mL)	24 µL
Normal Goat IgG (10mg/mL)	6 µL
Biotinylated antibody (0.5 mg/mL)	3.6 µL
DI H_2O	266.4 µL

The solution was freshly prepared prior to use and kept in dark.

APPENDIX B

RT-PCR REACTION MIXTURES AND AMPLIFICATION CONDITIONS

PCR reaction mixture for *MDR1* gene:		The Amplification Conditions:	
Water for PCR	36.5μl	Initial Denaturation	94 °C 5 min
10x Reaction Buffer	5μl	Denaturation	94 °C 30 sec
25mM $MgCl_2$	4μl	Annealing	55 °C 45 sec
25mM dNTP mixture	0.3μl	Extension	72 °C 1 min
MDR1 sense primer (100pmol/μl)	1μl	Final Extension	72 °C 5 min
MDR1 antisense primer (100pmol/μl)	1μl	Number of cycles	35
cDNA	2μl		
5U/μl Taq DNA Polymerase	0.2μl		
Total Volume	50μl		

PCR reaction mixture for *MRP1* gene:		The Amplification Conditions:	
Water for PCR	38.5μl	Initial Denaturation	94 °C 5 min
10x Reaction Buffer	5μl	Denaturation	94 °C 30 sec
25mM $MgCl_2$	3μl	Annealing	62 °C 45 sec
25mM dNTP mixture	0.3μl	Extension	72 °C 1 min
MRP1 sense primer (100pmol/μl)	0.5μl	Final Extension	72 °C 5 min
MRP1 antisense primer (100pmol/μl)	0.5μl	Number of cycles	35
cDNA	2μl		
5U/μl Taq DNA Polymerase	0.2μl		
Total Volume	50μl		

PCR reaction mixture for *BCRP* gene:		The Amplification Conditions:	
Water for PCR	36.3µl	Initial Denaturation	94 °C 5 min
10x Reaction Buffer	5µl	Denaturation	94 °C 50 sec
25mM $MgCl_2$	4µl	Annealing	59 °C 50 sec
25mM dNTP mixture	0.5µl	Extension	72 °C 20 sec
BCRP sense primer (100pmol/µl)	1µl	Final Extension	72 °C 10 min
BCRP antisense primer (100pmol/µl)	1µl	Number of cycles	34
cDNA	2µl		
5U/µl Taq DNA Polymerase	0.2µl		
Total Volume	50µl		

PCR reaction mixture for *LRP* gene:		The Amplification Conditions:	
Water for PCR	35.8µl	Initial Denaturation	94 °C 5 min
10x Reaction Buffer	5µl	Denaturation	96 °C 30 sec
25mM $MgCl_2$	4µl	Annealing	59 °C 45 sec
25mM dNTP mixture	1µl	Extension	72 °C 1 min
LRP sense primer (100pmol/µl)	1µl	Final Extension	72 °C 10 min
LRP antisense primer (100pmol/µl)	1µl	Number of cycles	30
cDNA	2µl		
5U/µl Taq DNA Polymerase	0.2µl		
Total Volume	50µl		

PCR reaction mixture for *Bcl-2* gene:		The Amplification Conditions:		
Water for PCR	37.3µl	Initial Denaturation	94 °C	5 min
10x Reaction Buffer	5µl	Denaturation	94 °C	1 min
25mM $MgCl_2$	3µl	Annealing	54 °C	2 min
25mM dNTP mixture	0.5µl	Extension	72 °C	1 min
Bcl-2 sense primer (100pmol/µl)	1µl	Final Extension	72 °C	5 min
Bcl-2 antisense primer (100pmol/µl)	1µl	Number of cycles	30	
cDNA	2µl			
5U/µl Taq DNA Polymerase	0.2µl			
Total Volume	50µl			

PCR reaction mixture for *Bcl-X_L* gene:		The Amplification Conditions:		
Water for PCR	35.8µl	Initial Denaturation	94 °C	5 min
10x Reaction Buffer	5µl	Denaturation	94 °C	30 sec
25mM $MgCl_2$	4µl	Annealing	59 °C	1 min
25mM dNTP mixture	1µl	Extension	72 °C	1 min
Bcl-X_L sense primer (100pmol/µl)	1µl	Final Extension	72 °C	10 min
Bcl-X_L antisense primer (100pmol/µl)	1µl	Number of cycles	30	
cDNA	2µl			
5U/µl Taq DNA Polymerase	0.2µl			
Total Volume	50µl			

PCR reaction mixture for *GCS* gene: The Amplification Conditions:

Water for PCR	34.7μl	Initial Denaturation	94 °C	2 min
10x Reaction Buffer	5μl	Denaturation	94 °C	1 min
25mM $MgCl_2$	4μl	Annealing	49 °C	1 min
25mM dNTP mixture	1μl	Extension	72 °C	2 min
GCS sense primer (100pmol/μl)	1μl	Final Extension	72 °C	10 min
GCS antisense primer (100pmol/μl)	1μl	Number of cycles	35	
cDNA	2μl			
5U/μl Taq DNA Polymerase	0.2μl			
Total Volume	50μl			

PCR reaction mixture for *SK-1* and *LASS-1* gene: The Amplification Conditions:

Water for PCR	34.7μl	Initial Denaturation	94 °C	5 min
10x Reaction Buffer	5μl	Denaturation	94 °C	1 min
25mM $MgCl_2$	4μl	Annealing	58 °C	1 min
25mM dNTP mixture	1μl	Extension	72 °C	2 min
Sense primer (100pmol/μl)	1μl	Final Extension	72 °C	10 min
Antisense primer (100pmol/μl)	1μl	Number of cycles	35	
cDNA	2μl			
5U/μl Taq DNA Polymerase	0.2μl			
Total Volume	50μl			

PCR reaction mixture for *β-actin* gene:		The Amplification Conditions:		
Water for PCR	37.3μl	Initial Denaturation	94 °C	5 min
10x Reaction Buffer	5μl	Denaturation	94 °C	30 sec
25mM $MgCl_2$	3μl	Annealing	50 °C	45 sec
25mM dNTP mixture	0.5μl	Extension	72 °C	1 min
SK-1 sense primer (100pmol/μl)	1μl	Final Extension	72 °C	5 min
SK-1 antisense primer (100pmol/μl)	1μl	Number of cycles	35	
cDNA	2μl			
5U/μl Taq DNA Polymerase	0.2μl			
Total Volume	50μl			

APPENDIX C

PROTEIN CONCENTRATION DETERMINATION BY BRADFORD

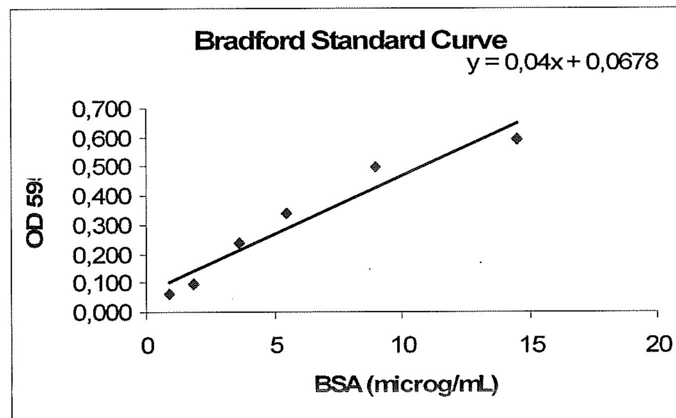

Figure C.1 Bradford standard curve

Protein concentration of the samples were calculated from the standard curve (Figure C.1). Optical densities of the samples at 595nm were inserted in the equation as y and concentration of the sample was calculated by solving the equation for x and multiplying by the dilution factor of the sample. Calculated protein concentrations of the samples were shown in Table C.1

Table C.1 Total protein concentrations

Protein	OD_{595}	DF	Protein(µg/ml)
RPMI-8226	0.574	110	1394
RPMI-8226/500µMPred	0.667	110	1650
RPMI-8226/50nMVinc	0.0.576	110	1400
RPMI-8226/1µMMelp	0.608	110	1488
U-266	0.0.660	110	1631
U-266/300µMPred	0.645	110	1590
U-266/2nMVinc	0.689	110	1711
U-266/1µMMelp	0.520	110	1246

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EDUCATION

Degree	Institution	Year of Graduation
PhD	METU Biology	2009
MS	METU Biotehcnology	2002
BS	METU Biology	2000
High School	TED Ankara College	1995

WORK EXPERIENCE

Year	Place	Enrollment
2008-Present	METU Health Center	Biolog
2002-2008	METU Health Center	Research Assistant
1999 July-October	Hacettepe University, Medical Biology	Summer Study

FOREIGN LANGUAGES

Advanced English

PUBLICATIONS

Papers published in international journals

1.**Kaya P,** Gündüz U, Arpacı F, Ural AU, Güran Ş. Identification of polymorphisms on the MDR1 gene among Turkish population and their effects on multidrug resistance in acute leukemia patients. American Journal of Hematology,2005; 80: p-26-34.

2.Baran Y, Gür Bala, **Kaya P,** Ural AU, Avcu F, Gündüz U. Upregulation of multidrug resistance genes in doxorubicin resistant human acute myelogeneous leukemia cells and reversal of the resistance. Hematology, 2007; 12(6): p-511-517.

3.Abaan OD, **Mutlu PK,** Baran Y, Atalay MC, Gündüz U. Multidrug resistance mediated by MRP1 gene overexpression in breast cancer patients. Cancer Investigation, 2009; 27(2): p-201-5 .

Abstracts published in international conferences

1. **Kaya P,** Gündüz U, Arpacı F, Ural AU, Güran Ş. Identification of polymorphisms in multidrug resistance gene in leukemia patients. Poster presentation, European Human Genetics Conference, May 3-6 2003 Birmingham-ENGLAND. European Journal of Human Genetics Vol (11-Suppl1) p-62-63.

2. **Kaya P,** Gündüz U, Arpacı F, Ural AU, Güran Ş. Identification of polymorphisms on MDR1 gene among Turkish population and their effects on multidrug resistance in acute leukemia patients. Oral presentation, 6th Balkan Meeting on Human Genetics, August 28-31 2004 Thessaloniki-GREECE. p-65.

3. Kars MD, İşeri ÖD, Atalay C, Baran Y, **Kaya P,** Gündüz U. Investigation of molecular mechanisms of multiple drug resistance. Poster presentation, The European 11th COST ACTION B16 Symposium on Multidrug Resistance Reversal, May 13-14 2005 Antalya-TURKEY. p-35.

4. Ural AU, İşeri ÖD, **Mutlu PK,** Kars MD, Avcu F, Gündüz U. Expression of multidrug resistance 1, lung resistance protein and breast cancer resistance protein genes in chronic lymphocytic and chronic myelogeneous leukemia. Oral presentation, American Society of Hematology 48th Annual meeting, December 9-12 2006 Orlando-Florida-USA, Blood Journal of American Society of hematology Vol 108 No:11 Part 2 p-169b.

Abstracts published in national conferences

1. **Kaya P,** Gündüz U, Arpacı F, Ural AU. Lösemi hastalarında çoklu ilaç dirençliliği genindeki polimorfizmlerin belirlenmesi. Poster sunumu, 29. Ulusal Hematoloji Kongresi, 26-28 Ekim 2002 Antalya. Turkish Journal of Hematology Vol:19 No:3 s-122-123.

2. **Kaya P,** Gündüz U, Arpacı F, Ural AU, Güran Ş. Türk popülasyonunda MDR1 genindeki polimorfizmlerin belirlenmesi ve bu polimorfizmlerin akut lösemi hastalarındaki çoklu ilaç dirençliliği üzerindeki etkileri. Poster sunumu, 2. Klinik Biyokimya ve Kanser Sempozyumu, 16-18 Nisan 2004 Bursa. s- 200.

3. **Kaya P,** Gündüz U, Arpacı F, Ural AU, Güran Ş. Türk toplumunda MDR1 Gen polimorfizmi ve akut lösemi olgularında ilaç dirençliliğine etkisi. Poster sunumu, 9. Ulusal Tıbbi Biyoloji Kongresi, 24-27 Kasım 2005 Manisa. s-210.

4. **Mutlu PK,** Gündüz U, Ural AU. Multipl myeloma hücre hatlarında çoklu ilaç dirençliliği mekanizmalarının moleküler yöntemlerle belirlenmesi. Sözlü sunum, Multidisipliner Kanser Araştırma Sempozyumu (Uluslararası katılımlı), 12-15 Mart 2006 Bursa. s-114.

5. Ural AU, İşeri ÖD, **Mutlu PK,** Kars MD, Avcu F, Gündüz U. Kronik lenfositik lösemi ve kronik miyelositer lösemi vakalarında çoklu ilaç direnç 1, akciğer direnç protein ve meme kanseri direnç protein genlerinin tanımlanması. Poster sunumu, 32. Ulusal Hematoloji Kongresi, 8-12 Kasım 2006 Antalya. Turkish Journal of Hematology Vol:23 No:3 s-131.

6. **Mutlu PK,** Ural AU, Gündüz U. Prednisol uygulanmış multipl myeloma hücre hatlarında çoklu ilaç dirençliliği mekanizmaları. Poster sunumu, 32. Ulusal Hematoloji Kongresi, 8-12 Kasım 2006 Antalya. Turkish Journal of Hematology Vol:23 No:3 s-177.