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RESEARCH ARTICLE

Frequent alterations of SLIT2–ROBO1–CDC42 signalling pathway in breast cancer: clinicopathological correlation

RITTWIKA BHATTACHARYA[1], NUPUR MUKHERJEE[1], HEMANTIKA DASGUPTA[1], MD. SAIMUL ISLAM[1], NEYAZ ALAM[2], ANUP ROY[3], PRIYOBRATA DAS[4], SUSANTA ROYCHOUDHURY[5] and CHINMAY KUMAR PANDA[1]*

[1]*Department of Oncogene Regulation, Chittaranjan National Cancer Institute, 37, S.P. Mukherjee Road, Kolkata 700 026, India*
[2]*Department of Surgical Oncology, Chittaranjan National Cancer Institute, Kolkata 700 026, India*
[3]*Department of Pathology, North Bengal Medical College and Hospital, 734 012, Siliguri, India*
[4]*Netaji Subhash Chandra Bose Cancer Research Institute, Kolkata 700 016, India*
[5]*Molecular and Human Genetics Division, Indian Institute of Chemical Biology, Kolkata 700 032, India*

Abstract

The aim of the study was to understand the role of SLIT2–ROBO1/2–CDC42 signalling pathways in development of breast cancer (BC). Primary BC samples ($n = 150$), comprising of almost equal proportion of four subtypes were tested for molecular alterations of SLIT2, ROBO1, ROBO2 and CDC42, the key regulator genes of this pathway. Deletion and methylation frequencies of the candidate genes were seen in the following order: deletion, *SLIT2* (38.6%) > *ROBO1* (30%) > *ROBO2* (7.3%); methylation, *SLIT2* (63.3%) > *ROBO1* (26.6%) > *ROBO2* (9.3%). Majority (80%, 120/150) of the tumours showed alterations (deletion/methylation) in at least one of the candidate genes. Overall, alterations of the candidate genes were as follows: SLIT2, 75.3% (101/150); ROBO1, 45.3% (68/150); ROBO2, 15.3% (23/150). Significantly, higher alteration of SLIT2 locus was observed in triple negative breast cancer (TNBC) over HER2 subtype ($P = 0.0014$). Similar trend is also seen in overall alterations of SLIT2 and/or ROBO1, in TNBC than HER2 subtype ($P = 0.0012$); of SLIT2 and/or ROBO2 in TNBC than luminal A ($P = 0.014$) and HER2 subtype ($P = 0.048$). Immunohistochemical analysis of SLIT2, ROBO1/2 showed reduced expression, concordant with their molecular alterations. Also, high expression of total CDC42 (49/52; 94.2%) and reduced expression of phospho Serine-71 CDC42 (41/52; 78.8%) was observed. Coalterations of SLIT2 and/or ROBO1, SLIT2 and/or ROBO2 had significant association with reduced expression of phospho Serine-71 CDC42 ($P = 0.0012$–0.0038). Alterations of SLIT2 and/or ROBO1, reduced expression of phospho Serine-71 CDC42 predicted poor survival of BC patients. Results indicate the importance of SLIT2–ROBO1–CDC42 signalling pathway in predicting tumour progression.

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Introduction

Breast cancer (BC) is the most common cause of cancer among women in both developed and developing countries. It has been reported that one million new cases of breast cancer occur each year worldwide (Mc Pherson *et al.* 2000). Determinants of poor prognosis of BC include young age (<40 years), early menstruation, late menopause, family history, multiparity, nulliparity, use of contraceptive pills, obesity etc. (Mukherjee *et al.* 2012). Study of molecular pathogenesis of breast cancer is essential for early diagnosis and development of individual-based treatment.

Cytogenetic analysis and chromosome mapping revealed deletions in several chromosomal regions like 3p12 and 4p15 to be associated with development of BC (Shivapurkar *et al.* 1999; Dallol *et al.* 2001, 2002; Alvarez *et al.* 2013). The candidate tumour suppressor genes *ROBO1*, located at 3p12.3 and *SLIT2*, located at 4p15.2 were shown to be altered in BC by different studies (Shivapurkar *et al.* 1999; Dallol *et al.* 2002). SLIT2 is the cognate ligand for different receptors including ROBO1/ROBO2 and could control different pathways (Dickinson and Duncan 2010). Interaction of SLIT2 with ROBO1 leads to SrGAP1 recruitment to ROBO1, resulting conversion of active CDC42–GTP to inactive CDC42–GDP complex (Wong *et al.* 2001).

*For correspondence. E-mail: ckpanda.cnci@gmail.com.

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SLIT2–ROBO1 signalling mediating intrinsic dephosphorylation of CDC42–GTP has been reported to prevent tumour progression in glioma (Yiin *et al.* 2009; Xu *et al.* 2010) and in medulloblastoma (Werbowetski-Ogilvie *et al.* 2006) with no prior report on the role of this pathway in BC progression.

Different studies showed frequent deletion (57–63%) and methylation (48–80%) of *SLIT2* locus in BC compared to *ROBO1* (deletion, 15–27%; methylation, 5–19%) (Shivapurkar *et al.* 1999; Dallol *et al.* 2001; Martinez *et al.* 2001; Dallol *et al.* 2002; Alvarez *et al.* 2013). Alterations of *ROBO2* (located 1.3 Mb telomeric to *ROBO1* at chromosome 3p12.3) is yet to be studied in BC, as its alterations have already been studied in head and neck squamous cell carcinoma (HNSCC) and carcinoma of the cervix (CACX) (Ghosh *et al.* 2009; Mitra *et al.* 2012). No somatic mutation in *SLIT2* and *ROBO1/2* genes was reported in BC (Dallol *et al.* 2001, 2002). In immunohistochemical (IHC) analysis, weak or absence of SLIT2 expression was seen in BC compared to ROBO1 expression in 45–59% samples (Wang *et al.* 2011). However, to the best of our knowledge, alterations of *SLIT2* and *ROBO1* were not analysed in the same set of samples to understand their role in development of BC.

IHC data showed upregulation of Rho GTPase Cdc42 protein (82.35% of studied BC population), without emphasizing on active CDC42–GTP expression in BC (Halon *et al.* 2013). It has also been evident that, besides SLIT2–ROBO1 mediated inactivation of CDC42, Serine-71 phosphorylation could inhibit the binding of downstream effector proteins like PAK1, WASP etc. (Taegun *et al.* 2000; Schwarz *et al.* 2012), resulting in deregulation of this pathway. The expression pattern of phosphorylated CDC42 at Serine 71 (pSer71 CDC42) has not been studied in BC. Therefore, it is imperative to analyse the alterations (deletion/methylation/mutation/expression) of *SLIT2* and *ROBO1/ROBO2* along with expressions of CDC42 and pSer71 CDC42 in same set of BC samples to understand the association of SLIT2–ROBO1–CDC42 pathway in the development of BC.

Thus, our study has been focussed on the following aspects in primary BC of Indian patients of different clinical stages: (i) analysis of alterations (deletion/methylation/mutation) of *SLIT2, ROBO1* and *ROBO2* genes, (ii) expression analysis of SLIT2, ROBO1 and ROBO2, CDC42, pSer71 CDC42 proteins by IHC, (iii) clinicopathological correlation of alterations of these genes with progression of BC. Our data showed frequent inactivation of *SLIT2* and *ROBO1/2* genes by deletion and/or methylation followed by their reduced expression along with pSerCDC42 in BC, indicating importance of this pathway in development of this tumour.

Materials and methods

Collection of clinical specimens

DNA was isolated from freshly operated 150 primary pretherapeutic BC samples and their adjacent normal tissues for molecular analysis. One part of each sample was fixed in formalin and paraffin embedded for IHC. Remaining part of the samples were stored at −80°C until further use. Informed consent and approval were obtained from patient for sample collection from the Research Ethics Committee of the institute. All tumours were staged according to the International Union against Cancer (UICC) tumour-node-metastasis (TNM) classification (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). Detailed clinicopathological history of the patients is provided in table 1.

Microdissection and DNA extraction: Prior to DNA extraction, the contaminant normal cells in the tumour specimen were removed by manual microdissection from cryosections using surgical knives under a dissecting microscope (Leica MZ 16, Bannockburn, USA). Tumours containing at least 70–80% tumour cells after microdissection were taken for DNA isolation. For each sample, DNA was isolated from tumour, adjacent normal tissue following a previously established protocol, i.e. proteinase K digestion followed by phenol–choloroform extraction (Dasgupta *et al.* 2002).

Deletion analysis

Deletion analysis of *SLIT2* and *ROBO1/2* loci for the tumours ($n = 150$) was carried out by microsatellite/exonic markers (table 2 in electronic supplementary material) Mitra *et al*. (2012). Briefly, polymerase chain reaction (PCR) was carried out by using a [γp32] ATP-lablled forward primer. PCR products were electrophoresed on 7% denaturing polyacrylamide gel and autoradiographed on X-ray film Dasgupta *et al*. (2002). The scoring of loss of heterozygosity (LOH), hemizygous deletion (HED), microsatellite size alterations of one allele (MAI), homozygous deletion (HD) were done on autoradiogram as described by Mitra *et al.* (2012).

Promoter methylation analysis

Promoter methylation analysis of *SLIT2* and *ROBO1/2* using methylation sensitive restriction analysis (MSRA) was carried out in the entire 150 primary BC and adjacent normal tissue pairs using methylation sensitive restriction enzyme *Hpa*II and its methylation insensitive isoschizomer *Msp*I following a previously established protocol (Singh *et al.* 2005). The β-3A adaptin gene (K1) was used as digestive control and RARβ2 (K2) was used as the control for DNA integrity (Loginov *et al.* 2004). Methylation status of the candidate genes were also checked in a BC cell line, MCF7, by MSRA. The methylation primers of *SLIT2* and *ROBO1/2* genes, and the control genes are described in table 1 in electronic supplementary material.

Methylation analysis was further validated in 15 randomly selected primary BC sample pairs by methylation-specific-PCR (MSP) (Herman *et al.* 1996) after bisulphite modification of DNA using primers listed in table 1 in electronic supplementary material. Genomic DNA (5 μg) was

Table 1. Clinicopathological correlations showing association of early age (≤40), menopausal status, grade, stage, lymph node involvement of the BC samples with their subtype.

	n (%)	Mean	Luminal A	Luminal B	HER2	TNBC
Age						
Early (≤40)	70 (46.6)	32.7 ± 8	14	19	21	16
Late (≥40)	80 (53.3)	53.4 ± 6	23	19	17	21
P value			0.293	0.772	0.297	0.77
Menopausal status						
Premenopausal	62 (41.3)	34 ± 7	18	15	15	14
Postmenopausal	88 (58.6)	55 ± 4	19	23	22	24
P value			0.397	0.937	0.936	0.645
Grade						
I/II	97 (64.6)	44.5 ± 5	24	28	24	21
III/IV	53 (35.3)	46.2 ± 4	13	10	13	17
P value			0.865	0.25	0.865	0.227
Stage						
I/II	55 (36.6)	44.7 ± 6.3	12	19	13	11
III/IV	95 (63.3)	44.2 ± 5.6	25	19	24	27
P value			0.675	0.246	0.979	0.343
Lymph node	Positive: 113 (75.3)	47 ± 5	28	26	30	29
	Negative: 37 (24.6)	44.2 ± 7	9	12	7	9
P value			0.869	0.354	0.474	0.956
Morbidity	*N* = 120		41.6% (*n* = 36)	36% (*n* = 25)	31.2% (*n* = 32)	37.03% (*n* = 27)

subjected to bisulphite modification followed by PCR amplification of the modified DNA using primers for nonmethylation (U) or methylation (M) specific alleles. Details of MSRA/MSP protocols have been described in electronic supplementary data.

Validation of methylation status of candidate genes in MCF7 cells: MCF7 cell line was cultured for five days at 5 μM and 10 μM concentrations of 5-Aza-2′-deoxycytidine (5-aza-dC) (Sinha *et al.* 2011). The cells were harvested followed by RNA isolation, cDNA preparation and real time quantitation of the candidate genes. Briefly, total RNA was isolated from the 5-aza-dC treated/untreated MCF-7 samples using TRIzol reagent according to manufacturer's protocol (Invitrogen, USA). The real time quantitation of RNA expression of *SLIT2, ROBO1/ROBO2* was done by Power SYBR-green PCR assay (Applied Biosystems, USA) using ddCt method (Livak and Schmittgen 2001; Schmittgen and Livak 2008), with β2-microglobulin gene as internal control. Details of this method have been described in electronic supplementary data.

Mutation analysis

Mutation analysis for the SLIT2–ROBO1 interacting regions (exons 9–14 of *SLIT2*; exons 2–4 of *ROBO1*) was carried out by SSCP in all 150 sample pairs followed by PCR amplification and direct sequencing of the target region (Maiti *et al.* 2015) in 10 random pairs of BC samples. Sequencing of both strands of each PCR product was done with ABI PRISMTM BD Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, USA) and electrophoresed in 3100-Avant Genetic Analyzer (PE Applied Biosystems). Sequence of the samples (tumour and normal) were analysed and compared with reference human genome sequence GRCh37 (Ensemble) to identify mutations in the regions, if any.

IHC

Molecular subtyping: Randomly collected BC samples from hospital section of Chittaranjan National Cancer Institute (CNCI) were subtyped for oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expressions by IHC (Reiner *et al.* 1990; Perrone *et al.* 2006). Briefly, about 5 μm paraffin sections of primary BC samples were dewaxed, rehydrated and reacted overnight with primary antibodies: mouse monoclonal IgG2a (sc-787) for ERα; rabbit polyclonal IgG (sc-7208) for PR and mouse monoclonal IgG2a (F-11, sc-7301) for HER2, Santa Curz Biotehcnology, USA. Horseradish peroxidase (HRP)-conjugated secondary antibodies (goat antimouse IgG (sc-2005); goat antirabbit IgG (sc-2004)) were added at 1:500 dilutions. The slides were developed using 3-3′ diaminobenzidine (DAB) as the chromogen and counterstained with hematoxylin. Scoring was done as per the recommended guidelines of American Society of Clinical Oncology (ASCO).

In the present study, a total of 150 BC samples were selected which comprised of comparable frequencies (24.6–25.3%) of the four different subtypes of BC samples for

Table 2. Correlations of alterations of SLIT2 and ROBO1 with various clinicopathological parameters (*$P \leq 0.05$).

	Deletion/allelic alteration (n =150)						Promoter methylation (n =150)						Overall alterations (n =150)					
	SLIT2		*ROBO1*		*ROBO2*		*SLIT2*		*ROBO1*		*ROBO2*		SLIT2		ROBO1		ROBO2	
	D+	D−	D+	D−	D+	D−	M+	M−	M+	M−	M+	M−	A+	A−	A+	A−	A+	A−
Grade																		
I/II	28	68	35	61	10	86	55	41	31	65	11	85	69	27	42	54	18	78
III/IV	13	41	15	39	1	53	33	24	11	43	3	51	43	11	26	37	5	49
P value	0.63		0.462		0.098		0.923		0.17		0.328		0.393		0.884		0.189	
Stage																		
I/II	18	37	13	42	19	36	41	14	14	41	4	51	40	15	24	31	9	46
III/IV	26	69	19	76	27	68	50	45	25	70	10	85	71	24	41	56	14	81
P value	0.611		0.751		0.548		0.013*		0.938		0.712		0.938		0.882		0.974	
Lymph node status																		
Node+	40	68	33	75	9	99	73	35	31	77	11	97	87	21	57	51	19	99
Node−	18	34	12	30	2	39	18	24	9	22	2	40	35	7	52	20	4	38
P value	0.902		0.606		0.727		0.0093*		0.849		0.354		0.873		0.889		0.431	
Age at onset																		
Early	28	43	19	52	8	63	40	31	21	50	7	64	57	14	33	38	8	63
Late	30	49	24	56	3	80	47	43	19	61	6	15	59	20	35	44	11	68
P value	0.987		0.795		0.137		0.718		0.531		0.067		0.533		0.918		0.808	

better comparative analysis of molecular alterations among the subtypes (figure 1 in electronic supplementary material).

Expression of candidate genes: Protein expressions of SLIT2, ROBO1 and ROBO2 were studied by IHC in 68 primary BC (from the same pool of BC samples) and in adjacent normal breast tissues using primary antibodies (goat polyclonal IgG sc-16611, sc-16615, sc-1661 for SLIT2, ROBO1 and ROBO2, respectively; mouse polyclonal sc-8401 for CDC42; rabbit polyclonal sc-135641 for phosphor Serine-71 CDC42) and HRP conjugated secondary antibodies (rabbit antigoat sc-2768; goat antimouse IgG (sc-2005); goat anti-rabbit IgG (sc-2004)) from Santa Cruz Biotehcnology, USA, following a previously established protocol (Mitra *et al.* 2012). Of the 68 samples, protein expression of total CDC42 and phospho Serine-71 CDC42 could be further carried out in 52 BC samples. Scoring for SLIT2, ROBO1, ROBO2, CDC42 and pSer71 CDC42 expression was carried out following the method of Perrone *et al.* (2006).

Statistical analysis

Fisher's exact test was used to determine different clinicopathological association with alterations of the tumours. All statistical tests were two-sided and considered significant at probability value, $P \leq 0.05$. Survival curves were obtained according to Kaplan–Meier. Cox proportional hazards regression model predicted patient's survival. Overall survival (OS) was measured from the date of surgery to the date of most recent follow-up or death (up to five years). The detailed follow-up records are available for 83 BC patients. All the statistical analyses were performed using statistical programs Epi Info 6.04, SPSS 10.0 (SPSS, Chicago, USA).

Results

Deletion analysis

In BC, *SLIT2, ROBO1* and *ROBO2* showed deletion and microsatellite size alteration of one allele (MA1) (figure 1a). However, differential frequencies of deletions of these genes have been seen in the following order: *SLIT2* (38.6%) > *ROBO1* (30%) > *ROBO2*: 7.3% (table 1 in electronic supplementary material). The frequencies of MA1 in *SLIT2* and *ROBO1* were infrequent in BC (0.6% for *SLIT2*, 1.3% for *ROBO1*) (table 1 in electronic supplementary material). A nonsignificant trend in deletion frequencies of *SLIT2* and *ROBO1/2* genes among different subtypes were observed, indicating deletions in these genes could be independent events in development of BC (figure 1b; table 3 in electronic supplementary material).

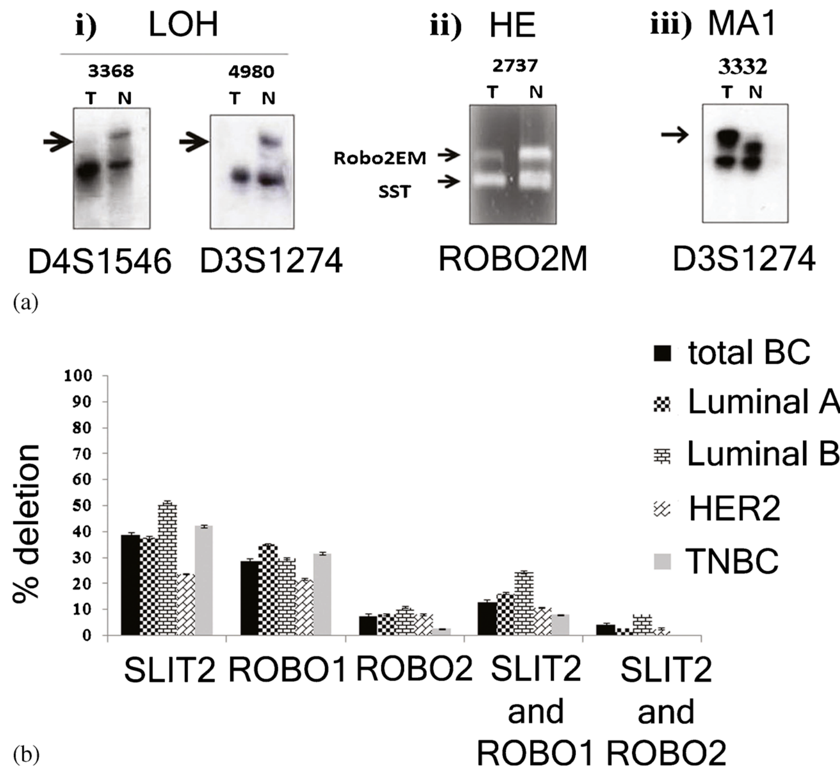

Figure 1. (a) Deletion and microsatellite size alterations of different marker loci of *SLIT2*, *ROBO1* and *ROBO2* (i) LOH, loss of heterozygosity; (ii) HE, hemizygous deletion; (iii) MA1, microsatellite size alteration of one allele. (b) Deletion pattern of individual candidate genes, *SLIT2–ROBO1* ligand–receptor pair, *SLIT2–ROBO2* ligand–receptor pairs in different subtypes. Bars represent percentage ±SD.

Methylation analysis

Variable frequencies of promoter methylation of *SLIT2, ROBO1* and *ROBO2* were seen in the BC samples (figure 2a; table 1 in electronic supplementary material). High frequency of methylation was seen in *SLIT2* (63.3%) followed by *ROBO1* (26.6%) and *ROBO2* (9.3%) in the BC samples (table 1 in electronic supplementary material; figure 2a). Comethylation frequencies of *SLIT2* and *ROBO1* varied from 5.4–26.3% whereas comethylation of *SLIT2* and *ROBO2* was observed only in luminal B and TNBC (7.8–13.1%) (figure 2a). MSP analysis in 15 randomly selected tumours from the same pool of BC samples also showed concordant results (figure 2 and table 4 in electronic supplementary material).

Significantly higher frequency of methylation of *SLIT2* was observed in TNBC compared to luminal A ($P = 0.041$) and HER2 subtype ($P = 0.0032$; figure 2a) of BC. A statistically significant association was seen methylation frequency between *ROBO1* and *ROBO2* genes in BC (table 3 in electronic supplementary material). In addition, the promoter methylation was analysed in MCF7 cell line by MSRA. MCF7, *SLIT2* and *ROBO1* genes were found to be methylated (figure 3a in electronic supplementary material).

Validation of promoter methylation of the genes

In confirmation of the promoter methylation of *SLIT2* and *ROBO1* in MCF7, it was evident that gradual increase in RNA expression of these genes were seen with increase in concentration of 5-aza-dC. At 10 μm 5-aza-dC treatment in MCF7 cells, the candidate genes showed increased expression in the following order *SLIT2*: 3.74 fold > *ROBO1*: 9.8 fold > *ROBO2*: 1.47 fold (figure 2b; figure 3b in electronic supplementary material).

Mutation analysis

No altered band was observed in the SLIT2–ROBO1 interacting domains of all the 150 sample pairs. The SSCP analysis followed by sequencing of 10 pairs of BC samples showed no somatic mutation in the *SLIT2–ROBO1* interacting domain (exons 9–14 of *SLIT2*; exons 2–4 of *ROBO1*). Thus, we restricted further screening of mutation by sequencing, considering that mutation of this region might be a rare event in development of BC.

Overall alteration of the candidate genes

Majority (80%; 120/150) of the tumours showed genetic/epigenetic alteration in at least one of the *SLIT2* and/or *ROBO1/ROBO2* genes, indicating importance of these genes in development of BC. Overall alterations (deletion and/or methylation) of the candidate genes were seen in the following order: *SLIT2*: 75.3% (101/150), *ROBO1*: 45.3% (68/150), *ROBO2*: 15.3% (23/150) (figure 2c). Frequency of overall alterations of *SLIT2* and/or *ROBO1* (80.6%) was high compared to that of *SLIT2* and/or *ROBO2* (46%) (figure 2c). Significantly, higher overall alteration of *SLIT2* gene was observed in TNBC compared to HER2 subtype ($P = 0.0014$) (figure 2c; table 2). Similar trend is also seen in overall alterations of *SLIT2* and/or *ROBO1*, in TNBC than HER2 subtype (P value: 0.0012) (figure 2c; table 2). Whereas, alterations of *SLIT2* and/or *ROBO2* was observed to be significantly higher in TNBC over luminal A (P value: 0.014) and HER2 subtype (P value: 0.0048) of BC patients (figure 2c). Overall alterations of the candidate genes did not show any significant difference with disease progression (figure 4 in electronic supplementary material).

Protein expression analysis

IHC analysis of SLIT2 and ROBO1/ROBO2 proteins showed expression of these proteins in the membrane and cytoplasm of luminal and myoepithelial cells of normal breast duct (figure 3, a(i), b(i), c(i)), while expression of these proteins were mainly localized in the cytoplasm of primary tumours (figure 3, a(ii, iii), b(ii, iii), c(ii, iii)). Reduced or absence of expression of SLIT2, ROBO1 and ROBO2 were observed in 82.4, 55.7 and 40% of BC samples ($n = 68$) respectively (table 3). Significant concordance was observed in the alterations (deletions and/or methylation) of SLIT2 and ROBO1 with their expression ($P = 0.026$ and $P = 0.00012$ for SLIT2 and ROBO1 respectively) (table 3).

Expression of total CDC42 and pSer71 CDC42 was localized in the membrane and cytoplasm of luminal and myoepithelial cells of normal breast duct (figure 4, a(i), b(i)) and in the cytoplasm of primary BC (figure 4, a(ii), b(ii)). Moderate to high expression of total CDC42 was observed in 94.2% (49/52) of BC tumour samples, while reduced expression of pSer71 CDC42 was observed in 78.8% (41/52) of the samples (table 3). Also, a significant association was observed with coalterations of *SLIT2* and/or *ROBO1*, *SLIT2* and/or *ROBO2* with reduced expression of pSer71 CDC42 (P value: 0.0012–0.0038) ($n = 52$) (table 3). It was observed that 88.4% (46/52) of the samples showed reduced expression of at least one of the candidate proteins (SLIT2, ROBO1, ROBO2 and pSer71-CDC42) (table 3).

Clinicopathological correlations and survival analysis among BC samples

The deletion and methylation of *SLIT2*, *ROBO1* and *ROBO2* did not show any significant association with age at onset, grade and lymph node involvement, except significant association of *SLIT2* methylation with early stage (P value: 0.013) and lymph node involvement (P value: 0.0093) (table 2). Expression pattern of SLIT2, ROBO1/2 showed no significant association with stage, grade, lymph node or age at onset among BC samples (table 2).

The Kaplan–Meier (K–M) survival analysis revealed poor prognosis of BC patients showing alterations of *SLIT2*

Table 3. Correlations between genetic alterations of SLIT2, ROBO1 and ROBO2 with expression and expression analysis of active CDC42 in primary BC with/without alterations of SLIT2, ROBO1 and ROBO2.

	SLIT2			ROBO1			ROBO2			CDC42T	pSer71CDC42
	D	M	E	D	M	E	D	M	E	E	E
6346	+	−	−	+	+	−	−	−	++	+	−
215	+	+	−	+	−	−	−	−	++	++	−
3785	−	+	−	+	−	−	−	−	+	+	−
4727	−	+	−	−	−	−	−	−	−	+	−
1889	+	−	−	−	−	−	−	−	−	+	−
6447	+	−	−	+	+	−	−	−	+	++	−
22	+	+	−	−	−	+	−	−	−	+	−
1772	−	−	+	−	−	+	−	−	+	+	+
5173	+	−	−	+	+	−	−	−	−	+	−
848L	−	−	−	−	−	+	−	−	+	+	+
5971	+	−	−	−	−	−	−	−	−	+	−
5521	−	+	−	+	−	−	−	−	+	+	−
1830	+	−	−	−	−	+	−	−	+	+	−
2804	−	+	−	+	−	−	−	−	−	+	−
2529	−	−	+	−	−	+	−	−	−	+	+
3295	−	−	−	+	−	−	−	−	+	+	−
588	−	+	−	−	−	+	−	−	−	+	+
6038	−	−	−	+	+	−	−	−	+	++	−
5848	+	+	−	+	−	−	+	−	−	++	−
2417	+	+	−	−	−	+	−	−	−	+	−
1809	−	+	+	−	−	+	−	−	+	+	+
933	−	+	+	−	−	+	−	−	+	+	−
2767	−	+	+	−	−	−	−	−	−	+	+
5836	−	+	+	−	−	++	−	−	+	+	+
2737	+	+	−	+	−	−	−	−	−	++	−
1284	+	+	−	−	+	+	−	−	+	+	−
4160	+	+	−	+	−	−	−	−	+	+	−
268	−	+	+	−	+	+	−	+	−	−	−
1289	−	+	+	−	−	+	−	−	+	+	+
5099	+	+	−	−	+	−	−	+	+	+	−
4800	−	+	−	+	−	−	−	−	+	+	−
1013	−	+	−	−	−	−	−	−	−	+	−
1553	−	+	−	−	+	−	−	−	−	+	−
5076	+	+	−	+	+	−	−	+	−	++	−
3332	+	+	−	+	+	−	−	−	+	+	−
5036	−	−	−	−	+	−	−	+	−	+	−
1089	−	+	−	−	+	−	−	−	+	+	−
148	−	+	−	−	−	+	−	−	+	−	−
5700	+	−	−	−	+	+	−	−	++	+	−
5287	−	+	−	−	−	++	−	−	++	++	−
324	+	+	−	−	−	+	−	−	+	−	−
5283	−	−	+	−	−	+	−	−	+	++	−
4928	−	+	−		+	−	−	−	−	+	−
6495	−	+	−	+	−	−	−	−	+	++	−
3218	−	−	−	+	−	−	−	−	−	+	−
5364	−	−	−	−	−	+	−	−	−	+	+
4892	−	+	−	−	−	−	−	−	+	++	−
2972R	−	+	−	−	+	+	+	−	+	++	−
2434	−	−	−	−	−	+	−	−	+	+	+
3368	+	+	−	+	+	−	−	−	−	++	−
5135	−	−	+	−	−	+	−	−	+	+	+
5901	−	+	−	−	−	+	−	−	−	ND	ND
3158	+	+	−	−	−	−	+	−	−	ND	ND
1144	−	+	−	−	+	−	−	−	+	ND	ND
4353	+	+	−	−	+	−	+	−	−	ND	ND
4953	−	+	−	−	−	+	−	−	+	ND	ND
5732	−	−	−	+	+	−	−	−	−	ND	ND
1206	+	+	−	−	−	+	−	−	+	ND	ND
4337	−	−	+	−	−	+	−	−	+	ND	ND
2445	+	+	−	−	+	−	−	+	−	ND	ND
5301	−	−	+	−	−	+	−	−	+	ND	ND

Table 3 (*contd*)

	SLIT2			ROBO1			ROBO2			CDC42T	pSer71CDC42
	D	M	E	D	M	E	D	M	E	E	E
1866	–	+	–	–	–	–	–	–	–	ND	ND
2929	–	+	–	–	+	–	–	–	–	ND	ND
2571	–	+	–	–	+	–	–	–	–	ND	ND
4604	–	+	–	–	–	+	–	–	+	ND	ND
Alt SLIT2 vs expression			0.026*								
Alt ROBO1 vs expression			0.00012*								
Alt SLIT2 and/or ROBO1 vs CDC42p Ser 71			0.0012*								
Alt SLIT2 and/or ROBO2 vs CDC42p Ser 71			0.0038*								

D, deletion; M, methylation; E, expression; ND, not done.
*$P \leq 0.05$.

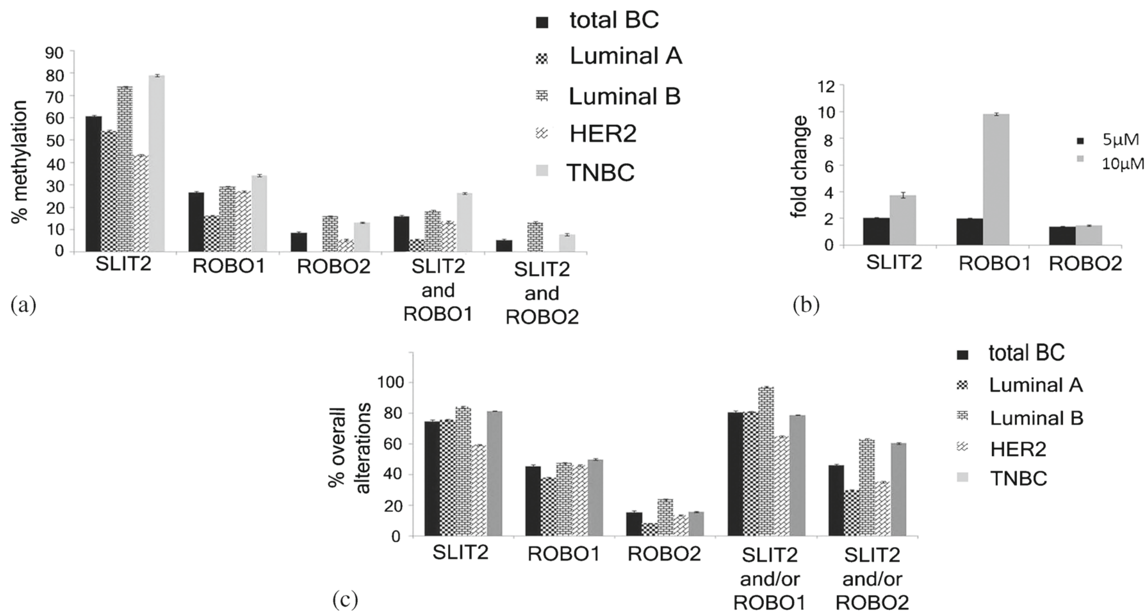

Figure 2. (a) Methylation pattern of *SLIT2*, *ROBO1*, *ROBO2*, *SLIT2–ROBO1* ligand–receptor pair, *SLIT2–ROBO2* ligand–receptor pair. (b) Confirmation of methylation status of the candidate genes in MCF7 cell line by 5-aza-dC mediated reactivation of RNA expression, as observed in qRT-PCR. (c) Overall alteration pattern of individual candidate genes, *SLIT2–ROBO1* ligand–receptor pair, *SLIT2–ROBO2* ligand–receptor pairs. Bars represent percentage ±SD (2a, 2c) and fold change ±SD (2b).

(*P* value: 0.0267) and/or *ROBO1* (*P* value: 0.006) (figure 5), indicating their prognostic significances. This also indicated that abrogation of these ligand–receptor interactions predicted poor prognosis in BC patients. Survival analysis also showed that BC patients with alterations of *SLIT2, ROBO1* and/or *ROBO2* and reduced expression of pSer71 CDC42 had poor disease prognosis compared to alterations positive or alterations negative BC with moderate level of expression of pSer71 CDC42 (*P* value: 0.0399; figure 6), indicating importance of pSer71 CDC42 in disease prognosis (figure 6). Together, these results suggested a bidirectional regulation mechanism in SLIT2–ROBO1–CDC42 mediated tumourigenesis (figure 7).

From our study, it is also evident that for both hormone receptor positive and negative subtypes, alterations of candidate genes predicted poor prognosis for the patient

Table 4. Cox multivariate analysis of genetic, clinical and aetiological parameters in predicting survival of BC patients.

Variable	*P* value	Overall survival		
		HR	95% CI for HR	
Alt SLIT2	0.0152*	2.4882	1.0835	5.7140
Alt ROBO1	0.0007*	3.2150	1.6384	6.3088
Alt ROBO2	0.9635	0.9811	0.4336	2.2198
Reduced pSer71-CDC42	0.0231	0.0976	0.0131	0.7266
Alt SLIT2 and ROBO1	0.0357*	2.6267	1.0668	6.4678
Alt SLIT2 and ROBO2	0.0197*	1.5957	1.0774	2.3634
Alt SLIT2 and reduced pSer71-CDC42	0.0483	0.2012	0.0410	0.9880
Alt ROBO1 and reduced pSer71-CDC42	0.0187*	1.1707	1.4662	2.9373
Grade	0.0197*	1.5957	1.0774	2.3634
Node	0.3907	1.4324	0.6305	3.2529
Stage	0.1217	1.4994	0.8977	2.5044

HR, hazard ratio; *$P \leq 0.05$ denotes statistical significance.

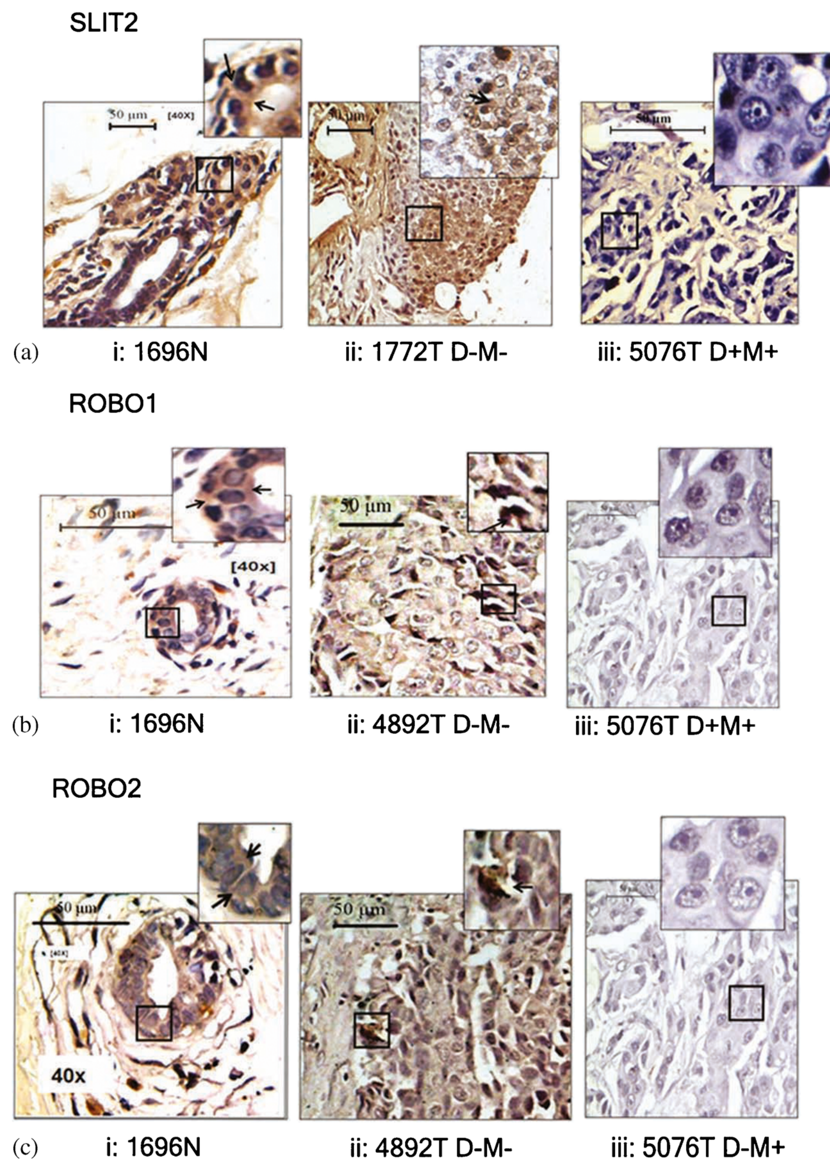

Figure 3. IHC to study alterations of SLIT2 (a), ROBO1 (b) and ROBO2 (c) expression in normal breast and in primary BC (magnification 20× (inset 40×); scale bar 50 μM). Arrows indicate expression of the candidate proteins.

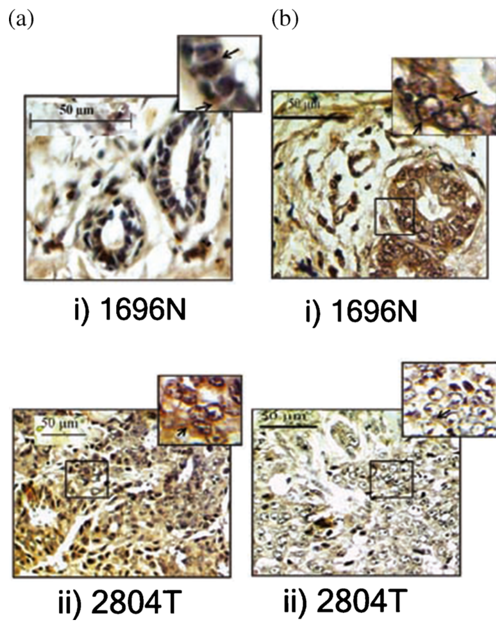

Figure 4. IHC to show expression profile of total CDC42 and phospho-CDC42 in normal a (i), b (i) and in BC a (ii) and b (ii), respectively. Arrows indicate expression of the candidate proteins.

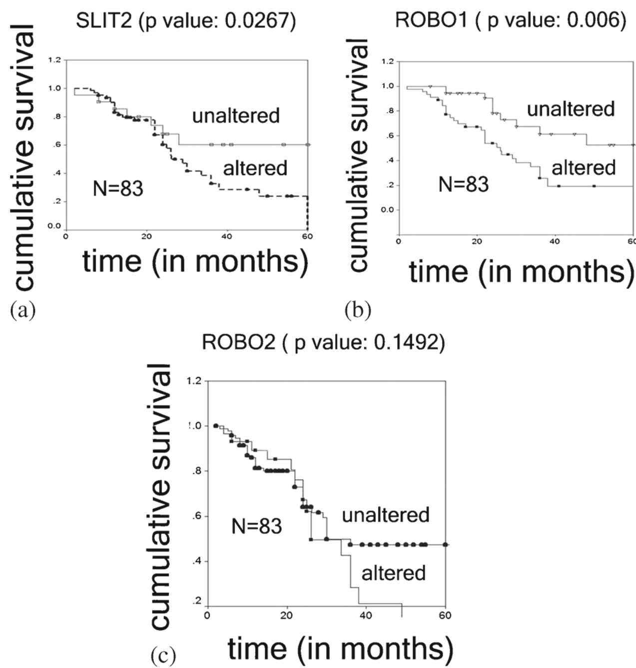

Figure 5. Kaplan–Meiyer analysis of survival of BC patients (up to 5 years) with/without alterations of *SLIT2* (a), *ROBO1* (b) and *ROBO2* (c).

(figure 5 in electronic supplementary material). The multivariate Cox model showed alterations of *SLIT2* ($P = 0.0268$; HR: 2.4882), ROBO1 ($P = 0.0007$; HR: 3.2180), coalterations of *SLIT2* and *ROBO1* ($P = 0.0357$; HR 2.62) as well as *SLIT2* and *ROBO2* ($P = 0.0125$; HR: 2.68), alterations of *ROBO1* and reduced expression of pSer71-CDC42

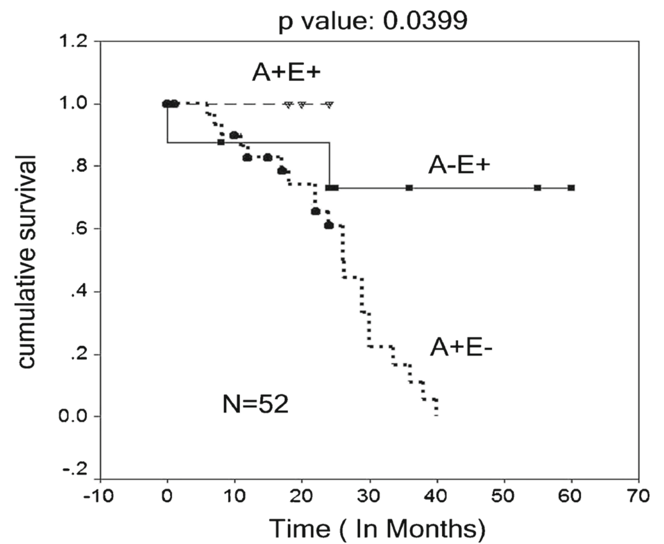

Figure 6. Survival analysis of BC patients with/without alterations of *SLIT2*, *ROBO1*, and/or *ROBO2* with reduced expression of pSer71 CDC42. A, alteration of *SLIT2*, *ROBO1*, and/or *ROBO2*; E, expression of pSer71 CDC42. +, With alterations and/or expressions of pSer71 CDC42. −, No alterations and/or reduced expression.

($P = 0.0187$; HR: 1.1707), high grade (grade III/IV) predicted poor outcome of the BC patients (table 4).

Discussion

The aim of this study was to understand the importance of SLIT2–ROBO1/ROBO2–CDC42 pathway in the development of BC. For this reason, the alterations (deletion and/or methylation) of *SLIT2* and *ROBO1/ROBO2* followed by protein expression of these genes along with CDC42 and pSer71-CDC42 were analysed in the primary BC samples. Our data showed low frequency of deletion and high promoter methylation in *SLIT2* (38.6 and 60.6% respectively), indicating the importance of epigenetic inactivation of this gene in development of BC. Unlike *SLIT2*, *ROBO1* had comparable frequencies of deletion (30%) and methylation (26.6%) while low frequencies were observed for overall alterations of *ROBO2* (7.3% deletion; 8.6% methylation). Overall alteration frequencies of the candidate genes showed maximum alterations in *SLIT2*, followed by *ROBO1* and very low frequency alterations in *ROBO2*. As confirmed by MSRA and 5-aza-dC mediated demethylation experiments, *SLIT2* and *ROBO1* were found to be methylated in BC. In conformity with our data, earlier studies also showed frequent deletion (57–63%) and methylation (48–80%) of *SLIT2* locus in BC compared to *ROBO1* (deletion: 15–27%; methylation: 5–19%) (Shivapurkar *et al.* 1999; Dallol *et al.* 2001; Martinez *et al.* 2001; Dallol *et al.* 2002; Alvarez *et al.* 2013). Frequent alterations of *SLIT2* and *ROBO1* have been reported in other malignancies namely CACX, HNSCC, hepatocellular carcinoma, glioma, prostate carcinoma

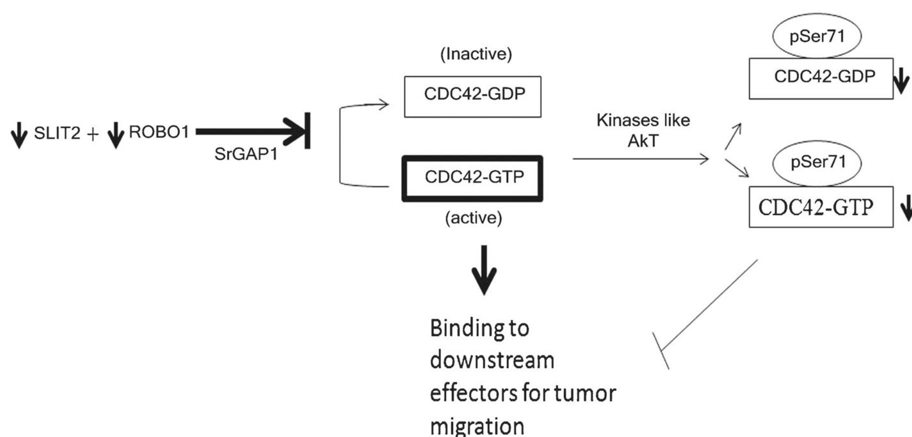

Figure 7. Schematic representation of SLIT2–ROBO1 signalling network and role of CDC42 as downstream effector in BC. Down arrow, down regulation in tumour; Pathway blocked in tumour is indicated as →| ; bold outline, pathway activated or blocked in tumour.

(Latil *et al.* 2003; Yiin *et al.* 2009; Zheng *et al.* 2009; Mitra *et al.* 2012; Maiti *et al.* 2015).

Absence of any somatic mutation in *SLIT2–ROBO1* interacting domain was also in conformity with earlier observation of Dallol *et al.* (2001, 2002) in a cohort study involving BC patients from UK, indicating mutation in this pathway as a rare event in development of BC.

To the best of our knowledge, our study is the first approach to study subtype specific association of the alterations of the candidate genes. From our data, it is evident that methylation frequency of *SLIT2* was significantly high in TNBC compared to luminal A (P=0.041) or HER2 (P = 0.0032) subtype of BC (figure 2a). Significantly, higher overall alterations of SLIT2 locus was observed in TNBC over HER2 subtype (P = 0.0014) (figure 2c). Our data showed higher frequencies of overall alterations of *SLIT2* and/or *ROBO1* in luminal B as well as in TNBC subtype over that of luminal A or HER2 subtype (figure 2c). Similar observation was reported by Guedj *et al.* (2012), showing higher frequencies of molecular alterations in luminal B as well as in basal-like tumours over other subtypes, owing to the high proliferative index of these subtypes (Guedj *et al.* 2012).

Alterations of *SLIT2* and *ROBO1/2*, correlated with their reduced protein expression profile from our study (reduced expression of SLIT2, ROBO1 and ROBO2 in 82.4, 55.7 and 40% of BC samples, respectively). Similar to our study, reduced expression of SLIT2, ROBO1/ROBO2 have been reported in several other malignancies like HNSCC, liver, lung, oral, cervical, breast, kidney (Dallol *et al.* 2001, 2002; Zabrovsky *et al.* 2002; Ghosh *et al.* 2009; Yiin *et al.* 2009; Zheng *et al.* 2009; Mitra *et al.* 2012; Maiti *et al.* 2015) though overexpression of these genes have also been reported in prostrate and breast cancers (Latil *et al.* 2003; Bieche *et al.* 2004). From our study, reduced expression of SLIT2 and ROBO1 in majority of primary BC with alterations of SLIT2 (63.2%), ROBO1 (45.5%) indicated the importance of genetic and/or epigenetic alterations in regulating gene expression (table 3). However, a moderate level of expression of ROBO2 was observed in 50% of the cases (n = 68). These indicate that ROBO2 alteration might be rare event in predicting BC progression.

Our next aim was to analyse the alterations in expression pattern of active CDC42, the downstream key regulator protein of SLIT2–ROBO1 signalling pathway. Earlier results had established that alterations of *SLIT2* correlated with poor tumour prognosis through upregulation of active CDC42 (Bieche *et al.* 2004; Yiin *et al.* 2009). Previous reports have shown that apart from SLIT2–ROBO1 pathway mediated deactivation of CDC42-GTP, AkT mediated Serine-71 phosphorylation of CDC42 results in inefficient downstream effector coupling through PAK1, N-WASP etc., leading to cell cycle arrest (Taegun *et al.* 2000; Schwarz *et al.* 2012). To understand whether *SLIT2–ROBO1* mediated deactivation as well as AkT mediated Serine-71 phosphorylation of CDC42 occur in tandem, we checked the expression pattern of total CDC42 and pSer71 CDC42, in the context of alteration of SLIT2/ROBO1/ROBO2. From our data, high expression of total CDC42 was observed in 94.2% of BC cases (49 of 52) whereas reduced expression of pSer71 CDC42 was observed in 78.8% of the cases, indicating phosphorylation-mediated inactivation is infrequent in BC and thus CDC42 is mostly retained in the active conformation, capable of binding to downstream effectors, namely PAK1, N-WASP etc., leading to tumour migration. Overexpression of CDC42 has also been reported in BC (Fritz *et al.* 2002; Halon *et al.* 2013), though the expression status of pSer71 CDC42 has not been reported in BC or any primary tumour.

In understanding the clinical importance of these alterations, our data showed significant association of methylation of *SLIT2* with early stage and with lymph node involvement. KM survival analysis showed alterations of *SLIT2* and/or *ROBO1* genes or alterations of *SLIT2*, *ROBO1/ROBO12* genes along with reduced expression of pSer71

CDC42 to be associated with poor prognosis in BC patients, indicating that alterations of *SLIT2–ROBO1* signalling are a necessary event for development of BC. Alterations of *SLIT2* and *ROBO1*, *SLIT2* and *ROBO2*, alterations of *ROBO1* along with reduced expression of pSer71-CDC42 were found to predict poor disease free survival, according to Cox multivariate analysis (table 4). In conformity with our observation, earlier reports have also established that alterations (deletion/methylation/expression) of *SLIT2* and *ROBO1* to be associated with poor prognosis of patients in BC and other cancers (Chang *et al.* 2012; Mitra *et al.* 2012). No earlier data have established the implication of pSer71-CDC42 in disease prognosis and in predicting patient outcome.

Thus, it was evident from our analysis that inactivation of *SLIT2*, *ROBO1/2* augment tumourigenesis through active CDC42 mediated downstream signalling.

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