

**ANALYSIS OF MEMBERS OF THE SLIT-ROBO PATHWAY AS
DIAGNOSTIC AND PROGNOSTIC TOOLS IN HEPATOCELLULAR
CARCINOMA WITH SPECIAL FOCUS ON ROBO2-ASSOCIATED
CELLULAR PHENOTYPE**

**A THESIS SUBMITTED TO
THE DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
AND THE INSTITUTE OF ENGINEERING AND SCIENCE OF
BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY**

**BY
MEHMET ENDER AVCI
AUGUST 2009**

Biricik eşim Duygu'ya ve en geniş anlamıyla aileme...

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope, and in quality, as a thesis for the degree of Doctor of Philosophy.

Assist.Prof. Dr.Tamer Yağcı

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope, and in quality, as a thesis for the degree of Doctor of Philosophy.

Assist.Prof. Dr.Ayşe Elif Erson

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope, and in quality, as a thesis for the degree of Doctor of Philosophy.

Assist.Prof. Dr.Özlen Konu

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope, and in quality, as a thesis for the degree of Doctor of Philosophy.

Assist.Prof. Dr.Kamil Can Akçalı

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope, and in quality, as a thesis for the degree of Doctor of Philosophy.

Assoc.Prof. Dr.Cengiz Yakıcıer

Approved for the Institute of Engineering and Science

Prof. Dr. Mehmet Baray

Director of the Institute of Engineering and Science

ABSTRACT

ANALYSIS OF MEMBERS OF THE SLIT-ROBO PATHWAY AS DIAGNOSTIC AND PROGNOSTIC TOOLS IN HEPATOCELLULAR CARCINOMA WITH SPECIAL FOCUS ON ROBO2-ASSOCIATED CELLULAR PHENOTYPE

Mehmet Ender Avcı

Ph.D. in Molecular Biology and Genetics

Supervisor: Assist. Prof. Tamer Yağcı

August 2009, 126 pages

Hepatocellular carcinoma is the sixth most common cancer in the world, with an annual incidence exceeding half a million. It is associated mainly with hepatitis B and C viral infections; and is the main cause of death among cirrhotic patients. Aflatoxin B1 exposure, chronic alcohol consumption and virtually all cirrhosis-inducing conditions are of the other etiologies. For early diagnosis of HCC, surveillance of the risk groups is a crucial task requiring the development of novel markers for HCC with stronger sensitivity and specificity. In addition, description of biomarkers specific to hepatocellular carcinoma subtypes could identify novel targets for therapy. In this study, we analyzed members of the *SLIT-ROBO* gene families as novel diagnostic and prognostic markers in hepatocellular carcinoma. We defined an expression signature for members of the *SLIT-ROBO* gene families in HCC cell lines and tissues by real-time quantitative RT-PCR analysis. We showed that *ROBO1* was overexpressed as stage and differentiation of the HCC proceeds. Furthermore, *ROBO4* downregulation and *SLIT2* overexpression marked late stage and poorly differentiated HCCs. Our results suggest that the expression of *ROBO1* and *ROBO4* can be used in early diagnosis of HCC. As another focus, we stably knockdowned *ROBO2* expression in a model *AFP* positive cell line Huh7 and characterized the associated cellular phenotype. *ROBO2* downregulation caused a significant decrease in proliferation rate whereas in wound-healing assay no significant difference in migration rate was observed. In addition, we performed a microarray experiment and found the differentially expressed genes between stable *ROBO2* knockdown and negative clones. In this analysis, we found an overexpression of *CK19*, *CD44*, *ABCG2/BCRP1* hepatic progenitor cell markers and *CD133* that is also a putative cancer stem cell marker of HCC, in stable *ROBO2* knockdown clones. In addition *KLF4* expression was augmented in these *ROBO2* knockdown clones. We propose a genetic association between SLIT-ROBO pathway and *CD133* at transcriptional level.

ÖZET

ROBO2'YE BAĞLI HÜCRESEL FENOTİPE ÖZEL ODAKLA SLIT-ROBO YOLAĞI ÜYELERİNİN HEPATOSELÜLER KARSİNOM TANI VE TAKİBİNDE ARAÇ OLARAK ANALİZİ

Mehmet Ender Avcı
Moleküler Biyoloji ve Genetik Doktora Tezi
Tez Yöneticisi: Yrd. Doç. Dr. Tamer Yağcı
Ağustos 2009, 126 Sayfa

Hepatoselüler karsinom, yıllık yarım milyonu aşan yeni vaka sayısı ile, dünya çapında en sık rastlanan altıncı kanser türüdür. Hepatoselüler karsinom, siroz hastalarının temel ölüm sebeplerinden hepatit B ve C enfeksiyonuyla ilişkilendirilmiştir. Aflatoxin B'ye maruz kalma, kronik alkol kullanımı ve sirozu tetikleyen bütün etkenler diğer etiyolojilerdir. Hepatoselüler karsinomun erken tanısı için risk gruplarının takibi önemlidir ve bu kansere karşı spesifitesi ve duyarlılığı daha yüksek yeni belirteçlerin geliştirilmesi gerekmektedir. Bunun yanında hepatoselüler karsinom alt tipleri için biyobelirteçlerin tanımlanması yeni terapi metodolojilerini de mümkün kılabilir. Bu çalışmada, *SLIT-ROBO* gen aileleri üyelerinin hepatoselüler karsinom tanı ve takibinde yeni belirteçler olarak kullanılabilirliğini inceledik. Gerçek zamanlı kuantitatif RT-PCR yöntemiyle *SLIT-ROBO* yolağı genlerinin hepatoselüler karsinomda ifadesini belirleyen bir imza tanımladık. *ROBO1* gen ifadesinin hepatoselüler karsinom derecesine ve diferensiyasyonuna bağlı olarak değiştiğini gösterdik. Bununla beraber *ROBO4* ifadesindeki azalma ve *SLIT2* ifadesindeki artış, ileri derece ve kötü diferansiye hepatoselüler karsinom dokularını belirledi. Sonuçlarımız, *ROBO1* ve *ROBO4* ifadelerinin hepatoselüler karsinomun erken tanısında kullanılabileceğine işaret etti. Diğer bir odak olarak; *AFP* ifadesi bulunan iyi diferansiye bir model olan Huh7 hücrelerinde, *ROBO2* gen ifadesini kalıcı bir şekilde baskıladık ve bu etkiyle ilişkili hücre fenotipini tanımladık. Bu şekilde elde edilen kalıcı tek-tip hücrelerde, *ROBO2* geninin baskılanması hücrelerin bölünme hızını etkilerken yara-iyileşmesi (wound-healing) deneylerinde ölçülen göç hızına istatistiksel olarak anlamlı etkisi görülmedi. Bağlantılı bir mikrodizi çalışmasında *ROBO2* ifadesi baskılanmış bu hücrelerle, *ROBO2*'yi ifade eden kontrol hücreler arasında farklı ifadelere sahip genleri inceledik. Bu incelemede *CK19*, *CD44*, *ABCG2/BCRP2* hepatik öncül hücre belirteçleri ve ayrıca insanda HCC kanser kök hücre belirteci olarak öne sürülen *CD133* ifadelerinin, *ROBO2* ifadesi baskılanmış hücrelerde arttığını bulduk. Bunlara ek olarak *KLF4* ifadesi *ROBO2* ifadesi baskılanmış bu hücrelerde arttı. *SLIT-ROBO* yolağı ve *CD133* arasında transkripsiyon seviyesinde genetik bir etkileşim bulunduğunu öne sürüyoruz.

ACKNOWLEDGEMENTS

I would like to thank the special people who had contributed this work in various ways.

I would like to express my gratitude to Assist. Prof. Tamer Yağcı for his supervision, support and valuable suggestions throughout the course of my studies. He always shared his knowledge and experience with me and directed me toward new horizons. I am grateful for his patience, motivation, enthusiasm and understanding.

I am also grateful to Assist. Prof. Özlen Konu for supporting me at every stage of my graduate education and doctoral work. Her un-ending energy has always inspired and motivated me.

I would like to thank Prof. Mehmet Öztürk for his guidance and for being an excellent archetype as a scientist.

I would also like to thank Assist. Prof. Can Akçalı, Assoc. Prof. İhsan Gürsel and Dr. Mayda Gürsel for sharing their experience and knowledge.

I want to thank all MBG supervisors Prof. Tayfun Özçelik, Assoc. Prof. Işık Yuluğ, Assoc. Prof. Rengül Çetin-Atalay, Assist. Prof. Uygur Tazebay, Assist. Prof. Ali Güre, Assist. Prof. Cengiz Yakıcıer for the knowledge and experience they shared through my graduate years.

For their friendship and insights in seemingly troublesome challenges in the lab; I am grateful to Ertuğrul Dalkıç, Koray Kaya, Şerif Şentürk, Hani Al-Otaibi, Nuri Öztürk, Emin Öztaş, Mine Mumcuoğlu, Fatih Semerci, Ceyhan Ceran, Sevgi Bağışlar, Ayça Arslan Ergül, Özge Gürsoy Yüzügüllü, Haluk Yüzügüllü, Bala Gür Dedeoğlu, Tolga Acun, Pelin Gülay, Elif Uz, Eralp Sayit, Tülay Arayıcı and Gonca Koçancı.

It is impossible to express my endless love and thanks to my family and my wife. I will forever be grateful to them. I dedicate this thesis to them.

For the first three years of my PhD study I was supported by a project grant given to Dr. Tamer Yağcı from TUBITAK and for the last two years, I was personally supported by TUBITAK PhD scholarship 2211.

TABLE OF CONTENTS

COVER PAGE.....	i
DEDICATION PAGE.....	ii
SIGNATURE PAGE.....	iii
ABSTRACT.....	iv
ÖZET.....	v
ACKNOWLEDGEMENTS.....	vi
TABLE OF CONTENT.....	vii
LIST OF TABLES.....	xii
LIST OF FIGURES.....	xiii
ABBREVIATIONS.....	xv
 1 INTRODUCTION.....	 1
1.1 Hepatocellular carcinoma.....	1
1.1.1 Pathogenesis of hepatocellular carcinoma	1
1.1.2 Molecular subtypes of hepatocellular carcinoma.....	2
1.1.3 Genetic aberrations described in HCC	3
1.1.3.1 Genome-wide alterations.....	4
1.1.3.2 Mutations.....	5
1.1.3.3 Genomic instability	5
1.1.3.4 Epigenetic alterations	5
1.1.4 Disrupted signaling pathways and molecular targets for HCC therapy	6
1.1.4.1 EGFR-Ras-MAPKK pathway	7
1.1.4.2 c-Met signaling.....	7
1.1.4.3 IGF signaling.....	8
1.1.4.4 PI3K/Akt/mTOR pathway.....	8
1.1.4.5 Wnt/ β -Catenin pathway.....	8
1.1.4.6 Hedgehog signaling.....	9
1.1.4.7 Apoptotic pathways.....	9
1.1.5 Novel markers used in early diagnosis of hepatocellular carcinoma	9
1.1.6 Epithelial-mesenchymal transition and HCC metastasis	11
1.1.7 Cancer stem cells in HCC	12

1.2	Slit and Robo gene families	13
1.2.1	Slit ligands.....	14
1.2.2	Robo receptors.....	15
1.2.3	Structural basis of Slit-Robo interaction	18
1.2.4	Role of Slit-Robo interactions in normal development and physiology	18
1.2.4.1	Regulation of commissural axon pathfinding	18
1.2.4.2	Role of Slit2-Robo2 interaction in kidney development.....	21
1.2.4.3	Role of Slit-Robo in lung development.....	22
1.2.5	Slit-Robo in cancer and other pathologies	23
1.2.5.1	<i>SLIT-ROBO</i> expression in tumors of various tissues	23
1.2.5.2	Epigenetic regulation of <i>SLIT-ROBO</i> genes	24
1.2.5.3	Role of <i>SLIT-ROBO</i> signaling in cancer cell migration and immune cell chemotaxis.....	24
1.2.5.4	Endothelial cell migration and angiogenesis.....	24
1.2.5.5	Disease association of mutations in <i>ROBO1</i> , <i>ROBO2</i> and <i>ROBO3</i> genes...	25
1.2.5.6	Regulation of <i>SLIT-ROBO</i> genes in hepatocarcinogenesis.....	25
2	OBJECTIVES AND RATIONALE	27
3	MATERIALS AND METHODS	29
3.1	MATERIALS	29
3.1.1	Reagents	29
3.1.2	Bacterial strains	29
3.1.3	Enzymes	29
3.1.4	DNA and protein molecular weight markers	29
3.1.5	Oligonucleotides.....	30
3.1.6	Plasmids	30
3.1.7	Electrophoresis, autoradiography, photography and spectrophotometer	30
3.1.8	Tissue culture reagents and cell lines	30
3.1.9	Transfection reagents	31
3.1.10	Kits	31
3.1.11	Antibodies	31
3.2	SOLUTIONS AND MEDIA	32
3.2.1	General solutions.....	32
3.2.2	RNA solutions	32
3.2.3	Microbiological media, reagents and antibiotics.....	32

3.2.4	Tissue culture solutions	33
3.2.5	Protein extraction, quantitation and western blotting solutions	34
3.2.6	Immunofluorescence solutions	35
3.2.7	BrdU incorporation assay solutions	36
3.3	METHODS	36
3.3.1	General methods	36
3.3.1.1	Bacterial transformation	36
3.3.1.1.1	Super-competent E.coli preparation	36
3.3.1.1.2	Super-competent E.coli transformation	37
3.3.1.2	Long term storage of bacterial strains	37
3.3.1.3	Plasmid DNA preparation	37
3.3.1.4	Preparation of genomic DNA from cultured cells	38
3.3.1.5	Extraction of total RNA from tissue culture cells	38
3.3.1.6	Quantification and qualification of nucleic acids and proteins	38
3.3.1.7	Restriction enzyme digestion of DNA	38
3.3.1.8	Agarose gel electrophoresis	39
3.3.1.8.1	Agarose gel electrophoresis of DNA	39
3.3.1.8.2	Agarose gel electrophoresis of RNA	39
3.3.2	Database and computer analysis	39
3.3.3	Vector construction	40
3.3.3.1	Construction of pSEC-shROBO2 vector using Ambion's Silencer Express system	41
3.3.3.2	Construction of pDEST26 mock vector to be used as control in pDEST26-ROBO2 overexpression experiments	44
3.3.4	Tissue culture techniques	44
3.3.4.1	Cell lines and stable clones	45
3.3.4.2	Thawing cryopreserved cells	45
3.3.4.3	Growth conditions of cell lines	45
3.3.4.4	Cryopreservation of cell lines	46
3.3.4.5	Transfection of cell lines	46
3.3.4.6	Wound-healing assay	47
3.3.5	cDNA synthesis	47
3.3.6	Primer design for expression analysis by semi-quantitative PCR	48
3.3.7	Fidelity and DNA contamination control in first strand cDNAs	50

3.3.8	Expression analysis of a gene by semi-quantitative PCR	50
3.3.8.1	Determination of optimal cycle of a gene for semi-quantitative PCR	50
3.3.8.2	<i>GAPDH</i> normalization	51
3.3.9	Real time quantitative RT-PCR.....	51
3.3.9.1	Real-time quantitative RT-PCR analyses of HCC cell lines.....	51
3.3.9.2	Real-time quantitative RT-PCR analyses of HCC tissues	52
3.3.10	Crude total protein extraction.....	54
3.3.11	Quantification of proteins.....	54
3.3.12	Western blotting	55
3.3.13	Immunofluorescence	55
3.3.14	BrdU incorporation assay	56
3.4	Microarray experiment and analysis	56
3.5	Statistical analysis	57
4	RESULTS.....	58
4.1	Expression analysis of members of the <i>SLIT-ROBO</i> gene families.....	58
4.1.1	Oncomine cancer profiling database results for <i>SLIT-ROBO</i> gene expression in liver	58
4.1.2	Onco.DB HCC database analysis for genomic gain/amplification or loss associated to <i>SLIT-ROBO</i> gene regions	59
4.1.3	<i>SLIT-ROBO</i> genes are expressed in hepatocellular carcinoma cell lines at different levels.....	60
4.1.4	AFP positivity is a distinctive marker for poorly- and well-differentiated HCC cell lines	61
4.1.5	<i>SLIT-ROBO</i> expression signature and their coordinate expression in HCC cell lines and liver tissues.....	62
4.1.5.1	Quantitative real-time RT-PCR data for <i>SLIT-ROBO</i> genes in HCC cell lines	62
4.1.5.2	Pearson's correlation analysis revealed genewise expression parallels in HCC cell lines	64
4.1.5.3	Cluster analysis identified two subgroups of HCC cell lines with respect to <i>SLIT-ROBO</i> expression.....	64
4.1.5.4	Quantitative real-time RT-PCR data for <i>SLIT-ROBO</i> genes in normal liver and hepatocellular carcinoma tissues	67

4.1.5.5	<i>SLIT-ROBO</i> genewise clustering observed in HCC cell lines translates into liver tissues.....	70
4.1.5.6	Pearson's correlation analysis revealed genewise expression parallels in liver tissues and a Mantel's permutation test confirmed the significance of correlation patterns in HCC cell lines and liver tissues.....	71
4.1.5.7	<i>ROBO1</i> expression differentiates normal tissues from tumors with respect to both stage and differentiation status.....	71
4.1.6	An alternatively spliced <i>ROBO2</i> isoform <i>ROBO2-tv2</i> , which was initially described in zebrafish (<i>Danio rerio</i>) and rat (<i>Rattus norvegicus</i>); is also expressed in human HCC cell lines	73
4.2	Characterization of <i>ROBO2</i> knockdown associated cellular phenotype in HCC	75
4.2.1	Stable knockdown of <i>ROBO2</i> expression in Huh7 cell line	75
4.2.2	Expression of the epithelial-to-mesenchymal transition (EMT) markers and E-cadherin suppressors in stable clones.....	77
4.2.3	In wound-healing migration assay, there was no significant difference between the migration rates of <i>ROBO2</i> knockdown and negative clones.....	80
4.2.4	Microarray analysis of the differentially expressed genes between stable <i>ROBO2</i> knockdown clones and negative clones.....	82
4.2.4.1	Pathway analysis of microarray results using Pathway-Express tool	83
4.2.4.2	Microarray verification of selected differentially expressed genes by semi-quantitative RT-PCR.....	86
4.2.5	Expression of the markers associated with side population, cancer stem cell and pluripotency phenotype was enriched in the stable <i>ROBO2</i> knockdown clones	87
4.2.6	Transient knockdown of <i>ROBO2</i> did not change the expression of side population, cancer stem cell and pluripotency markers	88
4.2.7	CD133 expression in HCC cell lines.....	91
4.2.8	Effect of <i>ROBO2</i> expression on CD133	92
4.2.9	Proliferation rate of stable <i>ROBO2</i> knockdown clones were diminished.....	94
5	DISCUSSION AND CONCLUSION.....	97
5.1	Expression signature of <i>SLIT-ROBO</i> genes in HCC.....	97
5.2	<i>ROBO2</i> knockdown phenotype in Huh7 hepatocellular carcinoma cells	100
6	FUTURE PERSPECTIVES	102
7	REFERENCES.....	106
8	PUBLICATIONS.....	115

LIST OF TABLES

Table 1.1: Key molecular aberrations described in hepatocellular carcinoma	4
Table 3.1: Target mRNA and oligo sequences for constructing siRNA expression cassettes (SECs)	41
Table 3.2: Precursor SEC amplification PCR profile	42
Table 3.3: SEC amplification PCR profile	43
Table 3.4: Application purposes and sequences of the primers used in this study	48
Table 3.5: PCR efficiencies of the primer pairs used in QRT-PCR relative quantification analysis	51
Table 3.6: Clinicopathological characteristics of normal liver and HCC samples	53
Table 3.7: Protein sample preparation for Bradford assay	54
Table 3.8: BSA dilutions for standard curve plot	55
Table 4.1: Significant differential expression results in liver obtained from Oncomine database	59
Table 4.2: Genomic locations of the <i>SLIT-ROBO</i> genes	60
Table 4.3: Relative expression values for <i>SLIT-ROBO</i> and <i>AFP</i> genes in HCC cell lines*	63
Table 4.4: Genewise correlation of <i>SLIT-ROBO</i> and <i>AFP</i> genes in HCC cell lines	64
Table 4.5: Relative expression values of <i>SLIT-ROBO</i> and <i>AFP</i> genes in normal liver and HCC samples*	68
Table 4.6: Genewise correlation of <i>SLIT-ROBO</i> and <i>AFP</i> genes in liver tissues	71
Table 4.7: Differentially expressed <i>SLIT-ROBO</i> genes between histopathological subgroups of liver tissues	73

LIST OF FIGURES

Figure 1.1: Histopathologic progression and molecular features of HCC.	2
Figure 1.2: Molecular targeted therapies in HCC.	6
Figure 1.3: Representation of the switch in the presentation of HCC in developed countries, as a result of implementation of surveillance among cirrhotic patients.	10
Figure 1.4: Basic structures of Slit and Robo proteins.	15
Figure 1.5: Model depicting the formation of the Slit-induced Robo/Abl–Ncadherin/β-catenin complex.	17
Figure 1.6: Commissural axon pathfinding in the mouse spinal cord and <i>Drosophila</i> ventral nerve cord.	19
Figure 1.7: Robo3 antagonizes Robo1 to allow crossing in mice.	20
Figure 1.8: A Robo code for lateral positioning in <i>Drosophila</i>	21
Figure 1.9: SLIT2-ROBO2 signaling is essential for proper kidney development in mice.	22
Figure 3.1: Overview of the Silencer Express procedure.	42
Figure 3.2: Ambion’s pSEC neo vector map.	43
Figure 3.3: Restriction map of the pDEST™26 Gateway® vector.	44
Figure 4.1: Semi-quantitative expression analysis of <i>SLIT-ROBO</i> genes in HCC cell lines. ...	61
Figure 4.2: Quantification of <i>AFP</i> transcript levels reveals two groups in HCC cell lines.	62
Figure 4.3: <i>SLIT-ROBO</i> expression establishes two groups, which predict <i>AFP</i> expression levels in HCC cell lines.	65
Figure 4.4: One-way ANOVA for <i>AFP</i> expression between Group1 and Group2 cell lines. .	66
Figure 4.5: Significant one-way ANOVA results of individual <i>SLIT-ROBO</i> genes between Group1 and Group2 HCC cell lines.	67
Figure 4.6: Significant one-way ANOVA results of individual <i>SLIT-ROBO</i> genes between normal and HCC tissues.	69
Figure 4.7: Genewise clustering of <i>SLIT-ROBO</i> genes in HCC cell lines translates into liver tissues.	70
Figure 4.8: Significant one-way ANOVA results of individual <i>SLIT-ROBO</i> genes between normal and HCC tissues.	72
Figure 4.9: Expression of transcript variant <i>ROBO2-tv2</i> (including the described conserved alternative exon, CAE) and <i>ROBO2-tv1</i> (lacking CAE) in HCC cell lines.	75
Figure 4.10: Relative <i>ROBO2</i> expression in pSEC transfected stable Huh7 clones.	76
Figure 4.11: ROBO2 protein levels in pSEC transfected stable Huh7 clones.	77

Figure 4.12: Evaluation of the EMT marker expression in stable clones.	78
Figure 4.13: Quantitative expression of <i>E-cadherin</i> and its suppressors <i>SLUG</i> and <i>SIP1</i> in stable clones.	79
Figure 4.14: β -catenin immunofluorescence staining in R2C10, NC4 clones and parental Huh7 cells.....	79
Figure 4.15: Wound-healing assay results for stable clones in normal and serum starved medium.....	81
Figure 4.16: Representative wound healing assay photographs.	81
Figure 4.17: Visualization of 18S and 28S rRNAs in total RNAs isolated from stable clones in formaldehyde agarose gel.....	82
Figure 4.18: Box- and MA-plots of shROBO2 RNA (2C) and negative RNA (NC) probed chips before and after RMA normalization.....	83
Figure 4.19: Significantly affected pathways identified in Pathway-Express.	85
Figure 4.20: Pathway-Express output of axon guidance pathway and the differentially expressed genes in the pathway.	86
Figure 4.21: Microarray verification with selected genes using semi-quantitative RT-PCR..	87
Figure 4.22: Semi-quantitative expression analysis of side population, cancer stem cell and pluripotency associated genes in stable clones.	88
Figure 4.23 Side population, cancer stem cell and pluripotency associated genes in shROBO2 transiently transfected cells.	89
Figure 4.24: CD133, ROBO2 and β -catenin staining in transient shROBO2 transfected Huh7 cells.....	91
Figure 4.25: CD133 expression in HCC cell lines.....	91
Figure 4.26: CD133 expression in selected stable clones.....	92
Figure 4.27: CD133 immunofluorescence staining in R2C10, NC4 clones and parental Huh7 cells.....	93
Figure 4.28: ROBO2 re-introduction into selected stable <i>ROBO2</i> knockdown clones had no apparent effect on CD133 expression.	94
Figure 4.29: Percent BrdU incorporation in stable clones after 24 hours incubation in the presence of BrdU.....	95
Figure 4.30: p15 expression in selected stable clones.....	96

ABBREVIATIONS

ABCG2	ATP-binding cassette, sub-family G (white), member 2
Abl	Abelson tyrosine kinase
ACTB	β -actin
AFP	Alpha fetoprotein
Amp	Ampicillin
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
APS	Ammonium persulphate
ASMA	α -smooth muscle actin
BCRP1	Breast cancer resistance protein 1
bp	Base pairs
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
Cdc42	Cell division cycle 42
cDNA	Complementary DNA
CK19	Cytokeratin 19
CNS	Central nervous system
CSC	Cancer stem cell
Ct	Cycle threshold
C-terminus	Carboxy terminus
DCC	Deleted in colorectal cancer
ddH ₂ O	Double distilled water
DGCP	Des- γ -carboxyprothrombin
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ds	Double strand
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EtBr	Ethidium bromide
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPC-3	Glypican-3
HCC	Hepatocellular carcinoma
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
HSC	Hepatic stellate cell
HSP70	Heat shock protein 70
Ig	Immunoglobulin
IGF2	Insulin-like growth factor-2
IGF2R1	IGF2 receptor
IGFBP	IGF-binding protein
Kan	Kanamycin
Kb	Kilobase
kDa	Kilodalton
KLF	Kruppel-like factor
LB	Luria-Bertani media
LRR	Leucine rich repeat
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
μg	Microgram
mg	Miligram
min	Minute
μl	Microliter
ml	Mililiter
μm	Micrometer
μM	Micromolar
mM	Milimolar
mRNA	Messenger RNA

mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEAA	Non-essential amino acid
NOD/SCID	Non-obese diabetic/severe combined immunodeficient
N-terminus	Amino terminus
Oligo(dT)	Oligodeoxythymidylic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3-K	Phosphatidylinositol 3-kinase
pmol	Picomole
PTEN	Phosphatase and tensin homolog
QRT-PCR	Quantitative real time RT-PCR
Rb	Retinoblastoma
RMA	Robust multichip average
RNA	Ribonucleic acid
ROBO	Roundabout
Rpm	Revolutions per minute
RT PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrilamide gel electrophoresis
Sec	Second
shRNA	short hairpin RNA
SIP1	smad interacting protein 1
siRNA	small interfering RNA
srGAP1	Slit–Robo Rho GTPase-activating protein 1
TAE	Tris-Acetate-EDTA buffer
TBS	Tris buffered saline
TERC	Telomerase RNA
TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor
Tm	Melting temperature

TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Tris	Tris (hydroxymethyl)- methylamine
Tv	Transcript variant
UV	Ultraviolet
v/v	Volume/volume
VEGF	Vascular endothelial growth factor
w/v	Weight/volume

1 INTRODUCTION

1.1 Hepatocellular carcinoma

Being the largest organ inside the body, liver has several physiological functions such as bile production and excretion; excretion of bilirubin, hormones, and drugs; metabolism of fats, proteins, carbohydrates; activation of enzymes; storage of glycogen, vitamins and minerals; synthesis of plasma proteins and clotting factors; blood detoxification and purification.

Liver cancer contains diverse, histologically distinct primary hepatic neoplasms, which include hepatocellular carcinoma (HCC), intrahepatic bile duct carcinoma (cholangiocarcinoma), hepatoblastoma, bile duct cystadenomacarcinoma, hemangiosarcoma and epitheloid hemangioendothelioma (Anthony 2002). Among these, hepatocellular carcinoma is the most common kind of liver cancers comprising 83% of all cases (American cancer society 2005 (Piper, Nurcombe et al. 2002)).

1.1.1 Pathogenesis of hepatocellular carcinoma

The molecular pathogenesis of HCC is a complex process involving different genetic and epigenetic alterations, chromosomal aberrations, gene mutations, and altered molecular pathways (Farazi and DePinho 2006). HCC arises in normal livers, abnormal but noncirrhotic livers, and in cirrhotic livers in 80% of cases resulting from several environmental risk factors (Llovet, Burroughs et al. 2003; Bruix and Sherman 2005).

Beginning from stem cells or mature hepatocytes, a multistep process occurs during transformation of cirrhotic liver into cancer by the accumulation of genetic alterations. Increased levels of transforming growth factor- α (TGF- α) and insulin-like growth factor-2 (IGF-2), lead to increased rate of hepatocyte proliferation in chronic HCV patients (Thorgeirsson and Grisham 2002; Bruix, Boix et al. 2004; Farazi and DePinho 2006; Villanueva, Newell et al. 2007). There is experimental evidence suggesting HCV core protein as a Wnt ligand, a transactivator of Ras signaling and a p53 inactivator (Branda and Wands 2006). In the case of chronic HBV infection; promoter activation of oncogenes, DNA rearrangements and chromosomal instability occur as a result of nonrandom HBV-DNA

integration (Ferber, Montoya et al. 2003). In addition, oxidative stress induced genomic and mitochondrial DNA damage arises because of the chronic inflammation of the liver (Hussain, Schwank et al. 2007). At preneoplastic stages, presence of genetic aberrations (allelic deletions) and epigenetic alterations (aberrant methylation) results in proliferation of selected cell populations into dysplastic nodules, which are HCC precursors. These cells gradually increase their telomerase activity in order to gain replicative potential. Ultimately, survival and proliferation related pathways are activated that brings on the malignant phenotype (Figure 1.1).

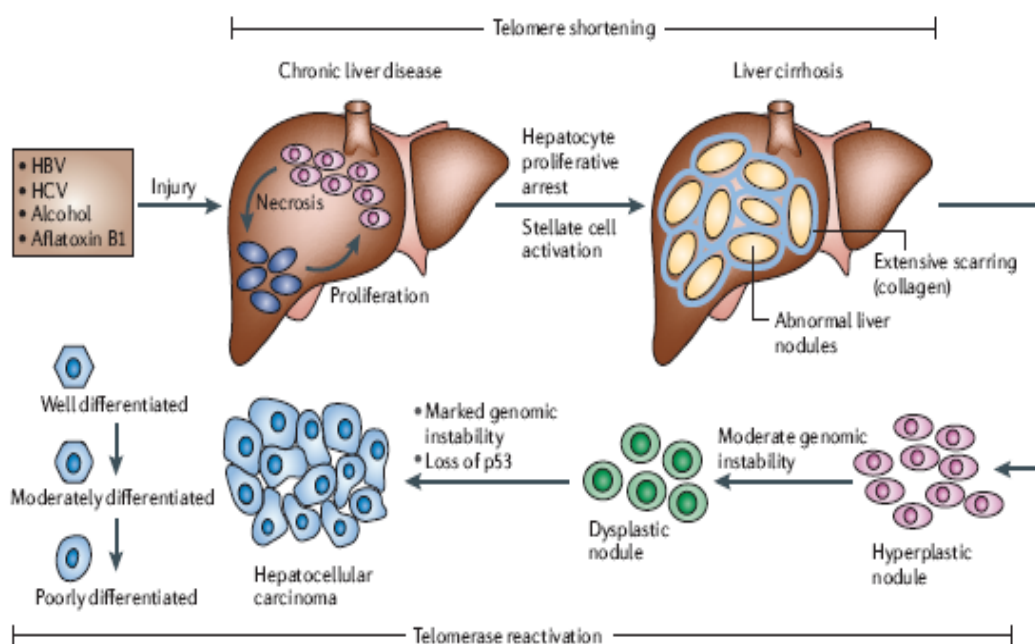


Figure 1.1: Histopathologic progression and molecular features of HCC. After hepatic injury incurred by any one of several factors (HBV, HCV, alcohol and aflatoxin B1) there is necrosis followed by hepatocyte proliferation. Continuous cycles of this destructive-regenerative process foster a chronic liver disease condition that culminates in liver cirrhosis. Subsequently, hyperplastic nodules are observed, followed by dysplastic nodules and ultimately hepatocellular carcinoma (HCC). Telomerase reactivation, loss and/or mutation of p53 and genomic instability also characterize hepatocarcinogenesis (Farazi and DePinho 2006).

1.1.2 Molecular subtypes of hepatocellular carcinoma

In an effort to combine Vogelstein's solid tumor development concept- stating the requirement of the disruption of three critical intracellular signaling networks- and the famous "Hallmarks of cancer" description of Hanahan and Weinberg; Llovet and Bruix proposed that HCCs have common disturbances and additionally more specific alterations identifying distinct classes (Hanahan and Weinberg 2000; Vogelstein and Kinzler 2004; Llovet and Bruix 2008). Common to half of the cases; first, the cell cycle checkpoint is inactivated through

TP53 point mutations or loss of heterozygosity, p16 or Retinoblastoma (Rb) gene silencing, or cyclinD1 overexpression. Secondly, in early stages of hepatocarcinogenesis, autocrine/paracrine vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), or angiopoietin-2 or VEGFA gene amplification results in abnormal angiogenesis. Third, aberrantly proliferating cells evade apoptosis through inactivation of p53 or overexpression of anti-apoptotic Bcl2. Finally, telomerase reverse transcriptase is reactivated to guarantee replicative immortality.

In gene expression studies, more specific alterations in cell proliferation and survival genes have defined molecular subtypes of HCC. One such class shows activation of Wnt signaling showing predominant mutations in *CTNNB1*. The protein product of *CTNNB1* gene; β -catenin escapes from ubiquitination through mutations or deletions in exon 3 of *CTNNB1*, resulting in nuclear translocation and target gene activation in the absence of Wnt ligands. Along with high proliferation and chromosomal instability, activation of RAS/mitogen-activated protein kinase kinase (MAPKK), insulin-like growth factor (IGF), c-met, or Akt/mammalian target of rapamycin (mTOR) signaling pathways defines another HCC subclass. *Stemness* characteristics, indicating progenitor cell origin, are associated with this subclass. Linked with leukocyte infiltration, interferon signaling represents a third class. The role of nuclear factor- κ B signaling, Janus kinase-signal transducer and activator of transcription (Jak-Stat), TGF- β , and hedgehog signaling activation is ambiguous. Another class represents the molecular mechanisms responsible for cell dissemination, invasiveness, and metastasis (Breuhahn, Vreden et al. 2004; Lee, Heo et al. 2006; Boyault, Rickman et al. 2007; Chiang, Villanueva et al. 2008; Llovet and Bruix 2008).

1.1.3 Genetic aberrations described in HCC

Contemporary use of high-throughput technologies like single-nucleotide polymorphism array, complementary cDNA microarray and protein mass spectrometry has brought a new perspective in our understanding of genetic aberrations in HCC. These aberrations can be grouped under four main titles: genome-wide alterations, mutations, genomic instability (being structural alterations) and epigenetic alterations. Table 1.1 summarizes the most frequent molecular aberrations in HCC that can be targeted therapeutically (Villanueva, Newell et al. 2007).

Table 1.1: Key molecular aberrations described in hepatocellular carcinoma

Function	Gene	Gene Expression	Mutations/Copy Alterations	Aberrant Methylation
Growth factors and receptors	<i>IGF-II</i>	Increased		
	<i>IGFR-II (M6PR)</i>	Decreased	25%/LOH 60%	
	<i>EGF</i>	Increased		
	<i>EGFR</i>	Increased	0%	
	<i>TGF-α</i>	Increased		
	<i>K-Ras</i>	-	11% (3%-42%)	
	<i>RASSF1</i>	Decreased		85%
	<i>PIK3CA</i>	-	12% (0%-35%)	
	<i>PTEN</i>	Decreased	0%-11%	
	<i>HGF/c-MET</i>	Increased		
Proliferation and differentiation	<i>β-catenin</i>	Increased	17% (0%-44%); 58% hepatoblastoma	
	<i>E-cadherin</i>	Decreased		Hyper: 46%
	<i>c-myc</i>	Increased		Hypo
	<i>APC</i>	Decreased		77%
	<i>VEGFA</i>	Increased	Amplification (5%)	
Angiogenesis	<i>VEGFR-2</i>	Increased		
	<i>Angiopoietin-2</i>	Increased		
Metastasis	<i>MMP-14</i>	Increased		
	<i>MMP-9</i>	Increased		
	<i>Topoisomerase 2A</i>	Increased		
	<i>Osteopontin</i>	Increased		
	<i>Rb</i>	Increased	15%	
	<i>cyclin D1</i>	Decreased	Amplification (7%)	
Cell cycle	<i>p53</i>	Decreased	27% (0%-67%)	
	<i>p16</i>	Decreased	13%	Hyper: 56%
	<i>p27kip</i>	Decreased		
	<i>Survivin</i>	Increased		

1.1.3.1 Genome-wide alterations

c-myc (8q), *Cyclin A2* (4q), *Cyclin D1* (11q), *Rb1* (13q), *AXIN1* (16p), *p53* (17p), *IGFR-II/M6PR* (6q), *p16* (9p), *E-Cadherin* (16q), *SOCS* (16p), and *PTEN* (10q) are among the candidate genes in hepatocarcinogenesis. Virtually, in all tumors chromosomal amplifications or deletions are found. The most prevalent amplifications are in 1q (58%-78%), 6p, 8q, 17q, and 20q, and deletions are in 4q, 8p, 13q, 16q, and 17p. The regions harboring cyclin D1 and VEGFA are found to have high-level amplifications: 11q13 (5% of cases) and 6p21 (4%-6% of cases), respectively (Thorgeirsson and Grisham 2002; Chiang, Villanueva et al. 2008).

1.1.3.2 Mutations

Somatic mutations of HCC are seen very scarcely and needs further investigation by the use of high-throughput sequencing technology (Villanueva, Newell et al. 2007). TP53 is mutated in 27% of cases (range 0%-67%); correlative to its functions in cell cycle control, DNA repair, apoptosis, and differentiation. In Africa and Asia, Aflatoxin B exposure is associated with *p53* G-to-T mutation at the third position of codon 249 (Puisieux, Lim et al. 1991). *β-catenin* mutations in exon 3 are present in 17% of cases of HCC, and in 58% of cases of hepatoblastoma. Somatic mutations of AXIN1, phosphoinositol 3-kinase A (PI3KA), and K-Ras have been described, rarely. In contrast to the other cancers with mutations of epidermal growth factor receptor (EGFR), Her2/nu phosphatase and tensin homolog (PTEN), or H-Ras; in HCCs these mutations are hard to find. In addition, till now no germline mutations were described for these genes in HCCs (Llovet and Bruix 2008).

1.1.3.3 Genomic instability

HBV integration in the TERT locus, amplification of the gene encoding the telomerase RNA component (TERC), or allelic loss of chromosome 10p, a region encoding a telomerase repressor culminates in increased telomerase activity, in nearly 90% of human HCCs. Being prevalent in HBV-related HCCs, high genomic instability has been associated with the proliferation subclass of HCC (Farazi, Glickman et al. 2003; Llovet and Bruix 2008).

1.1.3.4 Epigenetic alterations

Epigenetic silencing of gene expression occurs by abnormal methylation of gene promoter regions. Liver cancer cells have certain areas of dense hypermethylation over a background of global hypomethylation. Hypermethylation affects CpG islands localized in promoter regions of tumor suppressor genes like p16INK4a, E-cadherin, NORE1A, RASSF1, IGFR-II/MP6, and BRCA1 (Thorgeirsson and Grisham 2002; Bruix, Boix et al. 2004; Farazi and DePinho 2006; Villanueva, Newell et al. 2007).

1.1.4 Disrupted signaling pathways and molecular targets for HCC therapy

Disrupted signaling pathways put protein kinases forward as the main targets for liver cancer therapy. Upon the characterization of the whole kinome the emergence of novel oncology drugs has paced up. The key signaling pathways connected to the pathogenesis of HCC and the monoclonal antibodies and small molecules targeting these pathways are summarized in Figure 1.2 (Llovet and Bruix 2008).

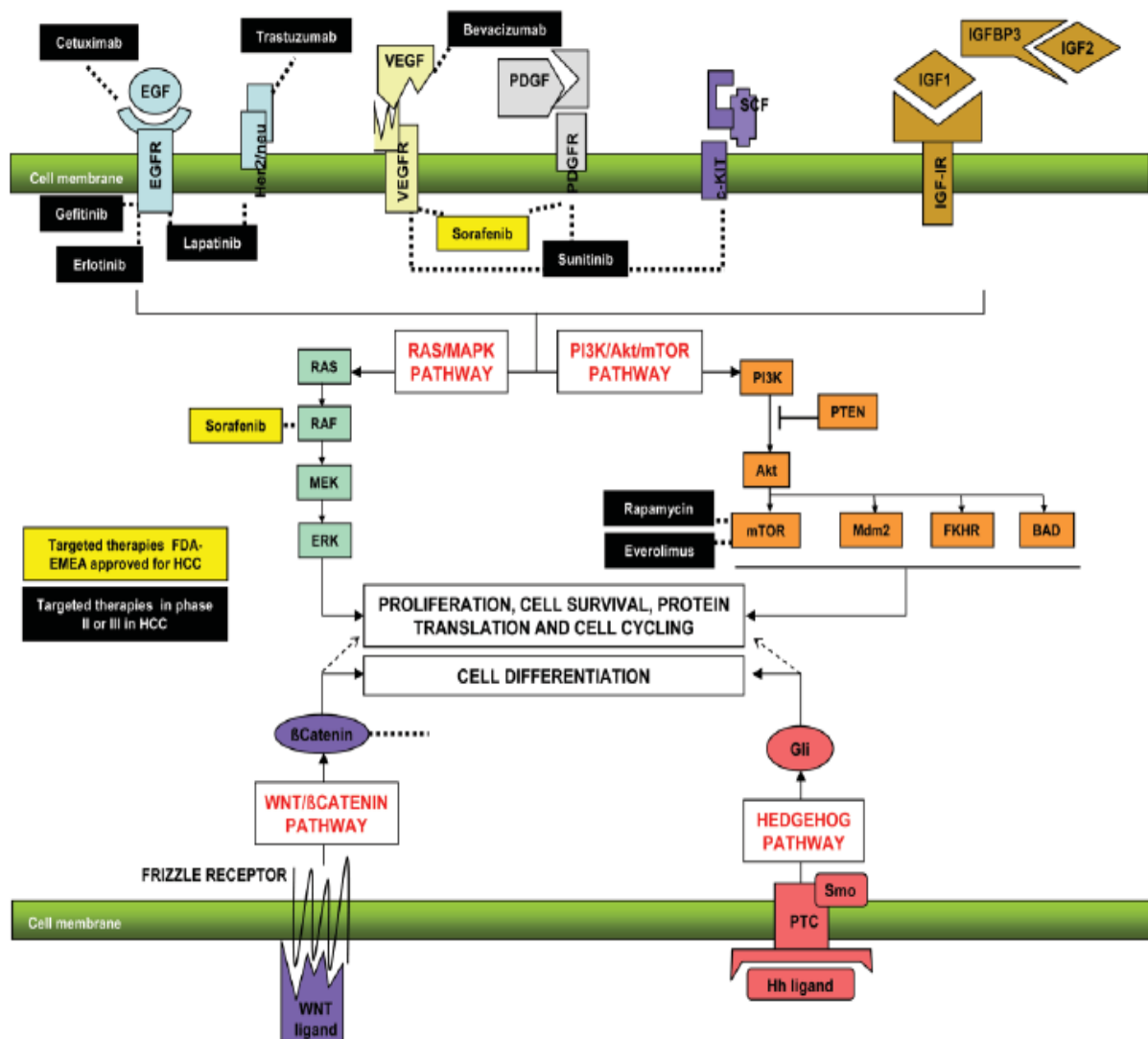


Figure 1.2: Molecular targeted therapies in HCC. Representation of monoclonal antibodies against ligands (VEGFR: bevacizumab; EGFR: cetuximab) tyrosine kinase inhibitors (VEGFR: sorafenib, sunitinib; PDGFR: sorafenib, sunitinib; EGFR: erlotinib, lapatinib; AEE788, Her2/nu: lapatinib, AEE788), serine/threonine kinase inhibitors (Raf: sorafenib; mTOR: rapamycin, everolimus; PI3K: XL-765) in preclinical studies or clinical trials in HCC (Llovet JM and Bruix J, 2008).

1.1.4.1 EGFR-Ras-MAPKK pathway

Binding of the cognate receptors by the ligands EGF, hepatocyte growth factor (HGF), PDGF, and VEGF activate the RAS/MAPK signaling pathway and induce transcription of genes of the AP1 family such as c-fos and c-jun, which are key elements for cell proliferation (Robinson, Wu et al. 2000). The EGFR is a member of a family of four related receptors (Her2/Neu, ErbB3, and ErbB4) that upon ligand binding trigger tyrosine kinase activity and consequently initiate signal transduction. Gain of function of EGFR classically occurs as a result of point mutations, amplification, or increase in ligand-receptor interaction (Baselga and Arteaga 2005).

Monoclonal antibodies against EGFR (cetuximab) or ErbB2/Her2/neu (trastuzumab) antagonize the tyrosine kinase activities of these receptor and thus blocks downstream signaling. Small molecule inhibitor erlotinib acts against the catalytic domain of EGFR and lapatinib acts against both EGFR and Her2 (Shepherd, Rodrigues Pereira et al. 2005; Bonner, Harari et al. 2006; Geyer, Forster et al. 2006; Jonker, O'Callaghan et al. 2007). In HCC, Ras/MAPK pathway activation might result from aberrant upstream signals (EGFR signaling, IGF signaling) or inactivation of tumor suppressor genes by aberrant methylation, as NORE1A (Calvisi, Ladu et al. 2006). Mutations of Raf and Ras are rare findings in HCC. Potent drugs blocking Ras/MAPK signaling are still at the exploratory phase, except for sorafenib, which has activity inhibiting B-Raf at nanomolar concentrations (Wilhelm, Carter et al. 2006; Llovet and Bruix 2008).

1.1.4.2 c-Met signaling

During injury-induced hepatocyte regeneration, hepatocyte growth factor is secreted by stellate cells and binds to the c-MET receptor (Fausto, Campbell et al. 2006). Although the direct link between HGF-cMet signaling and HCC has not been established, dysregulation of c-Met and HGF was observed in HCC (Takami, Kaposi-Novak et al. 2007; Villanueva, Newell et al. 2007). Several compounds have been developed that target the MET pathway, including antibodies against HGF or MET receptor, or selective small-molecule MET inhibitors, but none of them are yet in an advanced stage of research in HCC (Comoglio, Giordano et al. 2008).

1.1.4.3 IGF signaling

IGF-I has been associated with high risk of neoplasm development, but not in HCC. In fact, experimental studies suggest the potential role of this molecule in improving cirrhosis. IGF-II is overexpressed in around 30% of human HCCs and the tumor suppressors IGF binding protein-1 (IGFBP-1), IGFBP-3, and IGFBP-4 are down-regulated. More important, the tumor suppressor IGFR-II, whose main action consists of binding and degrading IGF-II, is down-regulated in a subgroup of HCCs as a result of chromosomal deletions in the IGFR-II locus (6q) or point mutations. Several monoclonal antibodies and small molecules blocking IGF-1R are under early clinical investigations (A12, XL228) (Breuhahn, Longerich et al. 2006; Llovet and Bruix 2008).

1.1.4.4 PI3K/Akt/mTOR pathway

Although the role of pAkt in HCC needs further investigation, recent studies have suggested a worse prognosis for tumors with activated Akt. An important mediator of the PI3K-Akt pathway is mTOR, which acts as a central regulator of cell growth and proliferation, by sensing nutritional status and allowing progression from G1 to S phase. The mTOR pathway is activated in a subset of HCCs, and its blockade with rapamycin or everolimus inhibits growth in HCC cell lines, and in experimental models. Everolimus (a rapamycin analog) and temsirolimus are approved for the treatment of renal cancer. Novel compounds are currently being tested in early clinical trials. These molecules (rapamycin and analogs) are already approved as immunosuppressive treatments after liver transplantation (Sabatini 2006; Llovet and Bruix 2008; Schmitz, Wohlschlaeger et al. 2008).

1.1.4.5 Wnt/ β -Catenin pathway

The Wnt- β -catenin pathway is implicated in colon cancer and HCC. Unfortunately, it constitutes the most undruggable pathway. Activation of the Wnt cascade has been shown in one third of HCCs, particularly in HCV-induced HCCs. Mutations in β -catenin occurs in around 17% of cases (ranging from 0%-44%) (Table 1.1); and association between Wnt pathway activation and the nuclear sequestration of β -catenin has been described by immunohistochemistry. Wnt canonical pathways can also be activated by aberrant methylation of the tumor suppressors APC (adenomatous polyposis coli) and E-cadherin or by increase of autocrine/paracrine secretion of Wnt ligands. Noncanonical activation of Wnt in

HCC deserves further research. New drugs targeting this pathway are under early clinical development (Laurent-Puig, Legoix et al. 2001; Moon, Kohn et al. 2004; Villanueva, Newell et al. 2007; Chiang, Villanueva et al. 2008; Llovet and Bruix 2008).

1.1.4.6 Hedgehog signaling

Overexpression of Sonic Hedgehog has been recently described in 60% of 115 human HCCs and its effective blockage leads to down-regulation of Gli related target genes. Moreover, tumorigenic activation of Smo can mediate c-myc overexpression, which plays an important role in hepatocarcinogenesis (Osipo and Miele 2006; Llovet and Bruix 2008).

1.1.4.7 Apoptotic pathways

Apoptosis is one of the mechanisms leading to cell death when cells have sustained damage to their DNA or cytoskeleton. Evasion of this mechanism is one of the hallmarks of cancer. Several proapoptotic receptor agonists targeting the extrinsic apoptosis pathway (including the ligand recombinant human Apo2L/tumor necrosis factor-related apoptosis inducing ligand [TRAIL]) and the intrinsic apoptotic pathway (BCL-2 antisense) are in development. Synergistic effects have been described with the combination of small molecules and agonists of Apo2L/TRAIL pathway in experimental models. For instance, sorafenib has been shown to sensitize resistant human cancer cells to TRAIL-induced death, providing the rationale for testing these combinations (Hanahan and Weinberg 2000; Ashkenazi and Herbst 2008; Llovet and Bruix 2008).

1.1.5 Novel markers used in early diagnosis of hepatocellular carcinoma

Cirrhosis is the strongest and the most common known risk factor for hepatocellular carcinoma (HCC), particularly cirrhosis related to hepatitis C virus (HCV) and hepatitis B virus (HBV) infections. In addition, HBV acquired in the perinatal period and early childhood is associated with increased risk of HCC even in the absence of cirrhosis (El-Serag, Marrero et al. 2008). Thus, there is a need for surveillance of the risk groups for early diagnosis of HCC.

Nowadays, early HCC diagnosis is feasible in 30–60% of cases in developed countries and this enables the application of curative treatments. In fact, while tumors less than 2 cm in diameter represented <5% of the cases in the early nineties in Europe, now they represent up to 30% of cases in Japan (Figure 1.3) (Llovet and Bruix 2008). However, detection of minute nodules of ~2 cm poses a diagnostic challenge as they are difficult to characterize by radiological or pathological examination. These nodules are defined as pre-neoplastic lesions. Dysplastic lesions should be followed by regular imaging studies, since one third of them will develop a malignant phenotype. Conversely, early tumors are treated with potentially curative procedures- albeit expensive- such as resection, transplantation and percutaneous ablation. Therefore, accurate diagnosis of small liver nodules is of paramount importance.

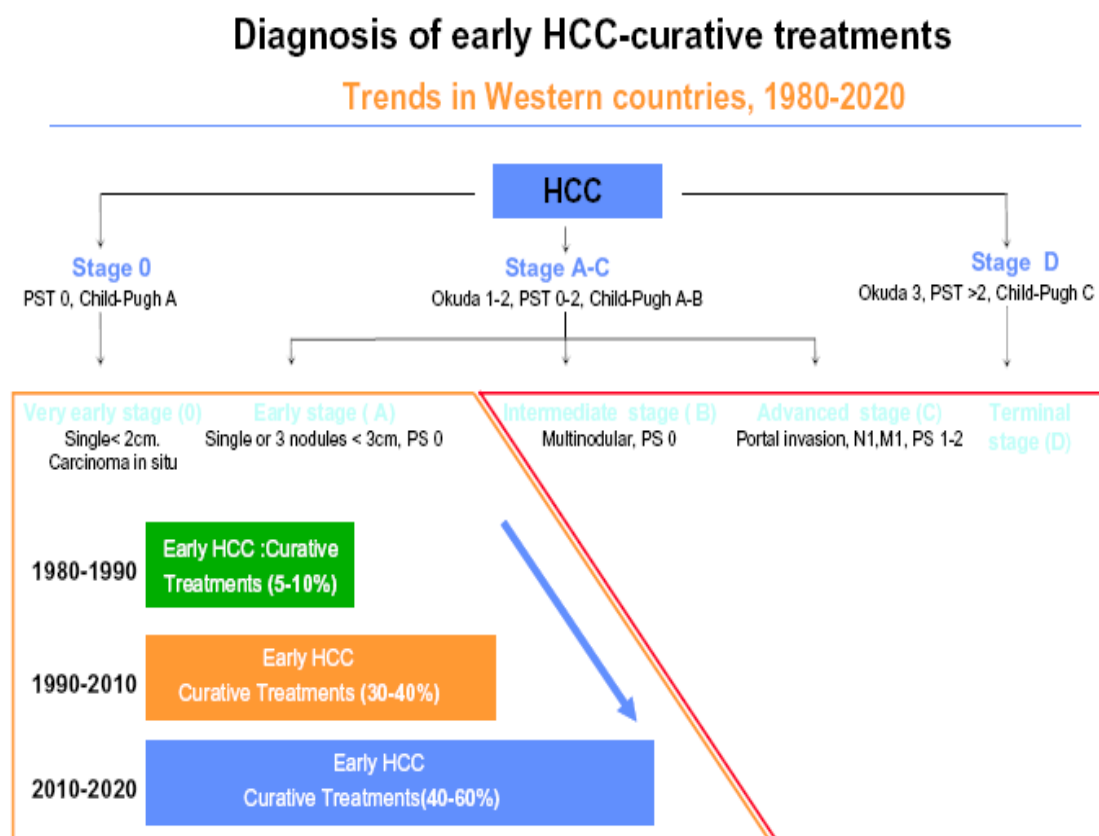


Figure 1.3: Representation of the switch in the presentation of HCC in developed countries, as a result of implementation of surveillance among cirrhotic patients. Estimates of applicability of potentially curative therapies have been divided in three periods: until 1990: 5–10% of cases; 1990–2010: 30–40% of cases; 2010–2020: 40–60% of cases (Llovet and Bruix 2008- Journal of Hepatology).

The major diagnostic techniques for HCC include serum markers, various imaging modalities and histological analysis (Gomaa, Khan et al. 2009). Immunostaining with CD34 and alpha fetoprotein (AFP) has significant diagnostic limitations (Park, Yang et al. 1998). Serum

biomarkers such as AFP, des- γ -carboxyprothrombin (DGCP) and AFP-L3 fraction are not accurate for the early diagnosis of HCC (Bruix and Sherman 2005). Non-invasive radiological criteria have been developed by using state-of-the-art radiological techniques, unfortunately their sensitivity is modest: only 30% of the HCC cases are confirmed by non-invasive criteria and this highlights the need for complementary tools. Recently, genome-wide DNA microarray or quantitative real time reverse-transcriptase polymerase chain reaction (RT-PCR) studies have attempted to identify markers of early HCC, such as heat shock protein 70 (HSP70), Glypican-3 (GPC3), telomerase reverse-transcriptase (TERT), serine/threonine kinase 15 (STK6) and phospholipase A2 (PLA2G1B) (Capurro, Wanless et al. 2003; Marrero and Lok 2004). A molecular index including a 13-gene set has also been proposed (including TERT, TOP2A and PDGFRA) (Paradis, Bieche et al. 2003). A microarray-generated signature of 120 genes was reported to discriminate between dysplastic nodules and HCC in HBV patients (Nam, Park et al. 2005). Proteomic studies in tissue have not identified informative HCC markers so far (Paradis, Degos et al. 2005). More recently a 3-gene set (Glypican- 3, LYVE1, and survivin) has been proposed as molecular diagnosis of early HCC with accuracy rates of 85–95% in training and validation sets in more than 70 samples. Immunostaining for Glypican- 3 was also identified as highly predictive of HCC in 75 samples (Llovet, Chen et al. 2006; Llovet and Bruix 2008).

1.1.6 Epithelial-mesenchymal transition and HCC metastasis

Epithelial to mesenchymal transition (EMT) is a process in which epithelial cells lose their phenotypic characteristic and acquire mesenchymal features. These include switch from an apical-basolateral, polarized epithelial phenotype to a spindle-shaped, fibroblast-like mesenchymal phenotype. In their natural state, epithelial cells exist as tight cell clusters that maintain cell-cell or cell-to-matrix contacts, whereas mesenchymal cells are loosely organized, unpolarized cells with reduced adhesion and enhanced migratory tendencies. EMT is well known in embryonic development, but has been recently recognized as a central event in cancer progression, leading to an invasive, mesenchymal-like phenotype important for tumor cell spreading and metastatic dissemination (Thiery and Sleeman 2006; Moustakas and Heldin 2007; van Zijl, Mair et al. 2009).

In the liver, hepatic stellate cells (HSC) are considered as the major fibrotic precursor cells that transdifferentiate to fibrogenic, extracellular matrix producing myofibroblasts in

inflammatory liver tissue upon TGF- β signaling, whereas hepatocytes undergo apoptosis upon signaling by this cytokine. However, identification of different fibrogenic populations apart from resident stellate cells, as well as convergent results of recent studies have challenged the paradigm of HSC as the essential source of liver myofibroblasts and inferred a prominent role for hepatocytes in liver fibrogenesis (Gressner, Rizk et al. 2008; Battaglia, Benzoubir et al. 2009). HCC progression frequently associates with an epithelial to mesenchymal transition (EMT) of hepatocytes caused by the collaboration of STAT5b with the hepatitis B protein, HBX, or by the cooperation of laminin 5 and TGF- β (Giannelli, Bergamini et al. 2005; Lee, Man et al. 2006). In addition, EMT has been reported to occur in cirrhotic liver-derived hepatocytes that exhibited enhanced cell survival dependent on MAPK signaling (Nitta, Kim et al. 2008; van Zijl, Mair et al. 2009).

A key feature in the initiation and execution of EMT is the downregulation of E-cadherin expression. TWIST, Snail1 (SNAI), SLUG and SIP1 (ZEB2) are known transcriptional repressors of E-cadherin (Thiery 2002; Zhou, Deng et al. 2004; Yook, Li et al. 2005; Matsuo, Shiraha et al. 2009). Vimentin, fibronectin and α -smooth muscle actin (ASMA) expression is characteristic of mesenchymal cells and they are not usually expressed in epithelial cells. The atypical expression of these mesenchymal markers in epithelial cancer cells might be associated with local invasiveness and metastasis potential.

1.1.7 Cancer stem cells in HCC

Cancer stem cells (CSCs) are a minority population of self-renewing stem cells that are entirely responsible for sustaining the tumor as well as giving rise to proliferating but progressively differentiating cells responsible for much of the cellular heterogeneity. With the development of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice for the xenografting of human tumors came the first good in vivo evidence for the existence of CSCs in leukaemia, breast and brain tumours and also in HCC (Alison, Islam et al. 2009).

Chiba and colleagues have reported that two of the four HCC cell lines that they studied had SP cells comprising 0.25% (for Huh7) and 0.8% (for PLC/PRF/5) of the cell population. These cells were highly proliferative and relatively resistant to apoptosis in vitro. Microarray analysis indicated that several genes implicated in stemness, like Wnt pathway genes, were substantially up-regulated in the SP cells in comparison to non- SP cells. Using the gold-

standard NOD/SCID mouse assay for CSCs, they found that transplanting 10^3 liver SP cells consistently yielded tumours, whereas transplantation of 10^6 non-SP cells failed to give rise to tumors (Chiba, Kita et al. 2006).

CD133 (PROM1) is a pentaspan membrane protein, whose function is as yet unclear, has been suggested as a CSC marker in many different tumors, including colon, pancreas, brain and prostate, although its utility has recently been called into question. Using the CD133/1 antibody, a number of studies have suggested that the CD133-positive fraction enriches for HCC CSCs. As might be expected of CSCs, they appear as a minority (<2%) population in primary tumors, although continued passaging has resulted in considerable enrichment of CD133+ cells in some HCC cell lines, up to 90% (Ma, Chan et al. 2007). HCCs with higher than the median number (1.32%) of CD133-positive cells are correlated with shorter survival, higher recurrence rates and higher tumor grade, and CD133+ cells appear highly resistant to conventional therapeutic drugs, such as 5-FU and doxorubicin (Ma, Lee et al. 2008; Song, Li et al. 2008). It has also been suggested that HCC CSCs are descendents of normal parenchymal stem cells that have lost sensitivity to the inhibitory growth effects of TGF- β , while cell selection based on Thy-1 (CD90), a disputed marker of oval cells, in combination with CD44, has also produced cells with aggressive tumorigenic potential (Tang, Kitisin et al. 2008; Yang, Ho et al. 2008; Yang, Ngai et al. 2008).

1.2 Slit and Robo gene families

The intricate patterning of nervous system is the result of an extraordinary ability of axons and dendrites to detect and identify their correct partners among a plethora of possible interactions. In adult humans, each of over a trillion neurons makes connections with, on average, over a thousand target cells. Axons and dendrites are equipped with a highly motile and sensitive structure called the growth cone at their very tips in order to navigate correctly in such a messy environment. Extracellular guidance cues can either attract or repel growth cones, and can operate either at close range or over a distance. By responding to the appropriate set of cues, growth cones are able to select the correct path toward their target (Tessier-Lavigne and Goodman 1996; Dickson 2002).

In 1970's and 80's, the introduction of various *in vitro* assays enabled scientist to uncover the mechanisms of axon guidance in developing nervous systems of both vertebrates and

invertebrates. As a result, in the early 1990's several conserved families of axon guidance molecules were discovered. Slit and Robo families are among these molecules interacting with each other and acting as repellent cues (Dickson 2002).

1.2.1 Slit ligands

Slit family of axon guidance cues consists of large extracellular and membrane-associated glycoproteins that are produced by cells of the central nervous system (CNS). *Drosophila slit* mutants were identified in screens for embryonic patterning and commissural axon pathfinding defects (Nusslein-Volhard C 1984; Seeger, Tear et al. 1993; Hummel, Schimmelpfeng et al. 1999). In these mutants, both ipsilateral and commissural axons unite in the midline rather than growing along it (Rothberg, Jacobs et al. 1990; Batty, Stevens et al. 1999; Kidd, Bland et al. 1999).

Although there is a single Slit in invertebrates, there are three vertebrate Slits: Slit1, Slit2, and Slit3. Vertebrate Slit is secreted from ventral floor plate. The distinctive feature of Slit is its four N-terminal leucine-rich repeat (LRR) domains, abbreviated as D1-D4. C-terminus to LRRs; six epidermal growth factor (EGF)-like domains, a laminin G (LG)-like domain and either one (in invertebrates) or three (in vertebrates) consecutive EGF-like domains, and a C-terminal cystein knot follow. *Drosophila slit* and mammalian Slit2 are proteolytically cleaved between their fifth and sixth EGF-like domains both *in vivo* and *in vitro*. *In vitro* studies have revealed that N-terminal cleavage product is biologically active whereas C-terminal fragment is inactive (Figure 1.4 B) (Brose and Tessier-Lavigne 2000; Hohenester 2008).

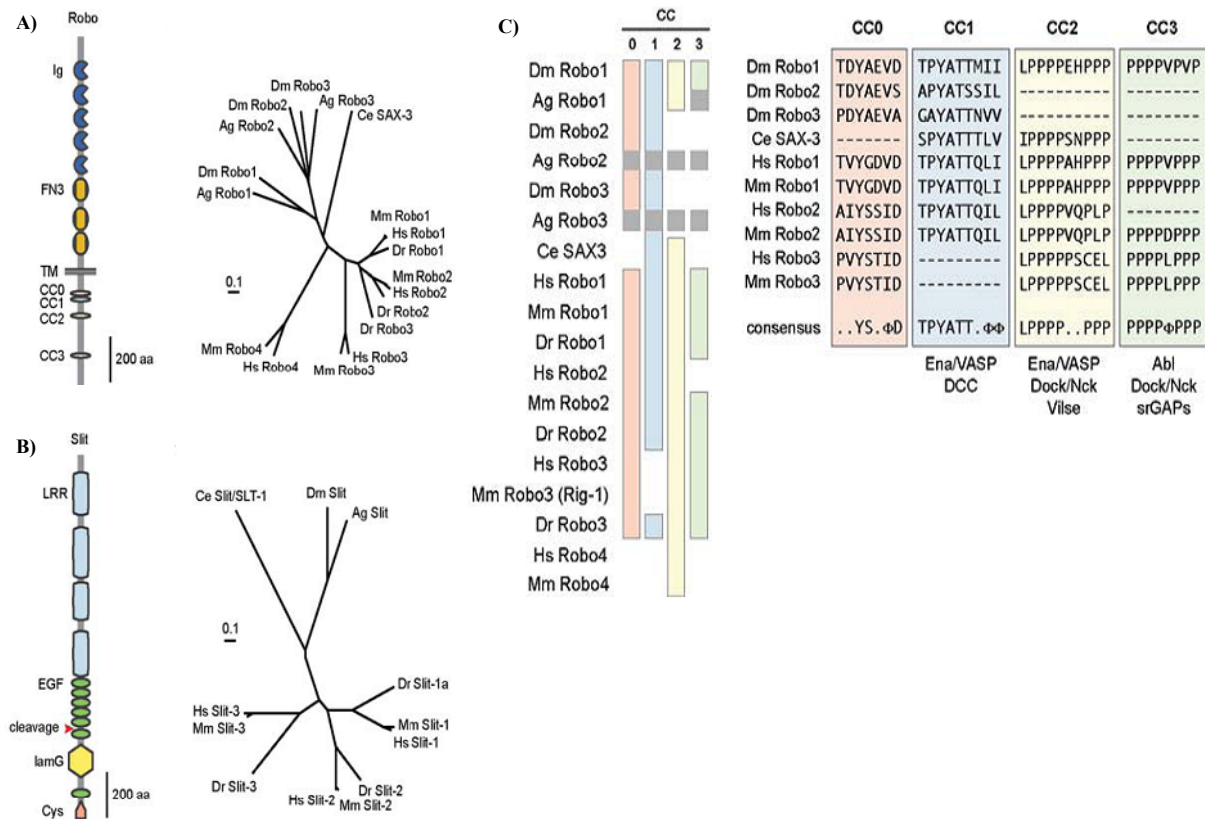


Figure 1.4: Basic structures of Slit and Robo proteins. A) Robo domain organization: Ig, immunoglobulin-like domain; FN3, fibronectin type 3 domain; TM, transmembrane region; CC0-3, conserved cytoplasmic motifs and phylogenetic analysis of Robo family proteins prepared by using extracellular sequences only. B) Slit domain organization: LRR, leucine-rich repeat; EGF, epidermal growth factor-like repeat; lamG, laminin G domain; Cys, cysteine-rich domain, arrowhead indicates cleavage site in vertebrate and *Drosophila* Slits and phylogenetic analysis of Slit family proteins. C) Organization of Robo cytoplasmic domains. Color bars indicate presence of individual CC motifs. Gray indicates that the relevant sequence is unavailable. Alignment of selected CC motifs, showing consensus sequences and known binding partners (Dickson and Gilestro, 2006).

1.2.2 Robo receptors

Similar to *slit*, *Drosophila robo* was identified in a screen for axon guidance defects in which both ipsilateral and commissural axons pass midline several times, thus given the name “roundabout” (Robo for short) (Seeger, Tear et al. 1993). There are four Robo genes in mammals: *Robo1*, *Robo2*, *Robo3* (*Rig-1*) and *Robo4* (*Magic Roundabout*). Subsequently, Slit was defined as a chemorepellent for Robo (roundabout) receptor, and this was evolutionarily conserved (Brose, Bland et al. 1999; Kidd, Bland et al. 1999).

In *Caenorhabditis elegans*, there is a single roundabout receptor known as Sax-3. In *Drosophila* three (Robo, Robo2, Robo3) and in vertebrates four Robo receptors (Robo1/Dutt1, Robo2, Robo3/Rig-1, Robo4/Magic roundabout) exist. *Drosophila robos* are expressed at midline and when bound to Slits they all act as repellent cues specifying lateral

positioning of the longitudinal axon tracts. In vertebrates, Robos are expressed at CNS floor plate, and Robo3 inhibit the repellent functions of Robo1 and Robo2 like the Commis sureless protein in *Drosophila* (Sabatier, Plump et al. 2004).

Except Robo4, all vertebrate Robos have the same extracellular domain organization resembling a cell adhesion molecule (CAM): from N-terminus to C terminus, five Ig domains and three fibronectin type 3 domains. Robo4 has only two Ig and two fibronectin type 3 repeats (Huminiecki, Gorn et al. 2002). A transmembrane domain link the extracellular part of the protein to the cytosolic part which shows considerable variation with only four conserved motifs, CC0-CC3. The conserved cytosolic domains are present in different combinations in different Robos. Since the cytosolic parts of Robos lack a uniform organization with a ~500 a.a. sequence, it is plausible that they interact with many adaptor proteins at the same time (Hohenester 2008) (Figure 1.4 A).

Indeed, the cytosolic domains of Robos are catalytically inactive. Abl (Abelson tyrosine kinase) is associated with Robo and plays an important role in mediating the interaction of Robo with N-cadherin (Bashaw, Kidd et al. 2000; Rhee, Mahfooz et al. 2002). Upon Slit binding to Robo, the adaptor protein Cables is recruited to Robo-associated Abl and forms a multimeric complex by binding directly to N-cadherin-associated β -catenin. Complex formation results in Abl-mediated phosphorylation of β -catenin on tyrosine 489, leading to a decrease in its affinity for N-cadherin, loss of N-cadherin function, and targeting of phospho-Y489- β -catenin to the nucleus. Nuclear β -catenin combines with the transcription factor Tcf/Lef and activates transcription. Thus, Slit induced formation of the Robo-N-cadherin complex results in a rapid loss of cadherin-mediated adhesion and has more lasting effects on gene transcription (Rhee, Buchan et al. 2007) (Figure 1.5).

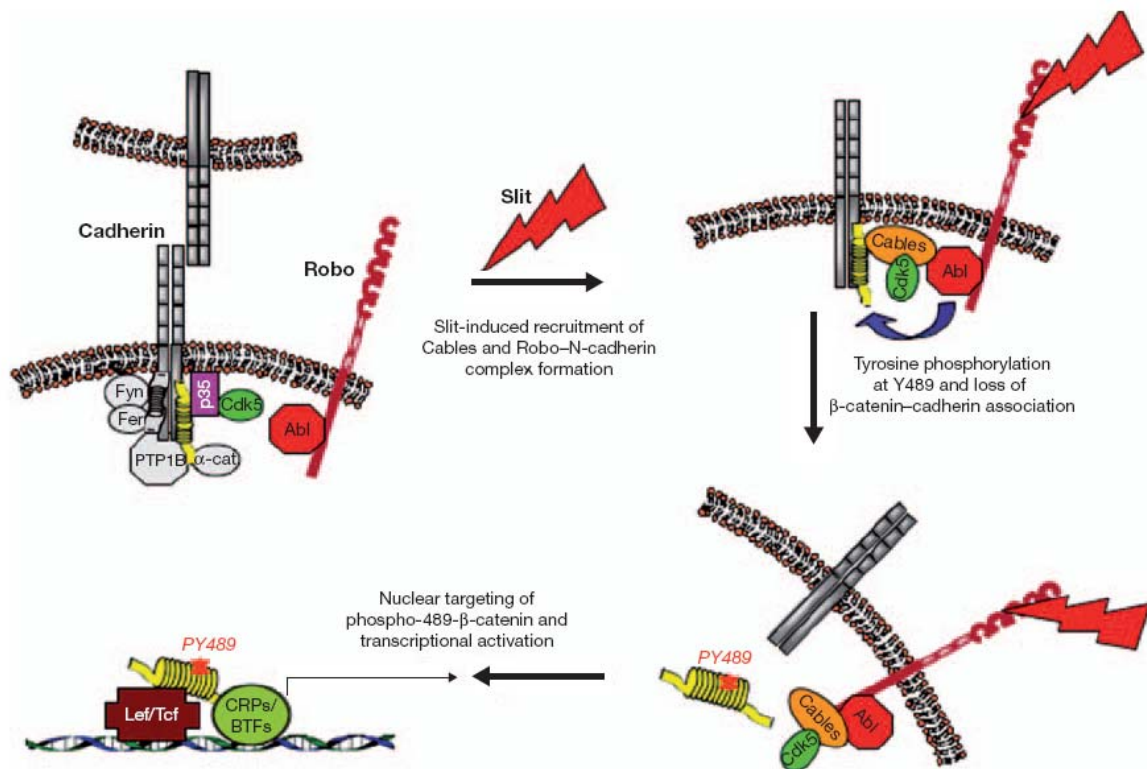


Figure 1.5: Model depicting the formation of the Slit-induced Robo/Abl-Ncadherin/β-catenin complex. Fyn and Fer are tyrosine kinases associated with the cadherin complex through p120-catenin^{25,32}. PTP1B, protein tyrosinephosphatase bound directly to N-cadherin; CRPs and BTFs, chromatin remodelling proteins and basal transcription factors, respectively, associated with transcriptionally active β-catenin (Rhee, Buchan et al. 2007).

srGAP1 (Slit–Robo Rho GTPase-activating protein 1) has been shown to bind to the proline-rich CC3 motif of mammalian Robo1 and mediate the Slit-dependent inactivation of the Rho family GTPase, Cdc42 (cell division cycle 42). The adaptor protein Dock/Nck also binds to CC3 (as well as to CC2) and mediates the Slit-dependent recruitment of SOS (Son of sevenless), which in turn activates another Rho GTPase, Rac (Wong, Ren et al. 2001; Fan, Labrador et al. 2003; Yang and Bashaw 2006). These examples illustrate how Slit–Robo signalling could alter cytoskeletal dynamics through regulation of Rho family members. In addition, Ena/VASP proteins bind to CC1 and CC2 (Bashaw, Kidd et al. 2000) and the Rho family GTPase activating protein (GAPs) Vilse/crGAP binds to CC2 (Lundstrom, Gallio et al. 2004; Hu, Li et al. 2005) (Figure 1.4 C). Inhibitor studies have also implicated PI3K (phosphoinositide 3-kinase), MAPKs (mitogen-activated protein kinases) and localized protein synthesis, but the adaptor molecules linking Robo activation to these pathways have yet to be identified (Wang, Xiao et al. 2003; Piper, Anderson et al. 2006). Furthermore, the cytosolic domains of DCC (deleted in colorectal cancer) and Robo receptors have been shown to interact physically, and Slit-activated Robo has been suggested to silence the attractive

Netrin-DCC signal once an axon has reached the CNS midline (Stein and Tessier-Lavigne 2001).

1.2.3 Structural basis of Slit-Robo interaction

All Robos, other than Robo4, were shown to bind to Slit ligands. This interaction is evolutionarily conserved (Brose, Bland et al. 1999). Concave face of Slit D2 domain was described as the minimum Robo binding domain and contains the common binding residues (*Drosophila Slit* residues Tyr402, Leu424, Tyr450 and His472) for all *Drosophila Robos* (Howitt, Clout et al. 2004). Also in vertebrates Slit D2 is responsible for Robo binding, containing the four conserved amino acids: Tyr356, Leu378, Tyr404 and His426 for human Robo1 (Morlot, Thielens et al. 2007) and it has the biological activity as a repellent demonstrated on *Xenopus* axon growth cones (Hussain, Piper et al. 2006).

Extracellular IG1 and IG2 domains of Robo proteins are required for Slit interaction (Liu, Patel et al. 2004) and the observed stoichiometry of minimum Slit-Robo interaction is 1:1 (Hussain, Piper et al. 2006). Although Robo IG2 lies far C-terminus to Slit interacting IG1, its deletion has dramatic effects on Slit binding, so the contribution of IG2 should further be clarified (Hohenester 2008).

1.2.4 Role of Slit-Robo interactions in normal development and physiology

1.2.4.1 Regulation of commissural axon pathfinding

The *slit* mutant was identified first in the classic genetic screen for embryonic patterning defects in *Drosophila* and subsequently in screens for commissural axon pathfinding defects. The mutants are characterized by the collapse of axon tracts onto the midline. *robo* was also initially identified through genetic studies in *Drosophila*; specifically, in the screen for mutants with commissural axon pathfinding defects. In *robo* mutant embryos, too many axons cross the midline, including both ipsilateral axons that aberrantly cross and commissural axons that recross (Nusslein-Volhard C 1984; Rothberg, Jacobs et al. 1990; Seeger, Tear et al. 1993; Kidd, Brose et al. 1998; Hummel, Schimmelpfeng et al. 1999; Kidd, Bland et al. 1999).

Vertebrate commissural neurons are born in the dorsal spinal cord, and their axons are first drawn to the midline by the chemoattractants netrin and sonic hedgehog, which emanate from the ventral floor plate (Kennedy, Serafini et al. 1994; Serafini, Kennedy et al. 1994; Charron, Stein et al. 2003). These axons then cross the floor plate and turn longitudinally on the opposite (contralateral) side, growing right alongside the floor plate, laterally. *Drosophila* commissural neurons also require netrins for crossing (Harris, Sabatelli et al. 1996; Mitchell, Doyle et al. 1996) (Figure 1.6).

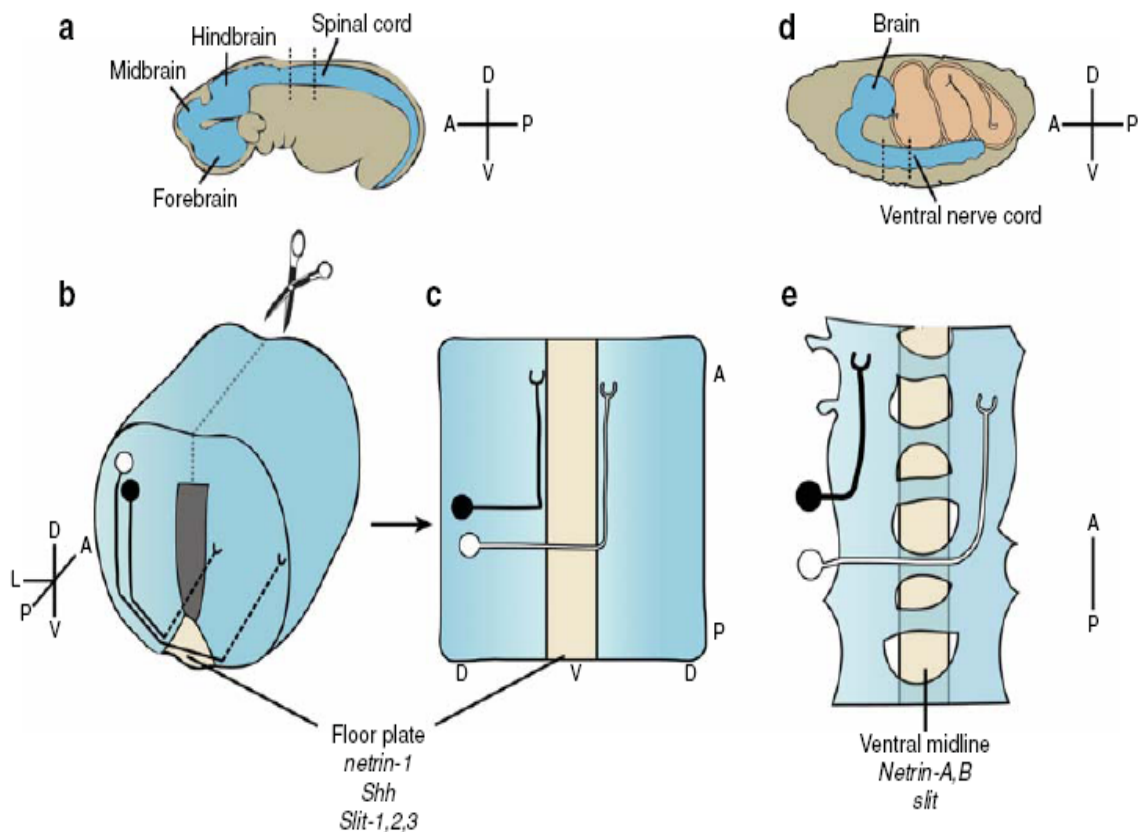


Figure 1.6: Commissural axon pathfinding in the mouse spinal cord and *Drosophila* ventral nerve cord. (a) Schematic of E9.5 mouse embryo. (b) Spinal cord section from E12.5 mouse embryo (as indicated by *dashed lines* in a). Examples of commissural (*white*) and ipsilateral (*black*) neurons are shown. (c) “Open-book” preparation, obtained by dissecting the spinal cord along the dashed line as indicated in b and laying it flat. (d) Stage 16 *Drosophila* embryo, at the end of embryogenesis. (e) Dorsal view of two segments of the ventral nerve cord, showing examples of intersegmental commissural (*white*) and ipsilateral (*black*) neurons. A, anterior; P, posterior; D, dorsal; V, ventral; L, lateral (Dickson and Gilestro, 2006).

In *Drosophila*, *commisureless* sorts *robo* to endosomes during midline crossing, thus prevents Slit’s repulsive effect (Keleman, Rajagopalan et al. 2002). In vertebrates, Robo3 has a similar effect and suppresses Robo1 activity in precrossing axons. However, in postcrossing axons Robo3 action is relieved allowing Robo1 to be expressed and arrest DCC activity. In

addition, Slit interaction with Robo1 blocks floor plate recrossing in these postcrossing axons (Stein and Tessier-Lavigne 2001; Sabatier, Plump et al. 2004) (Figure 1.7).

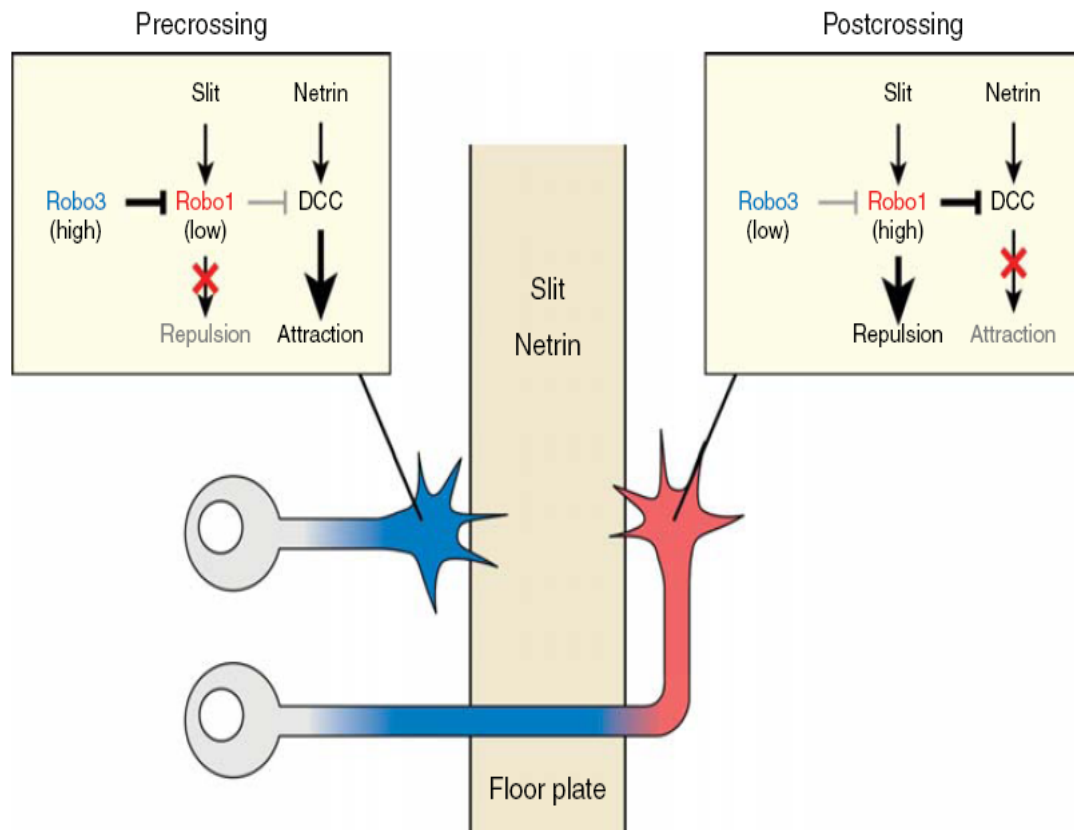


Figure 1.7: Robo3 antagonizes Robo1 to allow crossing in mice. In precrossing commissural axons, Robo3 levels are high, and Robo1 levels low. Robo3A is thought to inhibit Robo1-mediated repulsion in these axons so that they are instead attracted to the floor plate by netrin-1 and sonic hedgehog. After crossing, Robo3 levels are low, and Robo1 levels high. Axons are now repelled by signaling of Slit through Robo1. In addition, attraction to netrin-1 may be downregulated, possibly owing to a Slit-dependent interaction between Robo1 and the netrin receptor DCC (Deleted in colorectal cancer) (Dickson and Gilestro, 2006).

Lateral positioning of longitudinal axon fascicles are also determined by Robo homologs. In *Drosophila*, loss- and gain-of-function experiments have showed that there are mainly three longitudinal paths with respect to Robo expression: lateral fascicles express Robo1, Robo2 and Robo3; intermediate fascicles express Robo1 and Robo3; while medial fascicles express only Robo1 (Rajagopalan, Vivancos et al. 2000; Simpson, Bland et al. 2000) (Figure 1.8).

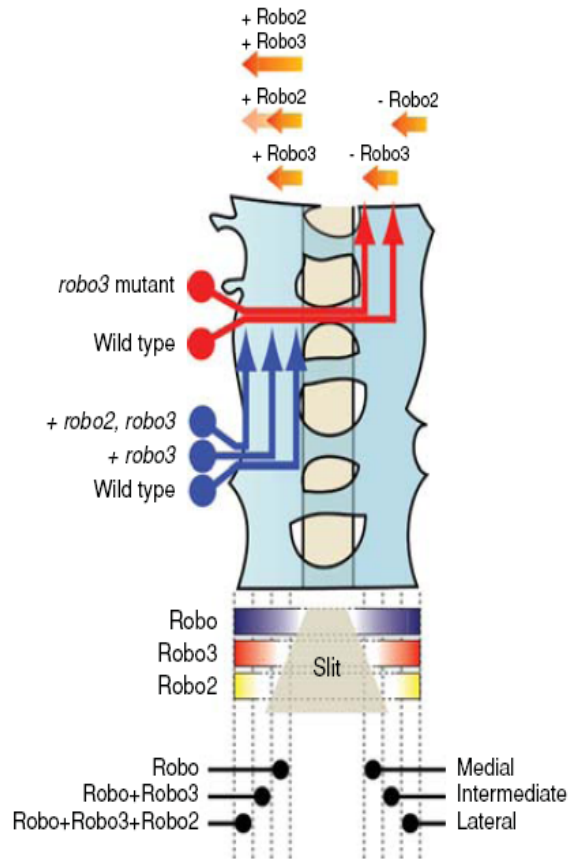


Figure 1.8: A Robo code for lateral positioning in *Drosophila*. Schematic showing the division of the longitudinal axon tracts into three major zones, according to the Robo code (Dickson and Gilestro, 2006).

1.2.4.2 Role of Slit2-Robo2 interaction in kidney development

Studies examining SLIT/ROBO signaling during kidney development bring this issue to the fore, with the recent analysis of *Slit2*^{-/-} mice suggesting a new role for SLIT2 signaling in regulating transcription (Grieshammer, Le et al. 2004). In contrast, efforts to demonstrate an established role for SLIT2 as a branching factor in kidney have been unsuccessful (Piper, Nurcombe et al. 2002).

In the absence of *Slit2* and also in *Robo2*^{-/-} mice multiple ureteric buds form in developing mouse kidney. In *Slit2*^{-/-} animals *Gdnf* gene dosage reduction results in rescue of the observed supernumerary bud phenotype. Expression analysis reveals *Slit2* transcripts in the nephric duct and in the anterior nephrogenic mesenchyme, where *Robo2* is also expressed. In contrast, *Gdnf* transcripts are expressed throughout the nephrogenic mesenchyme, but over the course of development they become progressively restricted to the posterior region where the ureteric bud forms. In the light of the available data, a model is proposed describing that SLIT2 signals through ROBO2 to suppress supernumerary bud formation (Figure 1.9). These

findings suggest that ROBO mediated signaling may have roles in cell fate specification by regulating transcription or translation of key determinants (Grieshammer, Le et al. 2004; Hinck 2004).

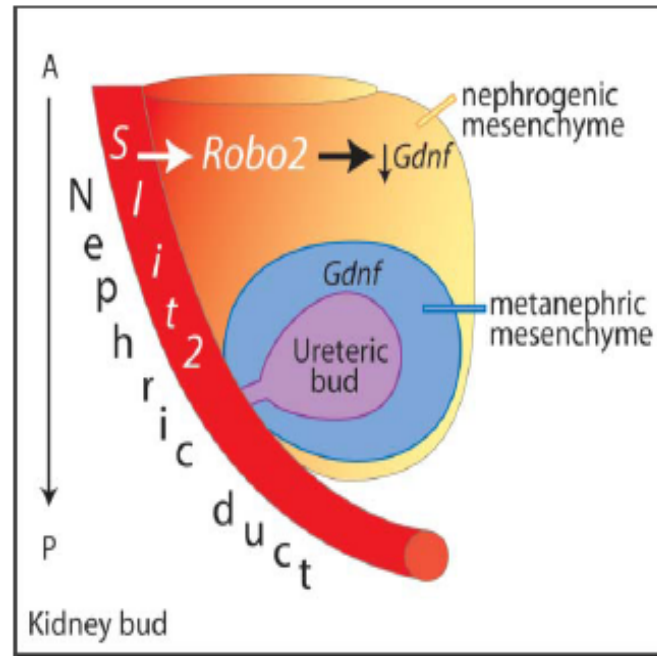


Figure 1.9: SLIT2-ROBO2 signaling is essential for proper kidney development in mice. SLIT2 signaling through ROBO2, restricts the anterior expression of *Gdnf*. In the posterior region, lack of ROBO2 prevents SLIT signaling, allowing *Gdnf* expression and consequently the appropriate outgrowth of the ureteric bud (Hinck L, 2004).

1.2.4.3 Role of Slit-Robo in lung development

Transcripts of *Slit2*, *Slit3*, *Robo1* and *Robo2* are expressed in the lung (Xian, Clark et al. 2001; Anselmo, Dalvin et al. 2003; Greenberg, Thompson et al. 2004). A small cell lung cancer line expresses a mutated *Robo1* gene termed *Dutt1*, for deleted in U2020, which is missing the first immunoglobulin repeat in the extracellular domain (Sundaresan, Chung et al. 1998). Mice engineered to express only *Dutt1* frequently die at birth due to respiratory failure, and histological analyses of their lungs reveal abnormally dense mesenchyme, surrounding smaller and irregularly shaped bronchioles (Xian, Clark et al. 2001). Since SLIT-ROBO interaction shown to promote axon branching in the nervous system, possibly these irregularly shaped bronchioles are a result of inappropriate branching (Wang, Brose et al. 1999; Ozdinler and Erzurumlu 2002; Hinck 2004).

1.2.5 Slit-Robo in cancer and other pathologies

1.2.5.1 *SLIT-ROBO* expression in tumors of various tissues

Although no more than a decade has passed from their discovery, there is mounting evidence on the involvement of SLITs and ROBOs in cancers. So far, no somatic point mutation of *ROBO1* (or of its ligands SLITs) was reported in tumors. A targeted mutation of mouse *robo1* was generated by deletion of exon 2, mimicking a deletion that naturally occurs in human small cell lung cancer cell line NIH-H219X, and resulted in the removal of Robo1 Ig1. In total, 63% of *Robo1*^{-/-} homozygous mice die in the first 24 hours because of respiratory failure due to abnormal lung development. A few homozygous mice survive up to 1 year and show epithelial bronchial hyperplasia, but no spontaneous tumor formation was detected. Later on tumor susceptibility of *Robo1* heterozygous mice was analyzed. During their second year of life, *Robo1* heterozygotes develop lymphoma and carcinomas, such as invasive lung carcinomas. In malignant tumor samples from *Robo1*^{+/-} mice, the expression of *Robo1* is undetectable. Moreover, the study of the remaining allele showed that its promoter is hypermethylated. Overall, these studies support a role for *Robo1* as a tumor suppressor gene, at least in the mouse (Xian, Clark et al. 2001; Xian, Aitchison et al. 2004; Chedotal, Kerjan et al. 2005).

The expression of *SLIT-ROBO* genes are analysed by quantitative RT-PCR in prostate tumor samples. *SLIT1* and *SLIT3* are found to be overexpressed in prostate tumors (Latil, Chene et al. 2003). *SLIT2* is expressed in many tumor cell lines such as human melanoma (A375), bladder squamous carcinoma (SCaBER), neuroblastoma (SK-N-SH), small cell lung cancer (NCI-H446), carcinoma of urinary bladder (T24), colon adenocarcinoma (LoVo), breast cancer (ZR-75-30), nasopharyngeal carcinoma (CNE), hepatocellular carcinoma (SMMC-7721), salivary gland carcinoma (Acc), rhabdomyosarcoma (A673) and primary tumors (melanoma, invasive breast carcinoma, colorectal carcinoma, etc.). *SLIT2-ROBO1* signaling increased microvessel density and tumor mass in a xenograft model (Wang, Xiao et al. 2003). *Slit2* acts as a potent chemoattractant for breast cancer cells specifically metastasizing to brain suggesting a role for *Slit/Robo* signaling in brain metastasis. In addition, *ROBO1* is overexpressed in breast carcinoma tissue samples (Schmid, Reznicek et al. 2007).

1.2.5.2 Epigenetic regulation of *SLIT-ROBO* genes

SLIT2 was proposed to be a tumor suppressor gene, which was silenced epigenetically in lung, breast, colon cancers and gliomas. In addition, *SLIT2* suppressed growth of colorectal carcinoma cells (Dallol, Da Silva et al. 2002; Dallol, Krex et al. 2003; Dallol, Morton et al. 2003). *SLIT3* was silenced by promoter hypermethylation in gliomas and colorectal cancers whereas *SLIT1* was inactivated epigenetically in only gliomas (Dickinson, Dallol et al. 2004). In these studies, epigenetic inactivation of *ROBO1* was shown in carcinomas of lung, breast and kidney (Dallol, Forgacs et al. 2002). Recently, gradual increase in promoter hypermethylation of *SLIT1*, *SLIT2*, *SLIT3*, *ROBO1* and *ROBO3* was reported during progression into cervical cancer (Narayan, Goparaju et al. 2006).

1.2.5.3 Role of *SLIT-ROBO* signaling in cancer cell migration and immune cell chemotaxis

Tumor cells often migrate to distant organs leading to secondary tumor formation and chemokines play a role in this process. *Slit2* was shown to be a potent inhibitor of stromal-derived factor (SDF)-1 induced leukocyte chemotaxis. This effect requires the interaction of CXCR4 with *Robo1* that are both expressed by leukocytes. Breast cancer cells and human melanoma also express CXCR4, *ROBO1* and *ROBO2* and chemokines such as CXCL12 that stimulate the migration of cancer cells. It has been shown that *Slit* inhibits CXCL12/CXCR4-induced breast cancer cell (DU4475) chemotaxis, chemoinvasion and adhesion. *Slit* inhibits CXCL12-induced phosphorylation of the focal adhesion component FAK and RAFTK/Pyk2 and paxillin. It also inhibits CXCL12-induced Src kinase and PI3-kinase activities, p44/42 MAP kinase and activity of the matrix metalloproteinase MMP-2 and MMP-9 (Wu, Feng et al. 2001; Prasad, Fernandis et al. 2004; Chedotal, Kerjan et al. 2005; Prasad, Qamri et al. 2007). *SLIT2* overexpression in MCF7 breast carcinoma cells induces tumor suppressive effect by increasing β -catenin/E-cadherin mediated cell-cell adhesion (Prasad, Paruchuri et al. 2008). *SLIT2-ROBO1* system suppresses HGF-dependent invasion in melanoma, endometrial and ovarian carcinoma cells (Stella, Trusolino et al. 2009).

1.2.5.4 Endothelial cell migration and angiogenesis

The most recently discovered member of the Roundabout family is *magic roundabout* or *ROBO4*. It was identified in a bioinformatics study for endothelial specific genes (Huminiacki

and Bicknell 2000; Huminiecki, Gorn et al. 2002). There are other SLIT-ROBO members which are expressed in endothelial cells. Slit2 and Slit3 mRNAs but not Slit1 were expressed in the rat endothelial cells (Wu, Feng et al. 2001). Robo1 was expressed on vascular endothelial cells and Slit2-Robo1 signaling was involved in angiogenesis. This increased microvessel density and tumor mass in a tumor xenograft model (Wang, Xiao et al. 2003). Recent findings indicated that SLIT2-ROBO4 interaction inhibited angiogenesis. It was shown that ROBO4 maintains the vascular integrity by inhibiting the pathologic angiogenesis and endothelial hyperpermeability. The mechanism of inhibition of pathologic angiogenesis is through the inhibition of VEGF-165 by Robo4/Slit2 axis. This inhibition also decreases the permeability levels of the vascular network (Jones, London et al. 2008).

1.2.5.5 Disease association of mutations in *ROBO1*, *ROBO2* and *ROBO3* genes

In 2004, mutations in *ROBO3* were described in patients with horizontal gaze palsy with progressive scoliosis (HGPPS) (Jen, Chan et al. 2004). Dyslexia- which is a difficulty in reading and writing despite normal intelligence, senses and adequate education- may be caused by partial haplo-insufficiency for *ROBO1* in rare families. It has shown that *ROBO1* gene was disrupted by a chromosome translocation in a dyslexic individual. In a large pedigree with 21 dyslexic individuals genetically linked to a specific haplotype of *ROBO1*, the expression of *ROBO1* from this haplotype was absent or attenuated in affected individuals (Hannula-Jouppi, Kaminen-Ahola et al. 2005). As its role in kidney development in mice, human *ROBO2* was shown to be essential for proper kidney and urinary tract development in human (Lu, van Eerde et al. 2007). Later in 2008, mutational gene variants of *ROBO2* were associated with Familial Vesicoureteral Reflux (Bertoli-Avella, Conte et al. 2008).

1.2.5.6 Regulation of *SLIT-ROBO* genes in hepatocarcinogenesis

Despite the compiling evidence of SLIT-ROBO deregulation in various tumors, only few reports with apparent controversies exist with regard to the expression pattern of these genes in hepatocellular carcinoma (HCC). The overexpression of ROBO1 in HCC was recently reported and this receptor was proposed as an HCC marker in human (Ito, Funahashi et al. 2006). In contrast, another study reported that Robo1 heterozygous mice developed spontaneous HCC tumors (Xian, Aitchison et al. 2004). It was shown by immunohistochemical staining that SLIT2 protein also was present in HCC tumor sections

(Wang, Xiao et al. 2003). SLIT1, SLIT2, SLIT3 are present in only poorly differentiated HCC samples at a low level (Ito, Funahashi et al. 2006).

Epigenetic silencing of *SLIT2* was recently described in HCC. Downregulation of *SLIT2* was detected in 6 of 8 (75%) HCC cell lines by quantitative real-time RT-PCR (qRT-PCR), and the downregulation of SLIT2 was generally dependent on the degree of methylation at the promoter region. Furthermore, expression of *SLIT2* was restored in relatively low-expressing cell lines after treatment with 5-aza-2-deoxycytidine (5-Aza-dC). Downregulation of *SLIT2* expression was also detected in 45 of 54 primary HCC samples (83.3%), and the decrease in expression was significantly correlated with CpG island hypermethylation. This decrease of *SLIT2* expression was also associated with lymph node metastasis in HCC. Moreover, overexpression of *SLIT2* in SMMC-7721 cells suppressed cell growth, migration, and invasion (Jin, You et al. 2009).

2 OBJECTIVES AND RATIONALE

Hepatocellular carcinoma is the sixth most common neoplasm in the world, with more than half a million new cases annually, and the main cause of death among cirrhotic patients (Llovet, Di Bisceglie et al. 2008). It is associated mainly with hepatitis B and C viral infections, Aflatoxin B1 exposure and chronic alcohol consumption etiologies. For early diagnosis of HCC, surveillance of the risk groups is a crucial task. The major diagnostic techniques for HCC include serum markers, various imaging modalities and histological analysis (Gomaa, Khan et al. 2009). Nowadays, early HCC diagnosis is feasible in 30–60% of cases in developed countries and this enables the application of curative treatments. Detection of minute nodules of ~2 cm poses a diagnostic challenge as they are difficult to characterize by radiological or pathological examination. These nodules are defined as pre-neoplastic lesions. Dysplastic lesions should be followed by regular imaging studies, since one third of them will develop a malignant phenotype. Therefore, accurate diagnosis of small liver nodules is of paramount importance. However, current molecular diagnostic methodologies have restraints. Immunostaining with CD34 and alpha fetoprotein (AFP) has significant diagnostic limitations (Park, Yang et al. 1998). Serum biomarkers such as AFP, des- γ -carboxyprothrombin (DGCP) and AFP-L3 fraction are not accurate for the early diagnosis of HCC (Bruix and Sherman 2005). Therefore, the development of novel markers for HCC with stronger sensitivity and specificity is important for the surveillance of patients with liver cancer risk.

Hepatocellular carcinoma is a complex and heterogeneous tumor with different genetic and epigenetic alterations, chromosomal aberrations, gene mutations, and altered molecular pathways (Farazi and DePinho 2006). With the implementation of high-throughput technologies like microarrays, molecular subclasses of cancers are better defined and led to the development of molecularly targeted therapies. A good example is the therapeutic use of trastuzumab (Herceptin) in ErbB2 positive breast cancers. Description of biomarkers specific to hepatocellular carcinoma subtypes could identify novel targets for therapy. In this study, we aimed to identify novel diagnostic and prognostic hepatocellular carcinoma markers; initially by database searches and then with expression analyses for validation and confirmation.

Our preliminary efforts directed us to investigate the *SLIT-ROBO* gene families in HCC. *SLIT-ROBO* gene family members initially described for their roles in axon guidance during development. Subsequently, their involvement in cancers of various tissues has been identified. *ROBO1* is located to a genomic region that is deleted in lung carcinomas. Immunoglobulin1 domain deletion of *ROBO1* was identified in lung cancer cell lines. Epigenetic inactivation of *SLIT1*, *SLIT2*, *SLIT3* and *ROBO1* genes were described in human tumor cell lines and tissues. *ROBO4* overexpression and *SLIT2-ROBO1* signaling is implicated in angiogenesis and tumor cell migration. Despite the compelling evidence of *SLIT-ROBO* deregulation in various tumors, only few reports with apparent controversies exist with regard to the expression pattern of these genes in hepatocellular carcinoma (HCC). We aimed to describe the expression of members of the *SLIT-ROBO* families in HCC cell lines and tissues. *ROBO2* gene expression signature emerged as a good distinction marker between *AFP* positive and negative HCC cell lines: with *ROBO2* overexpression in *AFP* positive cell lines. The absence of *ROBO2* expression in *AFP* negative cell lines with higher migration capacity and the role of *SLIT-ROBO* interaction as a chemokine signaling in cellular migration led us to hypothesize that *SLIT-ROBO* signaling may affect migratory behavior of HCC cells. We proposed that *ROBO2* gene knockdown in a model *AFP* positive cell line would unravel the cellular phenotype associated with *ROBO2*.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Reagents

All laboratory chemicals were analytical grade from Sigma-Aldrich (St.Louis, MO, USA), Farmitalia Carlo Erba (Milano, Italy) and Merck (Schuchardt, Germany) with the following exceptions: ethanol and methanol were from Riedel-de Haën (Germany). Agar, tryptone and yeast extract were obtained from Gibco (Carlsbad, CA, USA), BRL Life Technology Inc. (Gaithersburgs, MD, USA).

3.1.2 Bacterial strains

The bacterial strain used in this work was *E.coli* DH5 α .

3.1.3 Enzymes

Restriction endonucleases used for gene cloning were purchased from Jena Bioscience (Germany). BsrG1 restriction enzyme was purchased from New England Biolabs (Ipswich MA, USA-Catalog No: RO575S). T4 DNA ligase was purchased from Fermentas GmbH (Germany). DyNAzyme II DNA Polymerase was purchased from Finnzymes (Espoo, Finland).

3.1.4 DNA and protein molecular weight markers

DNA molecular weight standards were purchased from Fermentas GmbH (Germany) (100 bp-SM0241, 1kb-SM0311) and Jena Bioscience (Germany) (low range DNA ladder 50-1000 bp, high range DNA ladder 0.5-10kb). All protein molecular weight markers used in this study were obtained from Fermentas GmbH (Germany).

3.1.5 Oligonucleotides

The oligonucleotides used in polymerase chain reactions were synthesized and supplied from Iontek Inc. (Istanbul, Turkey) and Alpha DNA (Germany).

3.1.6 Plasmids

pSECneo vector (Cat #5774) was purchased from Ambion (Austin, TX, USA). pGIPZ shROBO1, shROBO2, and non-targeting vectors were purchased from OpenBiosystems (Huntsville, USA) while pDEST26-ROBO2 overexpression vector was purchased from ImaGenes GmbH (Berlin, Germany). pDEST™26 Gateway® Vector (Cat. No.11809019) was purchased from Invitrogen (Carlsbad, CA, USA).

3.1.7 Electrophoresis, autoradiography, photography and spectrophotometer

Electrophoresis grade agarose was obtained from Sigma Biosciences Chemical Company Ltd. (St. Louis, MO, USA). Horizontal electrophoresis apparatuses were from E-C Apparatus Corporation (Florida, USA). The power supply Power-PAC300 and Power-PAC200 was from Bio Rad Laboratories (CA, USA). The Molecular Analyst software used in agarose gel profile visualizing was from Vilber Lourmat (France). Mini-PROTEAN3 cell system and Trans-Blot SD semi-dry electrophoretic transfer cell were purchased from Bio-Rad (CA, USA). Immobilen-P transfer (PVDF) membrane was from Millipore (MA, USA) and 3MM filter paper was from Whatman International Ltd. (Madison, USA). The films used for autoradiography were Kodak and the development of the films was performed with Hyperprocessor (Amersham, UK). ECL-Plus Western Blotting detection reagent was purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). Beckman Spectrophotometer Du640 was purchased from Beckman Instruments Inc. (CA, USA) and Nanodrop ND-1000 Full-spectrum UV/Vis Spectrophotometer was purchased from Thermo Fisher Scientific (Wilmington, DE, USA).

3.1.8 Tissue culture reagents and cell lines

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, trypsin, non-essential amino acids, penicillin/streptomycin mixture and fetal calf serum were obtained from HyClone (South Logan, UT, USA). Tissue culture flasks, petri dishes and cryotubes were purchased

from Costar Corp. (Cambridge, England). Geneticin-G418 sulfate was purchased from GibcoBRL (Carlsbad, CA, USA).

3.1.9 Transfection reagents

FuGene HD (Roche, Mannheim, Germany) and Licofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfection reagents were used in this study. OptiMEM I serum free medium was purchased from Invitrogen (Carlsbad, CA, USA).

3.1.10 Kits

RNeasy Mini Kit was obtained from Qiagen (Hilden, Germany). NucleoSpin RNA II Kit was obtained from Macherey-Nagel (Duren, Germany). DyNAmo cDNA Synthesis Kit and DyNAmo™ HS SYBR® Green qPCR Kit were purchased from Finnzymes (Espoo, Finland). TissueScan Liver Cancer Tissue qPCR Array 1 was purchased from Origene Technologies (Rockville, MD, USA-Catalog No: LVRT501). Silencer Express Kits (Human H1 and Human U6) were purchased from Ambion (Austin, TX, USA). Qiaprep spin mini-prep kit (for small scale DNA isolation), Qiafilter plasmid midi kit (for medium-scale DNA isolation), Qiaquick PCR purification and gel extraction kits (for recovery and extraction of DNA from agarose gel) were from Qiagen (Hilden, Germany). UltraClean Tissue DNA Isolation Kit was purchased from MOBIO Laboratories (Carlsbad, CA, USA).

3.1.11 Antibodies

SLIT1, SLIT2, SLIT3, ROBO1, ROBO2, ROBO4 antibodies were purchased from Santa Cruz (USA). Additionally, mouse monoclonal anti-human ROBO2 antibody was purchased from RD Systems (USA). Rabbit polyclonal anti-human CD133 antibody was purchased from AbCam (USA). BrdU Mouse Monoclonal Antibody, used at a dilution of 1:500 in BrdU incorporation assay, was purchased from DAKO (Glostrup, Denmark). Anti-mouse and anti-rabbit HRP conjugated secondary antibodies were obtained from Sigma (USA). Anti-mouse and anti-rabbit AlexaFluor Red 568 and AlexaFluorGreen 488 secondary antibodies were purchased from Invitrogen, Carlsbad, CA, USA).

3.2 SOLUTIONS AND MEDIA

3.2.1 General solutions

Tris-acetic acid-EDTA (TAE): Stock solution (50XTAE) was prepared by addition of 121g Tris-base, 18.6g EDTA, and 28.55ml glacial acetic acid to 500ml ddH₂O. pH of the stock solution was adjusted to 8.5. Working solution (1XTAE) was prepared by dilution of 50XTAE to 1X with ddH₂O.

Ethidium bromide: 10mg/ml in water (stock solution), 30ng/ml (working solution).

6X Agarose gel loading dye: A mixture of 0.009g bromophenol blue (BFB), 0.009g xylene cyanol (XC), 2.8ml ddH₂O, 1.2ml 0.5M EDTA was prepared. The total volume was brought to 15ml by addition of glycerol.

3.2.2 RNA solutions

DEPC-treated water: 1ml DEPC was added to 1L ddH₂O and stirred under hood overnight. DEPC was inactivated by autoclaving.

Formaldehyde (FA) gel buffer: Stock solution (10XFA Gel Buffer) was prepared by dissolving 20.927g MOPS, 3.40g NaAc and 1.86g EDTA in 500ml ddH₂O. pH of the stock solution was adjusted to 7.0. Working solution was prepared by diluting the stock solution to 1X with ddH₂O.

5X RNA loading buffer: Bromophenol blue solution 16 µl, 500 mM EDTA pH 8.0 80µl, 37% formaldehyde 720µl, 100% glycerol 2ml, formamide 3084µl, 10XFA gel buffer 4ml, RNase free (DEPC treated) water to 10ml.

3.2.3 Microbiological media, reagents and antibiotics

Luria Bertani (LB) medium: Per liter; 10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl. For LB agar plates, 15g/L bacto agar was added.

Glycerol stock solution: A final concentration of 25% glycerol in LB was added to bacterial culture.

Ampicillin: 100mg/ml solution in ddH₂O, sterilized by filtration and stored at -20°C (stock solution). Working solution was 100µg/ml.

Kanamycin: 300mg/ml solution in ddH₂O sterilized by filtration and stored at -20°C (stock solution). Working solution was 30µg/ml.

SOB medium: Per liter; 20g tryptone (2%), 5g yeast extract (0.5%), 0.584g NaCl (10mM), 0.1864g KCl (2.5mM) autoclaved to sterilize. Then, 2.46g MgSO₄ and 2.03g MgCl₂ (10mM) are added.

SOC medium: SOB + 20mM glucose from filter sterilized 1M glucose stock solution in ddH₂O.

Transformation buffer (TB): 10mM PIPES, 55mM MnCl₂, 15mM CaCl₂, 250mM KCl. Filter sterilized and stored at 4°C.

3.2.4 Tissue culture solutions

DMEM/RPMI-1640 growth media: 10% FBS, 1% penicillin/streptomycin, 1% non-essential amino acid were added and stored at 4°C.

Freezing solution: 10% DMSO and 90% FCS were mixed freshly.

Phosphate buffered saline (PBS): Stock solution (10XPBS) was prepared by dissolving 80g NaCl, 2g KCl, 17.8g Na₂HPO₄·2H₂O, and 2.4g KH₂PO₄ in 1L ddH₂O. Working solution (1XPBS) was prepared by dilution of 10XPBS to 1X with ddH₂O. pH of the working solution was adjusted to 7.4.

Geneticin (G418) sulfate: 500mg/ml solution in ddH₂O. Sterilized by filtration and stored at -20°C (stock solution). 500µg/ml was used as working solution for stable cell line selection and 250µg/ml was used as working solution for maintenance of stable cell lines.

Puromycin: 2mg/ml solution in ddH₂O. Sterilized by filtration and stored at -20°C (stock solution). 2µg/ml was used as working solution for selection.

3.2.5 Protein extraction, quantitation and western blotting solutions

Radio immuno-precipitation assay (RIPA) buffer: 150mM NaCl, 50mM Tris-HCl pH 8.0, 1% NP-40, 0.1% SDS and 1X protease inhibitor mix (Roche, Basel, Switzerland) were mixed in ddH₂O.

Bradford stock solution: 17.5mg coomassie brilliant blue was dissolved in 4.75ml ethanol and 10ml phosphoric acid and completed to 25ml final volume with ddH₂O.

Bradford working solution: 1.5ml bradford stock solution was mixed with 0.75ml 95% ethanol and 1.5ml phosphoric acid and completed to final volume up to 25ml with ddH₂O. Filtered through whatman paper and prepared freshly.

Acrylamide-bisacrylamide solution: 29g acrylamide and 1g bisacrylamide were dissolved in 100 ml ddH₂O and stored in the dark at 4°C (stock solution).

10% Ammonium persulfate (APS): 0.1g APS was dissolved in 1ml of ddH₂O, prepared freshly.

1.5M Tris-HCl, pH 8.8: 54.45g Tris base (18.15g/100ml) ~150ml distilled water. Adjust to pH 8.8 with 1N HCl. Completed to 300ml with distilled water and stored at 4°C.

1M Tris-HCl, pH 6.8: 12.14g Tris base ~ 60ml distilled water. Adjust to pH 6.8 with 1N HCl. Completed to 100ml with distilled water and store at 4°C.

Coomassie brilliant blue solution: Per liter; 100mg Coomassie brilliant blue G-250, 50ml 95% ethanol, and 100ml 85% phosphoric acid. Filtered through whatman paper and stored at 4°C.

5X SDS-gel loading buffer: 5g SDS, 25mg bromophenol blue, 15.7ml 1M Tris pH 6.8, 21.8ml glycerol (from 87% stock) were mixed and completed to 50ml with ddH₂O. Before use, β-mercaptoethanol was freshly added to a final concentration of 5% to reach 1% when mixed with protein samples.

10X SDS-gel electrophoresis buffer: Per liter; 30.3g Tris base, 144.0g glycine, 10.0 g SDS. Diluted to 1X for working solution. Stored up to 1 month at 4°C.

Semi-dry transfer buffer: Per liter; 48mM Tris base, 39mM glycine, 0.037% SDS, 20% methanol.

Wet transfer buffer: 3.03g Tris and 14.4g glycine was mixed with 1ml 10% SDS and 20% methanol and completed to final volume of 1 liter. For high molecular weight proteins methanol was decreased by half.

10X Tris buffered saline (TBS): 30g Tris base, 80g NaCl and 2g KCl were dissolved in 1 liter of ddH₂O and the pH was adjusted to 8 (stock solution). Diluted to 1X and pH was adjusted to 7.6 with HCl just before use.

TBS-Tween (TBS-T): 0.1% Tween-20 was added into 1X TBS solution.

Blocking solution: 5% (w/v) non-fat milk, 0.1% Tween-20 in 1XTBS (prepared freshly).

3.2.6 Immunofluorescence solutions

H33258 fluorochrome dye: 1mg/ml solution in ddH₂O and stored at -20°C. Working solution was 1µg/ml.

DAPI (4', 6-diamidino-2-phenylindole): 0.1-1 µg/ml (working solution in ddH₂O).

2% paraformaldehyde: 2g paraformaldehyde dissolved in 100ml 1X PBS, pH 7.4. Stored in dark, at -20°C.

PBS-TritonX-100 (PBS-T): 0.1 Triton X-100 in 1X PBS.

Blocking solution: 2% BSA (bovine serum albumin) in 1X PBS.

3.2.7 BrdU incorporation assay solutions

BrdU stock solution: 10mg/ml BrdU in ddH₂O.

2N HCl: 9.1ml of stock 37% HCl (~11N), completed to 50ml with ddH₂O.

PBS-TritonX-100 (PBS-T): 0.1TritonX-100 in PBS.

3.3 METHODS

3.3.1 General methods

3.3.1.1 Bacterial transformation

3.3.1.1.1 Super-competent *E.coli* preparation

Super-competent *E.coli* preparation was described by Inoue (Inoue, Nojima et al. 1990). *E.coli* DH5 α cells were grown in SOB medium at 30°C to an O.D.₆₀₀ of 0.5-0.6 with vigorous shaking at 225rpm and cooled down on ice for 10 minutes. Cells were transferred to 500ml centrifuge bottles and centrifuged at 2500g for 10 minutes (Beckman JA10 rotor, pre-cooled to 4°C). The pellet was resuspended in ice-cold transformation buffer (1/3 of initial culture volume) by gently swirling and kept on ice for 10 minutes. The suspension was then centrifuged at 2500g for 10 minutes. The pellet was gently re-suspended in ice-cold transformation buffer (1/12.5 of initial culture volume) then DMSO was added with gently swirling to a final concentration of 7% and incubated on ice for 10 minutes. Super-competent

cells were aliquotted to 1.5ml eppendorf tubes, then immersed in liquid nitrogen to snap-freeze and stored at -80°C.

3.3.1.1.2 Super-competent E.coli transformation

Super-competent cells were thawed on ice for ~15minutes. From this, 150µl of cells were pipetted to pre-chilled transformation tubes and immediately mixed with the ligation products or 10ng intact plasmid. Then the complex was incubated on ice for 30 minutes, after which a heat-shock at 42°C for 30-45 seconds applied. Heat-shocked cells were immediately replaced on ice for 2 minutes and consequently 850µl LB medium was added. For antibiotic resistance gene to be expressed, the cells were rotated at 225rpm for 1hour in a 37°C shaker. If ligation product was used then the cells were pelleted and re-suspended in 100µl LB; otherwise when intact plasmid was used, 100µl from 1ml total culture was taken and spread on agar plates with suitable selection antibiotic. Under aseptic conditions, plates were left to drain for ~10minutes and then placed in an inverted position into 37°C incubators for overnight (12-16 hours). Next day the emerging colonies were collected for mini-prep.

3.3.1.2 Long term storage of bacterial strains

To keep bacterial cells including plasmid in it or as empty for future experiments and to have a stock of strain in a laboratory is necessary. The most frequently used method is “Glycerol-Stock” method. A single colony picked from either an agar plate or a loop-full of bacterial stock was inoculated into 5ml LB (with a selective agent if necessary) in 15ml screw capped tubes. Tubes were incubated overnight at 37°C and at 225rpm. For glycerol stock, 500µl of saturated culture was added into 700µl of 50% glycerol v/v. This mix was frozen/stored at -80°C.

3.3.1.3 Plasmid DNA preparation

Small scale isolation of plasmid DNA was performed with Qiaquick spin mini-prep kit according to the manufacturer’s instructions. This procedure yields approximately 200ng/µl of plasmid DNA for 1ml of LB culture. For large-scale preparation of pure plasmid DNA, the Qiafilter plasmid midi kit was used by following the manufacturer’s instructions. This procedure yields approximately 1µg/µl of plasmid DNA for 100 ml of LB culture.

3.3.1.4 Preparation of genomic DNA from cultured cells

Cultured cells were grown in 100mm tissue culture dishes to 70-80% confluency, trypsinized, and washed with 1XPBS. Genomic DNA was isolated by using UltraClean Tissue DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA) following manufacturer's instructions.

3.3.1.5 Extraction of total RNA from tissue culture cells

Exponentially growing monolayer cultures were washed twice with ice-cold PBS, scraped with a scraper, pelleted at +4°C and stored at -80°C until needed for RNA preparation. The total RNA isolation from cell line pellets was performed directly by use of NucleoSpin RNA II kit according to the manufacturer's instructions. The RNAs were eluted in a total volume of 40µl. The concentration of the isolated RNA and O.D.260/280 ratio were measured with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). Isolated RNAs were snap-frozen with liquid nitrogen and stored at -80°C.

3.3.1.6 Quantification and qualification of nucleic acids and proteins

Concentration and purity of the double stranded nucleic acids (plasmid and genomic DNAs) and total RNAs were determined by using the RNA and double-stranded(ds) DNA methods on Nanodrop ND-1000 Full-spectrum UV/Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Concentration and purity of proteins were determined by using the Beckman Instruments Du Series 600 Spectrophotometer software programs on the Beckman Spectrophotometer Du640 (Beckman Instruments Inc. CA. USA).

3.3.1.7 Restriction enzyme digestion of DNA

Restriction enzyme digestions were routinely performed in 20µl or 50µl reaction volumes and typically 5-10µg DNA was used. Reactions were carried out with the appropriate reaction buffer and conditions according to the manufacturer's recommendations. Digestion of DNA with two different restriction enzymes was also performed in the appropriate common reaction buffer and conditions recommended by the manufacturer.

3.3.1.8 Agarose gel electrophoresis

3.3.1.8.1 Agarose gel electrophoresis of DNA

DNA fragments were fractionated by horizontal gel electrophoresis in 1-2% (w/v) agarose gel by using 1XTAE buffer. DNA fragments less than 1 kb were generally separated on 1.0% or 2.0 % agarose gel, those greater than 1 kb (up to 11 kb) were separated on 1 % agarose gels. Agarose was completely dissolved in 1XTAE electrophoresis buffer in the desired percentages and ethidium bromide solution was added to final concentration of 30ng/ml. 6XDNA loading dye was added to 10µl of quantitative real time RT-PCR (QRT-PCR) products and 20µl of normal PCR products such that the final dye concentration will be 1X, and total volume was loaded to each well. Nucleic acids were visualized under ultraviolet light (long wave, 340nm) and GeneRuler (Fermentas) DNA size markers was used to estimate the fragment sizes. 1 kb DNA ladder was loaded for products sizes of over 1kb and 100 bp ladder for product sizes of below 1kb.

3.3.1.8.2 Agarose gel electrophoresis of RNA

Total RNA samples were fractionated by horizontal gel electrophoresis in 1.2% (w/v) agarose gel by using 1XFA gel buffer. After 1.2% agarose gel solution was prepared, the mixture was cooled and 1.8ml of 37% formaldehyde and 1µl of ethidium bromide from a stock of 10mg/ml solution were added into 100ml of gel. The gel was poured in a laminar flow hood. 2µg of total RNA from each sample was mixed with 1 volume of 5X loading buffer per 4 volumes of RNA sample and incubated for 5 minutes at 65°C, and chilled on ice then loaded to the gel. The gel was run at 5-7V/cm in 1XFA gel running buffer. Transilluminator (Bio-Rad, California, USA) was used to visualize the DNA bands under ultraviolet light (long wave, 340nm). MultiAnalyst (Bio-Rad, California, USA) software was used to take photographs of the gels.

3.3.2 Database and computer analysis

The sequences of *ROBO2*, *ROBO3*, *ROBO4* and *AFP* were obtained from NCBI (National Center for Biotechnology Information). The exon-intron information of these genes was derived using Ensembl Genome Browser (<http://www.ensembl.org>). Restriction endonuclease

maps of the plasmid DNAs were analyzed by using the online NEBcutter2 (<http://tools.neb.com/NEBcutter2/>) and Webcutter2 (<http://rna.lundberg.gu.se/cutter2/>) tools. Primers were designed by using Primer3 online primer design tool (<http://frodo.wi.mit.edu/>) (Rozen and Skaletsky 2000). The results of the DNA sequencing of engineered constructs were visualized using Chromas-v1.45 available for download at <http://www.technelysium.com.au/chromas14x.html>. The alignments of nucleic acid or protein sequence were performed by using the NCBI Blast2Sequences algorithm available at the web page <http://www.ncbi.nlm.nih.gov/BLAST/> and ClustalW algorithm provided by EMBL-EBI at <http://www.ebi.ac.uk/Tools/clustalw2/index.html> (Thompson, Gibson et al. 1997).

For ROBO2 siRNA target design, online siRNA Design Software was used (<http://i.cs.hku.hk/~sirna/software/sirna.php>). The secondary structure of the target siRNA sequence on ROBO2 mRNAs was analyzed by mFOLD program that is available online (<http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi>) (Mathews, Sabina et al. 1999; Zuker 2003). ROBO2 and ROBO2-AS (alternative spliced form described by (Dalkic, Kuscu et al. 2006) targeting sense and anti-sense oligos were purchased from Iontek (İstanbul, Turkey).

Human Plasma Membrane Receptome Database (<http://www.receptome.org/HPMR/>) was browsed for the members of 17 receptor families present (Ben-Shlomo, Yu Hsu et al. 2003). Oncomine Cancer Profiling Database (Rhodes, Yu et al. 2004) can be accessed after a free “academic” or “non-profit” registration (<http://www.oncomine.org/>). Selected members of Netrin, Plexin, Roundabout, and Ephrin receptor families with their cognate ligands, a total of 51 genes, are analyzed in Oncomine 2.0 for differential expression in HCC; specifically between normal liver and hepatocellular carcinoma of the liver. As a result, members of the SLIT-ROBO families were targeted.

Another database used in this study is OncoDB.HCC- Oncogenomic Database of Hepatocellular Carcinoma (Su, Chao et al. 2007). Possible genomic gain/amplification or loss associated to members of SLIT-ROBO families were analyzed using OncoDB.HCC.

3.3.3 Vector construction

3.3.3.1 Construction of pSEC-shROBO2 vector using Ambion's Silencer Express system

siRNA Expression Cassettes (SECs) were used to express siRNAs in mammalian cells (Castanotto et al 2002). SECs consist of an RNA polymerase promoter (either human H1 or U6) adjacent to a hairpin siRNA template (ROBO2, ROBO2-AS: alternatively spliced) and an RNA polymerase terminator. ROBO2 and ROBO2-AS mRNA target sequences identified by siRNA Design Software and their scrambled negative control sequences along with GAPDH target sequences (suggested in the kit's manual) are presented in Table 3.1.

Table 3.1: Target mRNA and oligo sequences for constructing siRNA expression cassettes (SECs)

Oligo ID	Oligo sequence 5'-3':
siROBO2 sense	AGACTACACAAATCTTAGTGTGTAATCGTCTCGGTGTTTCGTCCTTTCCACAAG
neg.ROBO2 sense	AATCTACACAAAATTATTCTTGTCTGTGGACCGGTGTTTCGTCCTTTCCACAAG
siROBO2-AS sense	AGGCTACACAAACCTGTTCTTGTAAGGCTGGCCGGTGTTTCGTCCTTTCCACAAG
neg.ROBO2-AS sense	ACTCTACACAAAAGTTACTTTGGGTCGCTGCCGGTGTTTCGTCCTTTCCACAAG
siROBO2 anti-sense	CGGCGAAGCTTTTTCCAAAAAAGACGATTACACACTAAGACTACACAAATCTT
neg.ROBO2 anti-sense	CGGCGAAGCTTTTTCCAAAAAAGTCCACAGACAAGAATAATCTACACAAAATTA
siROBO2-AS anti-sense	CGGCGAAGCTTTTTCCAAAAAACAGCCTTACAAGAACAGGCTACACAAACCTG
neg.ROBO2-AS anti-sense	CGGCGAAGCTTTTTCCAAAAAAGCAGCGACCCAAAGTAACTCTACACAAAAGTT
Oligo ID	mRNA target sequence:
siROBO2 sense	AGACGATTACACACTAAGA
neg.ROBO2 sense	GTCCACAGACAAGAATAAT
siROBO2-AS sense	CCAGCCTTACAAGAACAGG
neg.ROBO2-AS sense	GCAGCGACCCAAAGTAACT

Two step PCR amplification with two oligos (sense and anti-sense, separately) was used to produce the precursor SECs (pre-SECs) as described by the manufacturer (Figure 3.1 A). In the first PCR, sense oligos were used. Briefly, 5X mix for both human H1 and human U6 promoters were prepared by mixing 5µl promoter element, 5µl promoter primer, 10µl 10X PCR buffer, 10µl 2.5mM dNTP mix, 64µl nuclease free water and 1µl HotStar Taq polymerase (Qiagen). Then; 1µl from 10µM stocks of ROBO2, ROBO2-AS, Negative control or GAPDH sense oligo was added to 19µl aliquots of either H1 promoter mix or U6 promoter mix. In the second PCR, anti-sense oligos were used. Briefly, 4.5X mix for both human H1 and human U6 promoters were prepared by mixing 4.5µl promoter primer, 9µl 10X PCR buffer, 9µl 2.5mM dNTP mix, 57.6µl nuclease free water and 0.9µl HotStar Taq polymerase (Qiagen). Then; 1µl from 10µM stocks of ROBO2, ROBO2-AS, Negative control or GAPDH anti-sense oligo along with 1:100 diluted products from the 1st PCR was added to 18µl

aliquots of either H1 promoter mix or U6 promoter mix (PCR conditions for both reactions were the same and given in Table 3.2).

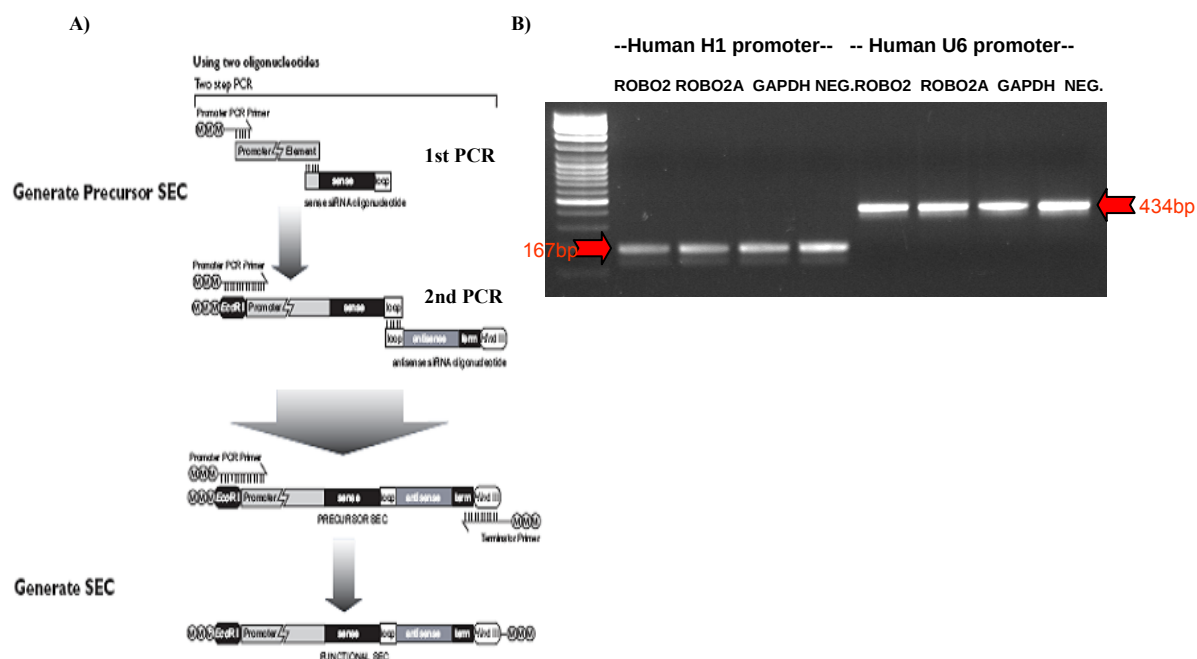


Figure 3.1: Overview of the Silencer Express procedure. A) SECs were produced with ROBO2, ROBO2-AS, Negative control or GAPDH sense (first PCR) and anti-sense (second PCR) oligos using two-step PCR procedure. Both H1 promoter and U6 promoter involving constructs were produced. B) PCR amplified SECs were reaction purified and run on 2% agarose gel for 25min at 100V and complete forms of SECs were observed at the expected molecular weights.

In a consequent PCR reaction, mature SECs were generated from pre-SECs. Briefly, a 10X reaction mix was prepared by mixing 100μl of 10X PCR buffer, 100μl 2.5mM dNTP mix, 665μl nuclease free water, 40μl terminator PCR primer and 5μl HotStar Taq polymerase (Qiagen). Then, 91μl was aliquotted from the mix and 5μl from 1:100 diluted pre-SEC PCR product along with the respective promoter PCR primer (either H1 or U6) added to the mix and the PCR reaction was run according to the profile in Table 3.3. Following the PCR reaction, SECs were reaction purified and run on 2% agarose gel for 25min at 100V (Figure 3.1 B).

Table 3.2: Precursor SEC amplification PCR profile

Hot start	95°C for 15 minutes
25 cycles	94°C for 30 seconds
	50°C for 30 seconds
	72°C for 30 seconds

Table 3.3: SEC amplification PCR profile

Hot start	95°C for 15 minutes
25 cycles	94°C for 30 seconds
	50°C for 30 seconds
	72°C for 30 seconds
Final extension	72°C for 5 minutes

Like siRNA prepared in vitro, SECs are quick and easy to synthesize, and they can be cloned into one of Ambion's pSEC™ siRNA expression vectors for long-term reduction of target gene expression. SECs were double digested using EcoRI and HindIII restriction enzymes and cloned into linearized (EcoRI, HindIII double digested) pSEC neo vector (Figure 3.2). For stable cell line generation, pSECneo plasmids containing ROBO2 targeting and scrambled negative SECs with H1 promoters were used.

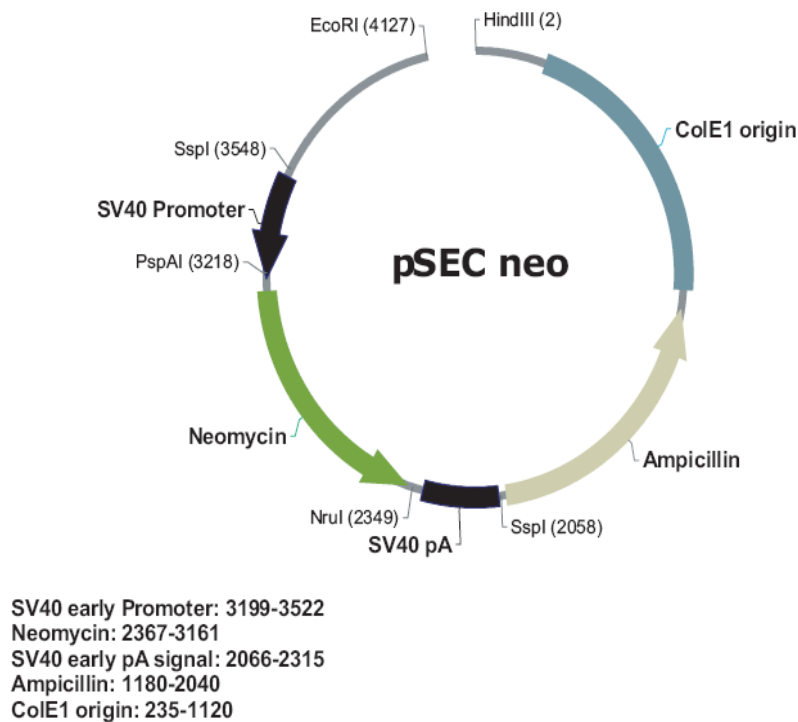


Figure 3.2: Ambion's pSEC neo vector map.

3.3.3.2 Construction of pDEST26 mock vector to be used as control in pDEST26-ROBO2 overexpression experiments

pDEST26-ROBO2 (IOH53663 clone) mammalian overexpression vector was purchased from ImaGenes GmbH (Berlin, Germany). pDEST™26 Gateway® Vector (Cat. No.11809019) was purchased from Invitrogen (Carlsbad, CA, USA) and the sequences between BsrG1 cut sites (678, 1961, 2363) that are required for recombinational cloning (*attR1* and *attR2*) and selection (*Cm^R*, *ccdB*) were removed by BsrG1 digestion (New England Biolabs, Ipswich MA, USA-Catalog No: RO575S) and the remaining vector backbone was agarose gel purified (Figure 3.3). Then, the purified vector backbone was re-ligated and used as mock control in ROBO2 overexpression experiments.

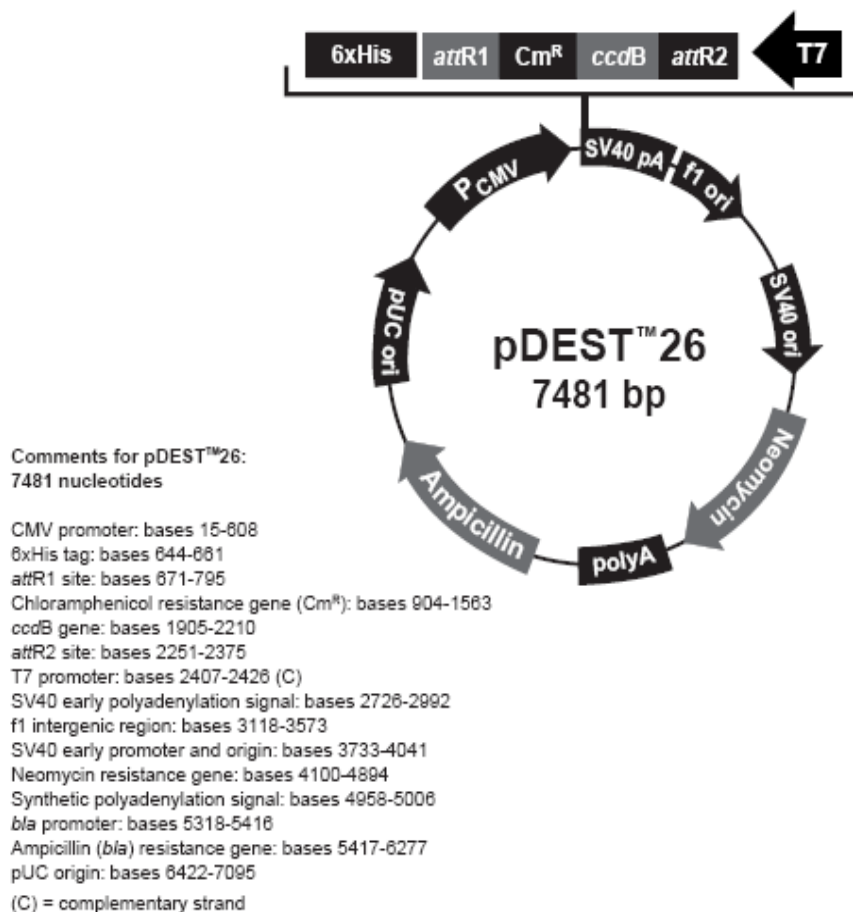


Figure 3.3: Restriction map of the pDEST™26 Gateway® vector. Restriction enzyme digestion with BsrG1 was done to remove *attR1*, *attR2*, *CmR* and *ccdB* regions and the remaining vector backbone was religated to be used as a mock control in ROBO2 overexpression experiments with pDEST26-ROBO2 vectors.

3.3.4 Tissue culture techniques

3.3.4.1 Cell lines and stable clones

13 hepatoma (Huh7, FOCUS, Mahlavu, Hep40, Hep3B, PLC/PRF/5, SK-Hep1, Snu182, Snu387, Snu398, Snu423, Snu449 and Snu475) and 1 hepatoblastoma (HepG2) cell lines were cultured as described previously (Celikkaya, Ciraci et al. 2007). The NTERA-2 cl.D1 cell line is a pluripotent human testicular embryonal carcinoma cell line derived by NTERA-2 cell line and it was grown in dishes and routinely scraped for splitting purposes since trypsinization causes differentiation of the cells (Andrews, Damjanov et al. 1984). Huh7-derived isogenic clones were obtained by G-418 selection after transfection with pSEC-H1-shNontargeting or pSEC-H1-shROBO2 plasmids. Cells were transfected using Lipofectamine2000 transfection reagent. 48 hours following transfection, cells were trypsinized and re-plated at lower density into 100cm plates. Cells were cultivated in the presence of geneticin G-418 sulfate (500µg/ml; GIBCO, Carlsbad, CA, USA) for 30 days; isolate single cell-derived colonies were selected by marking the bottom of the dishes under microscope, and then these colonies were picked with the help of a micropipette tip in the hood and expanded in the presence of 500µg/ml geneticin G-418 sulfate. For experiments where a defined number of cells were to be seeded, cell counting was performed. Following trypsinization, cells were resuspended in culture medium and counted manually with a hemocytometer.

3.3.4.2 Thawing cryopreserved cells

One vial of the frozen cells from the liquid nitrogen tank was taken and immediately put into ice. The vial was left 1 minute on the bench to allow excess nitrogen to evaporate and then placed into 37°C water bath until the external part of the cell solution was thawed (takes approximately 1-2 minutes). The cells were directly poured into a 15ml sterile tube containing 10ml cold fresh medium. The cells were centrifuged at 1500rpm at 4°C for 5 minutes. Supernatant was discarded and the pellet was resuspended in 10ml 37°C culture medium to be plated into 100mm dish. After overnight incubation in a humidified incubator at 37°C supplied with 5% CO₂, culture mediums were refreshed.

3.3.4.3 Growth conditions of cell lines

Focus, Hep40, Hep3B, Hep3B-TR, HepG2, HUH7, Mahlavu, PLC/PRF/5, SK Hep1 cells were cultured in low-glucose DMEM supplemented with 10% FBS, 100U/ml Penicillin-

Streptomycin, and 0.1mM non-essential amino acids (HyClone, Utah, USA). SNU387, SNU398, SNU423, SNU449, SNU475 cells were cultured in RPMI1640 medium supplemented with 10% FBS, 100 U/ml Penicillin-Streptomycin, 0.1mM non-essential amino acids (HyClone, Utah, USA). The growth medium was aspirated and the cells were washed once with 1XPBS. Trypsin was added to the flask to remove the monolayer cells from the surface. The fresh medium was added and the suspension was pipetted gently to disperse the cells. The cells were transferred to either fresh petri dishes or fresh flasks using different dilutions (from 1:2 to 1:10) depending on requirements. All media and solutions used for culture were kept at 4°C (except stock solutions) and warmed to 37°C before use. NTERA-2 cl.D1 [NT2/D1] cell line was grown in high-glucose medium supplemented with 10% FBS, 100 U/ml Penicillin-Streptomycin, 0.1mM non-essential amino acids (HyClone, Utah, USA). The NTERA-2 cl.D1 cell line was grown in dishes and routinely scraped for splitting purposes since trypsinization causes differentiation of the cells.

3.3.4.4 Cryopreservation of cell lines

Exponentially growing cells were harvested by trypsinization and neutralized with growth medium. The cells were counted and precipitated at 1500rpm for 5min. The pellet was suspended in a freezing solution (10%DMSO, 20%FCS and 70%DMEM for adherent cells) at a concentration of $\sim 4 \times 10^6$ cells/ml. 1ml of this solution was placed into 1ml screw capped cryotubes. The tubes were first frozen at -20°C for 0.5-1hours and then left at -80°C overnight. The next day, the tubes were transferred into the liquid nitrogen storage tank.

3.3.4.5 Transfection of cell lines

Transfections were performed using FuGene HD transfection reagent (Roche Diagnostics, Mannheim, Germany- Catalog No: 04709705001) according to the instructions of the supplier. Exponentially growing cells were plated in 6 well-plates at a concentration of $2.5-5.0 \times 10^5$ cells/well according to the cell type used, a day before the transfection. The cells were incubated overnight and reached to 70-80% confluency. Transfection was performed in cell culture medium lacking Penicillin/streptomycin with a 6:2, FuGene HD to DNA ratio. Briefly for one well, 2 μ g of plasmid was pipetted into OptiMem I Reduced-Serum medium (Invitrogen, Carlsbad, CA, USA- Catalog No: 11058-021) or cell culture medium lacking FBS to a final 100 μ l volume. Subsequently, the vial of FuGene HD was vortexed for 1 second

and 6µl was added directly into DNA/diluent mix without contacting with the walls of the eppendorf. The final mix was immediately vortexed for 1-2 seconds and spinned down and left to room temperature for the formation of transfection complex for 20-30 minutes. Transfection complex was pipetted dropwise onto the cells with fresh Penicillin/streptomycin lacking medium. The medium was replaced with fresh complete medium after 4-8 hours. After 48-72 hours incubation, cells were harvested and used for subsequent experiments.

3.3.4.6 Wound-healing assay

Cells were seeded onto 6 well plates at a density 3×10^5 cells/well. The day in advance, three vertical and two horizontal scratches with equal intervals were made in each well using 200µl micropipette tips. Immediately, the time 0 photos of the wounds were taken and the photo taken sites were marked. The medium was refreshed with either normal medium (10% fetal bovine serum) or medium lacking serum (serum starved condition). Then, the opened wounds were followed for 48 hours. At 24 and 48 hours photographs of the marked sites were taken. The relative migration distances were calculated by dividing the total healed wound distance (difference between initial and final wound sizes) by 2, for 10 different lines connecting horizontally the two sides of the wound. The average of these 10 calculations were used to construct the graphs and calculate the T-tests.

3.3.5 cDNA synthesis

cDNA was synthesized by using DyNAmoTM cDNA Synthesis Kit (Finnzymes, Espoo, Finland) according to manufacturer's instructions. Briefly; for 1X reaction 1µg of total RNA, 1µl of random hexamers and required amount of ddH₂O were mixed in a total 8µl volume. A minus RT control sample was also prepared; including RNA from the sample with highest RNA concentration, in the case of vector transfected samples RNA from the vector transfected sample was used. Then the mixes were incubated at 65°C for 5min and chilled on ice. Consequently, 10µl of 2X RT reaction buffer and 2µl of M-MuLV RNase H⁺ reverse transcriptase (including RNase inhibitor) were added to complete the reaction volume to 20µl. Instead of reverse transcriptase, nuclease free water was added to RT minus control. It contained all the reaction components except for the reverse transcriptase. Then the reaction mixes were incubated in thermal cycler programmed as follows: 25°C for 10 minutes, 37°C

for 60 minutes, 85°C for 5 minutes and 4°C hold. Each cDNA sample was diluted at a ratio of 1:2 with ddH₂O and stored at -20°C to be used as a PCR template for further experiments.

3.3.6 Primer design for expression analysis by semi-quantitative PCR

The primer pairs that have been used in expression profile analyses were designed carefully. PCR primers for human *SLITs*, *ROBO1* and *ROBO2* were previously described (Latil, Chene et al. 2003). Remaining primers were designed using Primer3 and targeting exon-exon junctions or different exons in order to prevent amplification of possible contaminating genomic DNA (Primer 3 reference). The primer pair was either be able to produce a longer amplicon from genomic DNA or not be able to amplify from the covered genomic DNA region in a given PCR condition (critical parameter was extension time). Primer pair for the housekeeping gene *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) was described before (Sayan, Sayan et al. 2001). *ACTB* (beta-actin) primer pair was supplemented in TissueScan Liver Cancer Tissue qPCR Array 1 (Origene Technologies, Rockville, MD, USA). Primers used for expression analysis have been designed strictly considering these criteria, and listed in Table 3.4.

Table 3.4: Application purposes and sequences of the primers used in this study

Application/property	Primer	5'-3' Sequence
Expression	SLIT1 F	GACGTGGTCTGTCCCCACAA
	SLIT1 R	AATCTCATTGTTATTCAATCGCAGTT
	SLIT2 F	TCCTAACTCCAAAGGGATTCAAATGT
	SLIT2 R	GGCTCCGTTTTTACACTTGTGTCT
	SLIT3 F	CCGCCTAACTACACAGGTGAGCTAT
	SLIT3 R	CGCTGTAGCCAGGGACACACT
	ROBO1 F	GCATCGCTGGAAGTAGCCATACT
	ROBO1 R	CTAGAAATGGTGGGCTCAGGAT
	ROBO2 F	GGGTACTACATCTGCCAGGCTT
	ROBO2 R	AGGTGGAGGTCTATCTGTCAAAACAT
	ROBO3-Old F	CAGTGTCCGATGGAAGAAGG
	ROBO3-Old R	GTCCATCTCCTGCACATTGG
	ROBO3-New F	TACACCCTCACCTCCTGACC
	ROBO3-New R	ATGGGCTGGGATACACTGAG
	ROBO3-MC F	GCAGTCCTCCGTGATGATTT
	ROBO3-MC R	TTGGAGGCTACGCACACATA
	ROBO4 F	GACACTTGGCGTTCCACCTC
	ROBO4 R	AGAGCAAGGAGCGACGACAG
	AFP F	AAATGCGTTTCTCGTTGCTT
	AFP R	CCAACACCAGGGTTTACTGG
Alternatively spliced ROBO2 expression	ROBO2-hRF	AGCACTGGACCAGACTCCTG
	ROBO2-hRR	AATGGGCTGGTAGGTGAG

	ROBO2-hRRA	TCCATCCAGCCTAAACCAG
PLXNC1 ligand	SEMA7A F	AAGACGCCATTGTTCCACTC
	SEMA7A R	GGCTGGATCTCCATGATGTT
Normalization	GAPDH F	GGCTGAGAACGGGAAGCTTGTCTAT
	GAPDH R	CAGCCTTCTCCATGGTGGTGAAGA
	ACTB F	CCAACCGCGAGAAGATGACC
	ACTB R	GGAGTCCATCACGATGCCAG
Array verification	PLXNC1 F	CATAACAGAGACGCCAACGA
	PLXNC1 R	TTTCAAGGGTGTGGATGACA
	SEMA3C F	TCAGCCGTGTGTGTGTATCA
	SEMA3C R	AATGCTCCTCCTGGACAAGT
	NEO1 F	TGGAAAACCAACTCCAAGTGA
	NEO1 R	TGATTTCAACAGACCCAAAAGTGA
	NRP1 F	GGCTCTCACAAGACCTTCTG
	NRP1 R	GAAGTTGCCATCTCCTGTGT
	NRP2 F	GATCAGTGCCTCATCTACCT
	NRP2 R	CACCTGGAGATACTCCTTGT
	ECadherin F	GACTCGTAACGACGTTGCAC
	ECadherin R	GGTCAGTATCAGCCGCTTTC
EMT marker	Twist F	CCGGAGACCTAGATGTCATTG
	Twist R	CACGCCCTGTTTCTTTGAAT
	SNAI1 F	CTCTAGGCCCTGGCTGCTAC
	SNAI1 R	TGACATCTGAGTGGGTCTGG
	Slug F	CCCTGAAGATGCATATTCGGAC
	Slug R	CTTCTCCCCGTGTGAGTTCTA
	SIP1 F	TCCTGTCTGTCTCGCAAAA
	SIP1 R	GCCTTGAGTGCTCGATAAGG
	Vimentin F	GCAGGAGGAGATGCTTCAGA
	Vimentin R	ATTCCACTTTGCGTTCAAGG
	ASMA F	TATCAGGGGGCACCCTATG
	ASMA R	GCTGGAAGGTGGACAGAGAG
CSC (Progenitor/Side population) marker	Fibronectin F	AATATCTCGGTGCCATTTGC
	Fibronectin R	CAGTAGTGCCTTCGGGACTG
	Podoplanin F	CATCGAGGATCTGCCAAGTT
	Podoplanin R	TTGTCTGTGTGTCTCCATCCA
	CD133 F	CGGCTCTAATTTTTCGGTA
	CD133 R	TGTTGTGATGGGCTTGTCTAT
	CK19 F	CCGCGACTACAGCCACTACT
	CK19 R	AGAGCCTGTTCCGTCTCAAA
	AFP F	AAATGCGTTTCTCGTTGCTT
	AFP R	CCAACACCAGGGTTTACTGG
	CEACAM6 F	ATGTGCCAAGCCCATAACTC
	CEACAM6 R	GTCTGGTCCAATCTGCCAGT
	Albumin F	ACTTTTATGCCCCGGAAGTC
	Albumin R	CAGCTTTGGGAAATCTCTGG
	CYP3A4 F	CAAGACCCCTTTGTGGAAA
	CYP3A4 R	CGAGGCGACTTTCTTTTCATC
	AREG F	GGGAGTGAGATTTCCCCTGT
	AREG R	CCATTTTTCCTCCCTTTT
	CK14 F	GACCATTGAGGACCTGAGGA
	CK14 R	GGCTCTCAATCTGCATCTCC
	MDR1 F	GCTCCTGACTATGCCAAAGC

	MDR1 R	TCTTCACCTCCAGGCTCAGT
	BCRP1 F	ATGGATTTACGGCTTTGCAG
	BCRP1 R	GAGATCGATGCCCTGCTTTA
	CD44 F	CGGACACCATGGACAAGTTT
	CD44 R	GAAAGCCTTGCAGAGGTCAG
	CD90 F	GTCCTTTCTCCCCAATCTC
	CD90 R	ACGAAGGCTCTGGTCCACTA
Pluripotency marker	OCT4 F	AGCGAACCAGTATCGAGAAC
	OCT4 R	TTACAGAACCACACTCGGAC
	SOX2 F	AGCTACAGCATGATGCAGGA
	SOX2 R	GGTCATGGAGTTGTACTGCA
	NANOG F	TGAACCTCAGCTACAAACAG
	NANOG R	TGGTGGTAGGAAGAGTAAAG
	MYC F	ACTCTGAGGAGGAACAAGAA
	MYC R	TGGAGACGTGGCACCTCTT
	KLF4 F	TCTCAAGGCACACCTGCGAA
	KLF4 R	TAGTGCCTGGTCAGTTCATC

3.3.7 Fidelity and DNA contamination control in first strand cDNAs

The fidelity and genomic DNA contamination of first strand cDNAs were checked before performing expression analyses. 2µl of diluted first strand cDNA was used for PCR amplification of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript. GAPDH primer pair for this analysis was designed to produce a 151 bp fragment from cDNA and 250 bp fragment from genomic DNA.

3.3.8 Expression analysis of a gene by semi-quantitative PCR

3.3.8.1 Determination of optimal cycle of a gene for semi-quantitative PCR

Using equal amount of templates for PCR amplifications of a gene of interest give comparable results at a certain number of PCR cycles. The number of optimal PCR cycle was determined by an initial study for each gene by performing 35-cycle PCR during which PCR amplicon samples were collected by 2-cycle intervals. Agarose gel analysis of samples with an equal load defined the minimum number of cycle to visualize the product on agarose gel and the saturation cycle. Agarose gels were analyzed by Densitometric Fluorescence-Chemiluminescence image analyzer and The Molecular Analyst software (BioRad, Hercules, CA, USA). The determined cycle number was used for amplification of the gene of interest.

3.3.8.2 *GAPDH* normalization

Equal volume (2µl) of all first strand cDNA samples was used for PCR amplification of *GAPDH* transcript using the pre-determined optimal cycle number for *GAPDH* (20 cycles). Then an equal volume of each sample was loaded onto agarose gel and intensity of each band was analyzed by Densitometric Fluorescence-Chemiluminescence image analyzer and The Molecular Analyst software. After intensities were determined, intensity of sample with the highest densitometric reading and 2 µl loading volume were used as reference points for normalization of input loading volume of other samples for expression analysis of both *GAPDH* and gene of interest by PCR amplification. Amplification products were analyzed by visualization under UV.

3.3.9 Real time quantitative RT-PCR

3.3.9.1 Real-time quantitative RT-PCR analyses of HCC cell lines

In cell lines and tissues, the relative expression ratio (R) of *SLIT-ROBO* and *AFP* transcripts (target gene) was measured based on a modified $\Delta\Delta C_t$ formula (Pfaffl 2001) and normalized to *GAPDH* or *ACTB* (reference gene). In $R = (E_{target})^{\Delta C_t_{target}^{(control-sample)}} / (E_{ref})^{\Delta C_t_{ref}^{(control-sample)}}$ formula, E_{target} and E_{ref} reflect PCR efficiencies of the primers for target genes and reference genes, respectively. PCR efficiency values for each primer pair was obtained by constructing a standard curve using threshold cycle (Ct) values derived from 6 data points, corresponding to 2-fold decrements of an original cDNA stock (duplicates were prepared for each dilution). The slope of the resulting curve was used to calculate the E value of primer pairs according to $E = 2^{-1/slope}$ formula. ΔC_t was the difference between the Ct values of controls and samples. PCR efficiencies of the genes ranged around 1.9-2.0 (Table 3.5).

Table 3.5: PCR efficiencies of the primer pairs used in QRT-PCR relative quantification analysis

PRIMER	EFFICIENCY
<i>ROBO1</i>	2.04
<i>ROBO2</i>	1.86
<i>ROBO4</i>	1.88
<i>SLIT1</i>	1.89
<i>SLIT2</i>	1.99
<i>SLIT3</i>	1.97

<i>GAPDH</i>	2.00
<i>CD133</i>	2.00
<i>AFP</i>	1.83
<i>ROBO2 hRF-hRRA</i>	1.97

In cell lines, *GAPDH* was the reference gene. ΔC_t values were obtained by subtracting C_t values of individual genes (sample) from the average C_t value of all cell lines for that gene (control). All reactions were performed in duplicates and repeated at least twice using different batches of RNA preparations. A no-template control of nuclease-free water was included in each run. Relative expression tables were established by representing $\Delta\Delta C_t$ values in log2 base, and in all subsequent analyses these values were used.

Quantitative expression analyses were performed using DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland) on an iCycler iQ real-time PCR detection system (Bio-Rad, Richmond, CA). The PCR reaction was set according to the manufacturer's recommendations. Briefly for 1X reaction; 10µl of 2X SYBR Green PCR Master Mix, 10µM forward and reverse primers, and 1µl of template cDNA were mixed in a total volume of 20µl. After an initial 15 minutes of denaturation at 95°C, thermal cycling was performed at 94°C for 30sec, 60-62°C for 30sec (optimized for each primer pair), 72°C for 30sec for a total of 50 cycles and a final extension step at 72°C for 10min. In order to validate the production of a single target-specific PCR product, the amplification was followed by a melt curve protocol with an initial step at 55°C for 30sec and 80 repeats of 0.5°C increments with 15sec dwell time, from 55°C to 95°C.

3.3.9.2 Real-time quantitative RT-PCR analyses of HCC tissues

The expression of *SLIT-ROBO* and *AFP* genes in HCC was analyzed using a 96-well plate format TissueScan Liver Cancer Tissue qPCR Array 1 (Origene Technologies, Rockville, MD), which contained tissue cDNAs normalized against beta-actin. Clinicopathological characteristics of the tissues were presented in Table 3.6. Real-time PCR protocol was applied as described by the manufacturer. Briefly, 30µl of reaction mix containing 15µl 2X SYBR Green PCR Master Mix and 10µM forward and reverse primers was directly added to PCR-plate wells. Plate was placed on ice for 15min for cDNAs to dissolve, and thermal cycling was performed according to above mentioned protocol. For each gene, mean C_t value of the

normal tissue cDNAs was set as the control group and relative quantitative expression values were calculated with the $\Delta\Delta C_t$ formula and were represented in log2 base by taking the *ACTB* C_t values as reference.

Table 3.6: Clinicopathological characteristics of normal liver and HCC samples

Sample #	Appearance	Diagnosis/Histology	Differentiation	Stage
N1	Normal	hcc	tumor-adjacent normal	0
N2	Normal	hcc/adenocarcinoma	tumor-adjacent normal	0
N3	Normal	hcc	tumor-adjacent normal	0
N4	Normal	hcc	tumor-adjacent normal	0
N5	Normal	granuloma	tumor-adjacent normal	0
N6	Normal	nodular hyperplasia of liver, focal	tumor-adjacent normal	0
N7	Normal	hcc	tumor-adjacent normal	0
N8	Normal	hcc	tumor-adjacent normal	0
T1	Tumor	hcc	well	1
T2	Tumor	hcc	moderate	1
T3	Tumor	hcc	well	1
T4	Tumor	hcc	N/A	1
T5	Tumor	hcc	poor	1
T6	Tumor	hcc	N/A	1
T7	Tumor	hcc	moderate	1
T8	Tumor	hcc	moderate	1
T9	Tumor	hcc	N/A	1
T10	Tumor	hcc	moderate	2
T11	Tumor	hcc/adenocarcinoma	moderate	2
T12	Tumor	hcc/invasive	N/A	2
T13	Tumor	hcc/adenocarcinoma	poor	2
T14	Tumor	hcc	well	2
T15	Tumor	hcc	moderate	2
T16	Tumor	hcc	well	2
T17	Tumor	hcc	moderate	2
T18	Tumor	hcc/adenocarcinoma	poor	2
T19	Tumor	hcc	well	2
T20	Tumor	hcc/invasive	N/A	2
T21	Tumor	hcc	well	3a
T22	Tumor	hcc	poor	3a
T23	Tumor	hcc	moderate	3a
T24	Tumor	hcc	moderate	3a
T25	Tumor	hcc	moderate	3a
T26	Tumor	hcc	well	3a
T27	Tumor	hcc	moderate	3a
T28	Tumor	hcc	moderate	3a
T29	Tumor	hcc	well	3a
T30	Tumor	hcc	poor	3a
T31	Tumor	hcc	moderate	3a
T32	Tumor	hcc	moderate	3a

T33	Tumor	hcc	moderate	3a
T34	Tumor	hcc	moderate	4
T35	Tumor	hcc/from omentum	poor	4

3.3.10 Crude total protein extraction

Adherent monolayer cells (both stable and parental cells) were grown to 80% confluency. For cytoplasmic or nuclear proteins analyses cells were collected by trypsinization. For membrane proteins cells were collected by scraping after washing twice with ice-cold PBS to remove any serum residue. Volume of the dry cell pellet was estimated and twice that volume of lysis buffer was pipetted onto the pellet. For nuclear proteins RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5% sodium deoxycholate, 1 % NP-40, 0.1% SDS and 1X Complete Protein Inhibitor mix (Roche Diagnostics, Mannheim, Germany)) was used, and for cytoplasmic and membrane proteins NP-40 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1X protease inhibitor cocktail (Roche) and 1.0% NP-40) was used. Complete lysis was achieved by pipetting of crude cell lysates several times and by incubating the lysates on ice for 30 min and with continuous agitation by vortexing at 5 minutes intervals. Then, the lysate was centrifuged at 10000G for 30 minutes. Total cell protein was collected as supernatant.

3.3.11 Quantification of proteins

After the cell lysates were prepared, their concentrations were detected by Bradford assay. Briefly, 2µl of the samples were diluted with 98µl deionised water and then 900µl of Bradford working solution was added to the samples and mixed well, as described in Table 3.7. Immediately, the protein amounts of the samples were measured at 595nm versus blank reagent (NP-40 lysis buffer was used as blank). Known concentrations of BSA were prepared according to Table 3.8 as a standard. After OD reading at 595nm, samples and standard values were plotted; unknown concentrations were calculated from the standard curve.

Table 3.7: Protein sample preparation for Bradford assay

Tube no	1	2	3	4	5	6
Sample (µl)	0	2	2	2	2	2
ddH ₂ O (µl)	98	98	98	98	98	98
Bradford (µl)	900	900	900	900	900	900
Lysis buffer (µl)	2	-	-	-	-	-

Table 3.8: BSA dilutions for standard curve plot

Tube no	1	2	3	4	5	6	7	8
Sample (μl)	0	2.5	5	7.5	10	12.5	15	20
ddH ₂ O (μl)	100	97.5	95	92.5	90	87.5	85	80
Bradford (μl)	900	900	900	900	900	900	900	900

3.3.12 Western blotting

After protein quantification, protein lysates were aliquoted into fresh tubes and, stored at – 80°C. 8% (25-200kD range), 10% (15-100kD range) or 12% (10-75kD range) resolving gel and 5% stacking gel was used in SDS-PAGE analysis of protein lysates. Mini Protean III (BioRad, Hercules, CA, USA) vertical gel system was set up according to manufacturer's instructions. The standard SDS-electrophoresis buffer system was used. Equal amounts of cell lysates were solubilized in 5X SDS gel-loading buffer with 5% β-mercaptoethanol, denatured at 100°C for 5 min and incubated on ice for 2 min. After a quick spin, samples were loaded onto SDS-polyacrylamide gel. After electrophoresis at 80V for 20 minutes followed by 120 V for 1-2 hours, proteins were transferred onto Immobilon-P PVDF transfer membrane with 0.45μm pore size (Millipore, Billerica, MA, USA) by using Transblot-Semi Dry (BioRad, Hercules, CA, USA) electroblotting apparatus according to the manufacturer's instructions at 15V for ~45 min. Membrane was immediately treated for an hour in blocking solution at room temperature and probed with primary antibody either for an hour at room temperature or overnight at 4°C. After washing 4 times for (5 min, 15 min, 5 min, 5 min) in TBS-T solution at room temperature, the membrane was incubated with appropriate HRP conjugated secondary antibody for 1 hr. The membrane was washed 3 times for 5 min in TBS-T solution at room temperature. After final wash, the blot was exposed to ECL western blot detection kit (GE Healthcare Life Sciences, Buckinghamshire, UK) according to manufacturer's instructions. The chemiluminescence emitted was captured on X-ray film within 15sec to 5min exposure times.

3.3.13 Immunofluorescence

Autoclaved-sterilized coverslips were placed into wells of 6well plates. $2-4 \times 10^5$ cells were seeded onto each coverslip depending on the cell line and grown overnight in 2ml growth medium. Cells were washed with PBS and fixed in 1ml of cold methanol or 2%

paraformaldehyde for 15 minutes. For paraformaldehyde fixed cells permeabilization with 0.3% TritonX-100 in 1X PBS was applied. After fixation cells were blocked in 1ml blocking solution (2% BSA in 1X PBS) for 1 hour at room temperature. Coverslips were probed with primary antibody in appropriate dilution for 1hr at room temperature. After washing cells twice with 1X PBS, either appropriate secondary antibody (AlexaFluor Red/Green) was applied for 1hour at room temperature. Cells were washed 2 times with 1X PBS and DNA counter staining was performed with DAPI for 3 minutes. After DAPI was aspirated, destaining was done in double-distilled water for 5 minutes. Immediately after, coverslips were taken out from the wells and excess water removed by tissue paper. Coverslips were mounted onto slides with 50% glycerol (stock glycerol was 1/2 diluted in ddH₂O). All steps after the addition of secondary antibody were performed in the dark. Stained cells were examined under fluorescence microscope (ZEISS) and pictures were captured in a digital ZEISS AxioCam MRc5 camera.

3.3.14 BrdU incorporation assay

Sub-confluent cells were labeled with the thymidine analogue BrdU (5-bromo-2-deoxyuridine) for 24 hours in freshly added culture medium and tested for BrdU positivity. Briefly, cells were washed twice with 1X PBS, fixed with 70% ice-cold methanol and washed for 10 minutes with 1X PBS. Then the cells were incubated in 2N HCl for 20 minutes and washed three times for 5 minutes with 1X PBS. Following blocking with PBS-T plus 2% BSA for 15 minutes, 1:250 Alexa 488 secondary antibody incubation was performed for an hour. Cells were washed 2 times with 1X PBS and DNA counter staining was performed with DAPI for 3 minutes. After DAPI was aspirated, destaining was done in double-distilled water for 5 minutes. Immediately after, coverslips were taken out from the wells and excess water removed by tissue paper. Coverslips were mounted onto slides with 50% glycerol (stock glycerol was 1/2 diluted in ddH₂O). All steps after the addition of secondary antibody were performed in the dark. Stained cells were examined under fluorescence microscope (ZEISS) and pictures were captured in a digital ZEISS AxioCam MRc5 camera.

3.4 Microarray experiment and analysis

Total RNAs were extracted from the stable clones. Quality of the RNAs was checked using Nanodrop ND-1000 Full-spectrum UV/Vis Spectrophotometer and by running in

formaldehyde gel and visual inspection of 28S and 18S rRNAs under UV. Pooled RNAs from eight stable *ROBO2* knockdown clones (R2C3, R2C7, R2C8, R2C9, R2C10, R2C13, R2C14, and R2C17) were hybridized to one Affymetrix HG-U133plus2.0 chip and pooled RNA from four negative clones (NC4, NC5, NC6, and NC7) were hybridized to another Affymetrix HG-U133plus2.0 chip. Microarray experiment was performed by Hilal Özdağ from Ankara University Biotechnology Institute. After the experiment, raw .cell files of the two chips were obtained; then quality controlled and RMA (robust multichip average) normalized in R environment. In MS Excel, using built-in functions, we obtained a fold change list and a differentially expressed gene list. To filter out non-relevant, noise-related genes we determined a cut-off of ± 0.8 fold; the probe sets having fold change values between +0.8 and -0.8 were eliminated. The obtained genelist was given as input file to Pathway Express tool in OntoTools suite for determining the associated pathways and their visualization (<http://vortex.cs.wayne.edu/projects.htm>).

3.5 Statistical analysis

Using one-way ANOVA in R, mean expression levels of each gene were compared between high and low *AFP* expressing groups of HCC cell lines; and also between normal and tumor tissues of liver with respect to differentiation or stage. Pairwise comparisons were made using Fisher's multiple pairwise comparison method in Minitab® 13.20 Statistical Software (Minitab Inc. 2000). Furthermore, two-way hierarchical cluster analysis was used to group cell lines and liver tissues with respect to the *SLIT-ROBO* expression patterns using Cluster and TreeView (Eisen, Spellman et al. 1998). Pairwise correlations between *SLIT-ROBO* gene expression levels were calculated using Pearson's correlation coefficient. Moreover, a Mantel's association test was applied to compare cell line and tissue correlation matrices in R (R development core team, 2005). For comparison of two groups with equal sample numbers, built-in t-test statistic was used in MS Excel (using two-sample with unequal variance, with a two tailed distribution mode). In R environment, Wilcoxon rank sum test was applied to liver tissue relative expression data to predict if the clusters appeared in cluster analysis was dependent on differentiation state or staging of tumors.

4 RESULTS

4.1 Expression analysis of members of the *SLIT-ROBO* gene families

4.1.1 Oncomine cancer profiling database results for *SLIT-ROBO* gene expression in liver

Human Plasma Membrane Receptome Database (<http://www.receptome.org/HPMR/>) was browsed for the members of 17 receptor families present (Ben-Shlomo, Yu Hsu et al. 2003). Oncomine Cancer Profiling Database (Rhodes, Yu et al. 2004) can be accessed after a free “academic” or “non-profit” registration (<http://www.oncomine.org/>). Selected members of Netrin, Plexin, Roundabout, and Ephrin receptor families with their cognate ligands, a total of 51 genes, are analyzed in Oncomine 2.0 for differential expression in HCC; specifically between normal liver and hepatocellular carcinoma of the liver. As a result, members of the *SLIT-ROBO* families were targeted.

Differential expression of *SLIT-ROBO* genes were analyzed using Diff/Ex function of Oncomine. Individual *SLIT-ROBO* genes were queried and then in the summary panel the significant differential expression data in liver was clicked. As default, P-value cutoff was selected 1E-4 by the database. The trend associated with each gene is summarized in Table 4.1 for the genes that are significantly differentially expressed (*ROBO2*, *ROBO3* and *ROBO4* did not have significant results with the above mentioned P-value). *SLIT1*, *SLIT2*, *SLIT3* and *ROBO1* mRNA expressions were found to be low in normal liver when compared to other tissues of normal histology (Su, Cooke et al. 2002; Shyamsundar, Kim et al. 2005; Roth, Hevezi et al. 2006). *SLIT2* mRNA was upregulated in cirrhotic liver when compared to normal counterpart (Wurmbach, Chen et al. 2007). *ROBO1* was overexpressed in hepatocellular carcinoma when compared to normal or dysplastic liver (Chen, Cheung et al. 2002; Wurmbach, Chen et al. 2007).

Table 4.1: Significant differential expression results in liver obtained from Oncomine database

Gene	Study	Class1	Class2	T-stat	P-value	Reporter	Regulation
<i>SLIT1</i>	Roth_Normal	Other Tissues	Liver	6.875	2.6E-8	1557615_a_at	Down
<i>SLIT2</i>	Roth_Normal	Other Tissues	Liver	16.911	8.3E-27	228850_s_at	Down
<i>SLIT2</i>	Wurmbach_Liver	Normal Liver	Cirrhotic Liver	-6.644	1.5E-6	209897_s_at	Up
<i>SLIT3</i>	Su_Normal	Other Tissues	Liver	25.808	2E-33	35324_at	Down
<i>SLIT3</i>	Shyamsundar_Normal	Other Tissues	Liver	8.462	1.7E-6	H41160	Down
<i>SLIT3</i>	Roth_Normal	Other Tissues	Liver	11.398	6.1E-6	203813_s_at	Down
<i>ROBO1</i>	Roth_Normal	Other Tissues	Liver	10.399	5.8E-5	213194_at	Down
<i>ROBO1</i>	Wurmbach_Liver	Normal Liver	Hepatocellular Carcinoma	-11.91	3.9E-15	213194_at	Up
<i>ROBO1</i>	Chen_Liver	Non-tumor Liver	Hepatocellular Carcinoma	-6.42	1.3E-9	IMAGE:595162	Up
<i>ROBO1</i>	Wurmbach_Liver	Dysplastic Liver	Hepatocellular Carcinoma	-6.866	3.2E-8	213194_at	Up

4.1.2 Onco.DB HCC database analysis for genomic gain/amplification or loss associated to *SLIT-ROBO* gene regions

The OncoDB.HCC (<http://oncodb.hcc.ibms.sinica.edu.tw>) is based on physical maps of rodent and human genomes containing quantitative trait loci of rodent HCC models and various human HCC somatic aberrations including chromosomal data from loss of heterozygosity and comparative genome hybridization analyses, altered expression of genes from microarray and proteomic studies, and finally experimental data of published HCC genes (Su, Chao et al. 2007). Onco.DB HCC database was searched for the regions that are lost or gained/amplified in hepatocellular carcinoma genomes. From the “Quick search” panel genomic regions of *SLIT-ROBO* genes were manually searched by analyzing comparative HCC regions section. There was no genomic loss or gains in the genomic regions corresponding to members of the *SLIT-ROBO* families. The genomic locations of the *SLIT-ROBO* genes are outlined in Table 4.2. Moreover, in karyotyping analyses chromosomal gains/amplifications or losses have not been associated with genomic regions of *SLIT-ROBO* genes (Midorikawa, Yamamoto et al. 2006; Midorikawa, Makuuchi et al. 2007).

Table 4.2: Genomic locations of the *SLIT-ROBO* genes

Gene	Genomic location
<i>SLIT1</i>	10q23.3-24
<i>SLIT2</i>	4p15.2
<i>SLIT3</i>	5q35
<i>ROBO1</i>	3p12
<i>ROBO2</i>	3p12.3
<i>ROBO3</i>	11q24.2
<i>ROBO4</i>	11q24.2

4.1.3 *SLIT-ROBO* genes are expressed in hepatocellular carcinoma cell lines at different levels

The expression of *SLIT-ROBO* genes were determined by semi-quantitative RT-PCR in 13 hepatocellular carcinoma and 1 hepatoblastoma cell line. After 40 cycles of PCR amplification which is a high cycle number possibly saturating the products, the PCR products were run in 2% agarose gel until the respective DNA molecular weight markers have separated and then photographed under UV light. All PCR products were at expected size. *SLIT-ROBO* transcripts were present in the investigated HCC cell lines at varying levels (Figure 4.1). *ROBO1* and *SLIT2* transcripts were present in all cell lines that were examined, except SNU398, which failed to display the *SLIT2* transcript. *ROBO2* and *SLIT1* showed a similar yet more restricted expression pattern. Their transcripts were mainly confined to cell lines with epitheloid phenotype including Hep40, HUH7, Hep3B, Hep3B-TR, HepG2 and PLC/PRF/5. However, except SNU398, very weak or no expression of *SLIT1* and *ROBO2* was found in diffusely spreading cell lines with fibroblastoid phenotype (Focus, Mahlavu, SK Hep1, SNU387, SNU423, SNU449 and SNU475). In striking contrast, sharp *SLIT3* transcripts were observed in all cell lines with fibroblastoid phenotype, except SNU398, and in only Hep40 belonging to cell lines with epitheloid appearance. The expression of *ROBO4* was variable among HCC cell lines, yet it tended to be enhanced in fibroblastoid cells. Except SNU398, *ROBO3* transcript was barely detectable in the investigated HCC cell lines.

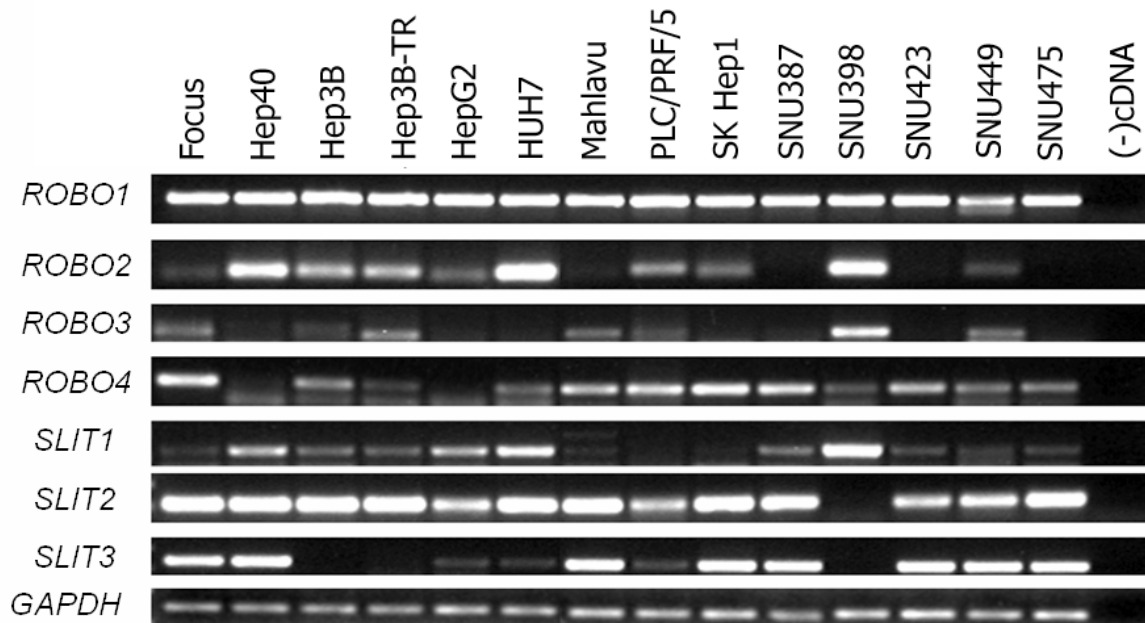


Figure 4.1: Semi-quantitative expression analysis of *SLIT-ROBO* genes in HCC cell lines. *GAPDH* equalized cDNA samples of HCC cell lines were amplified for 40 cycles with primers specific to *SLIT-ROBO* genes. Results are representative of two independent RT-PCR reactions, performed by cDNAs derived from different RNA preparations. A cDNA lacking negative control (-cDNA) was included in each experiment.

4.1.4 AFP positivity is a distinctive marker for poorly- and well-differentiated HCC cell lines

Previously, Lee and Thorgeirsson described two molecular subgroups of HCC cell lines that are characterized by low and high expression of AFP. They used 19 cell lines including the 13 cell lines (except Mahlavu) used in this study. In their microarray study, cell lines with low AFP profile (including Focus, SK Hep1, Snu387, Snu423, Snu449 and Snu475) showed upregulation of genes related to metastasis and cell motility whereas cell lines with high AFP profile (HepG2, Hep3B, Hep3B-TR, Hep40, HUH7 and PLC/PRF/5) did not. The latter group was clustered together with a hepatoblastoma cell line (HepG2) indicating their origin from progenitor cells, or dedifferentiation during tumorigenesis (Lee and Thorgeirsson 2002). To confirm the cellular origin and reliability of our HCC cell lines, we checked the *AFP* expression of the HCC cell lines in our hands.

AFP transcript was quantified using real-time quantitative RT-PCR. A total of 45 cycles was run. After each run, a melt-curve protocol was applied and relative quantity of *AFP*

expression was calculated using $\Delta\Delta C_t$ method. *AFP* expression in HCC cell lines was plotted in log2 base (Figure 4.2). The specificity of *AFP* PCR products were checked by both melt-curve analysis and agarose gel electrophoresis. The HCC cell lines were correctly grouped into two depending on their *AFP* expression as in the above mentioned study except Snu398, which was an outlier cell line stemming from the low-*AFP* expressing subgroup, was placed into low-*AFP* expressing cell lines in our analysis. In addition, Mahlavu expressed *AFP* at low levels.

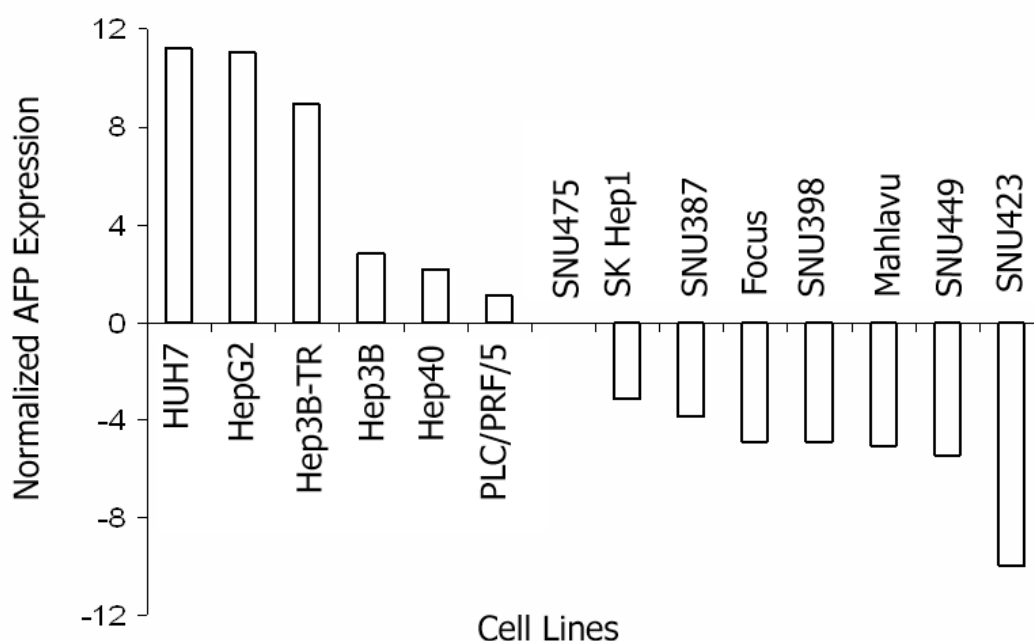


Figure 4.2: Quantification of *AFP* transcript levels reveals two groups in HCC cell lines. *GAPDH* normalized *AFP* expression was quantified by real-time RT-PCR and expression values of individual cell lines are calculated relative to the C_t average of all cell lines, and represented in log2 base. Samples were run in duplicates and the data are representative of two independent experiments.

4.1.5 *SLIT-ROBO* expression signature and their coordinate expression in HCC cell lines and liver tissues

4.1.5.1 Quantitative real-time RT-PCR data for *SLIT-ROBO* genes in HCC cell lines

Previously, the expression of *SLIT-ROBO* genes was analysed by quantitative RT-PCR in prostate tumor samples. *SLIT1* and *SLIT3* were found to be overexpressed in prostate tumors

(Latil, Chene et al. 2003). Using the described human *SLIT1*, *SLIT2*, *SLIT3*, *ROBO1* and *ROBO2* specific primers in that study and newly designed *ROBO3* and *ROBO4* primers, relative quantification of *SLIT-ROBO* genes were performed in HCC cell lines. Relative quantification was performed as described in materials and methods section by taking the mean expression values in all cell lines as the reference sample for each gene. The raw quantification values, along with the data for *AFP*, were log2 transformed and summarized in Table 4.3.

Table 4.3: Relative expression values for *SLIT-ROBO* and *AFP* genes in HCC cell lines*

	<i>ROBO1</i>	<i>ROBO2</i>	<i>ROBO4</i>	<i>SLIT1</i>	<i>SLIT2</i>	<i>SLIT3</i>	<i>AFP</i>
Focus	-1.53	-4.95	3.32	-1.72	1.85	-0.22	-4.86
Hep40	2.05	4.88	1.67	3.23	2.08	3.83	2.19
Hep3B	2.62	2.66	-0.78	1.10	2.88	-6.32	2.78
Hep3B-TR	2.67	4.46	0.40	0.23	4.95	-7.35	8.94
HepG2	2.52	-3.65	-2.27	1.35	-4.22	-4.77	11.03
Huh7	1.55	7.44	-1.62	4.60	4.25	-5.00	11.19
Mahlavu	-0.93	-3.61	-0.87	-3.30	2.33	3.90	-5.07
PLC	2.80	2.85	-0.89	-1.75	-3.95	-5.82	1.09
SKHep1	0.45	-1.99	3.58	-1.87	3.68	6.93	-3.14
Snu387	-5.58	-2.83	1.56	0.23	2.15	0.03	-3.86
Snu398	0.35	5.54	-2.18	6.28	-6.65	-4.67	-4.87
Snu423	-1.03	-3.56	-0.79	-2.05	-5.25	7.65	-9.94
Snu449	-4.38	-3.65	-0.23	-3.57	-4.40	6.63	-5.43
Snu475	-1.23	-3.93	-0.88	-1.37	-0.10	6.03	-0.04

* For each gene, average expression value of all cell lines was used as the control sample when calculating relative expression values.

Relative quantification data was mostly parallel to semi-quantitative expression data. *ROBO2* and *SLIT1* expression were up-regulated in high-*AFP* group of cells, except HepG2, which underexpressed *ROBO2*. On the other hand, both genes were found to be overexpressed in SNU398, one of the low-*AFP* group of cell lines. *SLIT3* transcript levels sharply contrasted those of *ROBO2* and *SLIT1*. Except SNU398, increased *SLIT3* expression was found among low-*AFP* expressing group of cell lines, and also in high-*AFP* expressing Hep40 cell line. Interestingly, widespread expression of *ROBO1* transcripts that we observed in RT-PCR turned out to be enhanced in cell lines with high-*AFP* background. *ROBO4* and *SLIT2* transcript levels remained variable among HCC cell lines and no phenotype-based association could be observed for these genes. Attempts to calculate PCR efficiency with two different *ROBO3* primer pairs failed, so there is no qRT-PCR data for *ROBO3*.

4.1.5.2 Pearson's correlation analysis revealed genewise expression parallels in HCC cell lines

Pearson's correlation analysis was performed in R environment for each possible gene pair using the relative expression values. The results are presented in Table 4.4. *ROBO1* expression showed a significant positive correlation with that of *ROBO2* and *AFP* whereas *ROBO1* expression showed a negative correlation with *SLIT3*. *ROBO2* correlated in a positive manner with *SLIT1* and in a negative manner with *SLIT3*. *SLIT1* expression also showed a significant negative correlation with *SLIT3*. *SLIT3* has a negative correlation with *AFP*, which is significant.

Table 4.4: Genewise correlation of *SLIT-ROBO* and *AFP* genes in HCC cell lines

Gene	<i>ROBO1</i>	<i>ROBO2</i>	<i>ROBO4</i>	<i>SLIT1</i>	<i>SLIT2</i>	<i>SLIT3</i>	<i>AFP</i>
<i>ROBO1</i>	1						
<i>ROBO2</i>	0.6060*	1					
<i>ROBO4</i>	-0.2721	-0.2653	1				
<i>SLIT1</i>	0.4027	0.7623*	-0.3288	1			
<i>SLIT2</i>	0.1241	0.1891	0.5143	-0.0039	1		
<i>SLIT3</i>	-0.5698*	-0.6072*	0.3681	-0.5451*	-0.1003	1	
<i>AFP</i>	0.6533*	0.5108	-0.3016	0.4579	0.3544	-0.6605*	1

* : $p < 0.05$.

4.1.5.3 Cluster analysis identified two subgroups of HCC cell lines with respect to *SLIT-ROBO* expression

Hierarchical clustering was performed using Cluster and TreeView tools. In terms of *SLIT-ROBO* expression, low-*AFP* expressing SNU449, SNU423, SNU475, Mahlavu, SK Hep1, SNU387, and Focus cells grouped together (Group I), while high-*AFP* expressing HepG2, PLC/PRF/5, HUH7, Hep3B-TR, HEP-3B and Hep40 cell lines (Group II) clustered separately from the first group (Figure 4.3). Exceptionally, SNU398 cell line displayed a *SLIT-ROBO* expression pattern concordant with Group II despite its low-*AFP* background. In ANOVA, the expression of *AFP* significantly differed between the two groups ($p = 0.0001$): Mean expression of *AFP* in Group I and Group II cells was -4.65 ± 2.75 and 6.20 ± 4.68 , respectively (Figure 4.4).

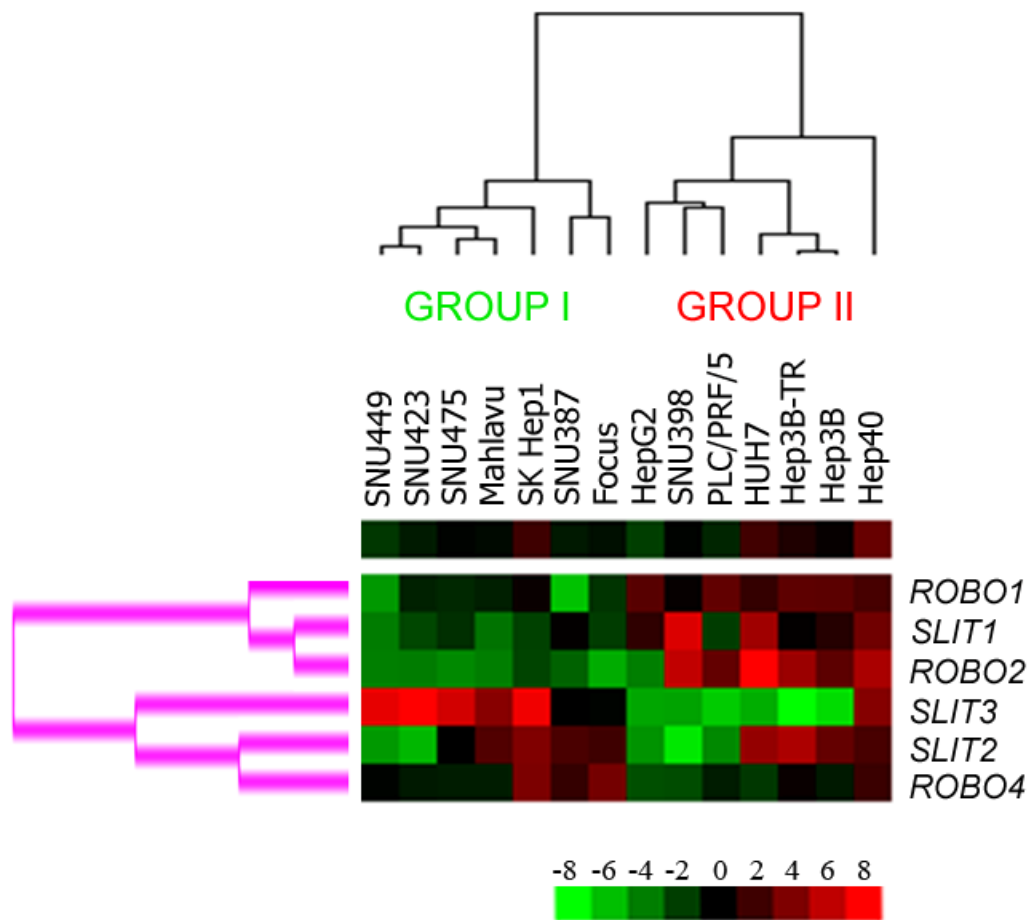


Figure 4.3: *SLIT-ROBO* expression establishes two groups, which predict *AFP* expression levels in HCC cell lines. The means of normalized expression values of individual *SLIT-ROBO* genes in each cell line were used to establish the hierarchical clustering in Cluster program, and the results were visualized as a heatmap by TreeView. Overexpression and underexpression of individual genes relative to Ct average of all cell lines are represented by *red* and *green* colors, respectively. Upper single-row heatmap displays the average expression of all six genes across all cell lines. The tree at the top of the heatmap represents samplewise clustering. Based on their *AFP* expression levels, HCC cell lines are represented in two main subgroups indicated as Group I (low-*AFP*) and Group II (high-*AFP*) cells. The tree on the left of the heatmap (*pink*) represents genewise clustering.

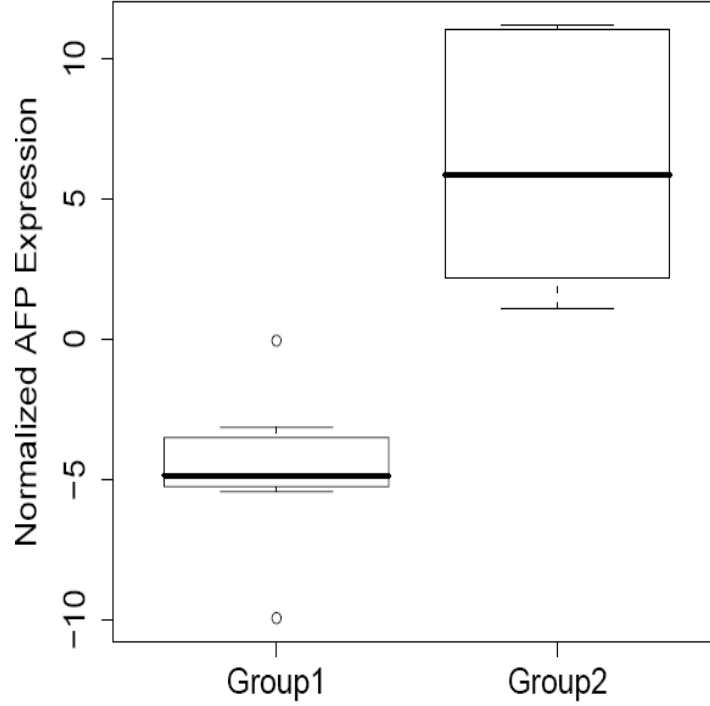


Figure 4.4: One-way ANOVA for *AFP* expression between Group1 and Group2 cell lines. *AFP* expression significantly differed between the two groups ($p = 0.0001$): Mean expression of *AFP* in Group1 and Group2 cells was -4.65 ± 2.75 and 6.20 ± 4.68 , respectively .

Hierarchical clustering analysis also revealed a genewise segregation of *SLIT-ROBO* genes. Coordinate expression was observed in two main clusters, one of which grouping together *ROBO1*, *ROBO2* and *SLIT1*, and the other *ROBO4*, *SLIT2*, and *SLIT3*, respectively (Figure 4.3). One-way ANOVA was performed to identify individual *SLIT-ROBO* genes, which significantly separate HCC cell lines with high- and low-*AFP* expression. In this analysis, *ROBO1* and *ROBO2* expression were up-regulated in high-*AFP* group ($p = 6.7 \times 10^{-4}$ and $p = 0.013$, respectively). Mean expression of *ROBO1* in Group I was -1.73 ± 2.15 while in Group II cells increased to 2.37 ± 0.48 . Similarly, *ROBO2* mean expression values were found as -2.37 ± 3.31 and 3.11 ± 3.73 , for Group I and Group II, respectively. In sharp contrast, *SLIT3* was overexpressed in cell lines with low-*AFP* profile (Mean_{Group1} = 3.28 ± 4.43 Mean_{Group2} = -4.24 ± 4.06 , $p = 6.9 \times 10^{-3}$) (Figure 4.5).

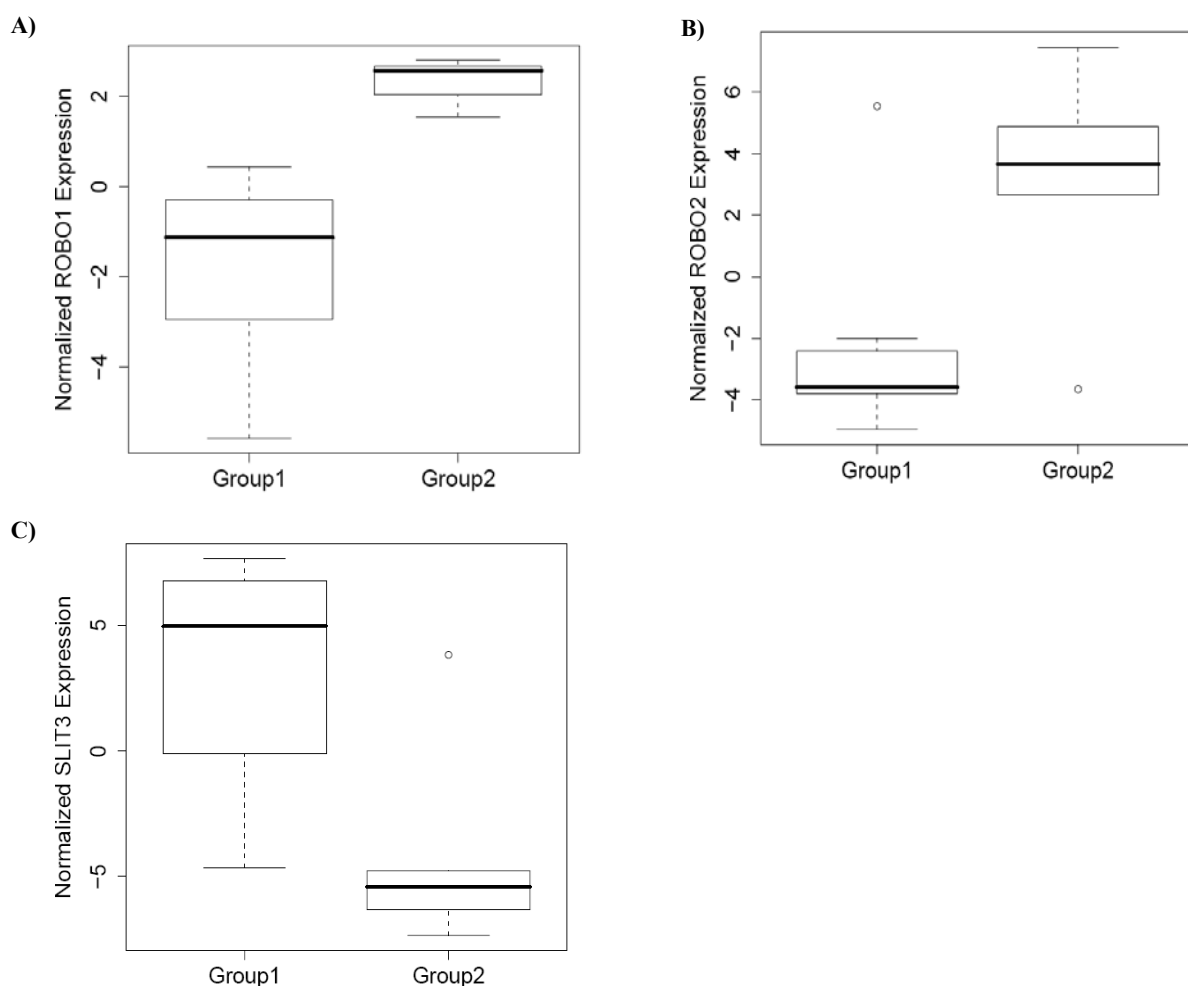


Figure 4.5: Significant one-way ANOVA results of individual *SLIT-ROBO* genes between Group1 and Group2 HCC cell lines. *ROBO1* and *ROBO2* expression were up-regulated in high-*AFP* group ($p = 6.7 \times 10^{-4}$ and $p = 0.013$, respectively). Mean expression of *ROBO1* in Group I was -1.73 ± 2.15 while in Group II cells increased to 2.37 ± 0.48 . Similarly, *ROBO2* mean expression values were found as -2.37 ± 3.31 and 3.11 ± 3.73 , for Group I and Group II, respectively. In contrast, *SLIT3* was overexpressed in cell lines with low-*AFP* profile (MeanGroup1= 3.28 ± 4.43 MeanGroup2= -4.24 ± 4.06 , $p = 6.9 \times 10^{-3}$).

4.1.5.4 Quantitative real-time RT-PCR data for *SLIT-ROBO* genes in normal liver and hepatocellular carcinoma tissues

SLIT-ROBO expression in tumor-adjacent normal liver and hepatocellular carcinoma tissues were analyzed in TissueScan Liver Cancer Tissue qPCR Array 1 (Origene Technologies, Rockville, MD, USA). The panel contained 48 tissue cDNAs normalized against β -actin. The *SLIT-ROBO* expression in 8 normal and 35 hepatocellular carcinoma cDNAs were analyzed, the remaining five cDNAs were of other unrelated pathologies (Table 3.6). Relative quantification was performed as described in materials and methods section by taking the mean expression values in normal cDNAs as the reference sample for each gene. The raw

quantification values, along with the data for *AFP*, were log2 transformed and summarized in Table 4.5.

Table 4.5: Relative expression values of *SLIT-ROBO* and *AFP* genes in normal liver and HCC samples*

Sample #	<i>ROBO1</i>	<i>ROBO2</i>	<i>ROBO4</i>	<i>SLIT1</i>	<i>SLIT2</i>	<i>SLIT3</i>	<i>AFP</i>
N1	-0.66	-2.49	0.15	2.76	0.03	1.09	2.06
N2	0.24	1.61	0.35	-1.04	1.63	1.09	1.98
N3	0.04	-2.39	1.35	-0.94	0.43	-0.21	1.23
N4	-0.66	-2.49	-0.75	-1.04	-1.88	0.09	-0.74
N5	-0.06	1.81	-0.55	1.76	-0.78	-1.21	-0.96
N6	1.34	3.71	1.05	-0.14	0.83	-0.21	-1.45
N7	-0.06	-0.69	-1.85	-0.84	-1.18	-2.41	-1.3
N8	-0.16	0.91	0.25	-0.54	0.93	1.79	-0.83
T1	1.84	-0.79	-1.75	0.66	-2.68	-3.81	5.46
T2	1.74	1.71	-0.65	-0.34	-1.48	-0.71	-2.24
T3	0.74	-0.69	-1.05	0.76	-0.58	0.39	0.13
T4	0.14	-1.59	-1.05	-0.14	-2.68	-2.41	0.08
T5	2.04	0.51	-3.15	-0.34	0.83	-1.11	5.48
T6	1.44	-0.99	0.25	0.46	2.63	1.89	0
T7	-0.16	-1.49	-0.15	0.86	0.33	-0.11	-1.01
T8	-0.16	-1.49	0.15	4.56	-0.38	-2.81	-1.77
T9	-0.46	-1.39	0.15	0.06	0.43	-0.71	-0.99
T10	3.84	0.91	-0.25	-1.04	0.73	1.59	8.68
T11	3.44	-2.09	-0.35	-0.64	-4.98	-0.91	4.75
T12	4.24	-2.69	-1.95	1.76	-7.68	-0.71	-0.09
T13	1.54	1.01	-2.75	3.56	-1.68	-0.51	6.72
T14	1.64	2.41	-0.35	-0.54	0.63	1.59	-0.07
T15	3.04	1.61	-3.25	2.76	-5.38	-4.11	11.63
T16	3.14	-0.99	0.35	0.46	-1.28	-0.01	5.09
T17	2.94	-0.99	-1.25	0.46	-7.88	-2.81	-6.19
T18	0.84	-2.89	0.45	-1.44	3.93	2.49	-1.81
T19	1.54	-1.59	0.65	-0.14	1.03	1.59	3.13
T20	-0.06	2.61	-0.95	0.06	-2.68	-3.01	0.87
T21	1.14	-0.29	-1.95	1.16	-0.98	-1.91	4.09
T22	4.34	-1.89	-0.35	-0.44	2.83	1.79	-0.73
T23	1.44	-0.79	-1.05	5.96	1.03	2.59	-6.07
T24	-1.06	2.41	-1.05	-0.34	1.13	5.09	-9.53
T25	4.54	1.41	-1.55	-0.24	-6.38	-3.11	-4.18
T26	-1.16	-3.49	1.15	-2.04	-1.28	-2.71	-10.13
T27	3.64	0.71	0.25	-1.24	-0.78	0.69	-3.99
T28	3.94	6.11	-1.95	-0.14	0.23	0.49	11.36
T29	-0.46	-0.99	-1.85	0.46	0.13	0.59	-0.17
T30	0.04	-0.99	-4.85	2.46	1.13	1.59	1.95
T31	-0.26	1.11	-0.45	-0.44	-1.08	-0.31	2.41
T32	1.44	-2.19	0.25	-0.74	0.63	1.29	1.94
T33	1.04	-1.29	0.95	0.16	1.43	2.29	-2.67

T34	0.94	-2.19	-0.25	-0.74	2.33	0.39	-5.27
T35	2.24	-1.99	-1.45	-0.54	-0.98	0.39	9.94

* For each gene, average expression value of normal samples was used as the control sample when calculating relative expression values.

In these analyses, only *ROBO1* was found to be significantly overexpressed in tumor tissues ($\text{Mean}_{\text{Normal}} = 0 \pm 0.63$ $\text{Mean}_{\text{Tumor}} = 1.57 \pm 1.63$, $p = 0.011$). Also, the *ROBO4* expression was downregulated in tumor tissues with a p-value near significance ($\text{Mean}_{\text{Normal}} = 0 \pm 1.03$ $\text{Mean}_{\text{Tumor}} = -0.89 \pm 1.30$, $p = 0.079$). *AFP* displayed a highly variable expression, yet it was overexpressed in more than half of the tumors (18/35) (Figure 4.6).

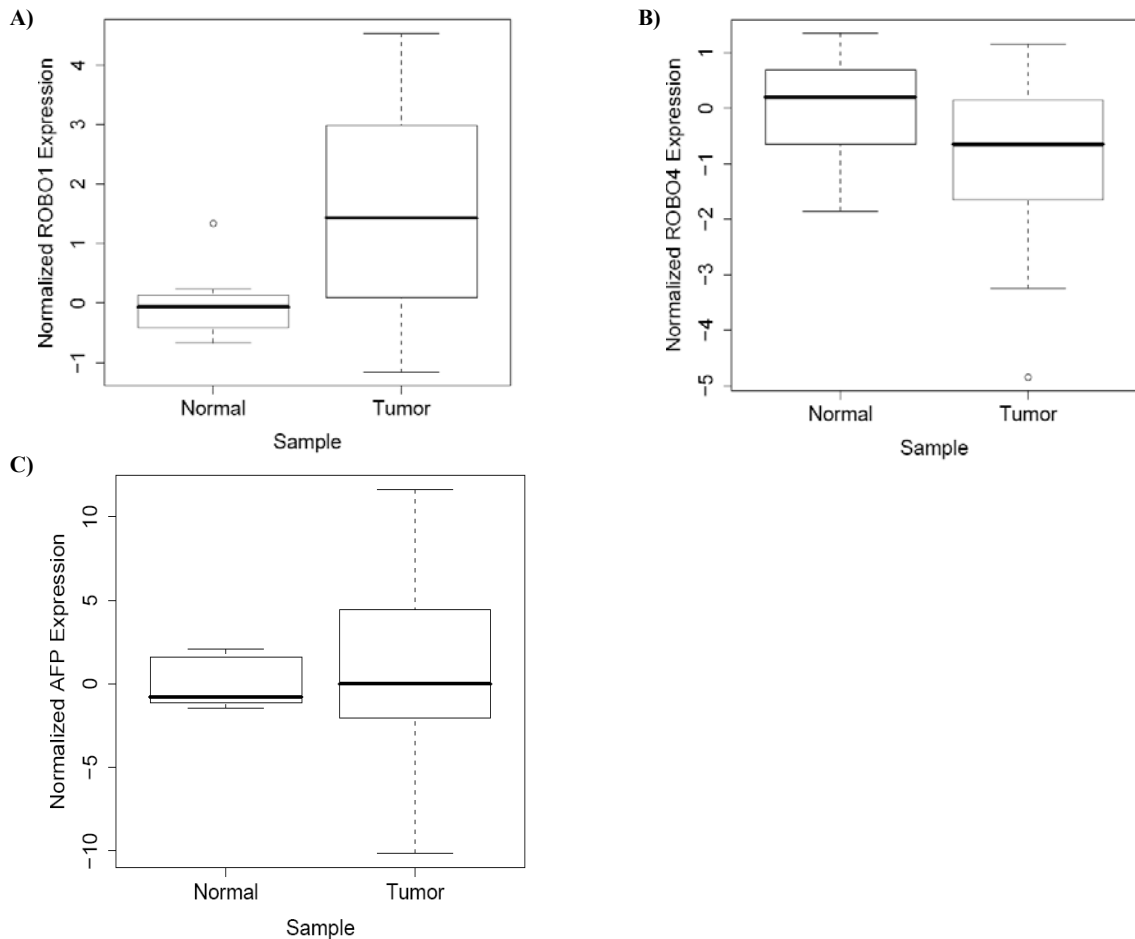


Figure 4.6: Significant one-way ANOVA results of individual *SLIT-ROBO* genes between normal and HCC tissues. *ROBO1* was found to be significantly overexpressed in tumor tissues ($\text{Mean}_{\text{Normal}} = 0 \pm 0.63$ $\text{Mean}_{\text{Tumor}} = 1.57 \pm 1.63$, $p = 0.011$) whereas *ROBO4* was downregulated in tumor tissues with a p-value near significance ($\text{Mean}_{\text{Normal}} = 0 \pm 1.03$ $\text{Mean}_{\text{Tumor}} = -0.89 \pm 1.30$, $p = 0.079$). *AFP* displayed a highly variable expression, yet it was overexpressed in more than half of the tumors (18/35).

4.1.5.5 *SLIT-ROBO* genewise clustering observed in HCC cell lines translates into liver tissues

Hierarchical clustering was performed using Cluster and TreeView tools. Hierarchical clustering grouped *SLIT-ROBO* genes in a similar manner as in HCC cell lines. *ROBO1*, *ROBO2* and *SLIT1* clustered together, whereas *ROBO4*, *SLIT2* and *SLIT3* formed another cluster (Figure 4.7).

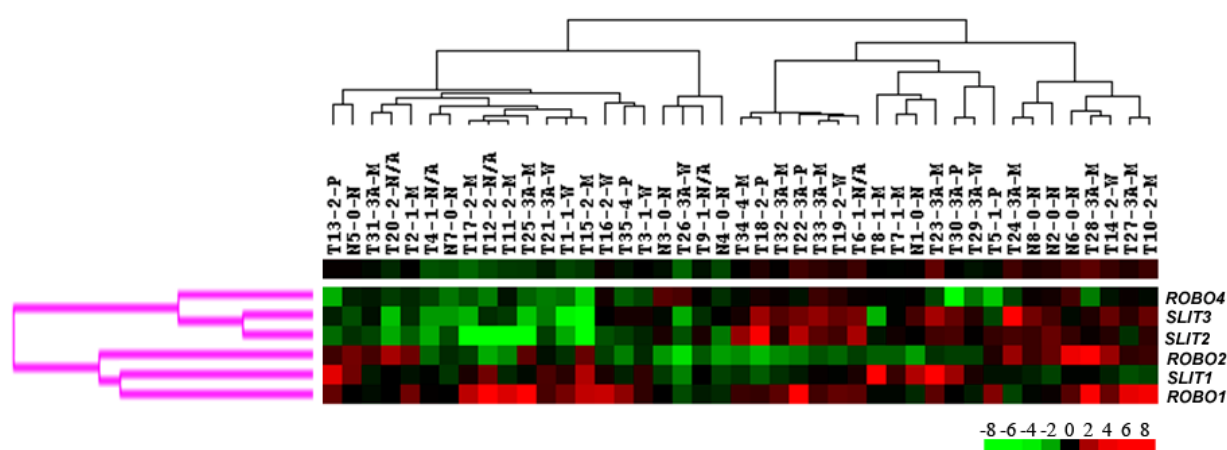


Figure 4.7: Genewise clustering of *SLIT-ROBO* genes in HCC cell lines translates into liver tissues. The normalized expression values of individual *SLIT-ROBO* genes in liver tissue samples (n = 43) were used to establish the hierarchical clustering in Cluster program and the results were visualized as a heatmap by TreeView. Overexpression and underexpression of individual genes relative to Ct average of normal tissues (n = 8) are represented by red and green colors, respectively. Upper single-row heatmap displays the average expression of all six genes across all tissues. The tree at the top of the heatmap represents samplewise clustering, and the tree on the left of the heatmap (pink) represents genewise clustering. T1-35: tumors; N1-8: normal tissues; 0: stage 0 (non-tumor); 1, 2, 3A, 4: stage 1, stage 2, stage 3A, stage 4 HCC, respectively; N: tumor-adjacent normal tissue; W: well-differentiated; M: moderately differentiated; P: poorly differentiated; N/A: not-assigned.

Two main clusters appeared among tissue samples; however, no significant association of clusters was found with differentiation state or staging of tumors (Wilcoxon rank sum test) and *AFP* expression (one-way ANOVA).

4.1.5.6 Pearson's correlation analysis revealed genewise expression parallels in liver tissues and a Mantel's permutation test confirmed the significance of correlation patterns in HCC cell lines and liver tissues

Expression correlation in individual *SLIT-ROBO* and *AFP* genes in liver tissues was analyzed by Pearson's correlation analysis in R (Table 4.6). Accordingly, *ROBO1* expression showed a significant positive correlation with that of *AFP* whereas *ROBO1* expression showed a negative correlation with *SLIT2*. *ROBO4* correlated in a positive manner with *SLIT2* and in a negative manner with *SLIT1* and *AFP*. *SLIT2* expression showed a highly significant positive correlation with *SLIT3*.

Table 4.6: Genewise correlation of SLIT-ROBO and AFP genes in liver tissues

Gene	<i>ROBO1</i>	<i>ROBO2</i>	<i>ROBO4</i>	<i>SLIT1</i>	<i>SLIT2</i>	<i>SLIT3</i>	<i>AFP</i>
<i>ROBO1</i>	1						
<i>ROBO2</i>	0.1835	1					
<i>ROBO4</i>	-0.1879	-0.2134	1				
<i>SLIT1</i>	0.0298	0.002	-0.3793*	1			
<i>SLIT2</i>	-0.3679*	-0.0078	0.3432*	-0.1389	1		
<i>SLIT3</i>	-0.0973	-0.0027	0.2596	-0.0965	0.6844***	1	
<i>AFP</i>	0.3679*	0.2324	-0.3699*	0.0924	-0.0769	-0.1638	1

***: $p < 0.001$; *: $p < 0.05$.

Mantel's permutation test was performed in R environment in order to compare gene expression correlation matrices of cell lines and tissues (described in Table 4.4 and Table 4.6, respectively), and indicated that gene-to-gene correlation patterns in both sample groups were significantly associated ($R = 0.49$, $p < 0.02$).

4.1.5.7 *ROBO1* expression differentiates normal tissues from tumors with respect to both stage and differentiation status

Using relative expression data of liver tissues, one-way ANOVA and subsequent Fisher's multiple pairwise comparisons were applied in order to explore whether gene expression

characteristics of *SLIT-ROBO* family members discriminate among liver tissues with respect to differentiation status and staging of the samples. The tissue samples lacking stage or differentiation information in the pathological reports were left out of analysis. With these criteria, a total of 43 liver tissue samples for stage and of 38 tissue samples for differentiation status were analyzed. According to one-way ANOVA, *ROBO1* differentiated liver tissue samples on both the stage ($p = 0.018$) and differentiation status ($p = 0.031$) dependent manner (Figure 4.8 A, B). *ROBO4* expression significantly discriminated tissues only with respect to their differentiation status ($p = 0.039$) (Figure 4.8 C). Moreover, Fisher's pairwise comparison analyses revealed that *ROBO1*, *SLIT2* and *ROBO4* significantly discriminated between different histopathological subgroups both in terms of differentiation status and/or tumor staging (Table 4.7).

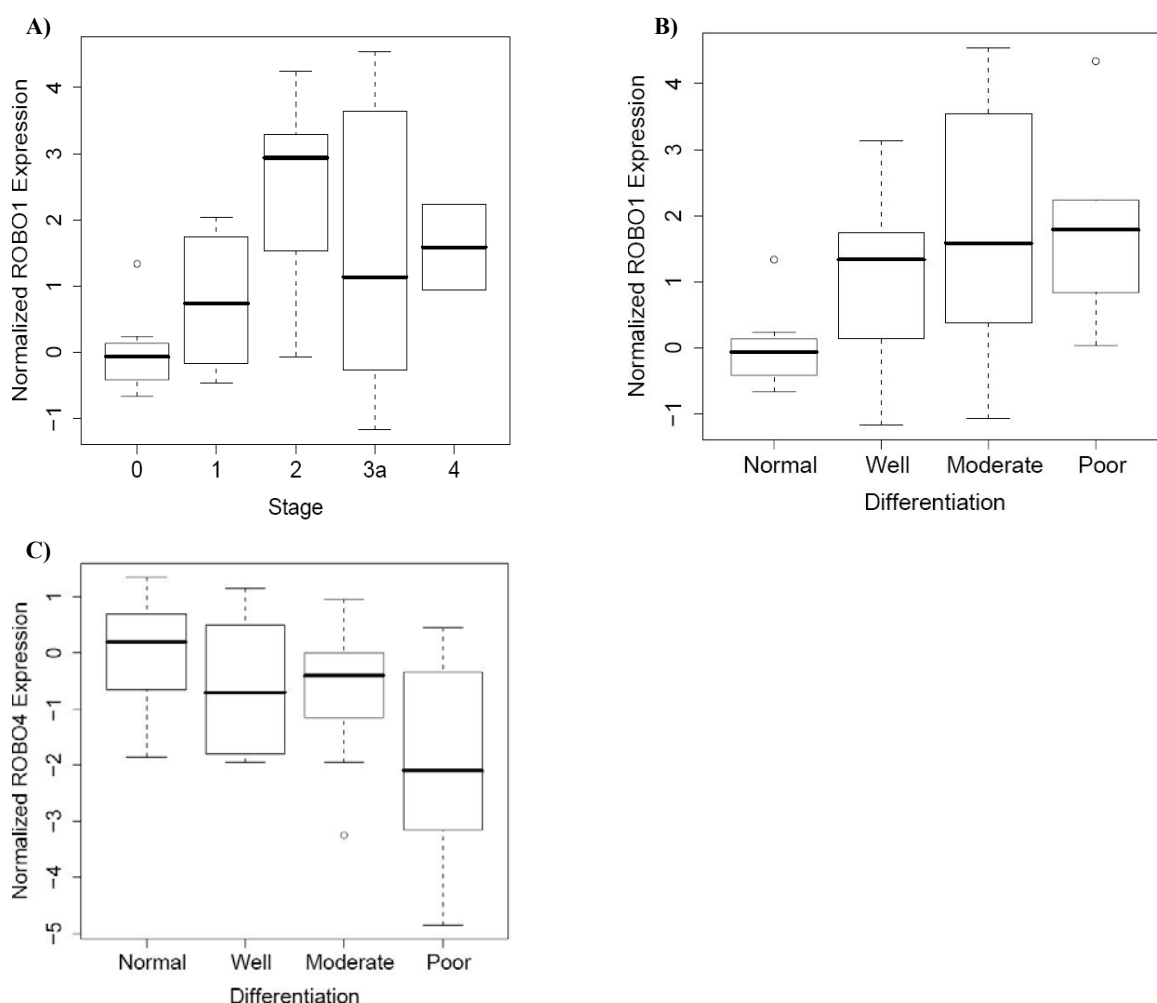


Figure 4.8: Significant one-way ANOVA results of individual *SLIT-ROBO* genes between normal and HCC tissues. *ROBO1* differentiated liver tissue samples depending on both A) the stage ($p = 0.018$) and B)

differentiation status ($p = 0.031$) dependent manner. C) *ROBO4* expression significantly discriminated tissues only with respect to their differentiation status ($p = 0.039$).

Table 4.7: Differentially expressed *SLIT-ROBO* genes between histopathological subgroups of liver tissues

Stage	<i>n</i>	Mean expression (SE)	Differentially expressed gene*
0	8	0 (0.22)	ROBO1
2	11	2.37 (0.41)	
0	8	0 (0.22)	ROBO1
3	13	1.43 (0.57)	
1	9	0.79 (0.33)	ROBO1
2	11	2.37 (0.41)	
2	11	-2.29 (1.15)	SLIT2
3	13	-0.96 (0.44)	
Differentiation state	<i>n</i>	Mean expression (SE)	Differentially expressed gene*
Normal	8	0 (0.22)	ROBO1
Moderate	16	1.89 (0.44)	
Normal	8	0 (0.22)	ROBO1
Poor	6	1.84 (0.60)	
Normal	8	0 (0.36)	ROBO4
Poor	6	-2.02 (0.80)	
Well	8	-0.60 (0.43)	ROBO4
Poor	6	-2.02 (0.80)	
Moderate	16	-0.66 (0.25)	ROBO4
Poor	6	-2.02 (0.80)	
Moderate	16	-1.28 (0.78)	SLIT2
Poor	6	1.01 (0.88)	

* Pairs of stage and differentiation subgroups were compared in Fisher's pairwise comparison analysis and only the genes that significantly discriminate between subgroups were represented ($p < 0.05$). SE: standard error.

4.1.6 An alternatively spliced *ROBO2* isoform *ROBO2-tv2*, which was initially described in zebrafish (*Danio rerio*) and rat (*Rattus norvegicus*); is also expressed in human HCC cell lines

In 2006; Dalkic, Kuscu et al. described a novel conserved alternative exon (CAE) of *robo2* genes of zebrafish and rat. CAE is a 186-bp sequence that corresponds to a region in zebrafish chromosome 15 (from 40,471,855 to 40,472,040 bp and in between 21st and 22nd exons of Ensdart00000014877). CAE is predicted to yield an in-frame insertion without stop codons. It corresponds to a region between the second and the third cytoplasmic domains (between the

1311th and 1312th residues of NM_131633) of the zebrafish robo2 protein sequence. Rat robo2 mRNA (XM_213677) was reported to include the homolog of the zebrafish CAE, although some of the Ensembl-predicted isoforms differed in their usage of it (Ensrnot00000044621, Ensrnot00000043725, Ensrnot00000042437, Ensrnot00000050397) (Dalkic, Kuscü et al. 2006).

With two sets of PCR primers, expression of *ROBO2-tv1* and *tv2* human orthologs was analyzed in HCC cell lines (Figure 4.9 B). ROBO2-hRF was a common forward primer. Reverse primer ROBO2-hRRA specifically complements to CAE and amplifies *ROBO2-tv2* (giving a 220bp PCR product) whereas reverse primer ROBO2-hRR spans CAE and amplifies both *ROBO2-tv1* and *tv2* in the same reaction (amplifying a 102bp fragment and a CAE spanning 285bp fragment, respectively). Another primer pair described by Latil, Chene et al. 2003 was used to confirm the results. All reactions were run at 60°C of annealing temperature and for 40 cycles of amplification. Human ortholog of CAE mapped to Genscan predicted putative exonic site near the 3' end of robo2 [human: Chr. 3: 77,764,315:77,764,497 bp, (Genscan00000045332)]. There are three different *ROBO2* transcripts present in Ensembl genome browser: ROBO2-201 (ENST00000332191), ROBO2-202 (ENST00000398467) and ROBO2-203 (ENST00000403211). Primer pair described in Latil, Chene et al. 2003 binds to and amplifies all three transcripts. However, ROBO2-hRF and hRR binding sites are present in only ROBO2-201 and ROBO2-203. Thus, observed *ROBO2-tv1* expression represents these two transcripts.

Both *ROBO2-tv1* and *ROBO2-tv2* were present in *AFP*-positive, well-differentiated subgroup of HCC cell lines (Hep40, Hep3B, Hep3B-TR, Huh7 and PLC). In addition, they were present in Snu398 which is a poorly differentiated cell line. *ROBO2-tv1* also was expressed in the poorly differentiated Snu387 and Snu475 cell lines. Expressions of the transcripts amplified by *ROBO2* control primer pair (Latil, Chene et al. 2003) were strongly parallel to the *ROBO2-tv2* expression. However, *ROBO2* control primer pair also gave products in SK Hep1 and Snu449 cell lines (Figure 4.9 A).

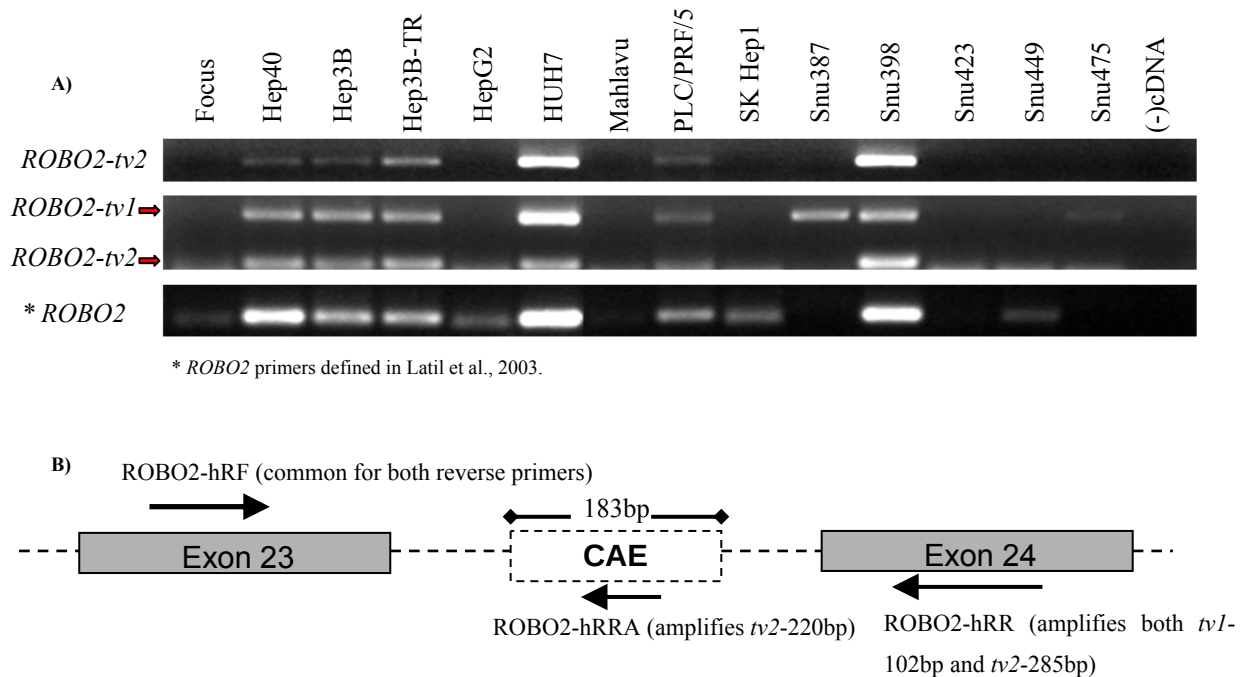


Figure 4.9: Expression of transcript variant *ROBO2-tv2* (including the described conserved alternative exon, CAE) and *ROBO2-tv1* (lacking CAE) in HCC cell lines. A) With two sets of PCR primers, *tv1* and *tv2* expression was analyzed in HCC cell lines. *ROBO2*-hRF was a common forward primer. Reverse primer *ROBO2*-hRRA specifically complements to CAE and amplifies *tv2* whereas reverse primer *ROBO2*-hRR spans CAE and amplifies both *tv1* and *tv2* in the same reaction. Another primer pair described by Latil et al., 2003 was used to confirm the results. B) Schematic representation of the used primers and the expected product sizes.

4.2 Characterization of *ROBO2* knockdown associated cellular phenotype in HCC

4.2.1 Stable knockdown of *ROBO2* expression in Huh7 cell line

The *SLIT-ROBO* expression data distinguished between *AFP*-positive and *AFP*-negative HCC cell lines correctly in the cluster analysis. When we analyzed the clustering pattern the most strikingly differentially expressed gene appeared as *ROBO2*. It was downregulated in poorly-differentiated *AFP*-negative cell lines whereas it was highly expressed in well-differentiated *AFP*-positive cell lines, except in HepG2-which is a hepatoblastoma derived cell line- it was negative. Another feature of the *ROBO2* downregulated cell lines was their motility. They overexpress genes associated with metastasis and motility (Lin, Ke et al. 1998; Seki, Kitada et al. 1999; Lee and Thorgeirsson 2002). The above mentioned literature data and the role of *SLIT-ROBO* interaction as a chemokine signaling in cellular migration led us to propose that interfering with *ROBO2* function in one of the well-differentiated HCC cell lines may affect

the migratory behavior these cells. To this end, *ROBO2* expression was stably knockdowned by transfecting Huh7 cells with *ROBO2* mRNA targeting shRNA vector and as a control nontargeting scrambled vector.

17 *ROBO2* shRNA expressing vector transfected clones (hereafter referred as R2C-clone number) and 8 nontargeting scrambled shRNA transfected clones (hereafter referred as NC-clone number) were obtained. *ROBO2* expression was checked in these clones by both real-time quantitative RT-PCR and western blot analysis (Figure 4.10 and Figure 4.11, respectively). In selected clones, *ROBO2* expression was found to be downregulated at both mRNA and protein levels. In addition we performed immunofluorescence experiments in R2C10 and NC4 clones (Figure 4.11 B).

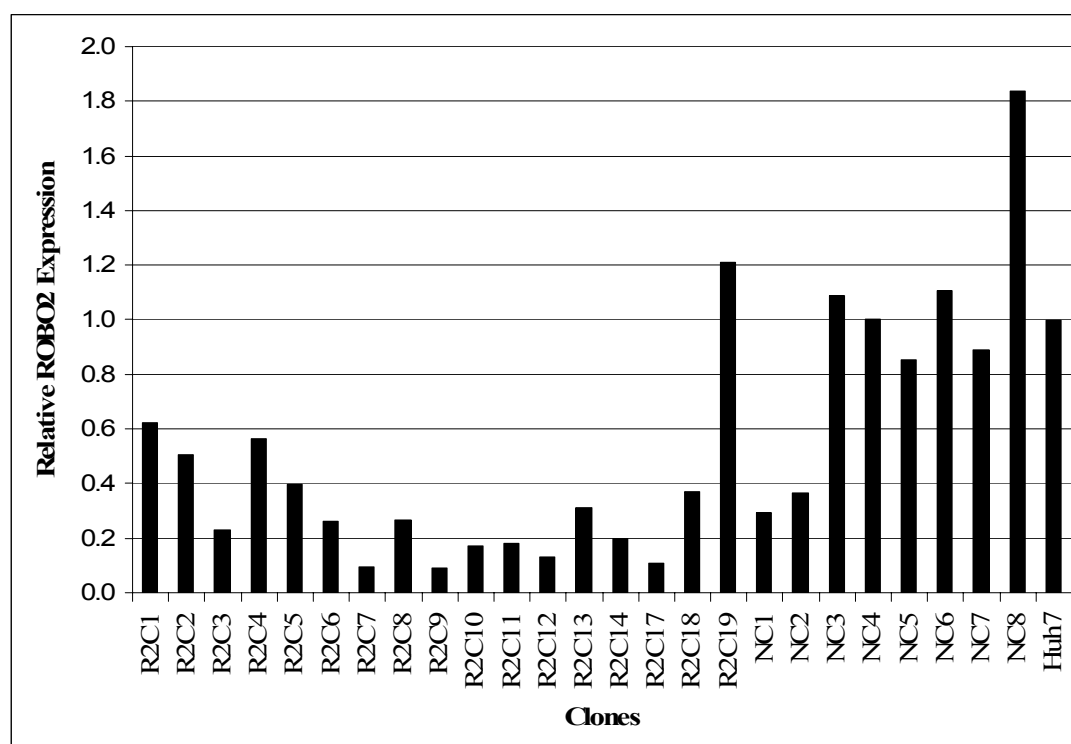


Figure 4.10: Relative *ROBO2* expression in pSEC transfected stable Huh7 clones. *ROBO2* expression was checked by real-time quantitative RT-PCR in Huh7 clones stably transfected with *ROBO2* targeting (R2C-) or nontargeting (NC-) shRNA vectors. Relative expression was calculated using $\Delta\Delta C_t$ method and *ROBO2* expression in Huh7 was taken as the reference.

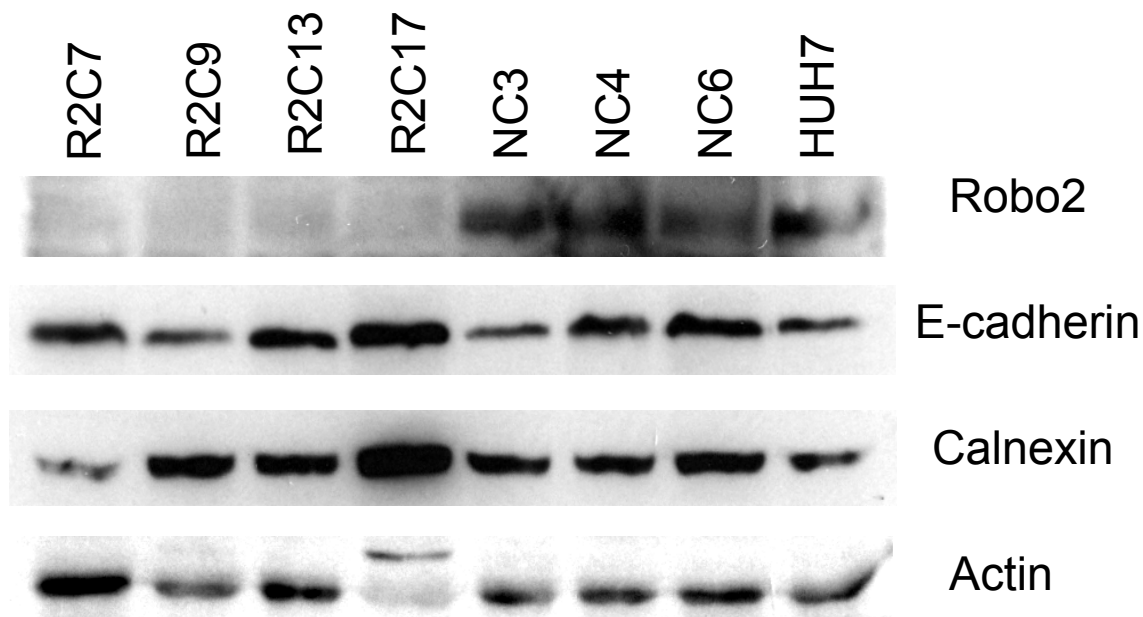


Figure 4.11: ROBO2 protein levels in pSEC transfected stable Huh7 clones. Western blots were performed to in selected stable clones. Membranes were probed with ROBO2 and E-cadherin antibodies. Equal loading was controlled with two different antibodies, Calnexin and Actin.

4.2.2 Expression of the epithelial-to-mesenchymal transition (EMT) markers and E-cadherin suppressors in stable clones

Epithelial-to-mesenchymal transition (EMT) is a process occurring during development for the proper dissemination of cell groups and subsequent tissue formation. EMT is also observed in tumor cells during distant metastasis. Invasive front of the solid tumor surpasses through EMT and launches from the primary tumor. After intravasation and systemic spread, cells find a proper microenvironment and settles, this time by a process which is the reverse of the EMT: mesenchymal-to-epithelial transition (MET). Next, we characterized the expression of EMT markers and E-cadherin transcriptional suppressors that are specifically upregulated during EMT. *Twist* and *SNAI* primer pairs did not give the expected in none of the clones and the parental Huh7 cell line. In RT-PCR experiments, there was no clear-cut differential expression of the analyzed genes between *ROBO2* knockdown and control clones (Figure 4.12). Only, *SLUG* was upregulated in R2C7, R2C9 and R2C17. We checked the expression of *E-cadherin* and its suppressors *SLUG* and *SIP1* further in QRT-PCR experiments (Figure 4.13). In these analyses, *E-cadherin* showed an upregulation whereas *SIP1* and *SLUG* showed a downregulation trend in stable *ROBO2* knockdown clones when compared to the negative clones. Individual clones differed in their expression patterns and there were outliers to the

general trend. β -catenin is known to make complex with E-cadherin in the cytosolic part of the membrane. Using a home-made antibody against β -catenin (9E10), we checked the cellular location of β -catenin in R2C10 and NC4 clones but both the location and signal did not change significantly (Figure 4.14).

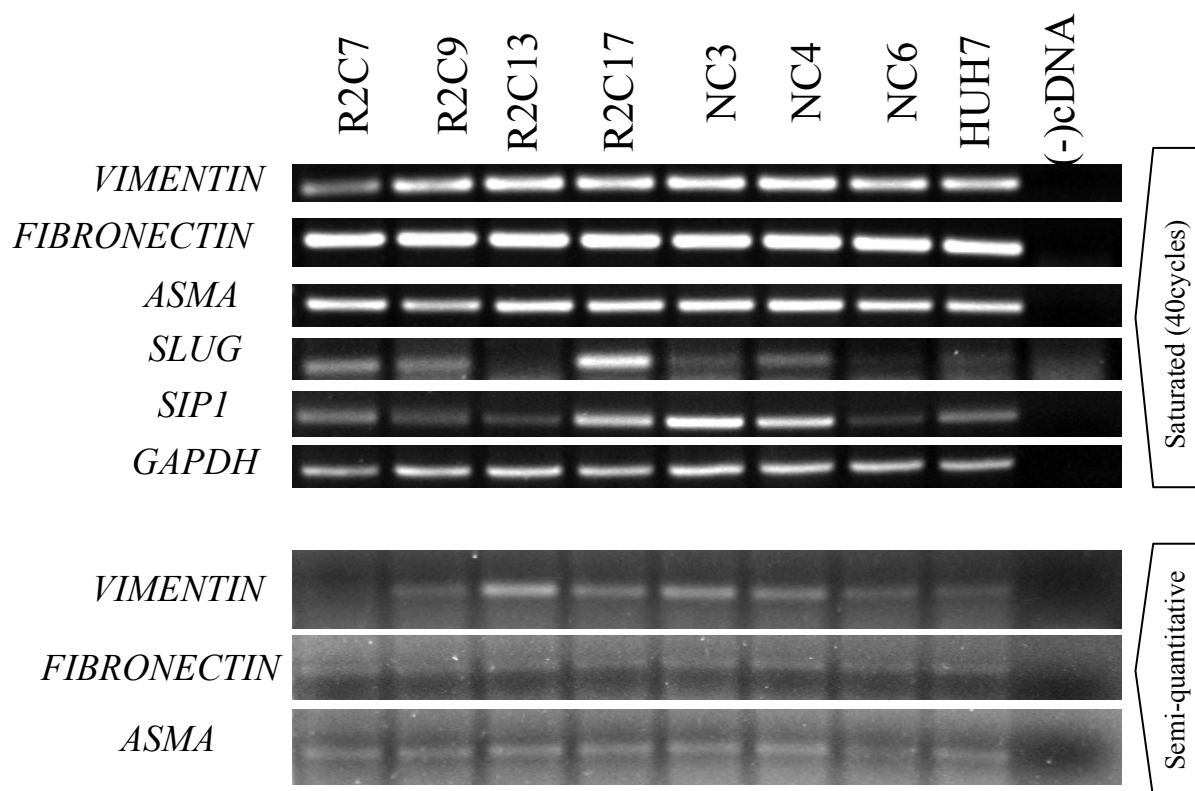


Figure 4.12: Evaluation of the EMT marker expression in stable clones. Mesenchymal markers vimentin, fibronectin, asma (alpha-smooth muscle actin) and E-cadherin suppressors slug and SIP1 expression was analyzed at 40 cycles. Since vimentin, asma and fibronectin products were saturated, semi-quantitative expressions at lower cycles were also analyze (20, 22 and 18 cycles respectively).

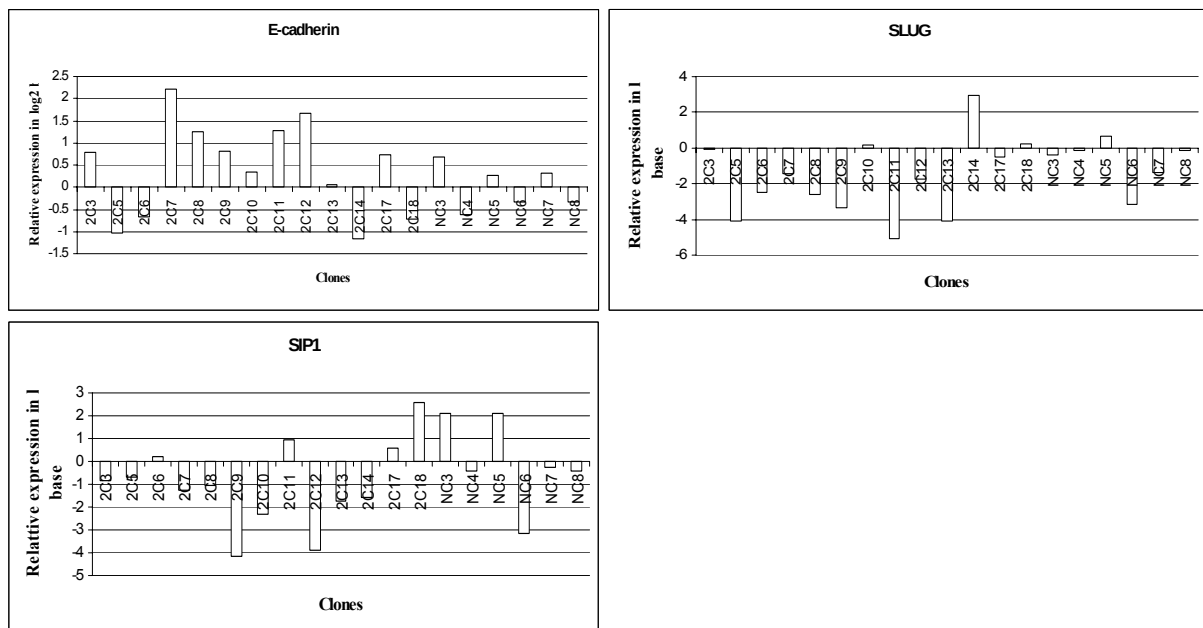


Figure 4.13: Quantitative expression of *E-cadherin* and its suppressors *SLUG* and *SIP1* in stable clones. Relative expressions of *E-cadherin*, *SIP1* and *SLUG* were measured taking the average expression in negative clones as the reference. Calculated values were plotted in log2 base.

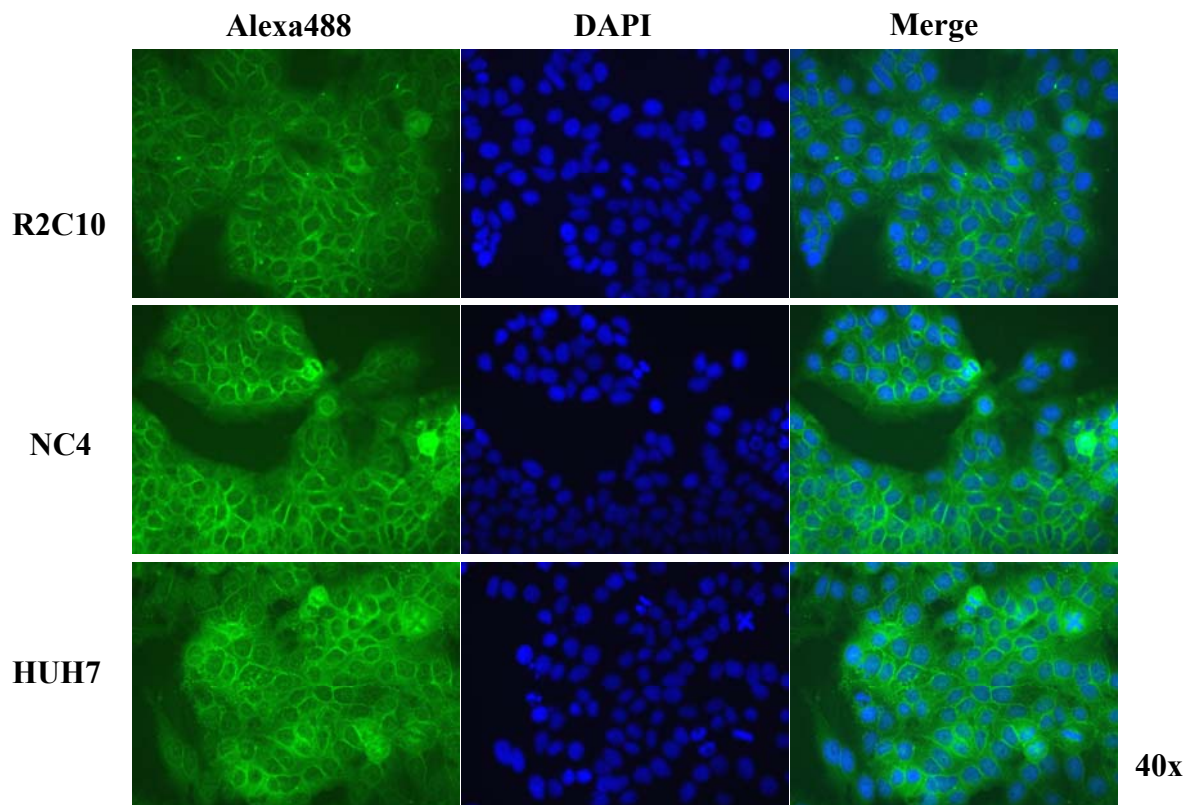


Figure 4.14: β -catenin immunofluorescence staining in R2C10, NC4 clones and parental Huh7 cells.

4.2.3 In wound-healing migration assay, there was no significant difference between the migration rates of *ROBO2* knockdown and negative clones

We addressed the migrational differences between stable *ROBO2* knockdown and negative clones. To this end, we performed wound-healing assay by simply scratching near-confluent cell layers with a 200 μ l micropipette tip both in the presence and absence of serum in the medium. Then, the opened wound was followed for 48 hours. At 0, 24 and 48 hours photographs were taken.

In serum positive conditions cells migrated faster as observed from the higher scale bar in the normal serum condition (Figure 4.15 A and B). The fastest healed clone was R2C17 and the slowest one was NC6 as can be seen in Figure 4.15 A. A representative assay photograph is given in Figure 4.16 showing the difference between these two fastest and slowest clones. Three *ROBO2* knockdown (R2C7, R2C9, R2C17) and three negative clones (NC3, NC4, NC6) were averaged for their relative migration capacities and compared by t-test at 24 hours (p-value = 0.78) and 48 hours (p-value = 0.37) in the presence of serum (Figure 4.15 B) but there was no significant difference in their migration. Likewise, in the serum absent conditions the migratory behavior of the clones was not significantly different (t-test, p-value = 0.31 for 24 hours and p-value = 0.55 for 48 hours) (Figure 4.15 C and D). In both normal and serum starved conditions, individual clones differed in their migratory capacities.

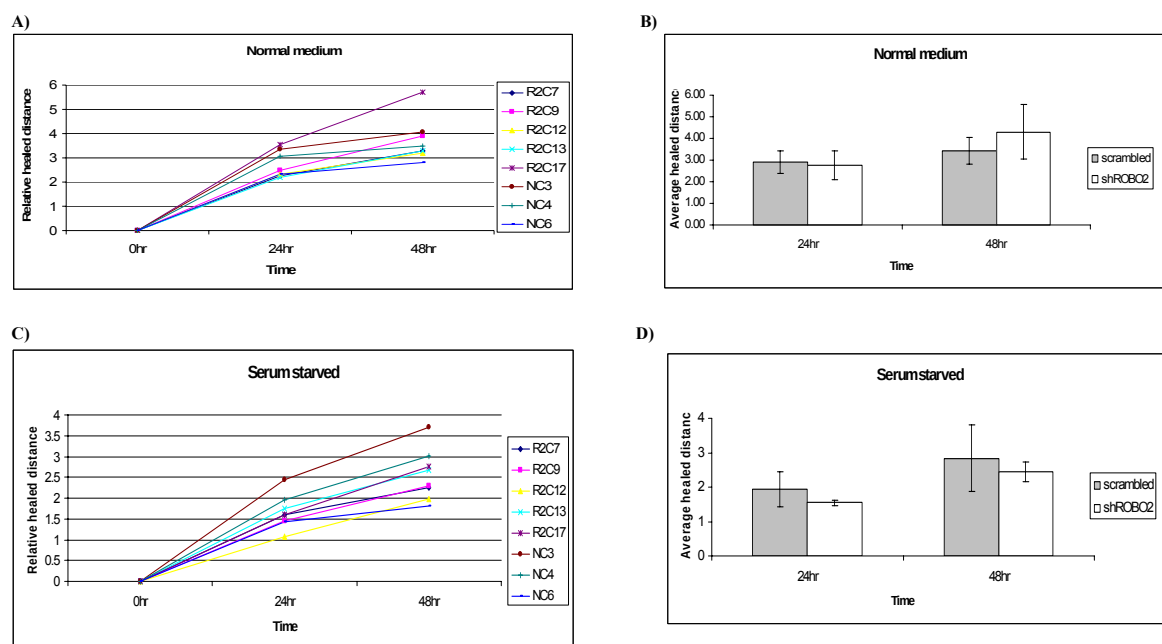


Figure 4.15: Wound-healing assay results for stable clones in normal and serum starved medium. Wounds were made at time 0 and photos were taken. Subsequently, at 24th and 48th hours photographs were taken and the relative healed distances were calculated by dividing the total healed wound distance (difference between initial and final wound sizes) by 2, for 10 different lines connecting horizontally the two sides of the wound. The average of these 10 calculations were used to construct the graphs and calculate the T-tests. In A), cells were incubated in normal medium, and in B) cells were serum starved. T-test was applied for deducing the significance of the migratory differences between *ROBO2* knockdown (R2C7, R2C9, R2C17 average values) and negative clones (NC3, NC4, NC6 average values) (C and D).

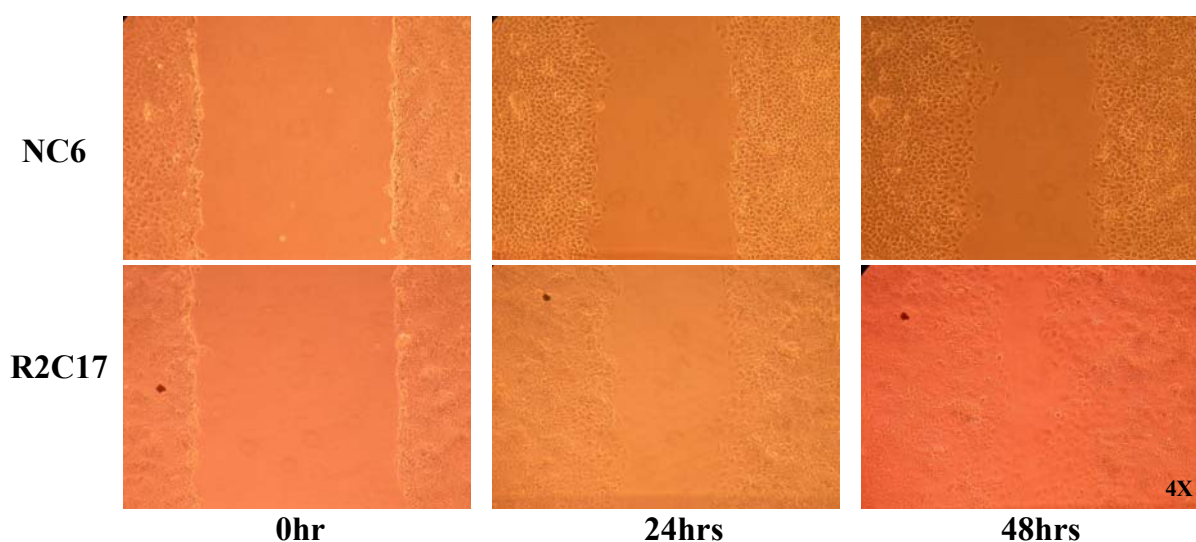


Figure 4.16: Representative wound healing assay photographs. In normal serum present conditions the most rapid and slow migrating clones were R2C17 and NC6, respectively. The wound-healing pattern of these clones are photographed.

4.2.4 Microarray analysis of the differentially expressed genes between stable *ROBO2* knockdown clones and negative clones

In order to observe the general picture related to *ROBO2* knockdown in stable clones, a microarray experiment was designed. Pooled RNAs from eight stable *ROBO2* knockdown clones (R2C3, R2C7, R2C8, R2C9, R2C10, R2C13, R2C14, and R2C17) were hybridized to one Affymetrix HG-U133plus2.0 chip and pooled RNA from four negative clones (NC4, NC5, NC6, and NC7) were hybridized to another Affymetrix HG-U133plus2.0 chip. Quality of the RNAs was checked using Nanodrop ND-1000 Full-spectrum UV/Vis Spectrophotometer and by running in formaldehyde gel and visual inspection of 28S and 18S rRNAs under UV (Figure 4.17). Microarray experiment was performed by Hilal Özdağ from Ankara University Biotechnology Institute. After the experiment, raw .cell files of the two chips were obtained; then quality controlled and RMA normalized in R environment (Figure 4.18).

In MS Excel, using built-in functions we obtained a fold change list and a differentially expressed gene list. To filter out non-relevant, noise-related genes we determined a cut-off of ± 0.8 fold; the probe sets having fold change values between +0.8 and -0.8 were eliminated. We have ended up with 668 upregulated and 744 downregulated probe sets.

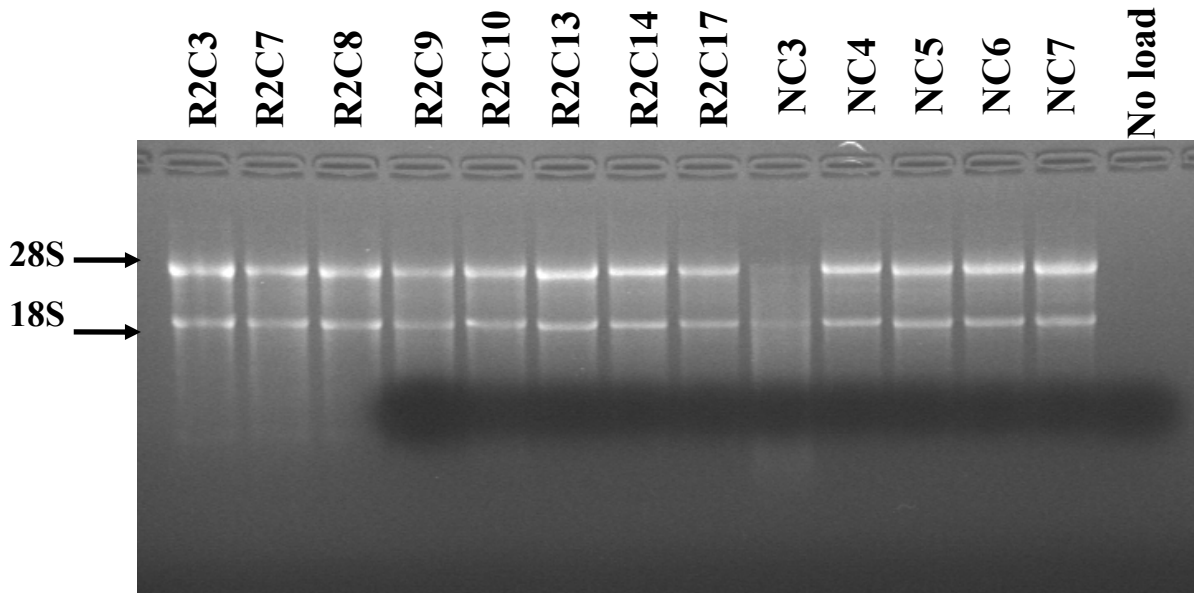


Figure 4.17: Visualization of 18S and 28S rRNAs in total RNAs isolated from stable clones in formaldehyde agarose gel. Before sending for microarray experiment total RNAs from stable clones were quality controlled in formaldehyde agarose gel. 18S and 28S rRNA bands were seen and no smear was observed, except in NC3, indicating the intactness of the RNA samples. Because of the observed smear pattern in NC3 total RNA sample, it was excluded from microarray experiment.

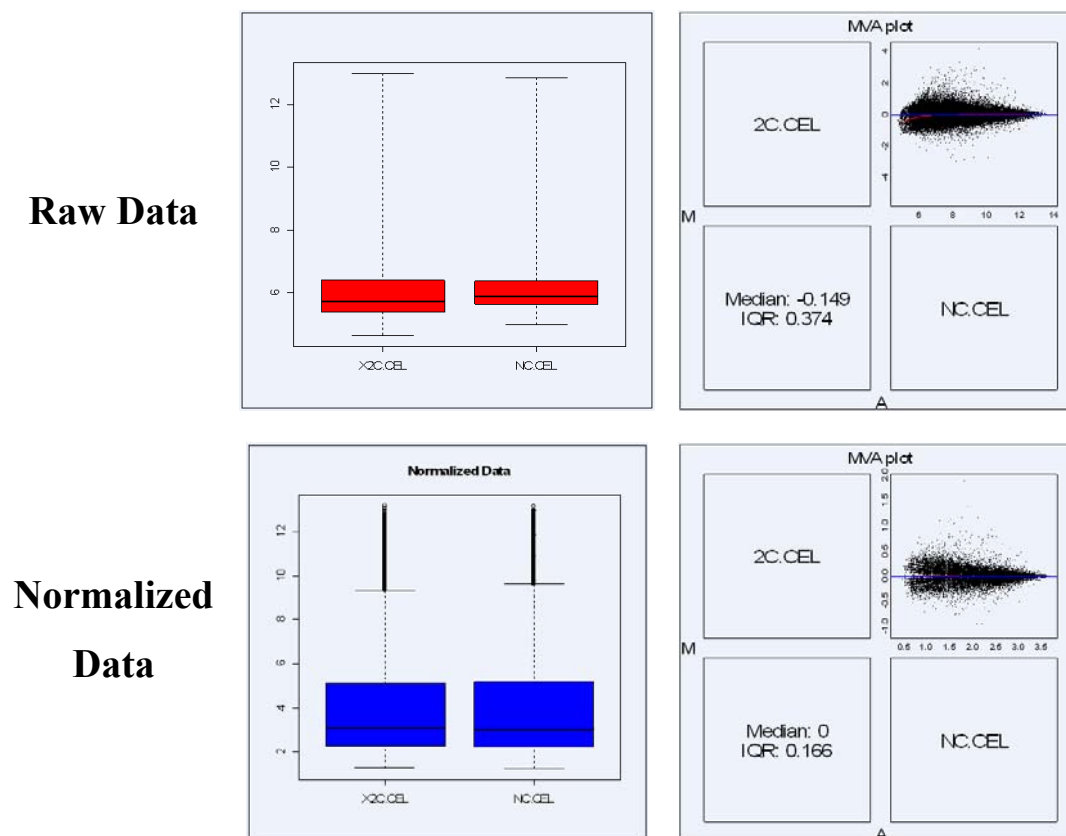


Figure 4.18: Box- and MA-plots of shROBO2 RNA (2C) and negative RNA (NC) probed chips before and after RMA normalization.

4.2.4.1 Pathway analysis of microarray results using Pathway-Express tool

Pathway-Express is a tool in the Onto-Tools ensemble that was designed to help in pathway analysis of microarray data. The goal of the Pathway-Express system is to automatically find interesting pathways. When the user submits a list of genes, the system performs a search and builds a list of all associated pathways. In principle, system weights a pathway associated with the gene list and gives an impact factor to the entire pathway. The impact factors of all pathways are used to rank the pathways before presenting them to the user. All pathways affected are presented regardless of their impact factors (Khatri, Bhavsar et al. 2004; Khatri, Sellamuthu et al. 2005). In addition, it also locates the differentially expressed genes into KEGG pathway charts and highlights down- and up-regulated genes with specific colors in order to ease in visualization.

In Pathway-Express, querying our differentially expressed gene list resulted in a significance associated pathway list (Figure 4.19). The most prominently disturbed pathway was cell adhesion molecules (CAMs) pathway including 15 differentially expressed genes from the input list. Then come “phosphatidylinositol signaling system” and “complement and coagulation cascades” that were not previously associated with any of the *SLIT-ROBO* genes. Following in the list were “axon guidance”, “ECM-receptor interaction”, “focal adhesion” and “regulation of actin cytoskeleton” in which *SLIT-ROBO* genes are directly or indirectly functional. As a representative Pathway-Express result, axon guidance pathway and the differentially expressed genes in the pathway is presented in Figure 4.20. The perturbation in *ROBO2* gene can be easily recognized with its blue color indicating downregulation. Interestingly; downstream to *ROBO2*, *srGAP1* gene was upregulated suggesting the compensatory activity of other possible redundant mechanisms.

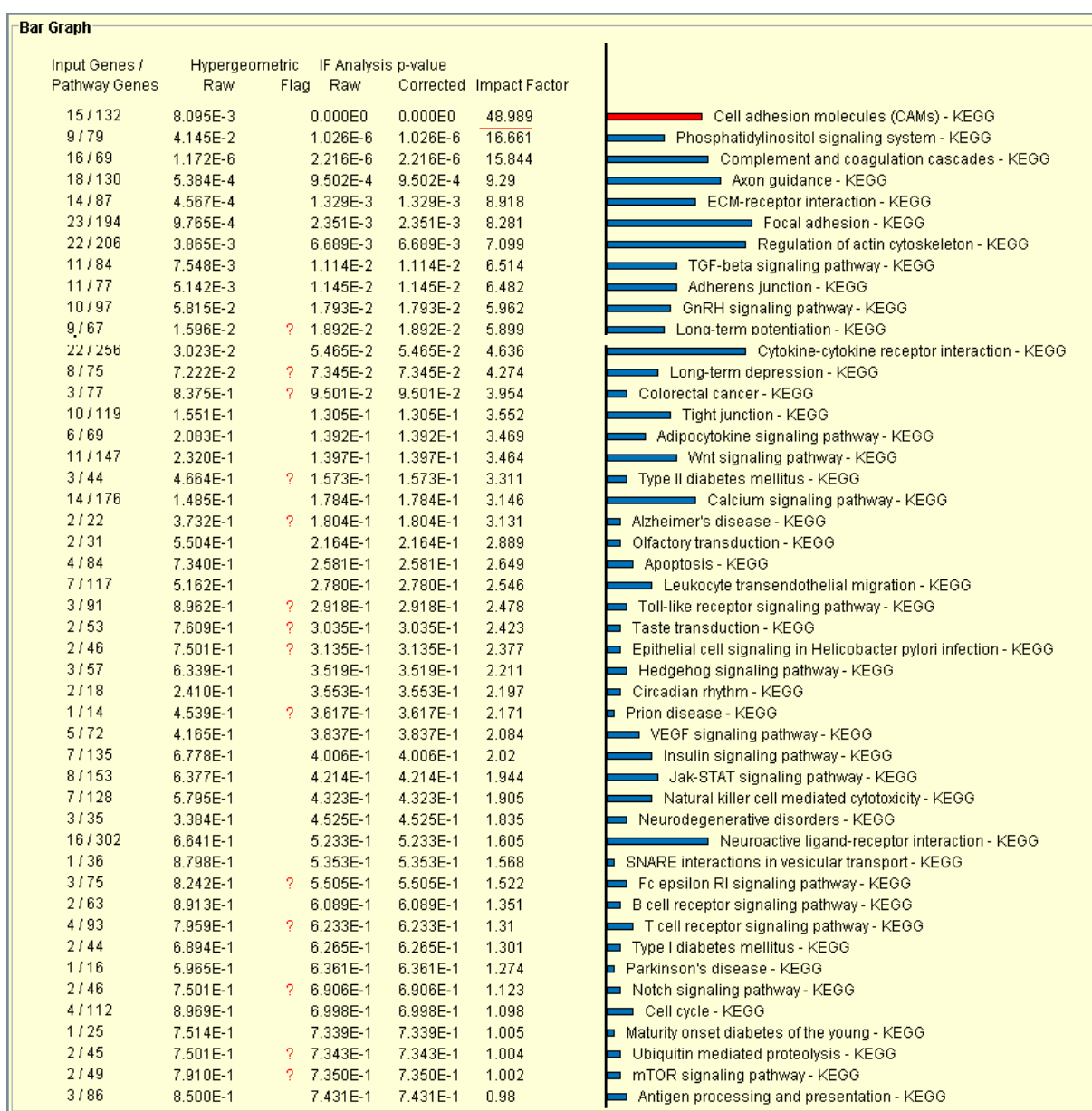


Figure 4.19: Significantly affected pathways identified in Pathway-Express.

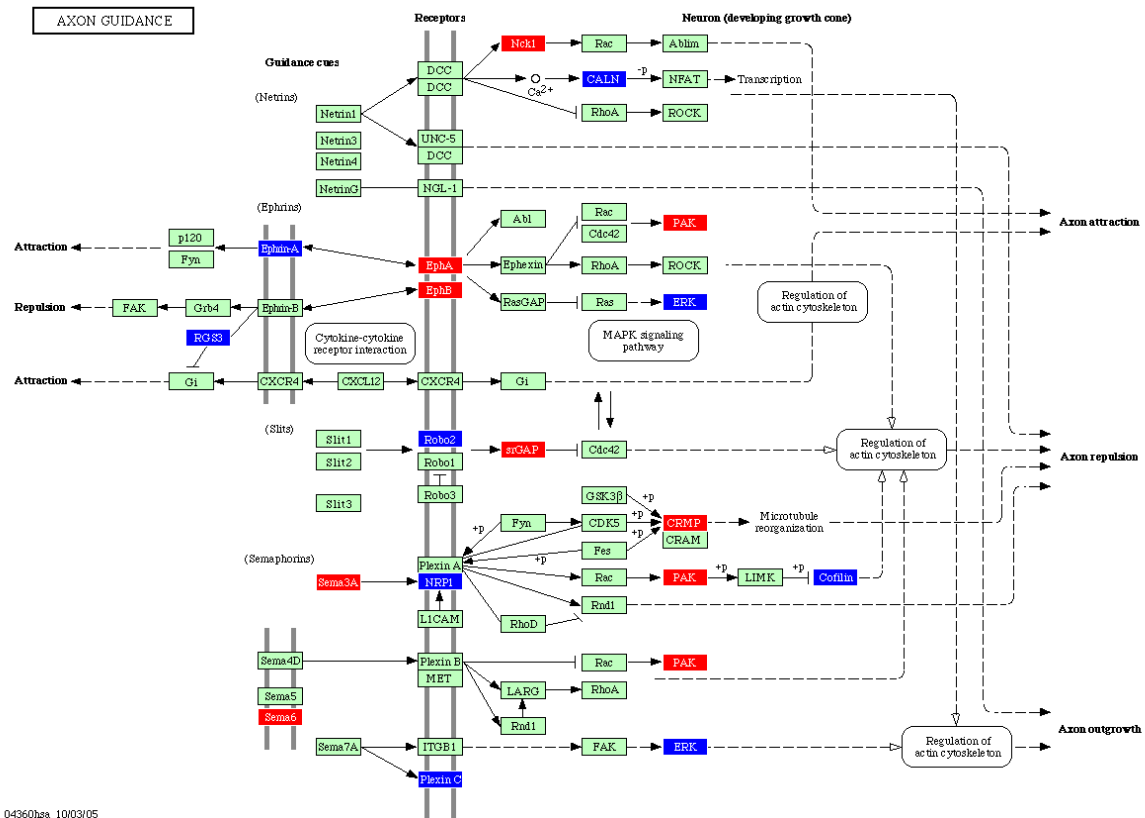


Figure 4.20: Pathway-Express output of axon guidance pathway and the differentially expressed genes in the pathway. A total of 18 genes were found to be differentially expressed. Upregulated genes were shown in red, and the downregulated ones were in blue.

4.2.4.2 Microarray verification of selected differentially expressed genes by semi-quantitative RT-PCR

With *CD133*, *CK19* and five other axon guidance gene primers microarray verification was performed in cDNAs of the stable clones by semi-quantitative RT-PCR (Figure 4.21). The fold change values associated with the genes were presented in the column at the right side of the figure. The observed expression pattern in semi-quantitative RT-PCR analyses was parallel to the fold change values obtained from microarray analysis.

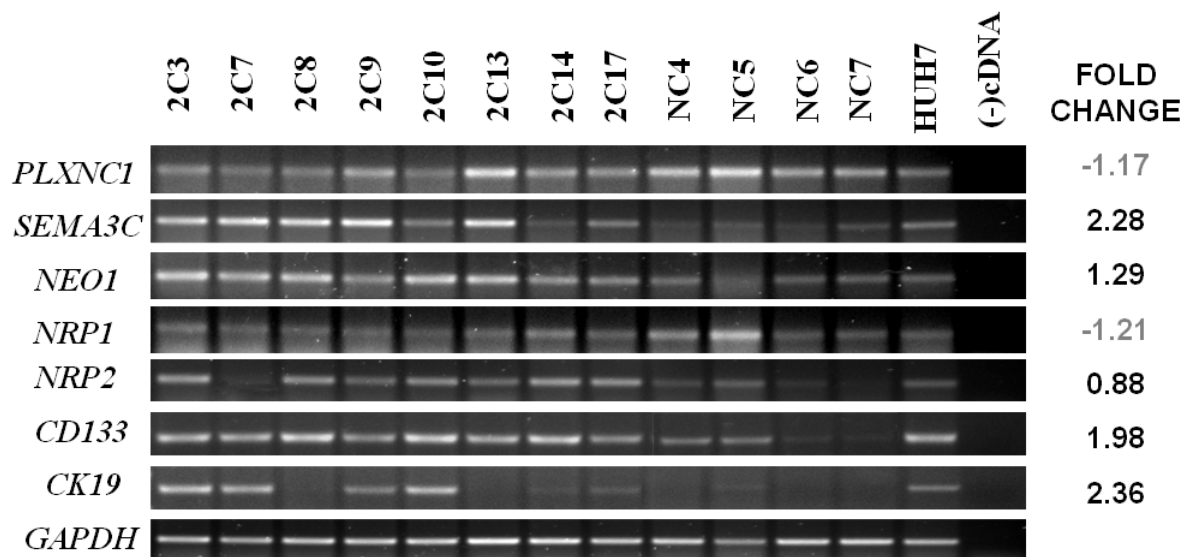


Figure 4.21: Microarray verification with selected genes using semi-quantitative RT-PCR. Selected genes were amplified at semi-quantitative cycles to observe the microarray predicted differential expression. Gene associated fold change values obtained in microarray analysis were given in the column, located at the right side of the figure.

4.2.5 Expression of the markers associated with side population, cancer stem cell and pluripotency phenotype was enriched in the stable *ROBO2* knockdown clones

Recently, side-population (SP) cells were described in 0.25-1% of Huh7 cell line and xenograft tumors of SP cells showed increased size and total number in SCID mouse. SP cells overexpress *BCRP1* (*ABCG2*), *MDR1*, *CK19* (*KRT19*) and *CD133*, and show increased invasion and chemoresistance capabilities (Chiba, Kita et al. 2006; Haraguchi, Utsunomiya et al. 2006). In another study, about 50% of Huh7 cells were found to be CD133+ and these cells formed tumor when xenografted to NOD/SCID mouse (Suetsugu, Nagaki et al. 2006). CD133+ Huh7 cells showed increased proliferation and clonogenic capacity. Even, 1×10^3 or 5×10^4 cells were sufficient for tumor development in SCID or nude mouse, respectively. β -catenin, Notch, SMO, Bmi and Oct3/4 genes are overexpressed in CD133+ subpopulation (Ma, Chan et al. 2007). *KLF4*, *SOX2*, *C-MYC*, and *OCT3/4* transduction in somatic cells have been shown to induce pluripotency (Takahashi, Tanabe et al. 2007). Hepatic progenitor cells or oval cells are liver stem cells implicated in origination of HCC, and BCRP1 (*ABCG2*), KRT19 (*CK19*), AFP and CD44 are among the markers used in identification of these cells in damaged liver (Alison, Islam et al. 2009). We observed 1.6 fold *BCRP1* (*ABCG2*), 2.4 fold *KRT19* (*CK19*), 2.6 fold *KLF4* and 2.0 fold *CD133* (*PROM1*) overexpression in our microarray analysis. With this lead, we checked the expression of *CD133*, *CK19*, *KLF4*,

MYC, *CD44*, *AFP*, *BCRP1*, *MDR1* and *SOX2* in the stable clones with semi-quantitative RT-PCR. We found that *CD133*, *CK19*, *KLF4*, *CD44*, *BCRP1*, *SOX2* and to some extent *MDR1* expression was enriched in *ROBO2* knockdown clones (Figure 4.22).

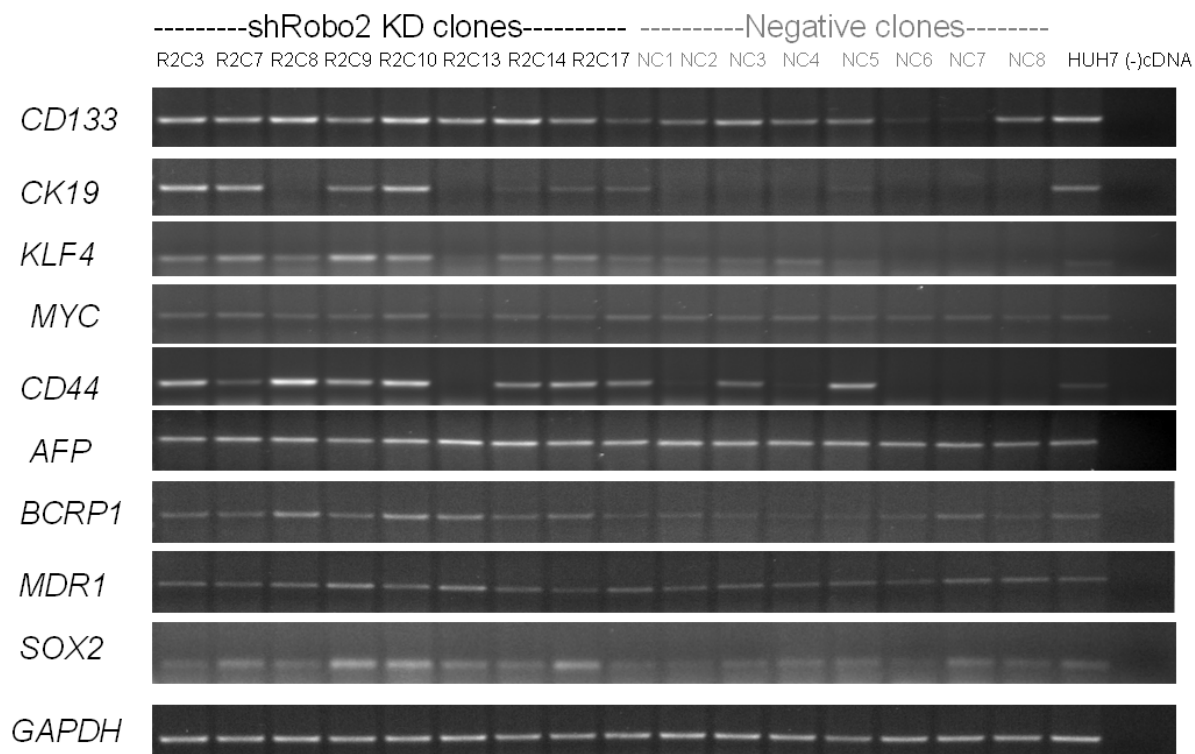


Figure 4.22: Semi-quantitative expression analysis of side population, cancer stem cell and pluripotency associated genes in stable clones. At semi-quantitative cycles, RT-PCR expressions of *CD133*, *CK19*, *KLF4*, *MYC*, *CD44*, *AFP*, *BCRP1*, *MDR1*, and *SOX2* genes were analyzed.

4.2.6 Transient knockdown of *ROBO2* did not change the expression of side population, cancer stem cell and pluripotency markers

Huh7 is a heterogeneous cell line meaning that individual single cell clones isolated from Huh7 may not always have the same genetic background. To investigate whether the observed enrichment of *CD133*, *CK19*, *KLF4*, *CD44*, *BCRP1*, *SOX2* and *MDR1* expression in *ROBO2* knockdown clones was dependent on *ROBO2* or was a by-product of this heterogeneous character of Huh7 clones, we transiently silenced the expression of *ROBO2* in parental Huh7. Two different *ROBO2* targeting shRNAmir vectors (R2-1 and R2-2, in pGIPZ backbone) were transfected either singly or together (Figure 4.23). pGIPZ vector with a scrambled-nontargeting shRNAmir construct was used as control. No change was observed in the expression of above listed genes in these transiently transfected Huh7 cells.

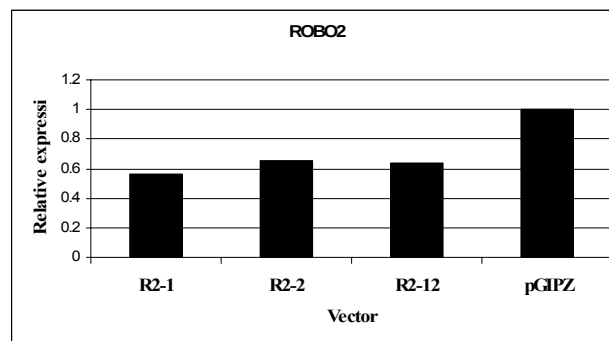
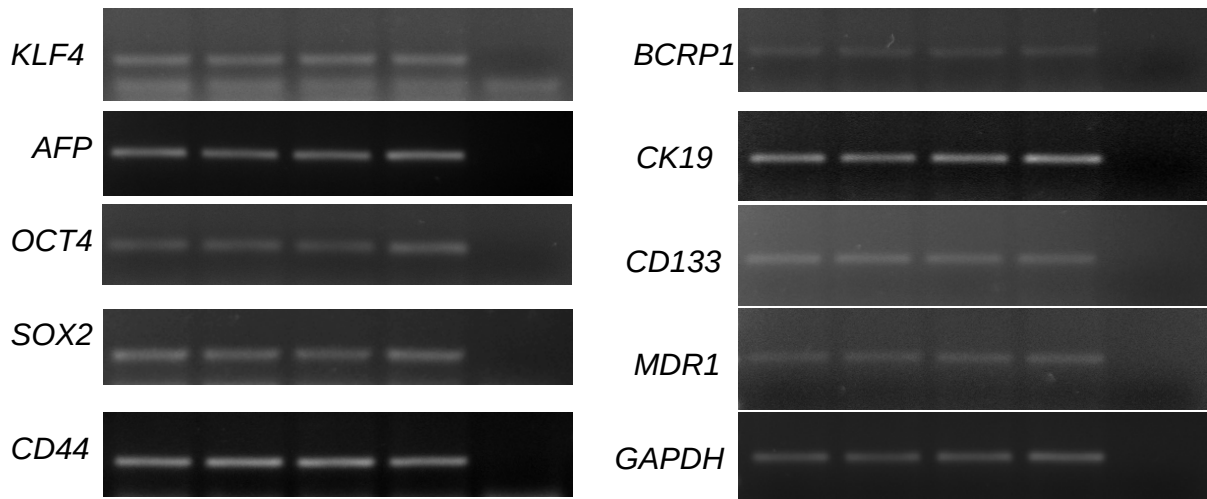


Figure 4.23 Side population, cancer stem cell and pluripotency associated genes in shROBO2 transiently transfected cells. *CD133*, *CK19*, *KLF4*, *CD44*, *BCRP1*, *SOX2* and *MDR1* expression was semi-quantitatively checked in shROBO transient transfected Huh7 cells. ROBO2 expression after transfections was determined by real-time quantitative PCR (down left corner).

In addition with immunoflourescence experiments, we checked the expression of ROBO2, CD133 and β -catenin (with 9E10 monoclonal antibody) in these transiently transfected Huh7 cells. The transfected shRNAmir constructs have internal GFP expression in front of the shRNAmir construct linked with and internal ribosome entry sequence (IRES), so the individual shRNAmir expressing cells were seen with green flourescence. ROBO2, CD133 and β -catenin stainings were performed with Alexa 588 secondary antibody so the signal given by them was red. DAPI was used for counterstaining the nucleus (Figure 4.24).

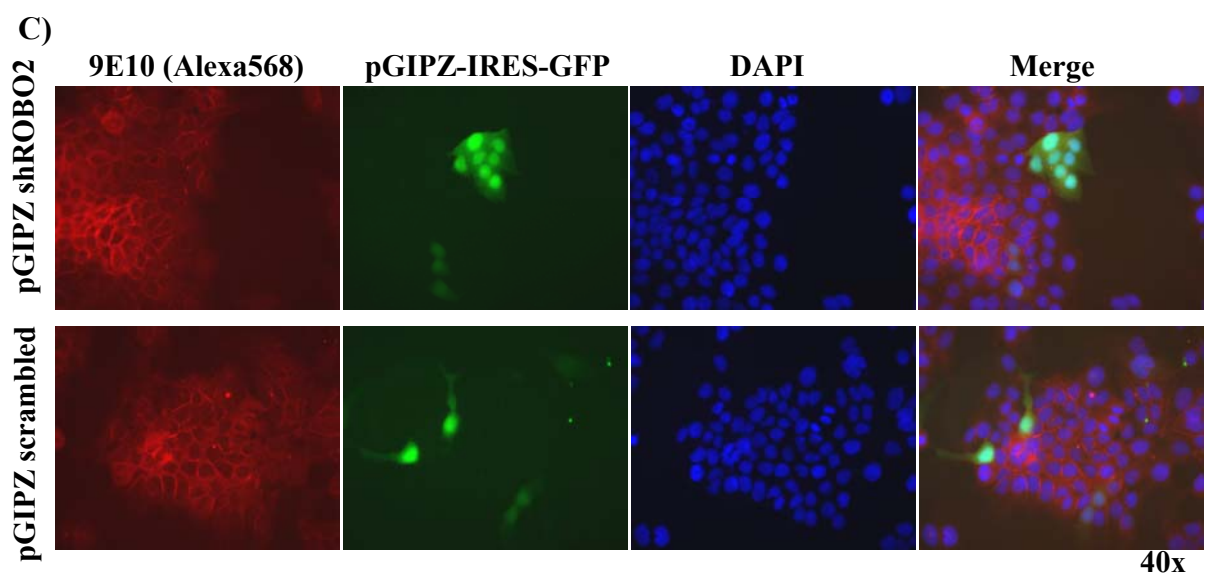
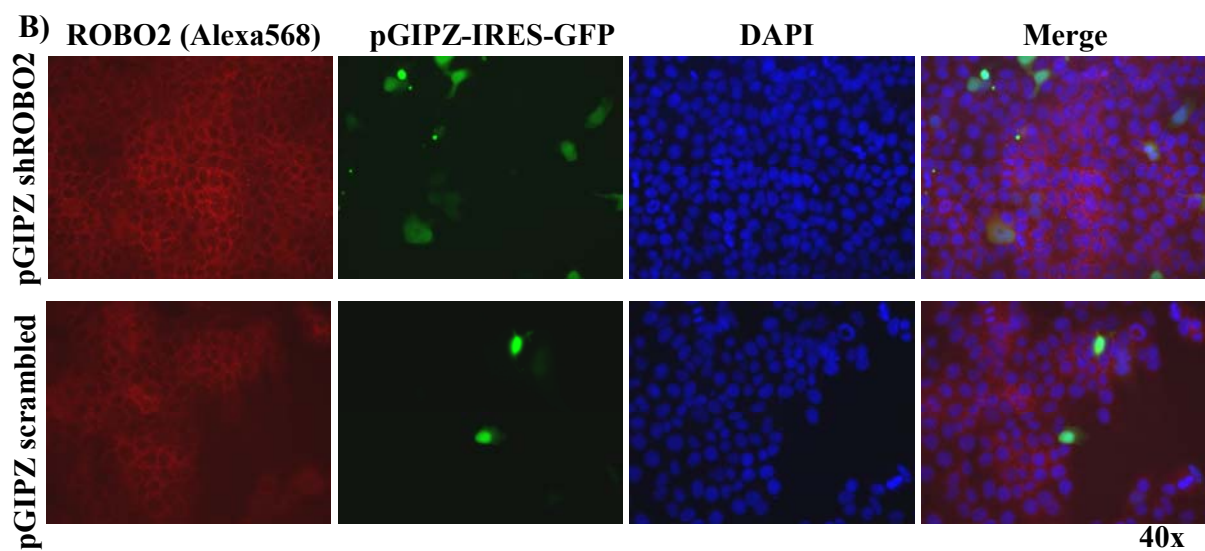
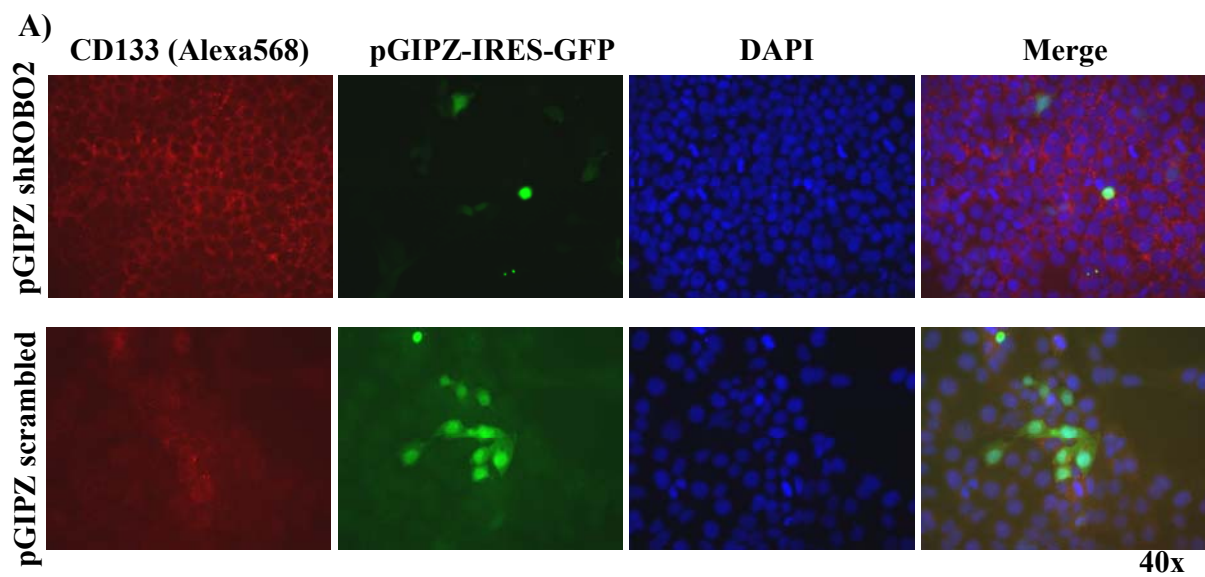


Figure 4.24: CD133, ROBO2 and β -catenin staining in transient shROBO2 transfected Huh7 cells. Transient shROBO2 transfected Huh7 cells were stained with either CD133 (A), ROBO2 (B), or β -catenin (C) specific antibodies and labelled with a red-flourescence giving Alexa568 secondary antibody. The shRNAmir expressing cells were producing green flourescence, expressed from pGIPZ vector's IRES-GFP sequence that is located 3' to shRNAmir. Nucleus was stained with DAPI.

4.2.7 CD133 expression in HCC cell lines

CD133 was proposed to be a HCC cancer stem cell marker and CD133+ cells tumor cells showed increased xenograft tumor size and number (Ma, Lee et al. 2008). We checked the expression of CD133 mRNA and protein in selected HCC cell lines representing well- and poor-differentiated cells. In addition, we used two other cell lines: HEK293 and a known CD133+ cell line NTERA-2 cl.D1. CD133 mRNA and protein was present in Hep3B, PLC and Huh7 (Figure 4.25). In QRT-PCR experiment NT2 was taken as the reference.

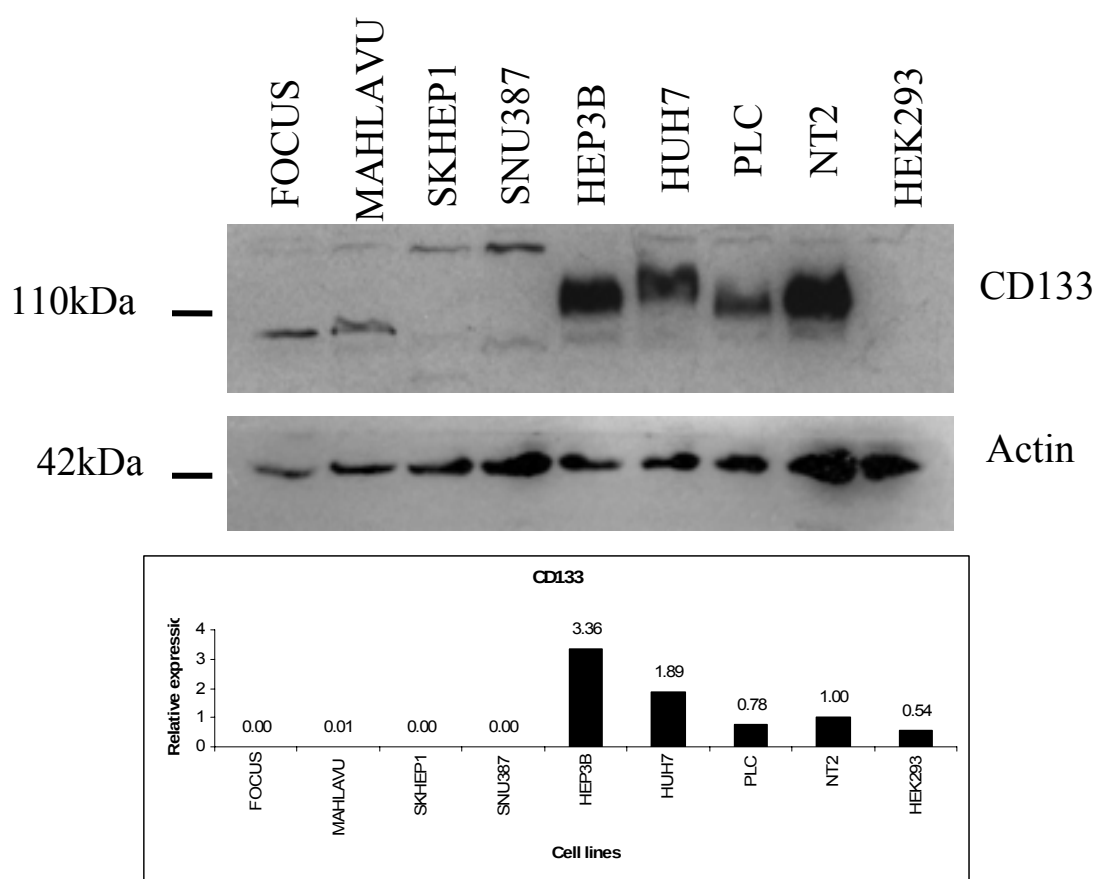


Figure 4.25: CD133 expression in HCC cell lines. The expression of CD133 was confirmed by both QRT-PCR and western blot. The results were parallel and of the analyzed HCC cell lines; Hep3B, Huh7 and PLC cell lines were positive for CD133 expression. In QRT-PCR experiment NT2 was taken as the reference.

4.2.8 Effect of *ROBO2* expression on CD133

In microarray analysis and semi-quantitative RT-PCR experiments, we observed a *CD133* upregulation in stable *ROBO2* knockdown clones. We decided to further characterize the effect of *ROBO2* on *CD133* expression. The expression of *CD133* was confirmed in stable clones by QRT-PCR and western blot experiments. Parallel to the results obtained in both microarray analysis and semi-quantitative RT-PCR experiments, *CD133* expression was found to be upregulated in stable *ROBO2* knockdown clones (Figure 4.26). In addition we performed immunofluorescence experiments in R2C10 and NC4 clones (Figure 4.27). *CD133* is known to be a membrane protein. Interestingly, in immunofluorescence experiments nucleus located multiple spots were observed in both NC4 and Huh7. These spots were also present in R2C10 but there was also membrane staining.

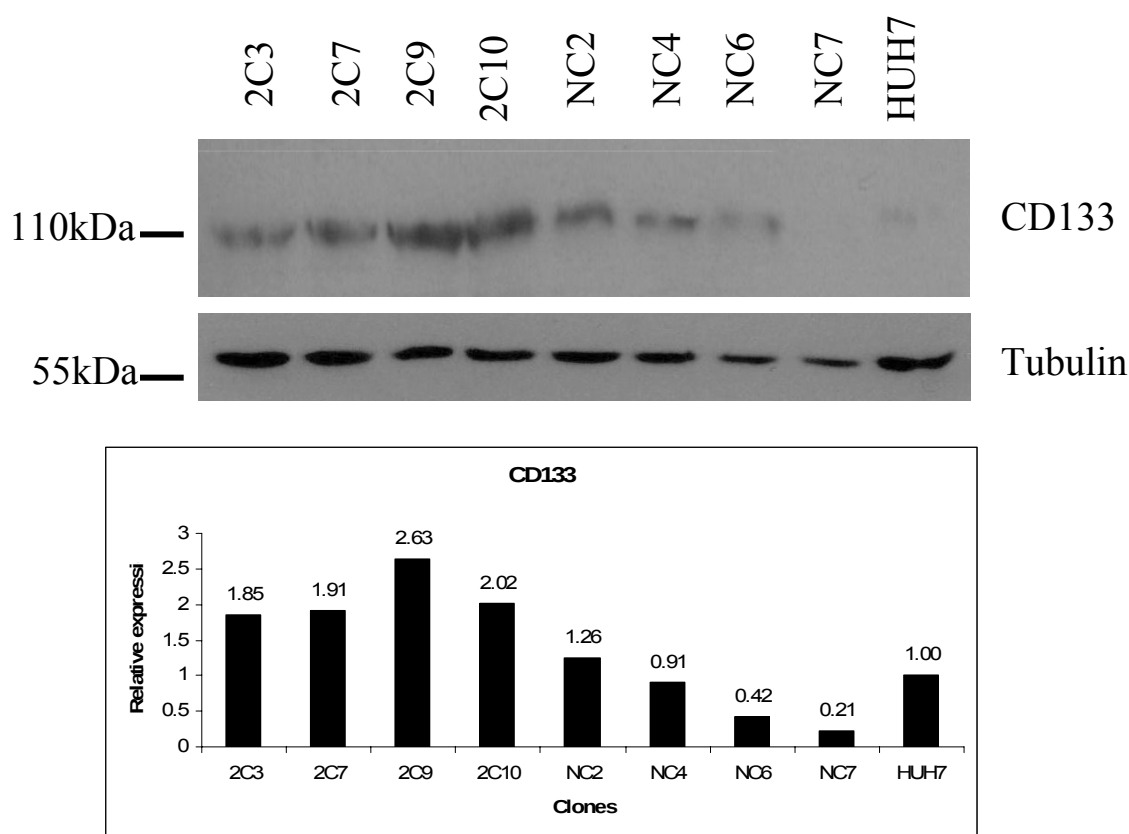


Figure 4.26: CD133 expression in selected stable clones. The expression of *CD133* was confirmed by both QRT-PCR and western blot. The results were parallel with the previous microarray and semi-quantitative PCR experiments. In QRT-PCR experiment parental Huh7 was taken as the reference.

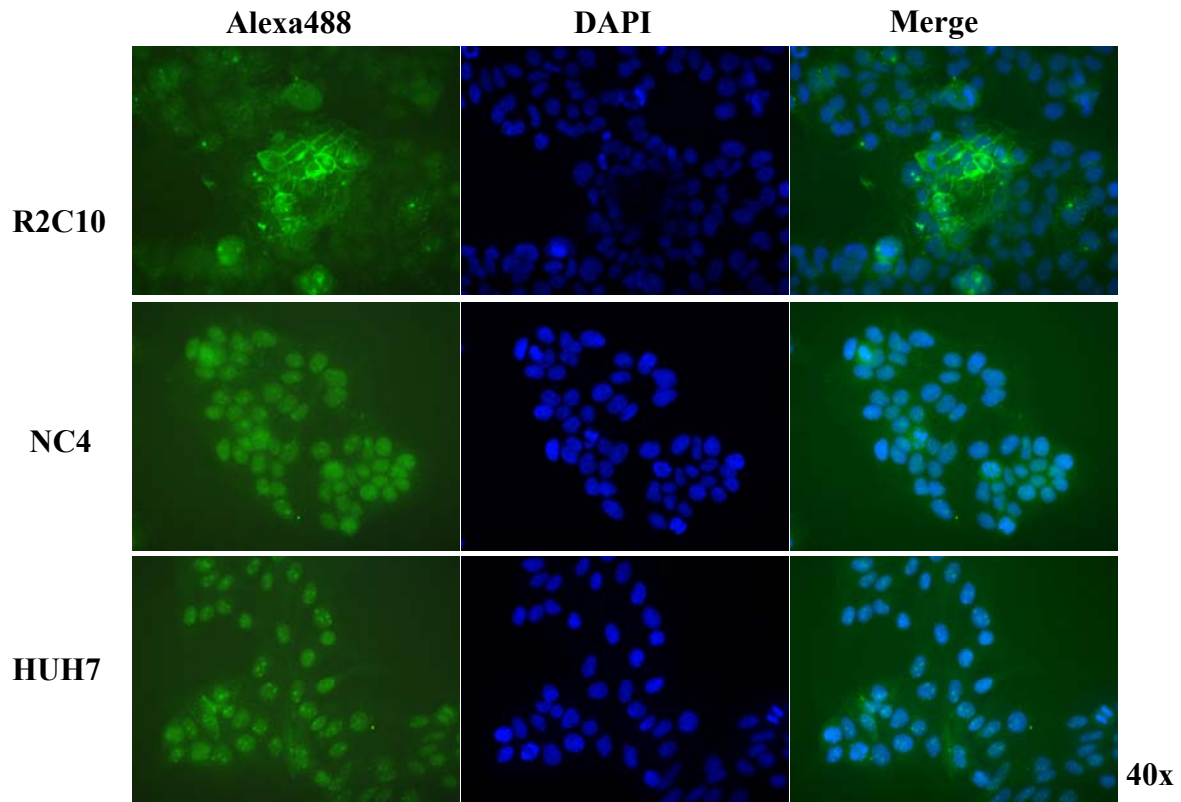


Figure 4.27: CD133 immunofluorescence staining in R2C10, NC4 clones and parental Huh7 cells.

In order to investigate the possible effect of *ROBO2* overexpression to CD133 expression in stable *ROBO2* knockdown clones, we re-introduced *ROBO2* into stable *ROBO2* knockdown clones with pDEST26-ROBO2 overexpression vector. pDEST26-ROBO2 plasmid was transfected into R2C7, R2C9 and R2C10 clones and SK Hep1, Snu449 (which were originally negative for ROBO2 expression) and parental Huh7 HCC cell lines. Re-introduction of ROBO2 into R2C7, RC9 and R2C10 stable clones had no apparent effect on CD133 expression (Figure 4.28). However in parental Huh7 cells ROBO2 overexpression decreased CD133 expression to some extent. The ROBO2 and CD133 non-expressing SK Hep1 and Snu449 cells were used as positive controls for ROBO2 overexpression.

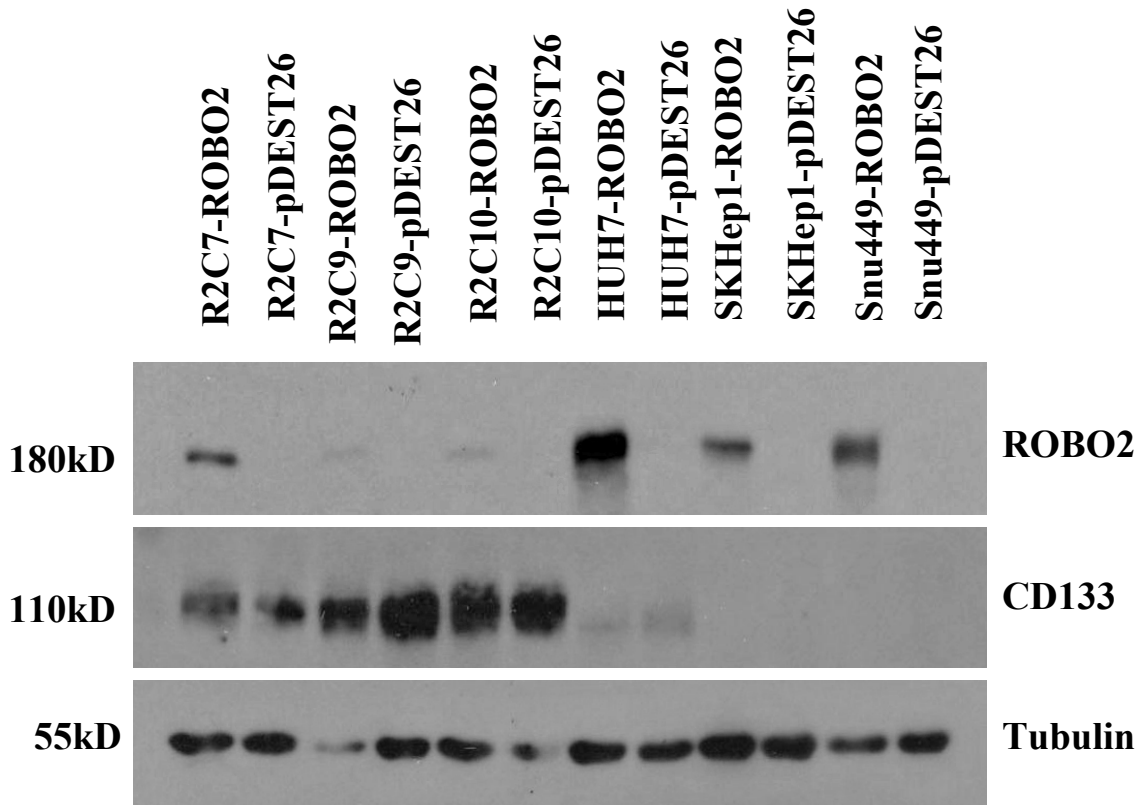


Figure 4.28: ROBO2 re-introduction into selected stable *ROBO2* knockdown clones had no apparent effect on CD133 expression. pDEST26-ROBO2 overexpression vector was transfected into R2C7, R2C9 and R2C10 clones and SK Hep1, Snu449 and parental Huh7 cell lines. Western blot experiments were performed using ROBO2 and CD133 antibodies. Tubulin was used for equal-loading.

4.2.9 Proliferation rate of stable *ROBO2* knockdown clones were diminished

It was taking longer for *ROBO2* knockdown clones to reach confluency than negative clones. This led us to test the proliferation rates of stable clones. We compared the proliferation rates of the stable clones by BrdU assay. We incubated our clones with BrdU for 24 hours and performed immunofluorescence staining using BrdU antibody. Cells that were positive as well as those that were negative for BrdU incorporation could clearly be distinguished under fluorescence microscope. Percent BrdU positivity was calculated and plotted for each stable clone (Figure 4.29 A). Average percent BrdU positivity of shROBO2 clones and shNon-targeting clones were calculated and T-test was applied (Figure 4.29 B). Proliferation rate of *ROBO2* knockdown clones was significantly lower than that of negative clones.

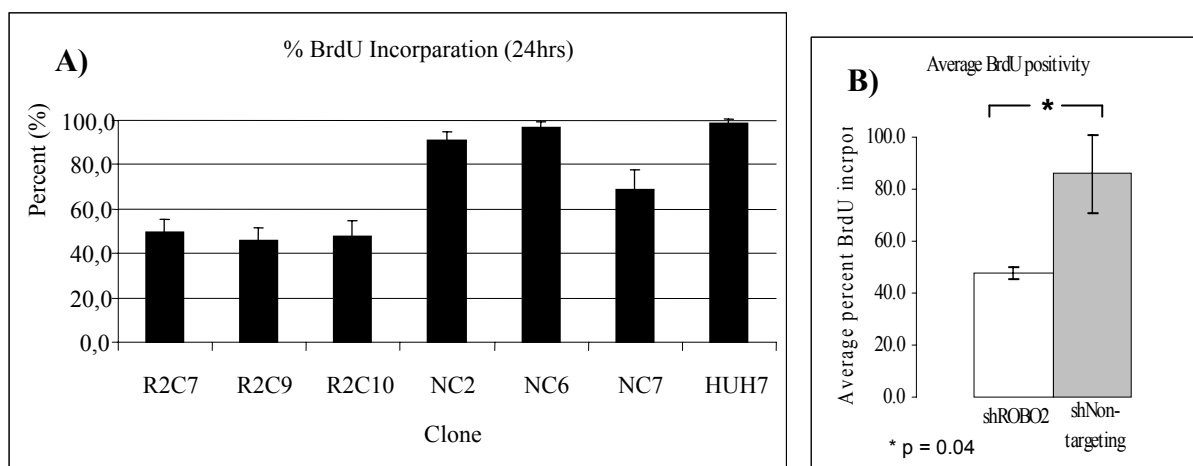


Figure 4.29: Percent BrdU incorporation in stable clones after 24 hours incubation in the presence of BrdU. Cells were incubated with BrdU for 24 hours and then stained with anti-BrdU antibody. A) Stained cells were counted and plotted against DAPI stained total cell number and given as percent BrdU positive cells. B) Average of three shROBO2 and shNon-targeting clones were plotted and T-test was applied to find significance.

In our microarray analysis *p15* (*CDKN2B*), which is a cell cycle inhibitor, was found to be upregulated in stable *ROBO2* knockdown clones by 1.3 fold. Thus, we suspected upregulation of *p15* as the cause of the observed decrease in proliferation rate of *ROBO2* knockdown clones. We checked *p15* expression in selected stable clones by western blot and QRT-PCR. There was not an apparent difference in *p15* protein levels between *ROBO2* knockdown and negative clones (Figure 4.30 A). Although clones expressed *p15* isoforms differentially, there was not any apparent trend associated with *ROBO2* knockdown (Figure 4.30 B).

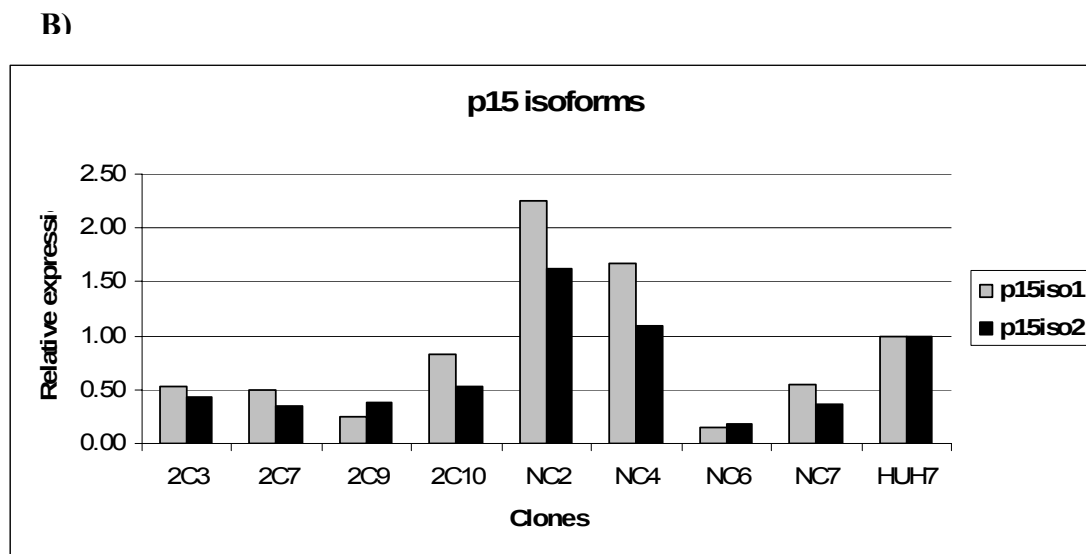
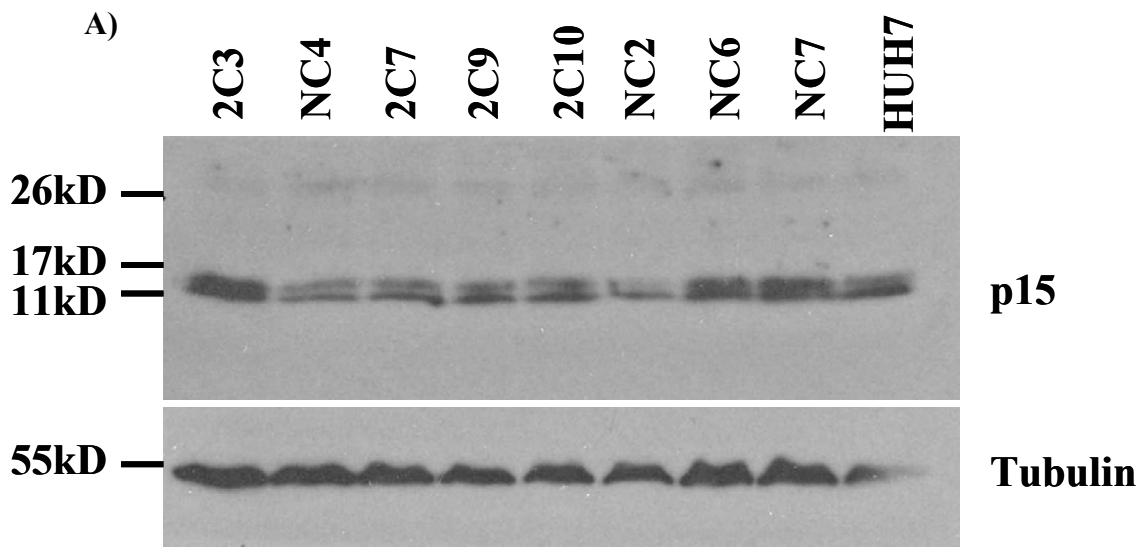


Figure 4.30: p15 expression in selected stable clones. The expression of p15 in selected stable clones and parental Huh7 was checked by western blot (A). Tubulin was used as equal loading control. In QRT-PCR experiments, expressions of both p15 isoforms were relatively quantified taking Huh7 as the reference sample (B).

5 DISCUSSION AND CONCLUSION

5.1 Expression signature of *SLIT-ROBO* genes in HCC

A collective analysis of expression of *SLIT-ROBO* family genes has not been assessed yet in liver tumor. In this study, we showed the co-regulation of *SLIT-ROBO* genes in HCC. In the HCC cell lines and liver tissues, *ROBO1*, *ROBO2*, *SLIT1*, and *ROBO4*, *SLIT2*, *SLIT3* showed coordinate expression as two distinct modules, yet displayed high variability at gene level within each module. Additionally, *SLIT-ROBO* expression was able to predict AFP status of HCC cell lines, and thereby establishing two groups with low- and high-AFP expressions. All the analyzed genes were expressed at different levels, except *ROBO3*. A preferential up- and down-regulation of *SLIT-ROBO* genes occurred depending on the *AFP* expression status of HCC cell lines. *ROBO1*, *ROBO2* and to a lesser extent *SLIT1* were overexpressed, whereas *SLIT3* was underexpressed in high-AFP group. *ROBO4* also tended to be downregulated in this group. However, *SLIT2* was expressed in most of the cell lines, regardless of the *AFP* expression status.

AFP is the main component of mammalian fetal serum and is synthesized by visceral endoderm of the yolk sac and by fetal liver. The expression of *AFP* is reactivated in liver tumors and germ cell tumors such as yolk sac tumor and teratocarcinomas. About 50% to 70% of HCC patients produce very high levels of AFP, whereas most hepatoblastoma patients produce AFP. Because *AFP* expression in germ cell tumors and hepatoblastoma is related to their cellular origin in yolk sac and fetal liver cells, it has been proposed that *AFP* expression in HCC could be related to its possible origin from progenitor liver cells, oval cells, or their close descendants (Sell 1993; Abelev and Eraisier 1999). Previously, Lee and Thorgeirsson described two molecular subgroups of HCC cell lines that are characterized by their *AFP* expression. They used 19 cell lines including the 13 cell lines (except Mahlavu) used in this study. In their microarray study, cell lines with low *AFP* profile (including Focus, SK Hep1, Snu387, Snu423, Snu449 and Snu475) showed upregulation of genes related to metastasis and cell motility whereas cell lines with high *AFP* profile (HepG2, Hep3B, Hep3B-TR, Hep40, HUH7 and PLC/PRF/5) did not. The latter group was clustered together with a hepatoblastoma cell line (HepG2) indicating their origin from progenitor cells, or dedifferentiation during tumorigenesis (Lee and Thorgeirsson 2002). In our clustering

analysis, HCC cell lines was subgrouped with respect to their *SLIT-ROBO* expression pattern and this clustering predicted the *AFP* positivity of the cell lines. HepG2, Snu398, PLC/PRF/5, HUH7, Hep3B-TR, Hep3B and Hep40 formed the *AFP*-positive group, namely Group2. These cell lines were mainly well-differentiated and possess the markers of their tissue of origin. Interestingly, Snu398 which was described as a poorly differentiated *AFP*-negative cell line clustered also in Group2. In this respect, Snu398 was a marginal cell line showing a quite unexpected *SLIT-ROBO* expression pattern. HepG2, another member of Group2, is a hepatoblastoma derived cell line. Co-clustering of other Group2 members with HepG2 indicates that they show a progenitor cell phenotype. Indeed, recent findings propose CD133 as a cancer stem marker and Hep3B, HUH7, PLC/PRF/5 to express this marker with differing levels. Our CD133 protein and mRNA expression analysis was in parallel to these findings and confirms the progenitor cell character of these cell lines in Group2. Hep3B-TR is a TGF- β resistant clone derived from Hep3B, so their close neighboring was expected in cluster analysis.

We also quantified *SLIT-ROBO* expression in 8 tumor adjacent normal liver tissues and 35 HCC tumors. We found that genewise clustering observed in HCC cell lines were conserved in tissues: *ROBO1*, *ROBO2*, *SLIT1* and *ROBO4*, *SLIT2*, *SLIT3* were coordinately expressed, respectively. In cluster analysis, two main subgroups in tissue samples were observed but this was not dependent on *AFP* expression, converse to the case in HCC cell lines. *AFP* and *ROBO1* expression was significantly correlated in both HCC cell lines and tissues. The observed difference between cell line and tissue data regarding AFP might be partly due to the heterogeneity of tissues. HCC cell lines were more homogenous when compared to tissue samples, which may contain stromal cells, endothelial cells, immune cells or any other tumor infiltrating cells. Moreover, our normal liver samples were tumor-adjacent tissues, which may harbor genetic changes of tumor microenvironment, and therefore may not reveal the actual molecular characteristics of a tumor free normal liver.

In the entire HCC cell lines studied, *ROBO1* transcript was present and it was significantly upregulated in the analyzed HCC tissues. In these tissues, *ROBO1* was overexpressed in later stages and as tumors progress to a less differentiated state. During the course of this study, a research article was published suggesting *ROBO1* as an HCC antigen and proposing it as both a diagnostic marker and therapeutic target for HCC. This result was in agreement with our findings in Oncomine database search and expression analyses. In most of the tissues and in

all of the HCC cell lines (although variable) *SLIT2* was expressed. Such a variance in HCC cell lines might explain the clustering of *SLIT2* in a different group than *ROBO1* and *ROBO2*, yet it is likely to be the main ligand for ROBO receptors. This does not exclude interactions between other SLIT and ROBO members. In addition, possible ligand independent activities of ROBO receptors still remain in HCC. *SLIT2* and *ROBO1* were both upregulated in HCCs with advanced stages and poor differentiation status (Figure 4.8 and Table 4.7). These findings also are in agreement with the expression of SLITs specifically in poorly differentiated HCCs (Ito, Funahashi et al. 2006). In a tumor xenograft model, *SLIT2*-*ROBO1* signaling was shown to have a role in angiogenesis, which supports tumor growth and metastasis (Wang, Xiao et al. 2003). Thus, collective behavior of *ROBO1*, *ROBO4* and *SLIT2* expression may be used as diagnostic markers in HCC progression.

Prior studies showed that *ROBO4* was specific to vasculature (Park, Morrison et al. 2003), but we observed varying levels of *ROBO4* transcript in HCC cell lines. The *ROBO4* transcripts in these cells might be partly explained by the presence of side population cells with stem cell characteristics that express markers of the vascular endothelium (Ma, Chan et al. 2007). *ROBO4* may be subject to a transcriptional regulational circuitry in liver tumors. In liver tissues, *ROBO4* expression was significantly downregulated in poorly differentiated tumors suggesting that *ROBO4* function is not essential for the maintenance of tumor at this step of hepatocarcinogenesis. Recent findings indicated that *SLIT2*-*ROBO4* interactions inhibited angiogenesis, which may be an explanation for its downregulation in our analysis (Jones, London et al. 2008).

Interestingly, we could not find *ROBO3* transcript virtually in all of the HCC cell lines. The *ROBO1* regulation in commissural axon guidance exerted by *ROBO3* resulting in functional inhibition of *ROBO1* may be a reason for this observation. Since *ROBO1* was overexpressed and seem to be essential for tumor cells, absence of a negative regulator of *ROBO1*- in this case *ROBO3*- seems to be plausible in HCC.

CAE of zebrafish was transcribed in most of the non-neuronal tissues while it was either absent or expressed at a very low level in the brain, eye, and heart. Furthermore, the isoform-specific expression of *robo2* appeared to be tightly controlled during development such that zebrafish larvae exhibited an expressional induction of *robo2_tv1* and *robo2_tv2* at around the mid-larval stages. These findings might implicate *robo2_tv2* in a role in tissue

morphogenesis, during which cell growth/migration is temporally and spatially regulated. The differential expression of robo2 isoforms in neuronal and non-neuronal tissues could become a source of variation in the function of robo2, possibly directed by different regulatory processes. These findings point to the existence of multiple robo2 proteins; thus, it is critical to identify which form is involved in a specific developmental process or event in pathogenesis (Dalkic, Kuscu et al. 2006). Regarding the recently defined CAE and new *ROBO2* transcript variant- *ROBO2-tv2*, this study is the first showing the presence of the *ROBO2-tv2* in a pathologic condition, in this case hepatocellular carcinoma. It is interesting that the CAE is conserved between *ROBO1*, *ROBO2* and *ROBO4* transcripts in *in silico* analysis. The only member lacking CAE is *ROBO3*.

5.2 *ROBO2* knockdown phenotype in Huh7 hepatocellular carcinoma cells

The expression of *ROBO2* in HCC cell lines was appealing such that it distinguishes the *AFP* positive and negative cell lines. *ROBO2* was overexpressed in *AFP* positive cell lines. This observation led us to propose that silencing of *ROBO2* may furnish the *AFP* positive cells with a phenotype resembling the *AFP* negative cells, possibly with higher migration ability since *AFP* negative SK Hep1 was shown to be highly motile. We knocked down *ROBO2* expression in Huh7 and obtained stable clones. In wound healing assays the migratory behavior of *ROBO2* knockdown and negative clones were not different. We found that E-cadherin expression was upregulated in *ROBO2* knockdown clones and in parallel expression of *SLUG*, which is a transcriptional suppressor of E-cadherin, was downregulated in these clones. These results were converse to our preliminary proposal that *ROBO2* knockdown may result in a more motile cellular phenotype, rather expression of E-cadherin which is a cell-to-cell adhesion molecule and conserved in epithelial cells was upregulated. But this may be because of the weaknesses of our experimental setup; the effect of secreted SLITs and receptor redundancy between ROBO members should be taken into account in other conditions where SLIT2 and/or *ROBO1* was depleted using shRNAs targeting them since *ROBO2* independent signaling may be in play.

We also analyzed the differentially expressed genes in a microarray experiment. We found that QRT-PCR experiments verify our microarray experiment. In the pathway analysis, cell adhesion molecules (CAMs), axon guidance, ECM-receptor interaction, focal adhesion and regulation of actin cytoskeleton pathway genes which may be structurally or functionally

related to SLIT-ROBO signaling were found to be enriched. Interestingly, phosphatidylinositol signaling system, and complement and coagulation cascades that were not previously associated with any of the SLIT-ROBO genes were also in the top levels of the list.

In the microarray analysis, we found that markers of the side population, cancer stem cell or pluripotency phenotypes were upregulated in *ROBO2* knockdown clones. We further confirmed these results by semi-quantitative RT-PCR. CD133 was recently proposed as a cancer stem cell marker. CD133 has seven transcripts and additionally it is posttranscriptionally modified and also known as AC133. We further confirmed its upregulation at protein level by western blot in *ROBO2* knockdown clones. In HCC cell lines, CD133 was expressed in well-differentiated, *AFP* positive cells parallel to the literature data. Huh7 was the cell line that we used to establish stable clones, but it is known that it has plasticity showing the ability to give rise to senescence or replication programmed clones (Ozturk, Erdal et al. 2006). In order to determine whether the CD133 and other pluripotency marker overexpression was dependent on clonogenicity or *ROBO2* knockdown we also performed transient transfection experiments but we could not confirmed the results that we obtained in stable cells. The effects of transient shROBO2 transfection in other HCC cell lines have not been investigated but this may result in a completely different scenario than we observed in Huh7 cells.

p15 (CDKN2B) which is a well-known cell cycle inhibitor was another upregulated transcript in our microarray gene list in *ROBO2* knockdown clones. We checked for its expression by both QRT-PCR and western blot but we could not confirm the upregulation. Furthermore, we found that stable *ROBO2* knockdown clones have significantly lower proliferation rates. This seems to not depend on p15 levels, but there may be other cell cycle regulators controlling the proliferation of these clones.

In the microarray experiment, we pooled total RNAs of 8 ROBO2 KD and 4 control clones, separately but we observed that individual clones differed in their expression of the analyzed genes. In addition, individual clones showed different EMT marker and p15 expression patterns. In upcoming investigations with these clones, may be it would be better to use a limited panel of ROBO2 KD and control clones that reflect the expected ROBO2 expression levels the utmost.

6 FUTURE PERSPECTIVES

Here, we defined the overexpression of *ROBO1* and the variable expression of other *SLIT-ROBO* family members in HCC. Our results help increase our understanding of pathological expression pattern of *SLIT-ROBO* family in HCC with potential for diagnostic applications. Elucidation of the mechanisms acting on the transcriptional regulation of *SLIT-ROBO* signaling pathway, such as alternative splicing, copy number variability and ligand/ receptor redundancy in both HCC and other pathophysiological contexts will contribute to a better understanding of hepatocarcinogenesis. We propose that the use of *ROBO1* and *ROBO4* expression as markers in early diagnosis of HCC shall have important implications.

In our cluster analysis, we found a conserved genewise clustering in both HCC cell lines and liver tissues. The founder members of the two gene clusters in these analyses seem to be *ROBO1* in one gene set and *SLIT2* in the other. Several reports suggest a role for *SLIT2* as a tumor suppressor and it was verified in different experimental setups that *SLIT2* was silenced by methylation in tumors of various tissues including human HCC which was described in a quite recent report (Jin, You et al. 2009). Converse to *SLIT2*, *ROBO1* was shown to be overexpressed in HCCs and proposed as a potential diagnostic and therapeutic target in HCC (Ito, Funahashi et al. 2006). Given the above mentioned results and our findings in HCC tissues, there appears a two way regulation among *SLIT-ROBO* genes during hepatocarcinogenesis. One is the possible oncogenic effect of genes clustered with *ROBO1* that are *ROBO2* and *SLIT1*. Second is the possible tumor suppressor effect of genes clustered with *SLIT2* that are *SLIT3* and *ROBO4*. In fact, in a recent report it was shown that *ROBO4* maintains the vascular integrity by inhibiting the pathologic angiogenesis and endothelial hyperpermeability (Jones, London et al. 2008). This is in parallel to our findings showing the downregulation of *ROBO4* in tumors when compared to normal tissues and additional decreasing trend in *ROBO4* expression as the tumors differentiate to a poor phenotype. Nevertheless, these possible proposed roles of *ROBO2*, *ROBO4*, *SLIT1* and *SLIT3* should be checked in the context of HCC. The effects of individual *SLIT-ROBO* genes and also their combinatorial effects should be described in detail by functional analyses. Further studies investigating potential methylation and inactivation of *SLIT3* and *ROBO4* should be done in order to rule out these presumptions.

In the light of all these findings, it is very likely that *SLIT2-ROBO1-ROBO4* might contribute to some of the variability associated with the differentiation status of HCC while expression of *ROBO1* and *SLIT2* also helps explain the stage differences in this cancer. However, the expression variability is high among liver tumors suggesting that a combinatorial code with a possibility of ligand redundancy might be at work in hepatocellular carcinoma, which prompts further functional studies that include knockdown and overexpression of the above mentioned individual genes.

A global gene expression analysis by microarray technology in 19 HCC cell lines revealed two molecular subtypes depending on their *AFP* expression level (Lee and Thorgeirsson 2002). Of 14 HCC cell lines that we studied, 13 were included in that study and the *SLIT-ROBO* dependent subgrouping in our analysis was parallel to the *AFP* subgrouping previously observed, verifying the reliability of our cell line panel. *ROBO1* and *SLIT3* were the genes that were most significantly correlated with *AFP* expression in a positive and negative manner, respectively. Genes regulating extracellular matrix establishment or remodeling and cell adhesion were differentially expressed between the HCC cell line subgroups. Cells that were defined to be more metastatic and motile correspond to cell lines that were clustered as Group I in our study. Given the important roles of SLIT-ROBO associated signaling molecules like ENA, ABL, and several GTPase activating proteins in cytoskeletal reorganization and cell motility, the connection between SLIT-ROBO signaling and HCC tumor cell invasion and metastasis remains to be further described (Bashaw, Kidd et al. 2000; Wong, Ren et al. 2001). SLIT-ROBO interaction represents another level of control on Abl kinase which is known to be translocated (forming Philadelphia chromosome) and activated in chronic myelogenous leukemia (CML) patients. Furthermore, in a limited number (1.6%) of acute lymphoblastic leukemia (ALL) cases, this has been related to poor prognosis (Pakakasama, Kajanachumpol et al. 2008). The association between SLIT-ROBO interaction and Abl activity may have crucial implications on treatment of the above mentioned leukemic cases.

Another observation related to CD133 was that its overexpression pattern in ROBO2 knockdown clones did not change during long-term cultivation in cell culture. This observation raises the possibility that the effect of ROBO2 may be upstream of the CD133 and at transcriptional level. A comparative genomics study on gene encoding CD133 (PROM1) showed that tandem TCF/LEF-binding sites were present within intron 2 of the

gene and they were conserved among human, chimpanzee, mouse, and rat orthologs (Katoh and Katoh 2007). Slit induced formation of the Robo-N-cadherin complex results in a rapid loss of cadherin-mediated adhesion and has more lasting effects on gene transcription through Abl-mediated phosphorylation of β -catenin on tyrosine 489, leading to a decrease in its affinity for N-cadherin, loss of N-cadherin function, and targeting of phospho-Y489- β -catenin to the nucleus (Rhee, Buchan et al. 2007). This established connection between SLIT-ROBO interaction and β -catenin may be effective on human CD133 gene expression through the described TCF/LEF-binding sites in intron 2 of the gene. This possible connection should be further examined and evaluated in an experimental setup for example using luciferase constructs containing several fragments of CD133 gene promoter and intron 2.

The absence of *ROBO3* transcript in HCC cell lines requires further investigation and explanation. It is plausible that *ROBO3* was downregulated in a microenvironment in which *ROBO1* was highly expressed since they are known to show contradictory roles in commissural axon guidance. The mechanism in which *ROBO3* expression was suppressed should be further characterized. Indeed, *ROBO3* promoter methylation analysis performed in our laboratory in HCC cell lines suspected epigenetic control as an operator. The situation in HCC tumor tissues from patients should be checked in following studies. In this respect, the CAE which codes for a short peptide that is present in ROBO1, ROBO2 and ROBO4 but lacks in ROBO3 requires further attention since the CAE is located in between conserved cytoplasmic regions of ROBOs and may have a role in the interaction with downstream molecules that have signaling activities.

The cellular phenotype associated with ROBO2 should be clarified in detail. BrdU incorporation assays had given promising results indicating a decrease in cellular proliferation in *ROBO2* knockdown clones. The mechanism of this change was not dependent on p15 which is a cell cycle inhibitor but the possible connection with other cell cycle regulators in the light of microarray results should further be followed. In wound healing assay, there was not a significant change in the migration rates of *ROBO2* knockdown clones and negative clones. However the effect of SLIT ligands in these clones should be analyzed in another experimental setup like transwell migration assays and using conditioned medium with ROBO ligands. It is known that SLIT-ROBO interaction has inhibitory effects on other migration inducing ligand-receptor interactions like c-met/HGF or SDF1/CXCR4. This interplay between different signaling receptors should be analyzed in HCC cell migration and

also invasion. In these experiments, another level of complication may be the possible redundancy between ROBO receptors. So, the contribution of the individual ROBO receptors to these processes is an open-end to be analyzed.

REFERENCES

- "R Development Core Team (2005). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing Vienna, Austria ISBN 3-900051-07-0."
- Abelev, G. I. and T. L. Erisir (1999). "Cellular aspects of alpha-fetoprotein reexpression in tumors." Semin Cancer Biol **9**(2): 95-107.
- Alison, M. R., S. Islam, et al. (2009). "Stem cells in liver regeneration, fibrosis and cancer: the good, the bad and the ugly." J Pathol **217**(2): 282-98.
- Andrews, P. W., I. Damjanov, et al. (1984). "Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Differentiation in vivo and in vitro." Lab Invest **50**(2): 147-62.
- Anselmo, M. A., S. Dalvin, et al. (2003). "Slit and robo: expression patterns in lung development." Gene Expr Patterns **3**(1): 13-9.
- Anthony, P. (2002). Pathology of the Liver. R. MacSween, Burt, A., Portmann, B., Ishak, K., Scheuer P. & Anthony, P. Churchill Livingstone, London, New York, Sydney, Toronto: 711-775.
- Ashkenazi, A. and R. S. Herbst (2008). "To kill a tumor cell: the potential of proapoptotic receptor agonists." J Clin Invest **118**(6): 1979-90.
- Baselga, J. and C. L. Arteaga (2005). "Critical update and emerging trends in epidermal growth factor receptor targeting in cancer." J Clin Oncol **23**(11): 2445-59.
- Bashaw, G. J., T. Kidd, et al. (2000). "Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor." Cell **101**(7): 703-15.
- Battaglia, S., N. Benzoubir, et al. (2009). "Liver cancer-derived hepatitis C virus core proteins shift TGF-beta responses from tumor suppression to epithelial-mesenchymal transition." PLoS One **4**(2): e4355.
- Battye, R., A. Stevens, et al. (1999). "Axon repulsion from the midline of the Drosophila CNS requires slit function." Development **126**(11): 2475-81.
- Ben-Shlomo, I., S. Yu Hsu, et al. (2003). "Signaling receptome: a genomic and evolutionary perspective of plasma membrane receptors involved in signal transduction." Sci STKE **2003**(187): RE9.
- Bertoli-Avella, A. M., M. L. Conte, et al. (2008). "ROBO2 gene variants are associated with familial vesicoureteral reflux." J Am Soc Nephrol **19**(4): 825-31.
- Bonner, J. A., P. M. Harari, et al. (2006). "Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck." N Engl J Med **354**(6): 567-78.
- Boyault, S., D. S. Rickman, et al. (2007). "Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets." Hepatology **45**(1): 42-52.
- Branda, M. and J. R. Wands (2006). "Signal transduction cascades and hepatitis B and C related hepatocellular carcinoma." Hepatology **43**(5): 891-902.
- Breuhahn, K., T. Longerich, et al. (2006). "Dysregulation of growth factor signaling in human hepatocellular carcinoma." Oncogene **25**(27): 3787-800.
- Breuhahn, K., S. Vreden, et al. (2004). "Molecular profiling of human hepatocellular carcinoma defines mutually exclusive interferon regulation and insulin-like growth factor II overexpression." Cancer Res **64**(17): 6058-64.
- Brose, K., K. S. Bland, et al. (1999). "Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance." Cell **96**(6): 795-806.
- Brose, K. and M. Tessier-Lavigne (2000). "Slit proteins: key regulators of axon guidance, axonal branching, and cell migration." Curr Opin Neurobiol **10**(1): 95-102.

- Bruix, J., L. Boix, et al. (2004). "Focus on hepatocellular carcinoma." Cancer Cell **5**(3): 215-9.
- Bruix, J. and M. Sherman (2005). "Management of hepatocellular carcinoma." Hepatology **42**(5): 1208-36.
- Calvisi, D. F., S. Ladu, et al. (2006). "Ubiquitous activation of Ras and Jak/Stat pathways in human HCC." Gastroenterology **130**(4): 1117-28.
- Capurro, M., I. R. Wanless, et al. (2003). "Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma." Gastroenterology **125**(1): 89-97.
- Celikkaya, H., C. Ciraci, et al. (2007). "Immunization with UV-induced apoptotic cells generates monoclonal antibodies against proteins differentially expressed in hepatocellular carcinoma cell lines." Hybridoma (Larchmt) **26**(2): 55-61.
- Charron, F., E. Stein, et al. (2003). "The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance." Cell **113**(1): 11-23.
- Chedotal, A., G. Kerjan, et al. (2005). "The brain within the tumor: new roles for axon guidance molecules in cancers." Cell Death Differ **12**(8): 1044-56.
- Chen, X., S. T. Cheung, et al. (2002). "Gene expression patterns in human liver cancers." Mol Biol Cell **13**(6): 1929-39.
- Chiang, D. Y., A. Villanueva, et al. (2008). "Focal gains of VEGFA and molecular classification of hepatocellular carcinoma." Cancer Res **68**(16): 6779-88.
- Chiba, T., K. Kita, et al. (2006). "Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties." Hepatology **44**(1): 240-51.
- Comoglio, P. M., S. Giordano, et al. (2008). "Drug development of MET inhibitors: targeting oncogene addiction and expedience." Nat Rev Drug Discov **7**(6): 504-16.
- Dalkic, E., C. Kusec, et al. (2006). "Alternatively spliced Robo2 isoforms in zebrafish and rat." Dev Genes Evol **216**(9): 555-63.
- Dallol, A., N. F. Da Silva, et al. (2002). "SLIT2, a human homologue of the Drosophila Slit2 gene, has tumor suppressor activity and is frequently inactivated in lung and breast cancers." Cancer Res **62**(20): 5874-80.
- Dallol, A., E. Forgacs, et al. (2002). "Tumour specific promoter region methylation of the human homologue of the Drosophila Roundabout gene DUTT1 (ROBO1) in human cancers." Oncogene **21**(19): 3020-8.
- Dallol, A., D. Krex, et al. (2003). "Frequent epigenetic inactivation of the SLIT2 gene in gliomas." Oncogene **22**(29): 4611-6.
- Dallol, A., D. Morton, et al. (2003). "SLIT2 axon guidance molecule is frequently inactivated in colorectal cancer and suppresses growth of colorectal carcinoma cells." Cancer Res **63**(5): 1054-8.
- Dickinson, R. E., A. Dallol, et al. (2004). "Epigenetic inactivation of SLIT3 and SLIT1 genes in human cancers." Br J Cancer **91**(12): 2071-8.
- Dickson, B. J. (2002). "Molecular mechanisms of axon guidance." Science **298**(5600): 1959-64.
- Eisen, M. B., P. T. Spellman, et al. (1998). "Cluster analysis and display of genome-wide expression patterns." Proc Natl Acad Sci U S A **95**(25): 14863-8.
- El-Serag, H. B., J. A. Marrero, et al. (2008). "Diagnosis and treatment of hepatocellular carcinoma." Gastroenterology **134**(6): 1752-63.
- Fan, X., J. P. Labrador, et al. (2003). "Slit stimulation recruits Dock and Pak to the roundabout receptor and increases Rac activity to regulate axon repulsion at the CNS midline." Neuron **40**(1): 113-27.
- Farazi, P. A. and R. A. DePinho (2006). "Hepatocellular carcinoma pathogenesis: from genes to environment." Nat Rev Cancer **6**(9): 674-87.

- Farazi, P. A., J. Glickman, et al. (2003). "Differential impact of telomere dysfunction on initiation and progression of hepatocellular carcinoma." *Cancer Res* **63**(16): 5021-7.
- Fausto, N., J. S. Campbell, et al. (2006). "Liver regeneration." *Hepatology* **43**(2 Suppl 1): S45-53.
- Ferber, M. J., D. P. Montoya, et al. (2003). "Integrations of the hepatitis B virus (HBV) and human papillomavirus (HPV) into the human telomerase reverse transcriptase (hTERT) gene in liver and cervical cancers." *Oncogene* **22**(24): 3813-20.
- Geyer, C. E., J. Forster, et al. (2006). "Lapatinib plus capecitabine for HER2-positive advanced breast cancer." *N Engl J Med* **355**(26): 2733-43.
- Giannelli, G., C. Bergamini, et al. (2005). "Laminin-5 with transforming growth factor-beta1 induces epithelial to mesenchymal transition in hepatocellular carcinoma." *Gastroenterology* **129**(5): 1375-83.
- Gomaa, A. I., S. A. Khan, et al. (2009). "Diagnosis of hepatocellular carcinoma." *World J Gastroenterol* **15**(11): 1301-14.
- Greenberg, J. M., F. Y. Thompson, et al. (2004). "Slit and robo expression in the developing mouse lung." *Dev Dyn* **230**(2): 350-60.
- Gressner, O. A., M. S. Rizk, et al. (2008). "Changing the pathogenetic roadmap of liver fibrosis? Where did it start; where will it go?" *J Gastroenterol Hepatol* **23**(7 Pt 1): 1024-35.
- Grieshammer, U., M. Le, et al. (2004). "SLIT2-mediated ROBO2 signaling restricts kidney induction to a single site." *Dev Cell* **6**(5): 709-17.
- Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." *Cell* **100**(1): 57-70.
- Hannula-Jouppi, K., N. Kaminen-Ahola, et al. (2005). "The axon guidance receptor gene ROBO1 is a candidate gene for developmental dyslexia." *PLoS Genet* **1**(4): e50.
- Haraguchi, N., T. Utsunomiya, et al. (2006). "Characterization of a side population of cancer cells from human gastrointestinal system." *Stem Cells* **24**(3): 506-13.
- Harris, R., L. M. Sabatelli, et al. (1996). "Guidance cues at the Drosophila CNS midline: identification and characterization of two Drosophila Netrin/UNC-6 homologs." *Neuron* **17**(2): 217-28.
- Hinck, L. (2004). "The versatile roles of "axon guidance" cues in tissue morphogenesis." *Dev Cell* **7**(6): 783-93.
- Hohenester, E. (2008). "Structural insight into Slit-Robo signalling." *Biochem Soc Trans* **36**(Pt 2): 251-6.
- Howitt, J. A., N. J. Clout, et al. (2004). "Binding site for Robo receptors revealed by dissection of the leucine-rich repeat region of Slit." *Embo J* **23**(22): 4406-12.
- Hu, H., M. Li, et al. (2005). "Cross GTPase-activating protein (CrossGAP)/Vilse links the Roundabout receptor to Rac to regulate midline repulsion." *Proc Natl Acad Sci U S A* **102**(12): 4613-8.
- Huminiecki, L. and R. Bicknell (2000). "In silico cloning of novel endothelial-specific genes." *Genome Res* **10**(11): 1796-806.
- Huminiecki, L., M. Gorn, et al. (2002). "Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis." *Genomics* **79**(4): 547-52.
- Hummel, T., K. Schimmelpfeng, et al. (1999). "Commissure formation in the embryonic CNS of Drosophila." *Development* **126**(4): 771-9.
- Hussain, S. A., M. Piper, et al. (2006). "A molecular mechanism for the heparan sulfate dependence of slit-robo signaling." *J Biol Chem* **281**(51): 39693-8.
- Hussain, S. P., J. Schwank, et al. (2007). "TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer." *Oncogene* **26**(15): 2166-76.

- Inoue, H., H. Nojima, et al. (1990). "High efficiency transformation of Escherichia coli with plasmids." Gene **96**(1): 23-8.
- Ito, H., S. Funahashi, et al. (2006). "Identification of ROBO1 as a novel hepatocellular carcinoma antigen and a potential therapeutic and diagnostic target." Clin Cancer Res **12**(11 Pt 1): 3257-64.
- Jen, J. C., W. M. Chan, et al. (2004). "Mutations in a human ROBO gene disrupt hindbrain axon pathway crossing and morphogenesis." Science **304**(5676): 1509-13.
- Jin, J., H. You, et al. (2009). "Epigenetic inactivation of SLIT2 in human hepatocellular carcinomas." Biochem Biophys Res Commun **379**(1): 86-91.
- Jones, C. A., N. R. London, et al. (2008). "Robo4 stabilizes the vascular network by inhibiting pathologic angiogenesis and endothelial hyperpermeability." Nat Med **14**(4): 448-53.
- Jonker, D. J., C. J. O'Callaghan, et al. (2007). "Cetuximab for the treatment of colorectal cancer." N Engl J Med **357**(20): 2040-8.
- Katoh, Y. and M. Katoh (2007). "Comparative genomics on PROM1 gene encoding stem cell marker CD133." Int J Mol Med **19**(6): 967-70.
- Keleman, K., S. Rajagopalan, et al. (2002). "Comm sorts robo to control axon guidance at the Drosophila midline." Cell **110**(4): 415-27.
- Kennedy, T. E., T. Serafini, et al. (1994). "Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord." Cell **78**(3): 425-35.
- Khatri, P., P. Bhavsar, et al. (2004). "Onto-Tools: an ensemble of web-accessible, ontology-based tools for the functional design and interpretation of high-throughput gene expression experiments." Nucleic Acids Res **32**(Web Server issue): W449-56.
- Khatri, P., S. Sellamuthu, et al. (2005). "Recent additions and improvements to the Onto-Tools." Nucleic Acids Res **33**(Web Server issue): W762-5.
- Kidd, T., K. S. Bland, et al. (1999). "Slit is the midline repellent for the robo receptor in Drosophila." Cell **96**(6): 785-94.
- Kidd, T., K. Brose, et al. (1998). "Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors." Cell **92**(2): 205-15.
- Latil, A., L. Chene, et al. (2003). "Quantification of expression of netrins, slits and their receptors in human prostate tumors." Int J Cancer **103**(3): 306-15.
- Laurent-Puig, P., P. Legoix, et al. (2001). "Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis." Gastroenterology **120**(7): 1763-73.
- Lee, J. S., J. Heo, et al. (2006). "A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells." Nat Med **12**(4): 410-6.
- Lee, J. S. and S. S. Thorgeirsson (2002). "Functional and genomic implications of global gene expression profiles in cell lines from human hepatocellular cancer." Hepatology **35**(5): 1134-43.
- Lee, T. K., K. Man, et al. (2006). "Signal transducers and activators of transcription 5b activation enhances hepatocellular carcinoma aggressiveness through induction of epithelial-mesenchymal transition." Cancer Res **66**(20): 9948-56.
- Lin, L. I., Y. F. Ke, et al. (1998). "Curcumin inhibits SK-Hep-1 hepatocellular carcinoma cell invasion in vitro and suppresses matrix metalloproteinase-9 secretion." Oncology **55**(4): 349-53.
- Liu, Z., K. Patel, et al. (2004). "Extracellular Ig domains 1 and 2 of Robo are important for ligand (Slit) binding." Mol Cell Neurosci **26**(2): 232-40.
- Llovet, J. M. and J. Bruix (2008). "Molecular targeted therapies in hepatocellular carcinoma." Hepatology **48**(4): 1312-27.

- Llovet, J. M. and J. Bruix (2008). "Novel advancements in the management of hepatocellular carcinoma in 2008." J Hepatol **48 Suppl 1**: S20-37.
- Llovet, J. M., A. Burroughs, et al. (2003). "Hepatocellular carcinoma." Lancet **362**(9399): 1907-17.
- Llovet, J. M., Y. Chen, et al. (2006). "A molecular signature to discriminate dysplastic nodules from early hepatocellular carcinoma in HCV cirrhosis." Gastroenterology **131**(6): 1758-67.
- Llovet, J. M., A. M. Di Bisceglie, et al. (2008). "Design and endpoints of clinical trials in hepatocellular carcinoma." J Natl Cancer Inst **100**(10): 698-711.
- Lu, W., A. M. van Eerde, et al. (2007). "Disruption of ROBO2 is associated with urinary tract anomalies and confers risk of vesicoureteral reflux." Am J Hum Genet **80**(4): 616-32.
- Lundstrom, A., M. Gallio, et al. (2004). "Vilse, a conserved Rac/Cdc42 GAP mediating Robo repulsion in tracheal cells and axons." Genes Dev **18**(17): 2161-71.
- Ma, S., K. W. Chan, et al. (2007). "Identification and characterization of tumorigenic liver cancer stem/progenitor cells." Gastroenterology **132**(7): 2542-56.
- Ma, S., T. K. Lee, et al. (2008). "CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway." Oncogene **27**(12): 1749-58.
- Marrero, J. A. and A. S. Lok (2004). "Newer markers for hepatocellular carcinoma." Gastroenterology **127**(5 Suppl 1): S113-9.
- Mathews, D. H., J. Sabina, et al. (1999). "Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure." J Mol Biol **288**(5): 911-40.
- Matsuo, N., H. Shiraha, et al. (2009). "Twist expression promotes migration and invasion in hepatocellular carcinoma." BMC Cancer **9**: 240.
- Midorikawa, Y., M. Makuuchi, et al. (2007). "Microarray-based analysis for hepatocellular carcinoma: from gene expression profiling to new challenges." World J Gastroenterol **13**(10): 1487-92.
- Midorikawa, Y., S. Yamamoto, et al. (2006). "Molecular karyotyping of human hepatocellular carcinoma using single-nucleotide polymorphism arrays." Oncogene **25**(40): 5581-90.
- Mitchell, K. J., J. L. Doyle, et al. (1996). "Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons." Neuron **17**(2): 203-15.
- Moon, R. T., A. D. Kohn, et al. (2004). "WNT and beta-catenin signalling: diseases and therapies." Nat Rev Genet **5**(9): 691-701.
- Morlot, C., N. M. Thielens, et al. (2007). "Structural insights into the Slit-Robo complex." Proc Natl Acad Sci U S A **104**(38): 14923-8.
- Moustakas, A. and C. H. Heldin (2007). "Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression." Cancer Sci **98**(10): 1512-20.
- Nam, S. W., J. Y. Park, et al. (2005). "Molecular changes from dysplastic nodule to hepatocellular carcinoma through gene expression profiling." Hepatology **42**(4): 809-18.
- Narayan, G., C. Goparaju, et al. (2006). "Promoter hypermethylation-mediated inactivation of multiple Slit-Robo pathway genes in cervical cancer progression." Mol Cancer **5**: 16.
- Nitta, T., J. S. Kim, et al. (2008). "Murine cirrhosis induces hepatocyte epithelial mesenchymal transition and alterations in survival signaling pathways." Hepatology **48**(3): 909-19.
- Nusslein-Volhard C, W. E., Kluding H (1984). "Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster." Roux's Arch Dev Biol **193**: 267-282.

- Osipo, C. and L. Miele (2006). "Hedgehog signaling in hepatocellular carcinoma: novel therapeutic strategy targeting hedgehog signaling in HCC." Cancer Biol Ther **5**(2): 238-9.
- Ozdinler, P. H. and R. S. Erzurumlu (2002). "Slit2, a branching-arborization factor for sensory axons in the Mammalian CNS." J Neurosci **22**(11): 4540-9.
- Ozturk, N., E. Erdal, et al. (2006). "Reprogramming of replicative senescence in hepatocellular carcinoma-derived cells." Proc Natl Acad Sci U S A **103**(7): 2178-83.
- Pakakasama, S., S. Kajanachumpol, et al. (2008). "Simple multiplex RT-PCR for identifying common fusion transcripts in childhood acute leukemia." Int J Lab Hematol **30**(4): 286-91.
- Paradis, V., I. Bieche, et al. (2003). "Molecular profiling of hepatocellular carcinomas (HCC) using a large-scale real-time RT-PCR approach: determination of a molecular diagnostic index." Am J Pathol **163**(2): 733-41.
- Paradis, V., F. Degos, et al. (2005). "Identification of a new marker of hepatocellular carcinoma by serum protein profiling of patients with chronic liver diseases." Hepatology **41**(1): 40-7.
- Park, K. W., C. M. Morrison, et al. (2003). "Robo4 is a vascular-specific receptor that inhibits endothelial migration." Dev Biol **261**(1): 251-67.
- Park, Y. N., C. P. Yang, et al. (1998). "Neoangiogenesis and sinusoidal "capillarization" in dysplastic nodules of the liver." Am J Surg Pathol **22**(6): 656-62.
- Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res **29**(9): e45.
- Piper, M., R. Anderson, et al. (2006). "Signaling mechanisms underlying Slit2-induced collapse of Xenopus retinal growth cones." Neuron **49**(2): 215-28.
- Piper, M., V. Nurcombe, et al. (2002). "Exogenous Slit2 does not affect ureteric branching or nephron formation during kidney development." Int J Dev Biol **46**(4): 545-50.
- Prasad, A., A. Z. Fernandis, et al. (2004). "Slit protein-mediated inhibition of CXCR4-induced chemotactic and chemoinvasive signaling pathways in breast cancer cells." J Biol Chem **279**(10): 9115-24.
- Prasad, A., V. Paruchuri, et al. (2008). "Slit-2 induces a tumor-suppressive effect by regulating beta-catenin in breast cancer cells." J Biol Chem **283**(39): 26624-33.
- Prasad, A., Z. Qamri, et al. (2007). "Slit-2/Robo-1 modulates the CXCL12/CXCR4-induced chemotaxis of T cells." J Leukoc Biol **82**(3): 465-76.
- Puisieux, A., S. Lim, et al. (1991). "Selective targeting of p53 gene mutational hotspots in human cancers by etiologically defined carcinogens." Cancer Res **51**(22): 6185-9.
- Rajagopalan, S., V. Vivancos, et al. (2000). "Selecting a longitudinal pathway: Robo receptors specify the lateral position of axons in the Drosophila CNS." Cell **103**(7): 1033-45.
- Rhee, J., T. Buchan, et al. (2007). "Cables links Robo-bound Abl kinase to N-cadherin-bound beta-catenin to mediate Slit-induced modulation of adhesion and transcription." Nat Cell Biol **9**(8): 883-92.
- Rhee, J., N. S. Mahfooz, et al. (2002). "Activation of the repulsive receptor Roundabout inhibits N-cadherin-mediated cell adhesion." Nat Cell Biol **4**(10): 798-805.
- Rhodes, D. R., J. Yu, et al. (2004). "ONCOMINE: a cancer microarray database and integrated data-mining platform." Neoplasia **6**(1): 1-6.
- Robinson, D. R., Y. M. Wu, et al. (2000). "The protein tyrosine kinase family of the human genome." Oncogene **19**(49): 5548-57.
- Roth, R. B., P. Hevezi, et al. (2006). "Gene expression analyses reveal molecular relationships among 20 regions of the human CNS." Neurogenetics **7**(2): 67-80.

- Rothberg, J. M., J. R. Jacobs, et al. (1990). "slit: an extracellular protein necessary for development of midline glia and commissural axon pathways contains both EGF and LRR domains." Genes Dev **4**(12A): 2169-87.
- Rozen, S. and H. Skaletsky (2000). "Primer3 on the WWW for general users and for biologist programmers." Methods Mol Biol **132**: 365-86.
- Sabatier, C., A. S. Plump, et al. (2004). "The divergent Robo family protein rig-1/Robo3 is a negative regulator of slit responsiveness required for midline crossing by commissural axons." Cell **117**(2): 157-69.
- Sabatini, D. M. (2006). "mTOR and cancer: insights into a complex relationship." Nat Rev Cancer **6**(9): 729-34.
- Sayan, A. E., B. S. Sayan, et al. (2001). "Acquired expression of transcriptionally active p73 in hepatocellular carcinoma cells." Oncogene **20**(37): 5111-7.
- Schmid, B. C., G. A. Reznicek, et al. (2007). "The neuronal guidance cue Slit2 induces targeted migration and may play a role in brain metastasis of breast cancer cells." Breast Cancer Res Treat **106**(3): 333-42.
- Schmitz, K. J., J. Wohlschlaeger, et al. (2008). "Activation of the ERK and AKT signalling pathway predicts poor prognosis in hepatocellular carcinoma and ERK activation in cancer tissue is associated with hepatitis C virus infection." J Hepatol **48**(1): 83-90.
- Seeger, M., G. Tear, et al. (1993). "Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline." Neuron **10**(3): 409-26.
- Seki, S., T. Kitada, et al. (1999). "Establishment and characteristics of human hepatocellular carcinoma cells with metastasis to lymph nodes." Hepatogastroenterology **46**(29): 2812-7.
- Sell, S. (1993). "The role of determined stem-cells in the cellular lineage of hepatocellular carcinoma." Int J Dev Biol **37**(1): 189-201.
- Serafini, T., T. E. Kennedy, et al. (1994). "The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6." Cell **78**(3): 409-24.
- Shepherd, F. A., J. Rodrigues Pereira, et al. (2005). "Erlotinib in previously treated non-small-cell lung cancer." N Engl J Med **353**(2): 123-32.
- Shyamsundar, R., Y. H. Kim, et al. (2005). "A DNA microarray survey of gene expression in normal human tissues." Genome Biol **6**(3): R22.
- Simpson, J. H., K. S. Bland, et al. (2000). "Short-range and long-range guidance by Slit and its Robo receptors: a combinatorial code of Robo receptors controls lateral position." Cell **103**(7): 1019-32.
- Song, W., H. Li, et al. (2008). "Expression and clinical significance of the stem cell marker CD133 in hepatocellular carcinoma." Int J Clin Pract **62**(8): 1212-8.
- Stein, E. and M. Tessier-Lavigne (2001). "Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex." Science **291**(5510): 1928-38.
- Stella, M. C., L. Trusolino, et al. (2009). "The Slit/Robo system suppresses hepatocyte growth factor-dependent invasion and morphogenesis." Mol Biol Cell **20**(2): 642-57.
- Su, A. I., M. P. Cooke, et al. (2002). "Large-scale analysis of the human and mouse transcriptomes." Proc Natl Acad Sci U S A **99**(7): 4465-70.
- Su, W. H., C. C. Chao, et al. (2007). "OncoDB.HCC: an integrated oncogenomic database of hepatocellular carcinoma revealed aberrant cancer target genes and loci." Nucleic Acids Res **35**(Database issue): D727-31.
- Suetsugu, A., M. Nagaki, et al. (2006). "Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells." Biochem Biophys Res Commun **351**(4): 820-4.

- Sundaresan, V., G. Chung, et al. (1998). "Homozygous deletions at 3p12 in breast and lung cancer." *Oncogene* **17**(13): 1723-9.
- Takahashi, K., K. Tanabe, et al. (2007). "Induction of pluripotent stem cells from adult human fibroblasts by defined factors." *Cell* **131**(5): 861-72.
- Takami, T., P. Kaposi-Novak, et al. (2007). "Loss of hepatocyte growth factor/c-Met signaling pathway accelerates early stages of N-nitrosodiethylamine induced hepatocarcinogenesis." *Cancer Res* **67**(20): 9844-51.
- Tang, Y., K. Kitisin, et al. (2008). "Progenitor/stem cells give rise to liver cancer due to aberrant TGF-beta and IL-6 signaling." *Proc Natl Acad Sci U S A* **105**(7): 2445-50.
- Tessier-Lavigne, M. and C. S. Goodman (1996). "The molecular biology of axon guidance." *Science* **274**(5290): 1123-33.
- Thiery, J. P. (2002). "Epithelial-mesenchymal transitions in tumour progression." *Nat Rev Cancer* **2**(6): 442-54.
- Thiery, J. P. and J. P. Sleeman (2006). "Complex networks orchestrate epithelial-mesenchymal transitions." *Nat Rev Mol Cell Biol* **7**(2): 131-42.
- Thompson, J. D., T. J. Gibson, et al. (1997). "The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools." *Nucleic Acids Res* **25**(24): 4876-82.
- Thorgeirsson, S. S. and J. W. Grisham (2002). "Molecular pathogenesis of human hepatocellular carcinoma." *Nat Genet* **31**(4): 339-46.
- van Zijl, F., M. Mair, et al. (2009). "Hepatic tumor-stroma crosstalk guides epithelial to mesenchymal transition at the tumor edge." *Oncogene*.
- Villanueva, A., P. Newell, et al. (2007). "Genomics and signaling pathways in hepatocellular carcinoma." *Semin Liver Dis* **27**(1): 55-76.
- Vogelstein, B. and K. W. Kinzler (2004). "Cancer genes and the pathways they control." *Nat Med* **10**(8): 789-99.
- Wang, B., Y. Xiao, et al. (2003). "Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity." *Cancer Cell* **4**(1): 19-29.
- Wang, K. H., K. Brose, et al. (1999). "Biochemical purification of a mammalian slit protein as a positive regulator of sensory axon elongation and branching." *Cell* **96**(6): 771-84.
- Wilhelm, S., C. Carter, et al. (2006). "Discovery and development of sorafenib: a multikinase inhibitor for treating cancer." *Nat Rev Drug Discov* **5**(10): 835-44.
- Wong, K., X. R. Ren, et al. (2001). "Signal transduction in neuronal migration: roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway." *Cell* **107**(2): 209-21.
- Wu, J. Y., L. Feng, et al. (2001). "The neuronal repellent Slit inhibits leukocyte chemotaxis induced by chemotactic factors." *Nature* **410**(6831): 948-52.
- Wurmbach, E., Y. B. Chen, et al. (2007). "Genome-wide molecular profiles of HCV-induced dysplasia and hepatocellular carcinoma." *Hepatology* **45**(4): 938-47.
- Xian, J., A. Aitchison, et al. (2004). "Targeted disruption of the 3p12 gene, Dutt1/Robo1, predisposes mice to lung adenocarcinomas and lymphomas with methylation of the gene promoter." *Cancer Res* **64**(18): 6432-7.
- Xian, J., K. J. Clark, et al. (2001). "Inadequate lung development and bronchial hyperplasia in mice with a targeted deletion in the Dutt1/Robo1 gene." *Proc Natl Acad Sci U S A* **98**(26): 15062-6.
- Yang, L. and G. J. Bashaw (2006). "Son of sevenless directly links the Robo receptor to rac activation to control axon repulsion at the midline." *Neuron* **52**(4): 595-607.
- Yang, Z. F., D. W. Ho, et al. (2008). "Significance of CD90+ cancer stem cells in human liver cancer." *Cancer Cell* **13**(2): 153-66.

- Yang, Z. F., P. Ngai, et al. (2008). "Identification of local and circulating cancer stem cells in human liver cancer." Hepatology **47**(3): 919-28.
- Yook, J. I., X. Y. Li, et al. (2005). "Wnt-dependent regulation of the E-cadherin repressor snail." J Biol Chem **280**(12): 11740-8.
- Zhou, B. P., J. Deng, et al. (2004). "Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition." Nat Cell Biol **6**(10): 931-40.
- Zuker, M. (2003). "Mfold web server for nucleic acid folding and hybridization prediction." Nucleic Acids Res **31**(13): 3406-15.

PUBLICATIONS

Research article

Open Access

Quantification of *SLIT-ROBO* transcripts in hepatocellular carcinoma reveals two groups of genes with coordinate expression

Mehmet Ender Avci, Ozlen Konu and Tamer Yagci*

Address: Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey

Email: Mehmet Ender Avci - aender@bilkent.edu.tr; Ozlen Konu - konu@fen.bilkent.edu.tr; Tamer Yagci* - tyagci@fen.bilkent.edu.tr

* Corresponding author

Published: 29 December 2008

Received: 14 July 2008

BMC Cancer 2008, 8:392 doi:10.1186/1471-2407-8-392

Accepted: 29 December 2008

This article is available from: <http://www.biomedcentral.com/1471-2407/8/392>

© 2008 Avci et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: SLIT-ROBO families of proteins mediate axon pathfinding and their expression is not solely confined to nervous system. Aberrant expression of *SLIT-ROBO* genes was repeatedly shown in a wide variety of cancers, yet data about their collective behavior in hepatocellular carcinoma (HCC) is missing. Hence, we quantified *SLIT-ROBO* transcripts in HCC cell lines, and in normal and tumor tissues from liver.

Methods: Expression of *SLIT-ROBO* family members was quantified by real-time qRT-PCR in 14 HCC cell lines, 8 normal and 35 tumor tissues from the liver. ANOVA and Pearson's correlation analyses were performed in R environment, and different clinicopathological subgroups were pairwise compared in Minitab. Gene expression matrices of cell lines and tissues were analyzed by Mantel's association test.

Results: Genewise hierarchical clustering revealed two subgroups with coordinate expression pattern in both the HCC cell lines and tissues: *ROBO1*, *ROBO2*, *SLIT1* in one cluster, and *ROBO4*, *SLIT2*, *SLIT3* in the other, respectively. Moreover, *SLIT-ROBO* expression predicted AFP-dependent subgrouping of HCC cell lines, but not that of liver tissues. *ROBO1* and *ROBO2* were significantly up-regulated, whereas *SLIT3* was significantly down-regulated in cell lines with high-AFP background. When compared to normal liver tissue, *ROBO1* was found to be significantly overexpressed, while *ROBO4* was down-regulated in HCC. We also observed that *ROBO1* and *SLIT2* differentiated histopathological subgroups of liver tissues depending on both tumor staging and differentiation status. However, *ROBO4* could discriminate poorly differentiated HCC from other subgroups.

Conclusion: The present study is the first in comprehensive and quantitative evaluation of *SLIT-ROBO* family gene expression in HCC, and suggests that the expression of *SLIT-ROBO* genes is regulated in hepatocarcinogenesis. Our results implicate that *SLIT-ROBO* transcription profile is bi-modular in nature, and that each module shows intrinsic variability. We also provide quantitative evidence for potential use of *ROBO1*, *ROBO4* and *SLIT2* for prediction of tumor stage and differentiation status.

Background

Drosophila slit and *roundabout (robo)* genes were identified in genetic screens of mutants for embryonic patterning and commissural axon pathfinding defects [1]. Subsequently, it was shown that SLIT acts as a ligand for ROBO receptor, preventing axons from recrossing the central nervous system (CNS) midline, and that this binding is conserved among vertebrates including mammals [2,3]. In mammals, three *SLIT* (*SLIT1-3*) and four *ROBO* (*ROBO1-4*) genes have been described [4,5].

SLIT and *ROBO* genes are mainly expressed in the CNS but there are affirmative data that they are also expressed in non-neuronal tissues, such as mouse lung and kidney [6,7]. Binding of SLIT2 to ROBO1 inhibits CXCL12-induced chemotaxis of leukocytes, T cells and monocytes [8-10]. However, ROBO4 expression has been found to be confined to vasculature and Robo4 signaling modulates endothelial cell migration [11].

On the other hand, like other developmental pathways, aberrant expression of the *SLIT-ROBO* genes has been observed in a wide variety of cancers. Mice with targeted homozygous deletion of first Ig domain of *Robo1/Dutt1* died at birth because of abnormal lung development, and few survivors eventually developed epithelial bronchial hyperplasia [6]. In breast carcinoma tissue samples *ROBO1* was shown to be overexpressed while SLIT2 induced migration of breast cancer cell lines [12]. SLIT2-ROBO1 signaling was involved in angiogenesis by increasing microvessel density and tumor mass in a tumor xenograft model [13]. In the same study, *SLIT2* exhibited overexpression in tumor cell lines and primary tumors of a variety of tissues. In contrast, *SLIT2* also was proposed to be a tumor suppressor gene, which was silenced epigenetically in lung, breast, colon cancers and gliomas [14-16]. *SLIT3* was silenced by promoter hypermethylation in gliomas and colorectal cancers [17]. *SLIT1* and *SLIT3* were overexpressed in prostate tumors [18], whereas along with *SLIT2* they were slightly expressed only in poorly differentiated HCC [19]. CXCL12 was reported to activate the migration of human melanoma and breast cancer cells that express CXCR4, ROBO1 and ROBO2, while SLIT2-ROBO interaction was demonstrated to inhibit chemotaxis, chemoinvasion and adhesion of breast cancer cells [20]. Furthermore, ROBO4 was overexpressed in tumor endothelial cells in comparison to normal adult endothelial cells [21].

Despite the compiling evidence of *SLIT-ROBO* deregulation in various tumors, only few reports with apparent controversies exist with regard to the expression pattern of these genes in hepatocellular carcinoma (HCC). The overexpression of ROBO1 in HCC was recently reported and this receptor was proposed as an HCC marker in humans

[19]. In contrast, another study reported that *Robo1* heterozygous mice developed spontaneous HCC tumors [22]. It was shown by immunohistochemical staining that SLIT2 protein also was present in HCC tumor sections [13]. Moreover, karyotyping analyses of HCC do not reveal any chromosomal gains or losses associated with *SLIT-ROBO* genes [23,24]. Therefore, in this study, we quantified *SLIT1*, *SLIT2*, *SLIT3*, *ROBO1*, *ROBO2* and *ROBO4* transcripts in HCC cell lines and tissues. We observed that *SLIT-ROBO* genes could be partitioned into two main clusters based on their expression in either the HCC cell lines or tissues. *SLIT-ROBO* expression also clustered the HCC cell lines in two groups according to their *AFP* expression pattern. In liver tissues, differential expression of *ROBO1*, *ROBO4* and *SLIT2* was found to be associated with clinicopathological parameters such as tumor staging and differentiation. Herein, we describe a comprehensive *SLIT-ROBO* expression signature in HCC.

Methods

HCC Cell Lines and Tissues

13 hepatoma and 1 hepatoblastoma (HepG2) cell lines were included in the study and cultured as previously described [25]. Focus, Hep40, Hep3B, Hep3B-TR, HepG2, HUH7, Mahlavu, PLC/PRF/5, SK Hep1 cells were cultured in low-glucose DMEM supplemented with 10% FBS, 100 U/ml Penicillin-Streptomycin, and 0.1 mM non-essential amino acids (HyClone, Utah, USA). SNU387, SNU398, SNU423, SNU449, SNU475 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml Penicillin-Streptomycin, 0.1 mM non-essential amino acids (HyClone, Utah, USA). TissueScan Liver Cancer Tissue qPCR Arrays, each containing 40 liver tumor and 8 tumor-adjacent normal tissue cDNAs, were purchased from Origene Technologies, (Rockville, MD, USA). 5 non-HCC tumor tissues consisting of 3 cholangiocarcinomas, 1 nodular hyperplasia and 1 liver adenoma were excluded from the present study. Clinicopathological characteristics of the tissues were presented in Additional file 1.

Primers

PCR primers for human *SLITs*, *ROBO1* and *ROBO2* were previously described [18]. Human *ROBO3*, *ROBO4* and *AFP* (*alpha-fetoprotein*) primers were designed using Primer3 and targeting exon-exon junctions in order to prevent amplification of possible contaminating genomic DNA [26]. Primer sequences were as follows: *ROBO3* forward 5'-CAGTGTCCGATGGAAGAAGG-3' and reverse 5'-GTCCATCTCCTGCACATTGG-3', *ROBO4* forward 5'-GACACTTGGCGTTCCACCTC-3' and reverse 5'-AGAGCAAGGAGCGACGACAG-3', *AFP* forward 5'-AAATGCGTTTCTCGTTGCTT-3' and reverse 5'-CCAACACCAGGGTTTACTGG-3'. Primer pair for the housekeeping gene *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) was described before [27]. *ACTB* (*beta-*

actin) primer pair was supplemented in TissueScan Liver Cancer Tissue qPCR Array 1 (Origene Technologies, Rockville, MD, USA).

RNA Isolation and cDNA Synthesis

Cell lines were grown to confluency in 100 mm dishes. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. cDNA was synthesized with random hexamers from 1 µg of total RNA using DyNAmo™ cDNA Synthesis Kit (Finnzymes, Espoo, Finland).

Real-time Quantitative RT-PCR Analyses of HCC Cell Lines

In cell lines and tissues, the relative expression ratio (R) of *SLIT-ROBO* and *AFP* transcripts (target gene) was measured based on a modified $\Delta\Delta C_t$ formula [28] and normalized to *GAPDH* or *ACTB* (reference gene). In

$$R = (E_{target})^{\Delta C_{t_{target}}(\text{control-sample})} / (E_{ref})^{\Delta C_{t_{ref}}(\text{control-sample})}$$

formula, E_{target} and E_{ref} reflect PCR efficiencies of the primers for target genes and reference genes, respectively. PCR efficiency values for each primer pair was obtained by constructing a standard curve using threshold cycle (C_t) values derived from 6 data points, corresponding to 2-fold decrements of an original cDNA stock (duplicates were prepared for each dilution). The slope of the resulting curve was used to calculate the E value of primer pairs according to $E = 2^{-1/\text{slope}}$ formula. PCR efficiencies of the genes ranged between 1.9 and 2.0. ΔC_t was the difference between the C_t values of controls and samples.

In cell lines, *GAPDH* was the reference gene. ΔC_t values were obtained by subtracting C_t values of individual genes (sample) from the average C_t value of all cell lines for that gene (control). All reactions were performed in duplicates and repeated at least twice using different batches of RNA preparations. Relative expression tables were established by representing $\Delta\Delta C_t$ values in log2 base, and in all subsequent analyses these values were used.

Quantitative expression analyses were performed using DyNAmo™ HS SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland) on an iCycler iQ real-time PCR detection system (Bio-Rad, Richmond, CA). The PCR reaction was set according to the manufacturer's recommendations. Briefly for 1× reaction; 10 µl of 2× SYBR Green PCR Master Mix, 10 µM forward and reverse primers, and 1 µl of template cDNA were mixed in a total volume of 20 µl. After an initial 15 minutes of denaturation at 95°C, thermal cycling was performed at 94°C for 30 sec, 60–62°C for 30 sec (optimized for each primer pair), 72°C for 30 sec for a total of 50 cycles and a final extension step at 72°C for

10 min. In order to validate the production of a single target-specific PCR product, the amplification was followed by a melt curve protocol with an initial step at 55°C for 30 sec and 80 repeats of 0.5°C increments with 15 sec dwell time, from 55°C to 95°C.

Real-time Quantitative RT-PCR Analyses of HCC Tissues

The expression of *SLIT-ROBO* and *AFP* genes in HCC was analyzed using a 96-well plate format TissueScan Liver Cancer Tissue qPCR Array 1 (Origene Technologies, Rockville, MD), which contained tissue cDNAs normalized against beta-actin. Real-time PCR protocol was applied as described by the manufacturer. Briefly, 30 µl of reaction mix containing 15 µl 2× SYBR Green PCR Master Mix and 10 µM forward and reverse primers was directly added to PCR-plate wells. Plate was placed on ice for 15 min for cDNAs to dissolve, and thermal cycling was performed according to above mentioned protocol. For each gene, mean C_t value of the normal tissue cDNAs was set as the control group and relative quantitative expression values were calculated with the $\Delta\Delta C_t$ formula and were represented in log2 base by taking the *ACTB* C_t values as reference.

Statistical Analysis

Using one-way ANOVA in R, mean expression levels of each gene were compared between high and low *AFP* expressing groups of HCC cell lines; and also between normal and tumor tissues of liver with respect to differentiation or stage [29]. Pairwise comparisons were made using Fisher's multiple pairwise comparison method in Minitab® 13.20 Statistical Software (Minitab Inc. 2000). Furthermore, two-way hierarchical cluster analysis was used to group cell lines and liver tissues with respect to the *SLIT-ROBO* expression patterns using Cluster and TreeView [30]. Pairwise correlations between *SLIT-ROBO* gene expression levels were calculated using Pearson's correlation coefficient. Moreover, a Mantel's association test was applied to compare cell line and tissue correlation matrices [29].

Results

QRT-PCR analyses reveal an association between *SLIT-ROBO* and *AFP* expression in HCC cell lines

We first investigated by RT-PCR the presence of *SLIT-ROBO* transcripts in 13 HCC and 1 hepatoblastoma cell lines. All genes were expressed at levels varying from none to strong after 40 cycles of amplification (Additional file 2). Since HCC cell lines were previously reported to cluster in two main molecular subtypes in terms of their *alpha-fetoprotein* (*AFP*) expression [31] we quantified *SLIT-ROBO* gene expression along with *AFP* transcript levels.

In our first set of qRT-PCR experiments, cell lines with fibroblastoid morphology, including Focus, Mahlavu, SK

Hep1, SNU387, SNU398, SNU423, SNU449 and SNU475, displayed low-AFP expression, while AFP was found to be overexpressed in epitheloid Hep40, Hep3B, Hep3B-TR, HepG2, HUH7 and PLC/PRF/5 cells (Figure 1). Second, we quantified *SLIT-ROBO* transcripts. *ROBO2* and *SLIT1* expression were up-regulated in high-AFP group of cells, except HepG2, which underexpressed *ROBO2* (Additional file 3). On the other hand, both genes were found to be overexpressed in SNU398, one of the low-AFP group of cell lines. *SLIT3* transcript levels sharply contrasted those of *ROBO2* and *SLIT1*. Except SNU398, increased *SLIT3* expression was found among low-AFP expressing group of cell lines, and also in high-AFP expressing Hep40 cell line. Interestingly, widespread expression of *ROBO1* transcripts that we observed in RT-PCR turned out to be enhanced in cell lines with high-AFP background (Additional file 2 and 3). *ROBO4* and *SLIT2* transcript levels remained variable among HCC cell lines and no phenotype-based association could be observed for these genes. Our attempts to calculate PCR efficiency with two different *ROBO3* primer pairs failed, and we discontinued qRT-PCR analysis of this gene.

A significant association of gene expression was found among *SLIT-ROBO* family genes and AFP based on Pearson's pairwise correlation coefficients (Table 1). In particular, expressions of *ROBO1* and *SLIT3* were significantly correlated with that of AFP, in a positive and negative manner, respectively.

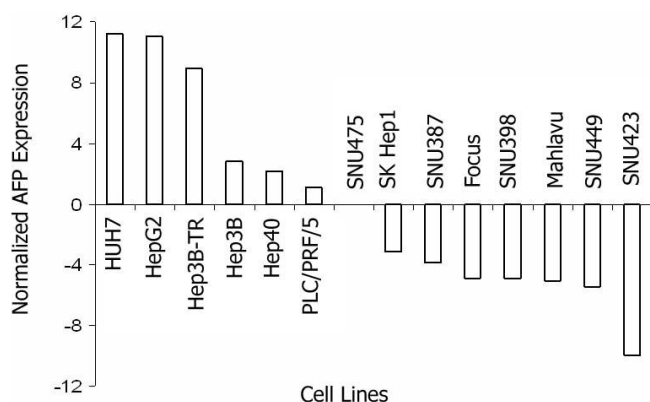


Figure 1
Quantification of AFP transcript levels reveals two groups in HCC cell lines. GAPDH normalized AFP expression was quantified by real-time RT-PCR and expression values of individual cell lines are calculated relative to the Ct average of all cell lines, and represented in log2 base. Samples were run in duplicates and the data are representative of two independent experiments.

Cluster analyses identify two subgroups in HCC cell lines with respect to *SLIT-ROBO* expression levels

Hierarchical clustering was performed in order to understand whether *SLIT-ROBO* expression predicts AFP-dependent grouping of HCC cell lines. In fact, in terms of *SLIT-ROBO* expression, low-AFP expressing SNU449, SNU423, SNU475, Mahlavu, SK Hep1, SNU387, and Focus cells grouped together (Group I), while high-AFP expressing HepG2, PLC/PRF/5, HUH7, Hep3B-TR, Hep3B and Hep40 cell lines (Group II) clustered separately from the first group (Figure 2). Exceptionally, SNU398 cell line displayed a *SLIT-ROBO* expression pattern concordant with Group II despite its low-AFP background. The expression of AFP significantly differed between the two groups ($p = 0.0001$): Mean expression of AFP in Group I and Group II cells was -4.65 ± 2.75 and 6.20 ± 4.68 , respectively.

Hierarchical clustering analysis also revealed a genewise segregation of *SLIT-ROBO* genes. Coordinate expression was observed in two main clusters, one of which grouping together *ROBO1*, *ROBO2* and *SLIT1*, and the other *ROBO4*, *SLIT2*, and *SLIT3*, respectively (Figure 2). We also performed one-way ANOVA to identify individual *SLIT-ROBO* genes, which significantly separate HCC cell lines with high- and low-AFP expression. We validated that *ROBO1* and *ROBO2* expression were up-regulated in high-AFP group ($p = 6.7 \times 10^{-4}$ and $p = 0.013$, respectively). Mean expression of *ROBO1* in Group I was -1.73 ± 2.15 while in Group II cells increased to 2.37 ± 0.48 . Similarly, *ROBO2* mean expression values were found as -2.37 ± 3.31 and 3.11 ± 3.73 , for Group I and Group II, respectively. In sharp contrast, *SLIT3* was overexpressed in cell lines with low-AFP profile ($\text{Mean}_{\text{Group I}} = 3.28 \pm 4.43$, $\text{Mean}_{\text{Group II}} = -4.24 \pm 4.06$; $p = 6.9 \times 10^{-3}$).

SLIT-ROBO family expression profile in liver tissues correlates with that in HCC cell lines

Next, we addressed the question of whether our cell line data translate to *in vivo* conditions. To this end, we quantified the expression of *SLIT-ROBO* and AFP genes using cDNAs of 8 tumor-adjacent normal and 35 tumor tissues (Additional file 4). Accordingly, only *ROBO1* was found to be significantly overexpressed in tumor tissues ($\text{Mean}_{\text{Normal}} = 0 \pm 0.63$, $\text{Mean}_{\text{Tumor}} = 1.57 \pm 1.63$; $p = 0.011$). We also observed a down-regulated *ROBO4* expression in tumor tissues with a p-value near significance ($\text{Mean}_{\text{Normal}} = 0 \pm 1.03$, $\text{Mean}_{\text{Tumor}} = -0.89 \pm 1.30$; $p = 0.079$). AFP displayed a highly variable expression, yet it was overexpressed in more than half of the tumors (18/35) (Additional file 4).

Hierarchical clustering grouped *SLIT-ROBO* genes in a similar manner as in cell lines. *ROBO1*, *ROBO2* and *SLIT1* clustered together, whereas *ROBO4*, *SLIT2* and *SLIT3*

Table 1: Genewise correlation of *SLIT-ROBO* and *AFP* genes in HCC cell lines

Gene	<i>ROBO1</i>	<i>ROBO2</i>	<i>ROBO4</i>	<i>SLIT1</i>	<i>SLIT2</i>	<i>SLIT3</i>	<i>AFP</i>
<i>ROBO1</i>	1						
<i>ROBO2</i>	0.6060*	1					
<i>ROBO4</i>	-0.2721	-0.2653	1				
<i>SLIT1</i>	0.4027	0.7623*	-0.3288	1			
<i>SLIT2</i>	0.1241	0.1891	0.5143	-0.0039	1		
<i>SLIT3</i>	-0.5698*	-0.6072*	0.3681	-0.5451*	-0.1003	1	
<i>AFP</i>	0.6533*	0.5108	-0.3016	0.4579	0.3544	-0.6605*	1

*: $p < 0.05$

formed another cluster (Figure 3). Two main clusters appeared among tissue samples; however, no significant association of clusters was found with differentiation state or staging of tumors (Wilcoxon rank sum test) and *AFP* expression (one-way ANOVA).

Expression correlation amongst individual *SLIT-ROBO* and *AFP* genes in liver tissues was analyzed by Pearson's correlation analysis in R (Table 2). Accordingly, expression of *ROBO1* in HCC samples was positively correlated with that of *AFP* whereas *ROBO4* and *AFP* were inversely correlated in terms of their expression. Mantel's permutation test, performed to compare gene expression correlation matrices of cell lines and tissues (described in Table 1 and Table 2, respectively), indicated that gene-to-gene correlation patterns in both sample groups were significantly associated ($r = 0.49$; $p < 0.02$).

***ROBO1* expression differentiates normal tissues from tumors with respect to both stage and differentiation status**

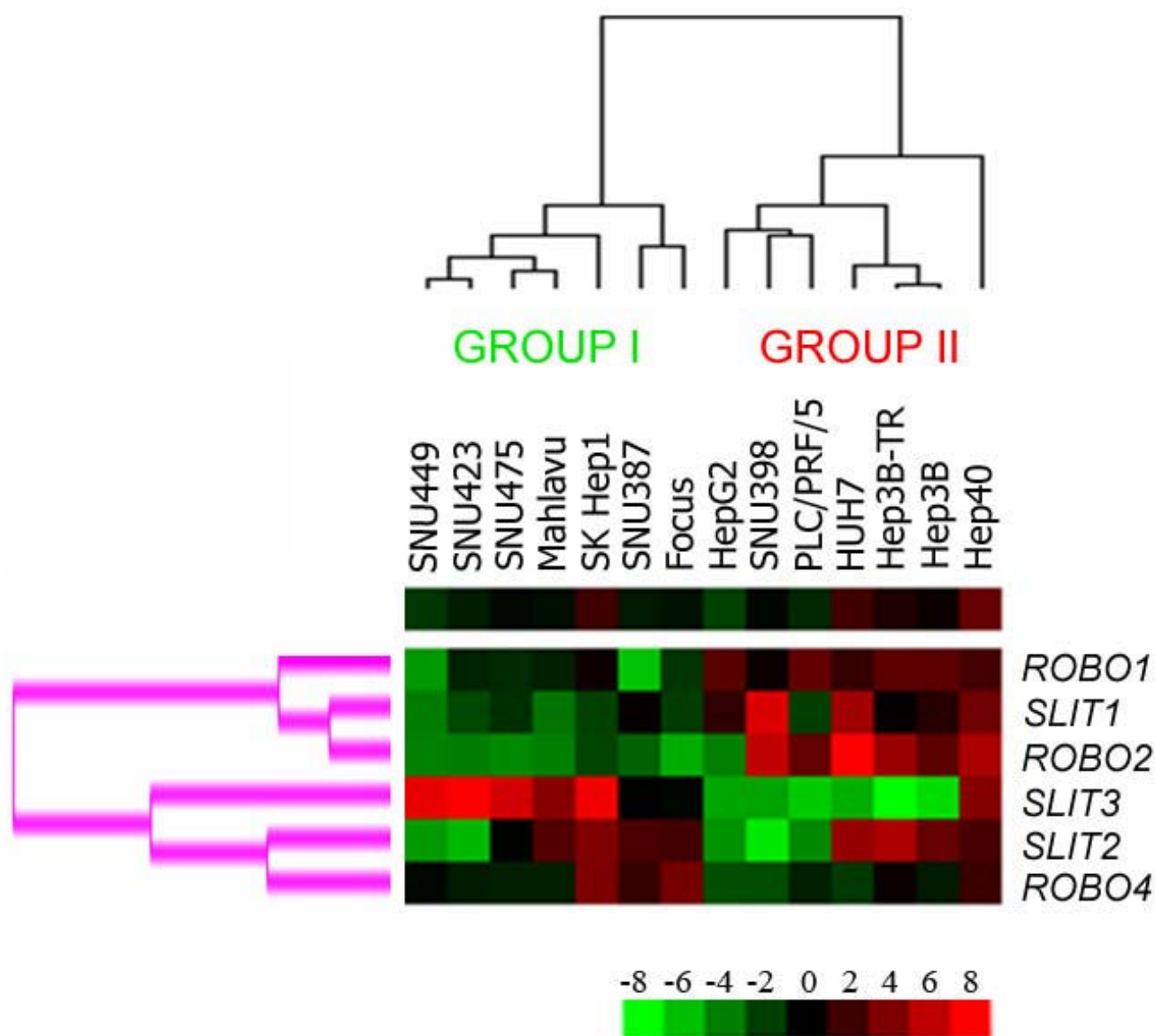
We also performed one-way ANOVA followed by Fisher's multiple pairwise comparisons in order to explore whether gene expression characteristics of *SLIT-ROBO* family members discriminate among liver tissues with respect to differentiation status and staging of the samples. The tissue samples lacking stage or differentiation information in the pathological reports were left out of analysis. With these criteria, a total of 43 liver tissue samples for stage and of 38 tissue samples for differentiation status were analyzed. According to one-way ANOVA, *ROBO1* differentiated liver tissue samples on both the stage ($p = 0.018$) and differentiation status ($p = 0.031$) dependent manner (Figure 4A, B). *ROBO4* expression significantly discriminated tissues only with respect to their

differentiation status ($p = 0.039$) (Figure 4C). Moreover, Fisher's pairwise comparison analyses revealed that *ROBO1*, *SLIT2* and *ROBO4* significantly discriminated between different histopathological subgroups both in terms of differentiation status and/or tumor staging (Table 3).

Discussion

HCC remains the fifth most common cancer worldwide and is at the third rank in cancer-caused deaths. The prognosis of patients is generally very poor with a 5-year relative survival of only 7% [32]. The elucidation of molecular mechanisms governing hepatocarcinogenesis is therefore of high priority not only for the better understanding of the disease, but also to develop more effective therapies. To achieve this goal, functional genomics studies could provide valuable information with regard to genes differentially expressed between HCC and normal liver. A collective analysis of expression signature of *SLIT-ROBO* family genes has not been assessed yet in liver tumor. Here, we showed the co-regulation of *SLIT-ROBO* genes in HCC. In both the HCC cell lines and liver tissues, *ROBO1*, *ROBO2*, *SLIT1*, and *ROBO4*, *SLIT2*, *SLIT3* showed coordinate expression as two distinct modules, yet displaying high variability at gene level within each module. Additionally, *SLIT-ROBO* expression was able to predict *AFP* status of HCC cell lines, and thereby establishing two groups with low- and high-*AFP* expressions.

Except *ROBO3*, all genes were found to be expressed at different levels in our analyses. A preferential up- and down-regulation of *SLIT-ROBO* genes occurred depending on the *AFP* expression status of HCC cell lines. *ROBO1*, *ROBO2* and to a lesser extent *SLIT1* were overexpressed, whereas *SLIT3* was underexpressed in high-*AFP*

**Figure 2**

***SLIT-ROBO* expression establishes two groups, which predict *AFP* expression levels in HCC cell lines.** The means of normalized expression values of individual *SLIT-ROBO* genes in each cell line were used to establish the hierarchical clustering in Cluster program, and the results were visualized as a heatmap by TreeView. Overexpression and underexpression of individual genes relative to Ct average of all cell lines are represented by red and green colors, respectively. Upper single-row heatmap displays the average expression of all six genes across all cell lines. The tree at the top of the heatmap represents samplewise clustering. Based on their *AFP* expression levels, HCC cell lines are represented in two main subgroups indicated as Group I (low-*AFP*) and Group II (high-*AFP*) cells. The tree on the left of the heatmap (pink) represents genewise clustering.

group. *ROBO4* also tended to be down-regulated in this group. However, *SLIT2* was expressed in most of the cell lines, regardless of the *AFP* expression status.

We also quantified *SLIT-ROBO* expression in 8 tumor-adjacent normal liver tissues and 35 HCC tumors. We found that genewise clustering observed in HCC cell lines

were conserved in tissues: *ROBO1*, *ROBO2*, *SLIT1* and *ROBO4*, *SLIT2*, *SLIT3* were coordinately expressed, respectively. We also noticed two main subgroups in tissue samples but the observed *AFP* dependent subgrouping in HCC cell lines did not translate into the tissue analysis, except that *AFP* and *ROBO1* expression was significantly correlated in both HCC cell lines and tissues. This discrep-

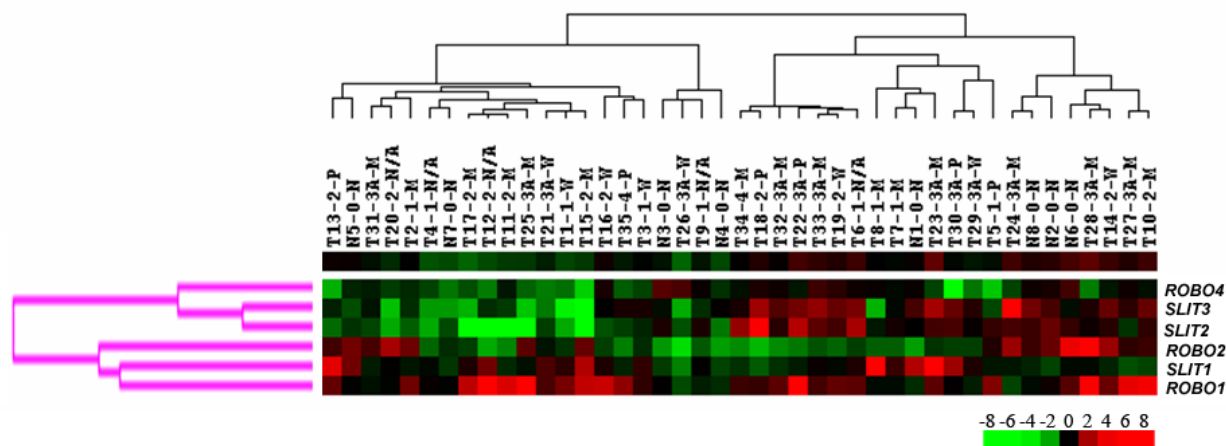


Figure 3
Genewise clustering of *SLIT-ROBO* genes in HCC cell lines translates into liver tissues. The normalized expression values of individual *SLIT-ROBO* genes in liver tissue samples ($n = 43$) were used to establish the hierarchical clustering in Cluster program and the results were visualized as a heatmap by TreeView. Overexpression and underexpression of individual genes relative to Ct average of normal tissues ($n = 8$) are represented by red and green colors, respectively. Upper single-row heatmap displays the average expression of all six genes across all tissues. The tree at the top of the heatmap represents sample-wise clustering, and the tree on the left of the heatmap (pink) represents genewise clustering. T1-35: tumors; N1-8: normal tissues; 0: stage 0 (non-tumor); 1, 2, 3A, 4: stage 1, stage 2, stage 3A, stage 4 HCC, respectively; N: tumor-adjacent normal tissue; W: well-differentiated; M: moderately differentiated; P: poorly differentiated; N/A: not-assigned.

any might be partly due to the heterogeneity of tissues. HCC cell lines were more homogenous when compared to tissue samples, which may contain stromal cells, endothelial cells, immune cells or any other tumor infiltrating cells. Moreover, our normal liver samples were tumor-adjacent tissues, which may harbor genetic changes of tumor microenvironment, and therefore may not reveal the actual molecular characteristics of a tumor-free normal liver.

ROBO1 transcript was present in all cell lines that were examined and it was significantly up-regulated in the analyzed HCC tissues, in which its overexpression culminated in later stages and as tumors progress to a less differentiated state. These data were in agreement with a recent report that demonstrated *ROBO1* as an HCC antigen and proposing it as both a diagnostic marker and therapeutic target for HCC [19]. *SLIT2* was present in most of the tumor tissues and HCC cell lines although at variable levels. Such variability might explain the clustering of *SLIT2*

Table 2: Genewise correlation of *SLIT-ROBO* and *AFP* genes in liver tissues

Gene	<i>ROBO1</i>	<i>ROBO2</i>	<i>ROBO4</i>	<i>SLIT1</i>	<i>SLIT2</i>	<i>SLIT3</i>	<i>AFP</i>
<i>ROBO1</i>	1						
<i>ROBO2</i>	0.1835	1					
<i>ROBO4</i>	-0.1879	-0.2134	1				
<i>SLIT1</i>	0.0298	0.002	-0.3793*	1			
<i>SLIT2</i>	-0.3679*	-0.0078	0.3432*	-0.1389	1		
<i>SLIT3</i>	-0.0973	-0.0027	0.2596	-0.0965	0.6844***	1	
<i>AFP</i>	0.3679*	0.2324	-0.3699*	0.0924	-0.0769	-0.1638	1

***: $p < 0.001$; *: $p < 0.05$

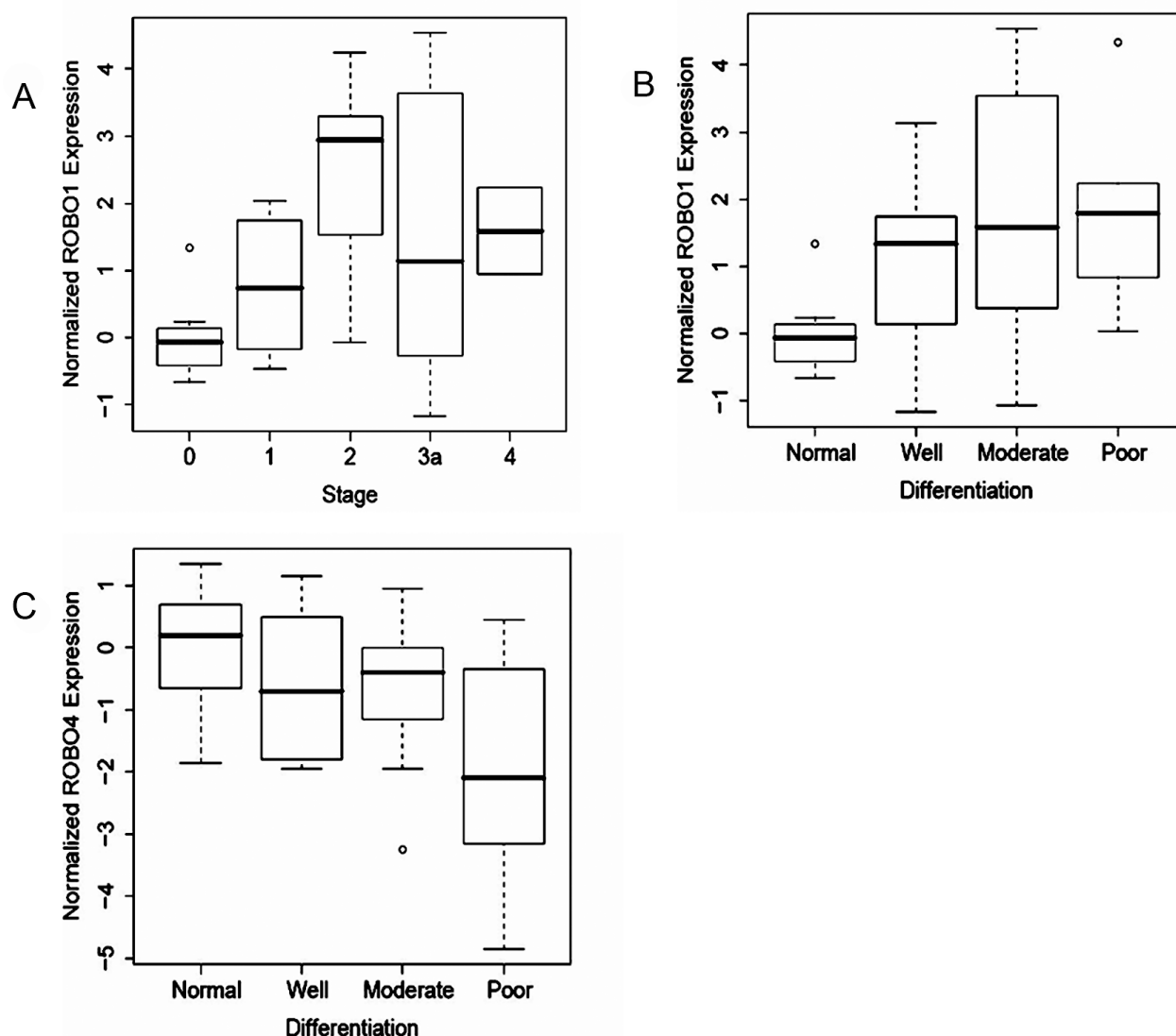


Figure 4

ROBO1 and ROBO4 expression discriminate between different stage and differentiation groups of liver tissues. Expression values of *SLIT-ROBO* genes were compared using one-way ANOVA in liver tissue samples that are stratified according to their stages and differentiation status and represented as boxplots. (A) *ROBO1* significantly differentiates one staging group from the others ($p = 0.018$). (B) The expression of *ROBO1* also discriminates between different differentiation status of liver tissues ($p = 0.031$). (C) *ROBO4* also significantly separates one differentiation group among all ($p = 0.039$).

in a different group than *ROBO1* and *ROBO2*, yet it is likely to be the main ligand for ROBO receptors. Nevertheless, this does not exclude interactions between other SLIT and ROBO members, nor it does the possible ligand-independent activities of ROBO receptors in HCC. Additionally, *SLIT2* and *ROBO1* were both upregulated in HCCs with advanced stages and poor differentiation status (Figure 3 and Table 3). These findings also are in agreement with the expression of SLITs specifically in poorly differentiated HCCs [19]. Furthermore, in a tumor xenograft model, SLIT2-ROBO1 signaling was shown to

have a role in angiogenesis, which supports tumor growth and metastasis [13].

Although *ROBO4* was shown to be specific to vasculature [11], we observed varying levels of *ROBO4* transcript in HCC cell lines. The *ROBO4* transcripts in these cells might be partly explained by the presence of side population cells with stem cell characteristics that express markers of the vascular endothelium [33]. One may also consider a possible regulation of *ROBO4* expression in liver tumors. In tissue expression analyses, we found that *ROBO4*

Table 3: Differentially expressed *SLIT-ROBO* genes between histopathological subgroups of liver tissues

Stage	n	Mean expression (SE)	Differentially expressed gene*
0	8	0 (0.22)	<i>ROBO1</i>
2	11	2.37 (0.41)	
0	8	0 (0.22)	<i>ROBO1</i>
3	13	1.43 (0.57)	
1	9	0.79 (0.33)	<i>ROBO1</i>
2	11	2.37 (0.41)	
2	11	-2.29 (1.15)	<i>SLIT2</i>
3	13	-0.96 (0.44)	
Differentiation state	n	Mean expression (SE)	Differentially expressed gene*
Normal	8	0 (0.22)	<i>ROBO1</i>
Moderate	16	1.89 (0.44)	
Normal	8	0 (0.22)	<i>ROBO1</i>
Poor	6	1.84 (0.60)	
Normal	8	0 (0.36)	<i>ROBO4</i>
Poor	6	-2.02 (0.80)	
Well	8	-0.60 (0.43)	<i>ROBO4</i>
Poor	6	-2.02 (0.80)	
Moderate	16	-0.66 (0.25)	<i>ROBO4</i>
Poor	6	-2.02 (0.80)	
Moderate	16	-1.28 (0.78)	<i>SLIT2</i>
Poor	6	1.01 (0.88)	

* Pairs of stage and differentiation subgroups were compared in Fisher's pairwise comparison analysis and only the genes that significantly discriminate between subgroups were represented ($p < 0.05$). SE: standard error.

expression is significantly down-regulated in poorly differentiated tumors, indicating that *ROBO4* function is not essential for the maintenance of tumor at this step of hepatocarcinogenesis. In fact, recent findings indicated that *SLIT2-ROBO4* interactions inhibited angiogenesis [34].

It is very likely that *SLIT2-ROBO1-ROBO4* might contribute to some of the variability associated with the differentiation status of HCC while expression of *ROBO1* and *SLIT2* also helps explain the stage differences in this cancer. However, the expression variability is high among liver tumors suggesting that a combinatorial code with a possibility of ligand redundancy might be at work in hepatocellular carcinoma, which prompts further functional studies that include knock-down and overexpression.

A global gene expression analysis by microarray technology in 19 HCC cell lines revealed two molecular subtypes depending on their *AFP* expression level [31]. Of 14 HCC cell lines that we studied, 13 were included in that study and the *SLIT-ROBO* dependent subgrouping in our analysis was parallel to the *AFP* subgrouping previously observed, verifying the reliability of our cell line panel. *ROBO1* and *SLIT3* were the genes that were most significantly correlated with *AFP* expression in a positive and negative manner, respectively. Genes regulating extracellular matrix establishment or remodeling and cell adhesion were shown to be differentially expressed between the HCC cell line subgroups [31]. Cells that were defined to be more metastatic and motile correspond to cell lines that cluster as Group I in our study. Given the important roles of *SLIT-ROBO* associated signaling molecules like *ENA*, *ABL*, and several GTPase activating proteins in

cytoskeletal reorganization and cell motility [35,36], the connection between SLIT-ROBO signaling and HCC tumor cell invasion and metastasis remains to be further described.

Conclusion

Here, we defined the overexpression of *ROBO1* and the variable expression of other *SLIT-ROBO* family members in HCC. Especially, downregulation of *ROBO4* and upregulation of *SLIT2* mark late stage and poorly differentiated HCCs. We have also shown that the collective expression of these genes occurs in a coordinated fashion in two main groups suggesting that SLIT-ROBO signaling is modular in nature, and that each module shows intrinsic variability. Our results help increase our understanding of pathological expression pattern of *SLIT-ROBO* family in HCC with potential for diagnostic applications. Elucidation of the mechanisms acting on the transcriptional regulation of SLIT-ROBO signaling pathway, such as alternative splicing, copy number variability and ligand/receptor redundancy in both HCC and other pathophysiological contexts will contribute to a better understanding of hepatocarcinogenesis.

Abbreviations

HCC: hepatocellular carcinoma; *ROBO*: roundabout; *AFP*: alpha-fetoprotein; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; *ACTB*: beta-actin.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MEA designed the primers, performed RT-PCR and real-time PCR experiments, and contributed to statistical analyses. OK participated in the design of the study, supervised the statistical analyses, and helped draft the manuscript. TY designed and coordinated the study, and finalized the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Clinicopathological characteristics of normal liver and HCC samples. This table gives Diagnosis/Histology, stage and differentiation information for normal and tumor tissues used in the study.

Click here for file

[<http://www.biomedcentral.com/content/supplementary/1471-2407-8-392-S1.pdf>]

Additional file 2

SLIT-ROBO genes are expressed in HCC cell lines at varying levels. Agarose gel electrophoresis image of SLIT-ROBO transcripts in 14 HCC cell lines after 40 cycles of RT-PCR amplification.

Click here for file

[<http://www.biomedcentral.com/content/supplementary/1471-2407-8-392-S2.tiff>]

Additional file 3

Relative expression of SLIT-ROBO and AFP genes in HCC cell lines. This table lists the $\Delta\Delta C_t$ values in log2 base for SLIT-ROBO and AFP transcripts in 14 HCC cell lines used in the study.

Click here for file

[<http://www.biomedcentral.com/content/supplementary/1471-2407-8-392-S3.pdf>]

Additional file 4

Relative expression of SLIT-ROBO and AFP genes in normal liver and HCC samples. This table lists the $\Delta\Delta C_t$ values in log2 base for SLIT-ROBO and AFP transcripts in 8 normal liver and 35 HCC samples used in the study.

Click here for file

[<http://www.biomedcentral.com/content/supplementary/1471-2407-8-392-S4.pdf>]

Acknowledgements

The authors are grateful to Dr. E. Oztas for his help in image processing. This work was supported by grant 104S243 from the Scientific and Technological Research Council of Turkey (TUBITAK), and funds from Bilkent University.

References

- Dickson BJ, Gilestro GF: **Regulation of commissural axon path-finding by slit and its Robo receptors.** *Annu Rev Cell Dev Biol* 2006, **22**:651-675.
- Kidd T, Bland KS, Goodman CS: **Slit is the midline repellent for the robo receptor in Drosophila.** *Cell* 1999, **96**:785-794.
- Brose K, Bland KS, Wang KH, Arnott D, Henzel W, Goodman CS, Tessier-Lavigne M, Kidd T: **Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance.** *Cell* 1999, **96**:795-806.
- Brose K, Tessier-Lavigne M: **Slit proteins: key regulators of axon guidance, axonal branching, and cell migration.** *Curr Opin Neurobiol* 2000, **10**:95-102.
- Huminiecki L, Gorn M, Suchting S, Poulsom R, Bicknell R: **Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis.** *Genomics* 2002, **79**:547-552.
- Xian J, Clark KJ, Fordham R, Pannell R, Rabbitts TH, Rabbitts PH: **Inadequate lung development and bronchial hyperplasia in mice with a targeted deletion in the Dutt1/Robo1 gene.** *Proc Natl Acad Sci USA* 2001, **98**:15062-15066.
- Grieshammer U, Le M, Plump AS, Wang F, Tessier-Lavigne M, Martin GR: **SLIT2-mediated ROBO2 signaling restricts kidney induction to a single site.** *Dev Cell* 2004, **6**:709-717.
- Wu JY, Feng L, Park HT, Havlioglu N, Wen L, Tang H, Bacon KB, Jiang Z, Zhang X, Rao Y: **The neuronal repellent Slit inhibits leukocyte chemotaxis induced by chemotactic factors.** *Nature* 2001, **410**:948-952.
- Prasad A, Qamri Z, Wu J, Ganju RK: **Slit-2/Robo-1 modulates the CXCL12/CXCR4-induced chemotaxis of T cells.** *J Leukoc Biol* 2007, **82**:465-476.
- Guan H, Zu G, Xie Y, Tang H, Johnson M, Xu X, Kevil C, Xiong WC, Elmetts C, Rao Y, Wu JY, Xu H: **Neuronal repellent Slit2 inhibits**

- dendritic cell migration and the development of immune responses. *J Immunol* 2003, **171**:6519-6526.
11. Park KW, Morrison CM, Sorensen LK, Jones CA, Rao Y, Chien CB, Wu JY, Urness LD, Li DY: **Robo4 is a vascular-specific receptor that inhibits endothelial migration.** *Dev Biol* 2003, **261**:251-267.
 12. Schmid BC, Reznicek GA, Fabjani G, Yoneda T, Leodolter S, Zeillinger R: **The neuronal guidance cue Slit2 induces targeted migration and may play a role in brain metastasis of breast cancer cells.** *Breast Cancer Res Treat* 2007, **106**:333-342.
 13. Wang B, Xiao Y, Ding BB, Zhang N, Yuan X, Gui L, Qian KX, Duan S, Chen Z, Rao Y, Geng JG: **Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity.** *Cancer Cell* 2003, **4**:19-29.
 14. Dallol A, Da Silva NF, Viacava P, Minna JD, Bieche I, Maher ER, Latif F: **SLIT2, a human homologue of the Drosophila Slit2 gene, has tumor suppressor activity and is frequently inactivated in lung and breast cancers.** *Cancer Res* 2002, **62**:5874-5880.
 15. Dallol A, Morton D, Maher ER, Latif F: **SLIT2 axon guidance molecule is frequently inactivated in colorectal cancer and suppresses growth of colorectal carcinoma cells.** *Cancer Res* 2003, **63**:1054-1058.
 16. Dallol A, Krex D, Hesson L, Eng C, Maher ER, Latif F: **Frequent epigenetic inactivation of the SLIT2 gene in gliomas.** *Oncogene* 2003, **22**:4611-4616.
 17. Dickinson RE, Dallol A, Bieche I, Krex D, Morton D, Maher ER, Latif F: **Epigenetic inactivation of SLIT3 and SLIT1 genes in human cancers.** *Br J Cancer* 2004, **91**:2071-2078.
 18. Latif A, Chene L, Cochant-Priollet B, Mangin P, Fournier G, Berthon P, Cussenot O: **Quantification of expression of netrins, slits and their receptors in human prostate tumors.** *Int J Cancer* 2003, **103**:306-315.
 19. Ito H, Funahashi S, Yamauchi N, Shibahara J, Midorikawa Y, Kawai S, Kinoshita Y, Watanabe A, Hippo Y, Ohtomo T, Iwanari H, Nakajima A, Makuuchi M, Fukayama M, Hirata Y, Hamakubo T, Kodama T, Tsuchiya M, Aburatani H: **Identification of ROBO1 as a novel hepatocellular carcinoma antigen and a potential therapeutic and diagnostic target.** *Clin Cancer Res* 2006, **12**:3257-3264.
 20. Prasad A, Fernandis AZ, Rao Y, Ganju RK: **Slit protein-mediated inhibition of CXCR4-induced chemotactic and chemoinvasive signaling pathways in breast cancer cells.** *J Biol Chem* 2004, **279**:9115-9124.
 21. Seth P, Lin Y, Hanai J, Shivalingappa V, Duyao MP, Sukhatme VP: **Magic roundabout, a tumor endothelial marker: expression and signaling.** *Biochem Biophys Res Commun* 2005, **332**:533-541.
 22. Xian J, Aitchison A, Bobrow L, Corbett G, Pannell R, Rabbitts T, Rabbitts P: **Targeted disruption of the 3p12 gene, Dutt1/Robo1, predisposes mice to lung adenocarcinomas and lymphomas with methylation of the gene promoter.** *Cancer Res* 2004, **64**:6432-6437.
 23. Midorikawa Y, Makuuchi M, Tang W, Aburatani H: **Microarray-based analysis for hepatocellular carcinoma: from gene expression profiling to new challenges.** *World J Gastroenterol* 2007, **13**:1487-1492.
 24. Midorikawa Y, Yamamoto S, Ishikawa S, Kamimura N, Igarashi H, Sugimura H, Makuuchi M, Aburatani H: **Molecular karyotyping of human hepatocellular carcinoma using single-nucleotide polymorphism arrays.** *Oncogene* 2006, **25**:5581-5590.
 25. Celikkaya H, Ciraci C, Oztas E, Avci ME, Ozturk M, Yagci T: **Immunization with UV-induced apoptotic cells generates monoclonal antibodies against proteins differentially expressed in hepatocellular carcinoma cell lines.** *Hybridoma (Larchmt)* 2007, **26**:55-61.
 26. Rozen S, Skaletsky H: **Primer3 on the WWW for general users and for biologist programmers.** *Methods Mol Biol* 2000, **132**:365-386.
 27. Sayan AE, Sayan BS, Findikli N, Ozturk M: **Acquired expression of transcriptionally active p73 in hepatocellular carcinoma cells.** *Oncogene* 2001, **20**:5111-5117.
 28. Pfaffl MW: **A new mathematical model for relative quantification in real-time RT-PCR.** *Nucleic Acids Res* 2001, **29**:e45.
 29. R Development Core Team: **R: A Language and Environment for Statistical Computing.** R Foundation for Statistical Computing Vienna, Austria ISBN 3-900051-07-0 2005.
 30. Eisen MB, Spellman PT, Brown PO, Botstein D: **Cluster analysis and display of genome-wide expression patterns.** *Proc Natl Acad Sci USA* 1998, **95**:14863-14868.
 31. Lee JS, Thorgeirsson SS: **Functional and genomic implications of global gene expression profiles in cell lines from human hepatocellular cancer.** *Hepatology* 2002, **35**:1134-1143.
 32. Bosch FX, Ribes J, Diaz M, Cleries R: **Primary liver cancer: worldwide incidence and trends.** *Gastroenterology* 2004, **127**:S5-S16.
 33. Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, Zheng BJ, Guan XY: **Identification and characterization of tumorigenic liver cancer stem/progenitor cells.** *Gastroenterology* 2007, **132**:2542-2556.
 34. Jones CA, London NR, Chen H, Park KW, Sauvaget D, Stockton RA, Wythe JD, Suh W, Larrieu-Lahargue F, Mukoyama YS, Lindblom P, Seth P, Frias A, Nishiya N, Ginsberg MH, Gerhardt H, Zhang K, Li DY: **Robo4 stabilizes the vascular network by inhibiting pathologic angiogenesis and endothelial hyperpermeability.** *Nat Med* 2008, **14**:448-453.
 35. Bashaw GJ, Kidd T, Murray D, Pawson T, Goodman CS: **Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor.** *Cell* 2000, **101**:703-715.
 36. Wong K, Ren XR, Huang YZ, Xie Y, Liu G, Saito H, Tang H, Wen L, Brady-Kalnay SM, Mei L, Wu JY, Xiong WC, Rao Y: **Signal transduction in neuronal migration: roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway.** *Cell* 2001, **107**:209-221.

Pre-publication history

The pre-publication history for this paper can be accessed here:

<http://www.biomedcentral.com/1471-2407/8/392/prepub>

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

