Supplementary data:

Materials and methods

RNA expression analysis

Freshly collected tissue was taken in TRIzol reagent for total RNA isolation according to the manufacturer's protocol. The cDNA synthesis was carried out in 1 μg total RNA using Random hexamer (Invitrogen, Carlsbad, USA) and Superscript III (Invitrogen). SYBR-green PCR assay was used for real-time quantitation of the candidate genes and β2-microglobulin as control. Briefly 2 μL of c-DNA was amplified in 25 μL reaction volume containing 12.5 μL of 2X SYBR-green master mix (Applied Biosystems, Foster City, USA) and 1 μL of each primer. PCR reaction was carried out for 40 cycles on an ABI prism 7500 machine (Applied Biosystems). After each run, melting curves were analysed to confirm amplification of specific transcripts. The relative level of gene expression was determined by comparative threshold cycle (Ct) method (Livak *et al.* 2001; Schmittgen *et al.* 2008).

Microdissection and DNA extraction

Cryosections (5 μm) were made from frozen tissues, representative sections were stained with hematoxylin-eosin and then examined for the tumour-rich zones. The sections representative of the above stained and marked portion were microdissected (Halon et al. 2013) under dissecting microscope (Leica MZ 16, Germany) and from these dissected portions high molecular weight DNA was isolated using phenol-chloroform (Dasgupta et al. 2002)

Methylation analysis

Methylation sensitive restriction analysis (MSRA) utilized the ability of methylation-sensitive restriction enzymes, namely *Msp*I/*Hpa*II (Roche Diagnostics, Germany) (Singh et al. 2007) to cleave sites with unmodified cytosines and not those with 5-methylcytosines. About, 100 ng of genomic DNA samples were digested overnight with methyl-sensitive restriction enzyme *Hpa*II and its methylation insensitive isoschizomer *Msp*I separately; the digested DNA was PCR amplified, run on 2% agarose gel and scored for methylation positivity. Mock digestion was done with each sample devoid of

enzyme. The 445-bp fragment of *β-3A adaptin gene (K1*) and 229-bp fragment of *RARβ2* exon-1 (*K2*) were used as digestion and integrity controls, respectively (Loginov *et al.* 2004).

The methylation data obtained by MSRA was validated by methylation-specific PCR (MSP) in 25 randomly selected primary BC lesions after bisulphite modification of the DNA (Herman et al. 1996). Briefly, 5 μg of genomic DNA was denatured with 0.2 M NaOH for 15 min at 37°C. Cytosines were sulphonated in the presence of 3.0 M sodium-bisulphfite (Sigma) and 10 mM hydroquinone (Sigma) for 16 h at 50°C. Thereafter, the DNA samples were desalted using Wizard DNA clean-up system (Promega, Madison, USA) and desulphonated in 0.3M NaOH at 37°C for 15 min. Finally, the treated DNA samples were precipitated with ethanol and resuspended in TE (pH 7.4). The modified DNA was further amplified with methylation specific/unmethylation specific (M/U) primers set (table 1) according to the standard procedure (Mitra *et al.* 2012). PCR products were analysed on 2% agarose gels, visualized under UV illumination and photographed.

Deletion analysis

For deletion analysis of microsatellite markers, a standard polymerase chain reaction (PCR) containing (γ-p32) ATP-end labelled forward primer was done in a 20 μL reaction volume. PCR products were electrophoresed on 7% denaturing polyacrylamide sequencing gel containing 8 M urea and autoradiographed. Loss of heterozygosity (LOH) was detected by densitometric scanning (Bio-Rad GS-800, USA) and the scoring of LOH and microsatellite size alterations (MA) was done as described previously (Mitra et al. 2012). LOH was scored if there was complete loss of one allele / 50% reduction in band intensities of one allele in the tumour compared to the same allele in normal. The value was calculated as ratio of band intensities of the larger to the smaller alleles in tumour divided by the same ratio in the normal sample. A LOH index >1.5 (loss of smaller allele) or <0.67 (loss of larger allele). MA was detected as a shift in one (MA-I) or both (MA-II) alleles compared to their normal alleles. MA of one allele and loss of the other was denoted as LOH+MA. In case of noninformative microsatellite markers, deletion was analysed by multiplex PCR, using (γ-P^{32})-end labelled forward primer of noninformative and a control marker (SST (3q27.3) and D4S2376 (4q31.21)). PCR products were run in

7% denaturing polyacrylamide sequencing gel containing 8 M urea and autoradiographed (Mitra *et al.* 2012). For exonic markers (EM) multiplex PCR was done using the concerned EM along with a control (SST EM at 3q27.3). The products were electrophoresed in 2% agarose gel, stained with ethidium bromide, visualized and scanned in Gel Documentation System (Bio-Rad, USA). SST was used as control locus for the noninformative markers of chr. 3 (D3S2515, D3S1604) and D4S2376 served as control locus for D4S1372.

The number of alleles at the locus of interest was evaluated from the signal intensities by densitometric scanning (Bio-Rad GS-800, USA). Deletion, amplification or retention of both alleles was calculated as follows (Mitra et al. 2012).

$$\text{Allelic number} = 2 \times \frac{\text{LOI (T)} / \text{CL (T)}}{\text{LOI (N)} / \text{CL (N)}} .$$

Where T is tumour DNA and N, normal DNA. LOI, locus of interest; CL, control locus (SST and D4S2376). The allele values of <0.6, 0.9 -1.3, >1.7 -< 5.0 and >5.0 were considered as: homozygous, hemizygous deletion, retention of both alleles and amplification of alleles, respectively.

Immunohistochemical analysis

About 3-5 μm paraffin sections of normal breast tissue and primary BC samples (n=15) were dewaxed, rehydrated and reacted overnight with primary antibodies (goat polyclonal IgG sc-16611, sc-16615, sc-1661 for SLIT2, ROBO1 and ROBO2; mouse polyclonal sc-8401 for CDC42; rabbit polyclonal sc-135641 for phosphor Serine-71 CDC42) and HRP conjugated secondary antibodies (sc-2768) from Santa Cruz Biotehcnology, USA) at a dilution of 1:100 at 4^{o}C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit antigoat sc-2768; goat antimouse IgG sc-2005); goat antirabbit IgG sc-2004) from Santa Cruz Biotechnology, USA, were added at 1:500 dilutions. The slides were developed using 3-3' diaminobenzidine (DAB) as the chromogen and counterstained with hematoxylin. The staining intensity (1, weak; 2, moderate; 3, strong) and the percentage of positive cells (<1 = 0, 1-20 = 1, 20-50 =2, 50-

80 = 3 and >80 =4) were detected by two observers independently and by combining the two scores, final evaluation of expression was done (0-2, low; 3-5, intermediate; 6-7, high) (Perrone *et al*. 2006).

Table 4. Confirmation of methylation status of BC samples by MSP.

Sample number	MSRA result			MSP result		
	SLIT2	*ROBO1*	*ROBO2*	*SLIT2*	*ROBO1*	*ROBO2*
#1553	+	+	--	+	+	--
#215	+	--	--	+	--	--
#3332	+	+	--	+	+	--
#2804	+	--	--	+	--	--
#5971	--	--	--	--	--	--
#5099	+	+	+	+	+	+
#933	+	--	--	+	--	--
#5965	--	--	--	--	--	--
#2417	+	--	--	+	--	--
#1809	+	--	--	+	--	--
#1830	--	--	--	--	--	--
#2529	--	--	--	--	--	--
#6447	--	+	--	--	+	--
#3368	+	+	--	+	+	--
#3885	--	+	+	--	+	+

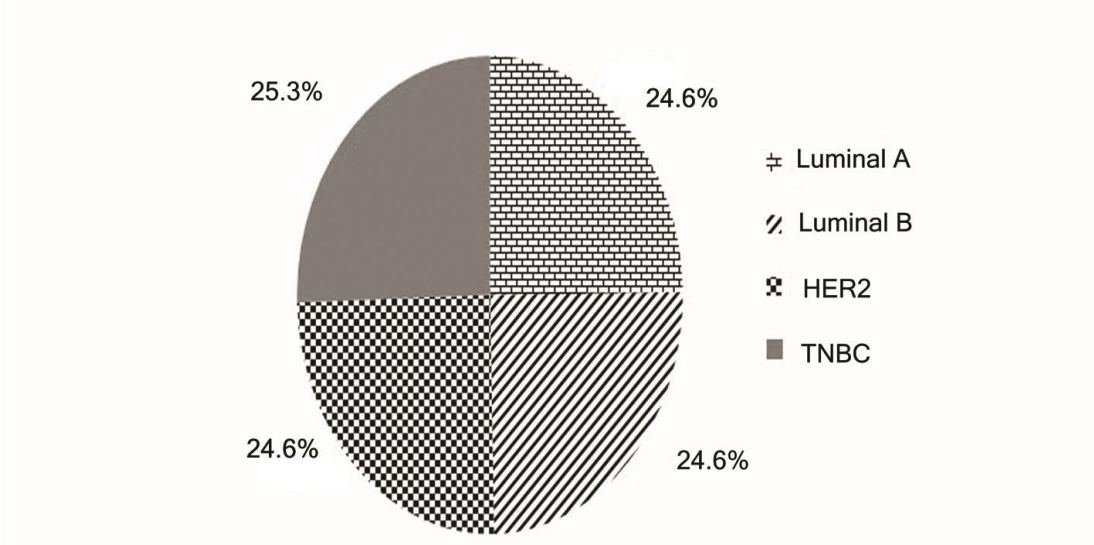

Figure 1. Subtype specific distribution of the BC samples under study.

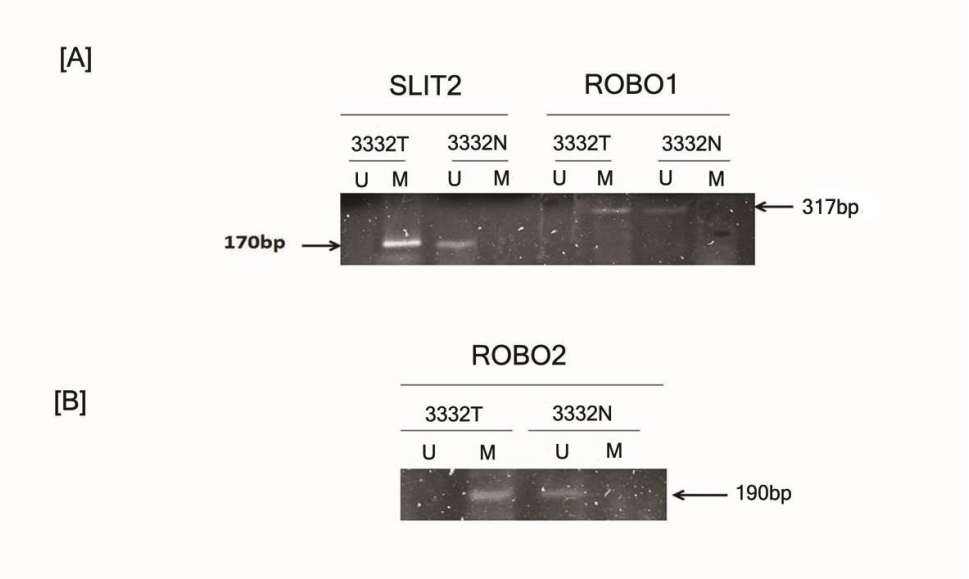

Figure 2. Representative tumour samples showing methylation status of the candidate genes by MSP. U, amplicons obtained with primer for bisulphite modified unmethylated DNA; M, amplicons obtained with primer for bisulphite modified methylated DNA; T, tumor DNA; N, DNA from corresponding normal tissue.

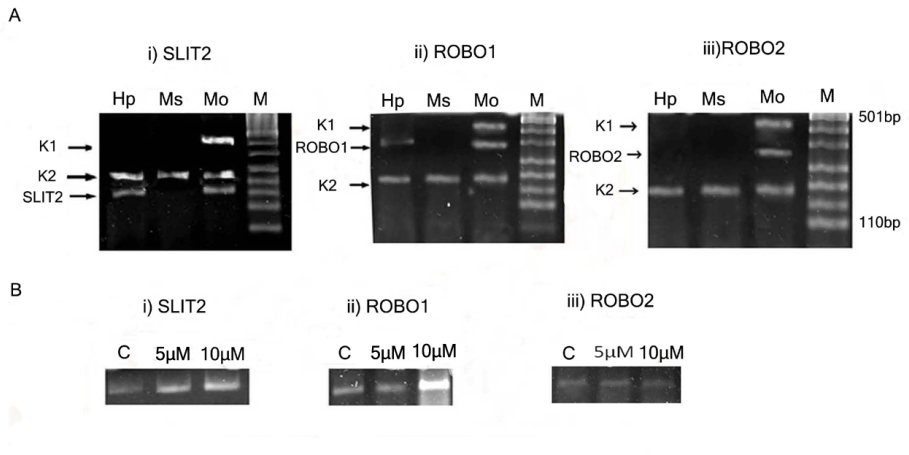

Figure 3. Analysis of promoter methylation of *SLIT2, ROBO1* and *ROBO2* by MSRA of MCF7: A (i,ii,iii); M, MW marker: *Hpa*II digested pUC19; size range 34-501 bp. Ms, *Msp*I digested, Hp: *Hpa*II digested; Mo, Mock Digested PCR product. B, 5-aza-DC mediated restoration of RNA expression of *SLIT2* (i) *ROBO1* (ii) and *ROBO2* (iii) genes in MCF7 as observed in RT-PCR.

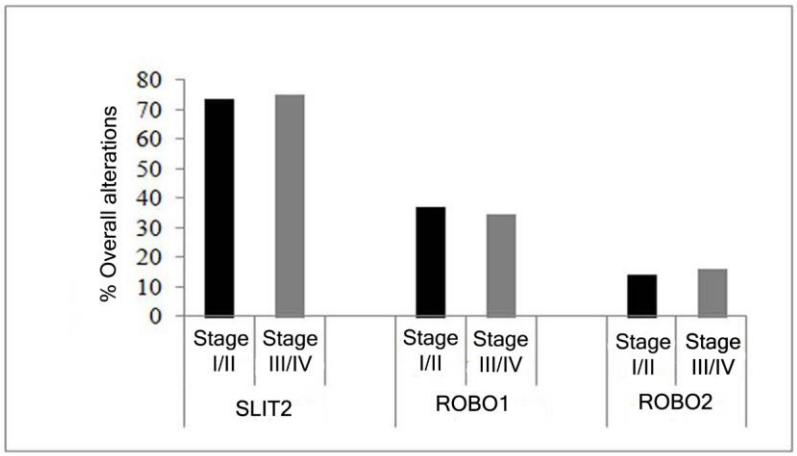

Figure 4. Overall alterations of *SLIT2* and *ROBO1* along disease progression.

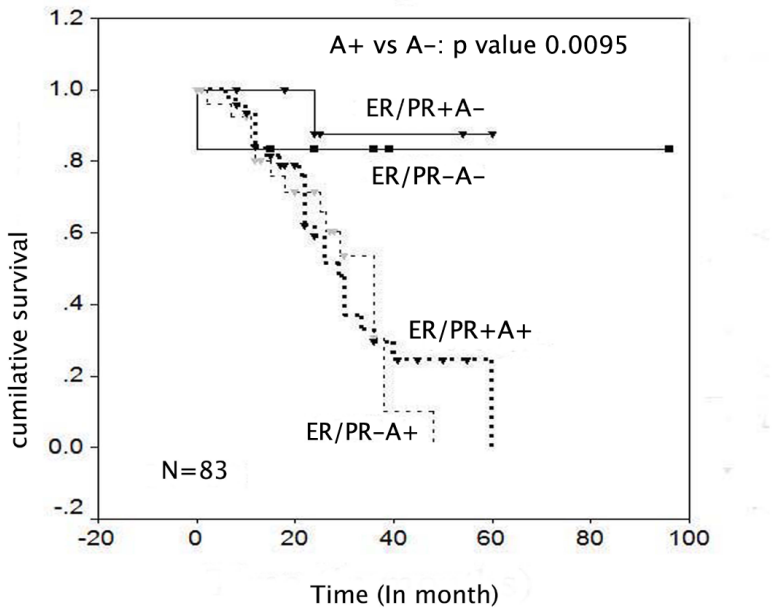

Figure 5. Survival of BC patients with/ ithout alteration of SLIT2, ROBO1 and/or ROBO2 stratifi ed for oestrogen receptor/progesterone receptor (ER/PR) status. A, alteration; N, sample size. +, alterations and/or ER/PR positive; -, alterations and/or ER/PR negative.