Supplementary Data

Loss of SLIT2 Causes β-catenin/E-cadherin Deregulation and is Associated with Poor Prognosis of Lung Cancer

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Materials and Methods

Clinical characterization of patients and DNA/RNA preparations

Paired tumor and normal lung tissues were obtained from 92 NSCLC patients recruited at the Taipei Veterans General Hospital between 2002 and 2004 after appropriate institutional review board permission and informed consent from patients were obtained. Overall survival was calculated from the day of surgery to the date of death or the last follow-up. Disease-free survival was calculated from the day of surgery to the date of recurrence, either locoregional or distant. The metastasis group was defined as when the metastases were noted at least 1 year after resection of the primary lung cancer. The mean follow-up period was 44.2 months (range: 1-74 months) for the 87 patients for whom overall survival data was available. A total of 18 (21%) of these patients relapsed, with the follow-up period of 28.1 months (range: 1-54 months) for disease-free survival analysis. For methylation assay, genomic DNA

from primary lung tumor tissues was prepared using proteinase K digestion and phenol–chloroform extraction. For RNA expression assay, total RNA was prepared from paired tumor lung and normal lung tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using SuperScript^{TM} reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

Immunohistochemical analysis

Paraffin blocks of tumors were sectioned into 5 *u*m slices and processed using standard deparaffinization and rehydration techniques. Polyclonal antibodies for β-catenin (1:1500) (Transduction Laboratories, Lexington, KY), βTrCP (1:800) (Santa Cruz Biotechnology, Santa Cruz, CA), SLIT2 (1:1000) (Chemicon, Temecula, CA), and ROBO1 (1:1000) (Rockland, Philadelphia, PA) were used as the primary antibodies to detect the protein expression. In addition, staining was scored 3, 2, 1, or 0 if >70%, 36-70%, 5-35%, or <5%, respectively, of tumor cell nuclei or cytoplasm stained positive for βTrCP. A score of 1 or 0 indicated the presence of little or no SLIT2, ROBO1, and βTrCP. Staining detected at >60% in cell nuclei and cytoplasm indicated β-catenin accumulation. The samples from positive- and negative-expression were processed together in batches, without knowledge of which were positive and negative from the previous assay, to eliminate the possibility of information bias. Each sample was assayed by repeating analyses three times.

Immunoprecipitation assay

Catch and Release Reversible Immuonprecipitation System kit (Upstate Chemicon, Temecula, CA) was used for protein-protein interaction analysis. One mg cell protein lysate was incubated with anti-E-cadherin, anti-β-catenin or normal mouse-IgG, and 10 µl affinity ligand was added, then 1× wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM α-glycerol-phosphate, 1% NP-40, 5 mM EDTA) was added to a final volume of 500 µl. After incubation at 4^{o}C overnight, immune complexes were washed thrice with wash buffer. Proteins were eluted by boiling in 4× sample buffer, separated by 8% SDS-PAGE, then blotted with appropriate antibodies.

Western blot analysis

Cells were lysed on ice using RIPA buffer (0.05M Tris-HCl, pH 7.4, 0.15M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, and 10 µg/ml aprotinin). Lysates were centrifuged at 13,000 rpm. for 10 min at 4°C, SDS gel loading buffer (60 mM Tris base, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol) was added and samples containing 50 µg of protein were separated on an 8% SDS-PAGE then electro-blotted onto Immobilon-P membranes (Millipore Co., Bedford, MA) in a transfer buffer. Immunoblotting was performed using antibodies against β-catenin (1:800), βTrCP

(1:500), SLIT2 (1:500), ROBO1 (1:500), AKT (1:800) (Cell Signaling, Danvers, MA), phospho-AKT (1:800) (Cell Signaling), GSK3β (1:800) (Cell Signaling), phospho-GSK3β (1:800) (Abcam Ltd., Cambridge, UK), E-cadherin (1:200) (Cell Signaling) and SNAI1 (1:500) (Santa Cruz Biotechnology). β-actin (1:5000, Abcam Ltd.) was used as loading control.

mRNA expression analysis

Primers for reverse-transcriptase polymerase chain reaction (RT–PCR) analysis are listed in **Supplementary Table 1**. Reactions were carried out in a volume of 25 μL solution with 1 μL of cDNA and 0.25 *p*mol of primers in a DNA Thermal Cycler. Tumor cells expressing *SLIT2* and *ROBO1* mRNA were normalized with *GAPDH* as the internal control. Those expressing levels <50% that of normal cells were deemed to have an abnormal pattern. Samples showing altered expression were assayed by repeating analyses three times.

Methylation-Specific PCR (MSP) assay

Primers for the MSP assay are listed in **Supplementary Table 1**. Positive control samples with unmethylated DNA from IMR90 normal lung cell and *Sss*I methyltransferase-treated methylated DNA were included in each PCR set. Hypermethylated genes were defined as those which produced amplified of methylation

(M) products from the tumor samples. Samples showing altered methylation pattern were assayed by repeating analyses three times.

5-aza-2'-deoxycytidine (5-aza-dC) treatment of lung cancer cells

CL1-5 human lung cancer cells (with high migration ability) (kindly provided by P.C. Yang, Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan) were plated at 10^5 per 100 mm culture dish on the day before treatment. The cultures were treated three doubling times with 2 μM 5-aza-dC. The cells were then harvested for MSP, RT-PCR, Western blot, and migration assays.

Conditioned medium assay

Conditioned medium was collected from low motility CL1-0 cells (kindly provided by P.C. Yang) with SLIT2 expression. High motility CL1-5 cells were incubated with a mixture of conditioned medium and fresh medium containing 30% serum, and relative migration ability of treated cells was measured after 48 hrs. Four independent experiments were performed. The protein concentration of SLIT2 in condition medium was measured by human SLIT2 ELISA kit (Uscnlife Co., Wuhan, China).

SLIT2 protein

Human SLIT2 protein was purchased from Abcam Ltd. Cells were plated at 10^5 per 6-mm culture dish on the day before treatment. The cultures were treated for 48

hrs with 5ng/ml SLIT2 protein. The cells were then harvested for an analysis of Trnaswell assay and Western blot analysis.

Plasmid Construction

To generate pcDNA-SLIT2, the pcDNA3.1(-) and pCR-XL-TOPO-SLIT2 were purchased from Invitrogen and GENDISCOVERY (Open Biosystem, Drive Huntsville, Australia). The pCR-XL-TOPO-SLIT2 was digested with XhoI/KpnI and subcloned into XhoI/KpnI-digested pcDNA3.1(-) to generate pcDNA-SLIT2.

Knockdown or ectopically expressed SLIT2 and knockdown AKT analysis

We used pGIPZ lentiviral vetor (empty vector without an shRNA insert)-mediated shRNA-*SLIT2* (Open Biosystem, Drive Huntsville, Australia) to generate knockdown clones for the *SLIT2* gene. The siRNA-*AKT* was obtained from Invitrogen. Generation of the pcDNA-*SLIT2* construct is described in the supplemental data. CL1-5 and CL1-0 cells (1×10^5) were transfected with 5 μg of shRNA-*SLIT2*, siRNA-*AKT* or pcDNA-*SLIT2* using ExGen 500 transfection reagent (Fermentas, Flamborough, Ontario, Canada) as recommended by the manufacturer. After 24-48 hours, cells were confirmed by RT-PCR, Western blot, and migration assays.

Transwell migration assay

The transwell migration assay was performed to determine the migratory ability of

shRNA-SLIT2 transfected cells and tumor cells treated with 5-Aza-dC, purified SLIT2 and ectopically expressed SLIT2. The transwell (Falcon, BD Labware, Bedford, MA) consists of upper and lower chambers separated by a layer of millipore membrane with pore size of 8μm. Cells with high migration capacity seeded in the upper chamber can migrate through the membrane to the lower chamber. $1x10^5$ of 5-Aza-dC treated cells, $1x10^6$ of shRNA-*SLIT2* transfected cells were seeded onto the upper chamber of the transwell with serum free DMEM medium and the lower chamber containing 10% FBS/DMEM attractant medium. After incubation for 24 hours (5-Aza-dC), or 24 hours (shRNA-*SLIT2* knockdown), or 48 hours (purified SLIT2), cells attached to the reverse phase of the membrane were stained by crystal violet or observed for cell with fluorescent and counted under microscope in 10 randomly selected fields. The experiment was carried out four times to reduce the possible effects of biological variability.

Wound healing assay

SLIT2 knock-down CL1-0 cells and control cells were then plated at a density of 1×10^6 cells in 100-mm culture dish and incubated at 37ºC. The following day, cells were cultured in fresh medium with 10% serum. A scratch in the form of a lane was made through the confluent monolayers with a plastic pipette tip. Photograph the views for observation the cell migration ability at 24 hours after scratch. In ectopically

expressed *SLIT2*, we performed the wound healing by Culture-Insert (Ibidi, D-82152 Martinsried, Germany). A cell-free gap of 500 μm was created after removing the Culture-Insert. We determined the area of cell-free gap by ImageJ. The migrated cells in to the wound area were calculated as (24 hr area×500 μm)/0 hr area. Migration Area = 500 μm - (24 hr area×500 μm)/0 hr area. Four independent experiments were performed.

Cell-extracellular matrix adhesion assay

The 96-well microplate will be pre-coated with extracellular matrix (ECM) proteins (ex: collagen type I, 4μg/well; collagen type IV, 1ug/well; fibronectin, 1ug/well; laminin, 1ug/well; vitronectin, 1ug/well; or Matrigel^{TM}, 40x dilution) (Sigma Chemical Co, Louis, MD) then blocked by 1% bovine serum albumin at 4^{o}C. The 5x10^{4} cells/well *SLIT2* knock-down CL1-0 cells or control cells will be seeded onto pre-coated wells after 2 hours recover in DMEM medium containing 20% FBS at 37^{o}C incubator with rotation. Then the seeded cells will be incubated for 30 minutes at 37^{o}C with 5% CO_2. Unattached cells will be removed by PBS wash for trice. Attached cells will be fixed and stained with 1% crystal violet/MeOH for 10 minutes at room temperature then lysed by DMSO. The absorbance at 590 nm was correlated to the number of cells attached toward coated ECM proteins onto the wells. The relative adhesion was measured by comparing the averaged attached cells in the

shRNA-*SLIT2* cells to that in the neo control cells. The experiment was carried out four times to reduce the possible effects of biological variability.

Cell growth assay

Cells (1×10^4), seeded on 24 well plates, were cultured in DMEM supplemented with 10% FBS. The number of viable cells was quantified at various time points. The experiment was carried out three times to reduce the possible effects of biological variability.

Detection of Apoptosis

TUNEL activity was detected in CL1-5 cells with or without 5-aza-dC treatment and CL1-0 cells transfected shRNA-*SLIT2* or empty vector using TumorTACS *In Situ* Apoptosis Detection kit (Trevigen, Gaithersburg, MA) according to the manufacturer's instruction.

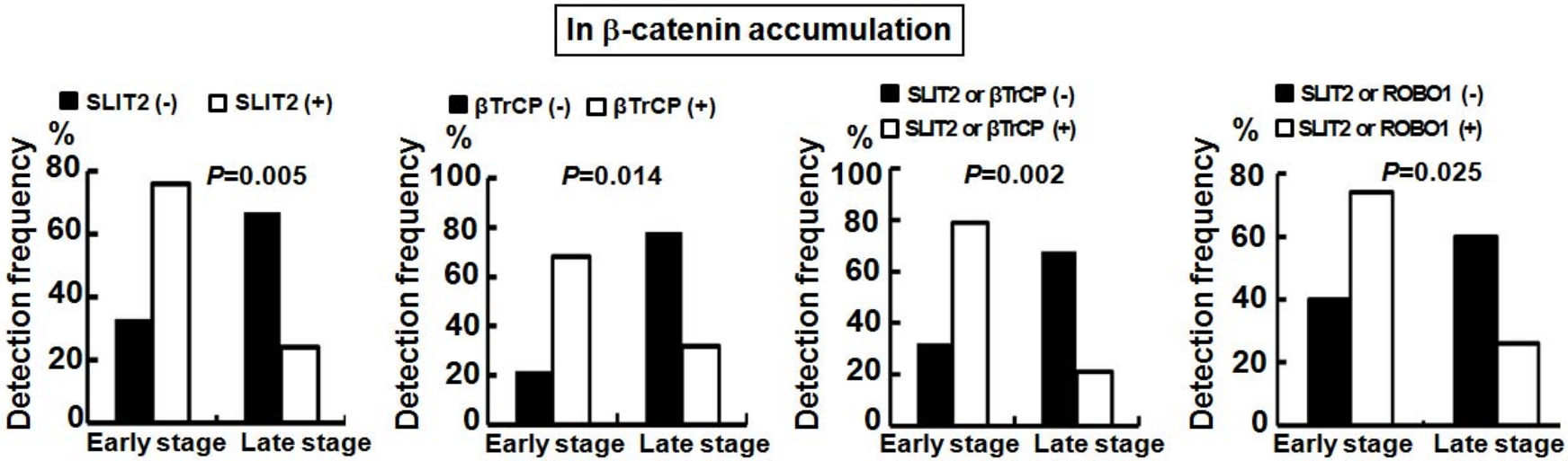
Analysis of cell cycle distribution

Adherent and floating cells were collected from CL1-5 cells with or without 5-aza-dC treatment and CL1-0 cells transfected shRNA-*SLIT2* or empty vector, wash once with PBS, and fixed with ice-cold 80% ethanol for at least overnight at -20^{o}C until analysis. Fixed cells were collected by centrifugation and wash once with PBS to discard ethanol. Cells were resuspended in 1 ml of PBS mixture (contain 20 μg/ml propidium

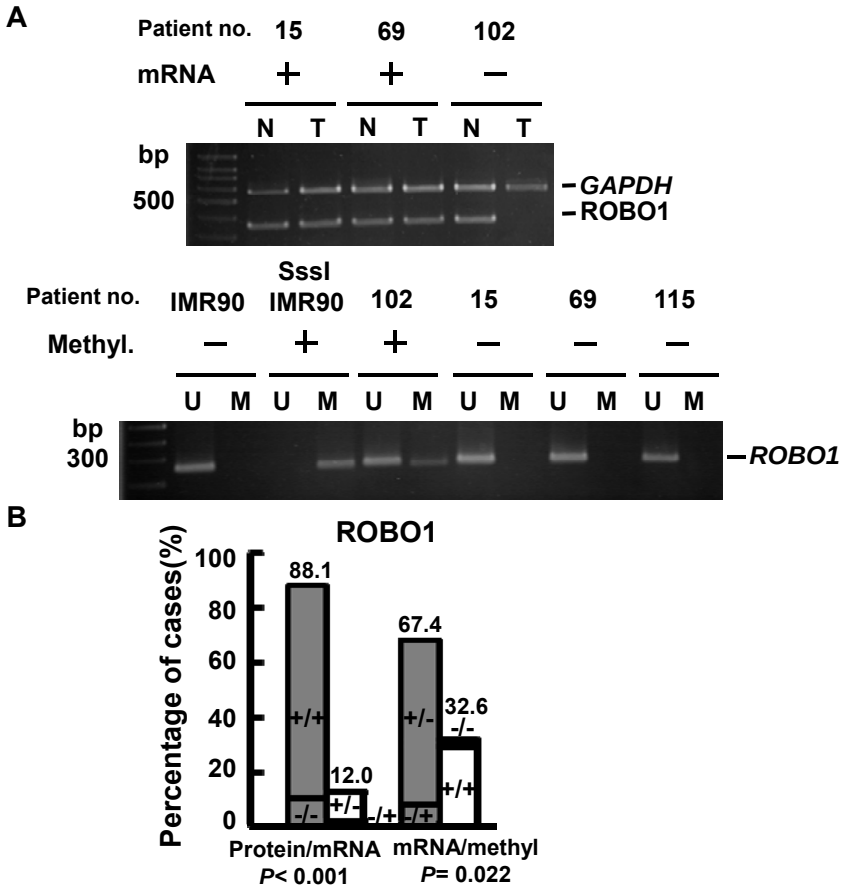
iodide (Sigma) 200 μg/ml RNase A, and 1 μl Triton X-100, preheated at 37^{o}C for 20 minutes) and then incubated at 37^{o}C in the dark for 15 minutes. Determination of cell cycle distribution was performed by FACScan Flow cytometer (BD, Franklin Lakes, NJ) and calculated using ModFIT LT 2.0 version software (BD).

Statistical analysis

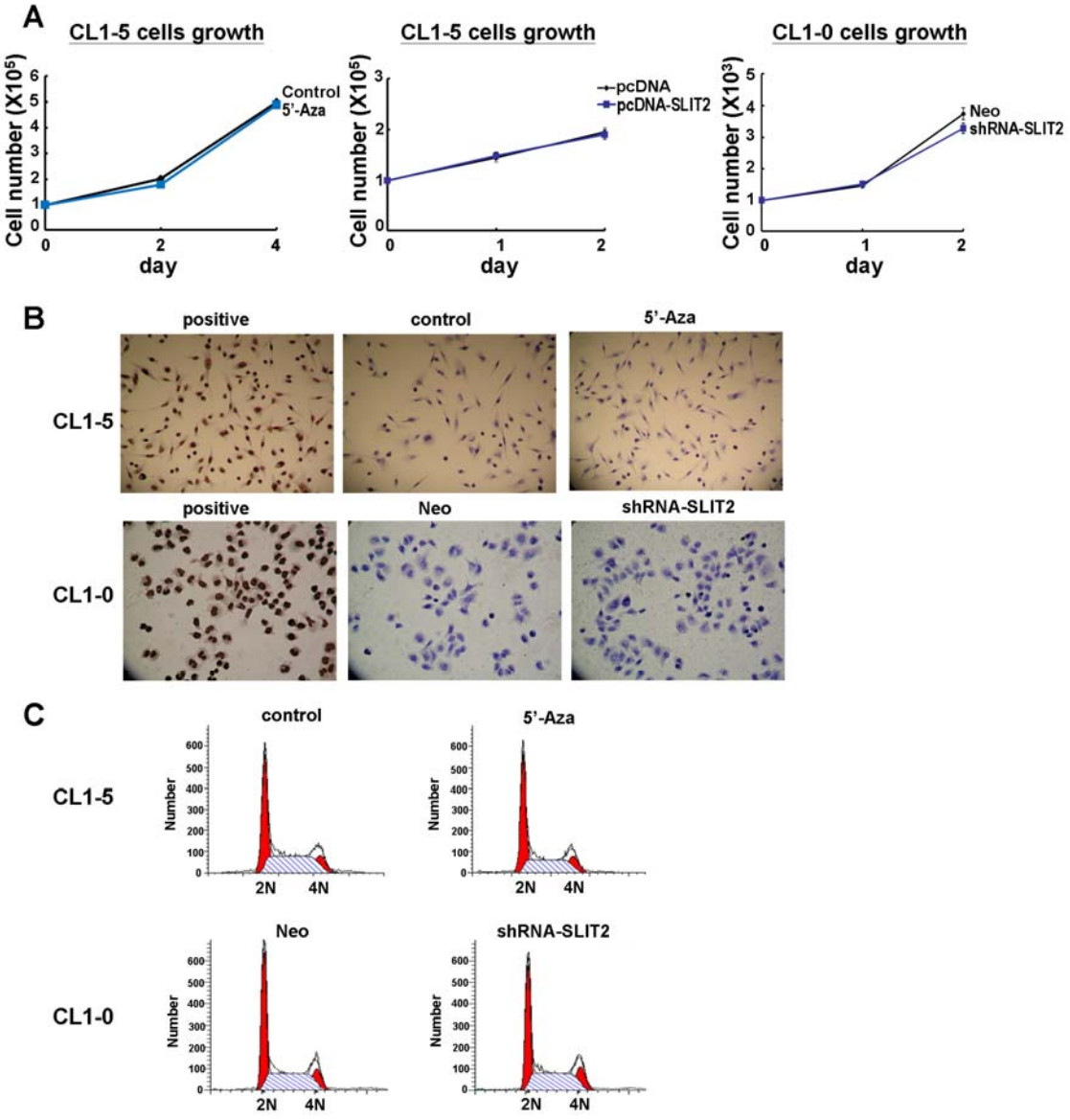
Pearson's χ^2 test was used to compare frequency of protein alterations in NSCLC patients at different disease stages. Type III censoring was performed on subjects who were still alive or without cancer recurrence at the end of the study. Overall survival curves and disease-free survival curves were calculated according to the Kaplan-Meier method, and comparison was performed using the log-rank test. $P \leq 0.05$ was considered statistically significant. SPSS version 13.0 (SPSS, Inc., Chicago, IL) was used for all statistical analyses.



Supplementary Figure 1. Histograms show frequency of the expression of indicated proteins in β-catenin accumulation patients with different stages. Black bar: patient group with altered expression pattern; white bar: patient group with normal expression pattern. P value for each comparison is indicated above.



Supplementary Figure 2. Epigenetic analyses of *ROBO1* in non-small cell lung cancer (NSCLC) patients. **(A)** The mRNA (by RT-PCR, upper panel) and promoter methylation (by MSP, lower panel) analyses of *ROBO1* in representative NSCLC patients with (+) and without (–) methylation expression. N: normal lung tissue; T: tumor lung tissue. The primer sets used for amplification; M: methylated genes. IMR90 is a normal lung cell line used as an unmethylated control and SssI-treated IMR90 as a methylated control. **(B)** Concordance analysis between protein, mRNA and promoter methylation for the *SLIT2* gene. Y-axis: percent of cases; X-axis: type of comparison. Positive (+) and negative (-) expression status and methylated (+) and unmethylated (-) promoter status are noted. The percentage in the concordant group (gray section) and non-concordant group (white section) is indicated above. P values are shown at the bottom.



Supplementary Figure 3. (A) The cell growth assay was performed in 5-aza-dC treatment, SLIT2 overexpression or *SLIT2* knock-down analysis. **(B)** TUNEL assay confirms the presence of apoptotic cells in CL1-5 treated or not with 5-aza-dC and CL1-0 transfected with shRNA-*SLIT2* or empty vector. TUNEL-positive cells have dark brownish nuclear staining, whereas the nuclei of TUNEL negative cells stain blue. "Positive" indicated the nuclease–treated control cells. It confirms that the permeabilization and labeling reaction have worked. **(C)** Flow cytometry assay indicates that cells treated with 5-aza-dC or shRNA-*SLIT2* did not affect cell cycle distribution.

Supplementary Table 1. List of primer sequences and their reaction conditions used in the present study.

Gene	Primer	5'→3' sequences	PCR size (bp)	Tm (°C)
SLIT2-cDNA	Forward	GGTGTCCTCTGTGATGAAGAG	387	63
	Reverse	GTGTTTAGGACACACACCTCG		
SLIT2-methyl-U	Forward	TGGTTTAGGTTGTGGTGGAGTTGAGGGT	158	62
	Reverse	CACAAAAACCCAACAAACCCATAACAAAACACA		
SLIT2-methyl-M	Forward	CGGTTTAGGTTGCGGCGGAGTCGAGGGC	158	68
	Reverse	CGCGAAAACCCAACGAACCCGTAACAAAACGCG		
ROBO1-cDNA	Forward	ATGATTGCGGAGCCCGCTCA	362	65
	Reverse	TTGTGGCTCACAGCCTCTCCAA		
ROBO1-methyl-U	Forward	GGGTGTTTAGAAGATGTGTGAGTGTTT	246	63
	Reverse	CAACAAACCCAATCAACAACAAA		
ROBO1-methyl-M	Forward	CGTTTAGAAGACGTGCGAGTGTTC	246	63
	Reverse	GACGAACCCAATCAACAACGAA		
GAPDH-cDNA	Forward	AATCCCATCACCATCTTCCA	588	55
	Reverse	CCTGCTTCACCACCTTCTTG		