

# Frequent alterations of *hMLH1* and *RBSP3/HYA22* at chromosomal 3p22.3 region in early and late-onset breast carcinoma: clinical and prognostic significance

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Young age can be an independent prognostic factor for adverse prognosis in women with breast carcinoma (BC). In younger women, BC exhibited more aggressive pathological features than older women, indicating differences in biology. Frequent alterations in chromosomal (chr.) 3p22.3 in different malignancies indicated the existence of multiple candidate tumor suppressor genes (TSG) in this region, yet its association with BC remains unclear. In an effort to understand the differences in molecular pathogenesis in two age groups of BC, detailed analysis of alterations at chr.3p22.3 region was carried out in 47 early onset (group-A:  $\leq 40$  years) and 59 late-onset (group-A:  $> 40$  years) BC samples. Deletion mapping of the four candidate TSG, *hMLH1*, *APRG1*, *ITGA9* and *RBSP3/HYA22*, located within 1 Mb of chr.3p22.3 showed high deletion in *hMLH1* and *RBSP3/HYA22* genes. Frequent methylation was also observed in these genes and significantly associated with their deletion. Quantitative messenger RNA (mRNA) expression and immunohistochemical analysis showed down-regulation of these genes. Alterations (deletion/methylation) of *hMLH1* were significantly associated with *RBSP3/HYA22* in group-A ( $P = 0.02$ ). Significant poor survival in group-A patients with alterations in *hMLH1* and *RBSP3/HYA22* and the same in group-B patients with *hMLH1* alterations indicated their importance as prognostic markers. Differential association of alterations of these genes with higher histological grades, more advanced stages and positive lymph node involvement were also seen. Thus, the present study suggests *hMLH1* and *RBSP3/HYA22* to be candidate TSG associated with development of both early and late-onset BC undergoing frequent genetic and epigenetic alteration and having significant prognostic implications. (*Cancer Sci* 2008; 99: 1984–1991)

**B**reast cancer (BC), is the third most prevalent malignancy and primary cause of death in women worldwide.<sup>(1)</sup> In India, it accounts for 19% of all cancers among women, and is the second most common cancer when both sexes are considered together, whereas about 23% of eastern Indian women suffer from this disease.<sup>(2,3)</sup> Considering the age at onset of tumor, BC can be classified as early onset (group-A:  $\leq 40$  years) and late-onset (group-B:  $> 40$  years).<sup>(4,5)</sup> However, the cut-off value for early onset BC varies among investigators, ranging from 35–50 years.<sup>(6,7)</sup> Despite this discrepancy, younger women with BC exhibit more aggressive pathological features, including large tumor size of higher grade, presence of positive lymph nodes, absence of steroid receptors and high S-phase fraction, than older women with BC.<sup>(6,7)</sup> Another report showed that, in an Asian population, BC patients below 40 years have tumors with a poorer prognostic profile. However, this did not translate into poorer overall survival and might be attributable to more aggressive adjuvant treatment of younger patients.<sup>(8)</sup> All these reports suggested early onset BC to be a biologically separate disease

that independently predicts more adverse outcomes. Thus, the molecular analysis of BC in the two age groups is essential to ascertain the differences in pathogenesis, if any. Our previous studies on chromosomes (chrs.) 1p/q, 9p/q, 11p/q, 13q and 17p/q have shown differences in pattern of molecular alterations in the two age groups.<sup>(6,7,9,10)</sup> So, to find out the differences in pathogenesis in early and late-onset BC, detailed molecular analysis is pertinent.

Cytogenetic analyses detected frequent loss of chromosome 3p (chr.3p) in a variety of malignancies.<sup>(11–16)</sup> Loss of heterozygosity (LOH) at chrs.3p25, 3p22–24, 3p21.3, 3p21.2–21.3, 3p14.2, 3p14.3 and 3p12 reported in breast cancers suggest the presence of tumor suppressor genes (TSG) within these deleted regions.<sup>(17,18)</sup> Among these deleted regions, chr.3p22.3 harbors multiple candidate TSG viz. *hMLH1*, *APRG1*, *ITGA9* and *RBSP3/HYA22* within a 1-megabase (Mb) region.

The DNA mismatch repair gene (MMR) *hMLH1*, located 37 Mb from chr.3p-ter, was reported to be altered in a variety of malignancies including BC. Varying frequency of mutations (10–47%) and methylation (32–44%) of *hMLH1* have been reported in both breast cancer cell lines and primary BC.<sup>(18–23)</sup> Association between mutation and methylation was seen in sporadic BC resulting in microsatellite instability (MSI).<sup>(19)</sup> Moreover, loss in expression of *hMLH1* in BC with no apparent methylation point towards other alternative mechanisms contributing to loss of function.<sup>(24)</sup> The *APRG1* gene located 0.4 Mb centromeric to *hMLH1* has been implicated in cell membrane interactions and reported to be a candidate TSG associated with BC.<sup>(25)</sup> Another candidate TSG, *ITGA9* (integrin  $\alpha 9$  subunit) gene located 0.4 Mb further down-stream of *APRG1*, is a member of the  $\beta 1$  integrin family expressed by stratified squamous epithelium and associated with cell differentiation and growth.<sup>(26)</sup> The *RBSP3/HYA22* (*CTDSPL*) gene located 190 kilobase (Kb) downstream of *ITGA9* was reported to undergo frequent homozygous (HD) and hemizygous deletion (HED) in various malignancies including BC.<sup>(26,27)</sup> *RBSP3/HYA22* is suggested to be a member of a previously uncharacterized family of small C-terminal domain (CTD) phosphatases that catalyzes the dephosphorylation of Ser 5 within the consensus repeat of the largest RNA polymerase II subunit, thus affecting gene transcription.<sup>(29)</sup> Missense and nonsense mutations of *RBSP3/HYA22* in different tumors have been reported. *RBSP3/HYA22* plays a direct role in dephosphorylation of ppRB, presumably leading to blocks in the cell cycle at the G<sub>1</sub>-S boundary.<sup>(27)</sup> It is also

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**Table 1. Clinico-pathological parameters of early and late-onset breast cancer**

Clinical features	Group A		Group B	
	Case	Average age (years)	Case	Average age (years)
Histological type				
Ductal	45	34	59	53
Lobular	2	28	0	–
Clinical stage				
Stage I	1	40	2	48
Stage II	7	33	21	51
Stage III	36	35	33	53
Stage IV	3	37	3	57
Sex				
Male	0	–	3	52
Female	45	35	56	52
Tumor differentiation				
Well	4	32	8	52
Moderate	32	33	39	47
Poor	11	38	12	56
Lymph node				
Positive	33	35	48	52
Negative	14	31	11	51

reported to have tumor suppressive role in both *in vitro* and *in vivo* systems.<sup>(27)</sup>

Hence, it is evident that cumulative increase in genetic instability and cellular proliferation could be a consequence of genetic and epigenetic alterations of these genes. Study of coalterations of these genes could be useful in devising a diagnostic and prognostic marker. So, to delineate the molecular pathogenesis of early and late-onset BC, attempts were made in the present study to analyze the alterations (deletion, methylation and expression) in these genes and correlate them with different clinico-pathological parameters.

## Materials and Methods

**Patients, tumor tissues and cell lines.** Forty-seven early-onset (group-A) and fifty-nine late-onset (group-B) sporadic BC samples and their corresponding normal tissues or peripheral blood leukocytes (PBL) were randomly collected over a period of 9 years (1999–2007) from 104 unrelated patients undergoing surgery at the hospital of Chittaranjan National Cancer Institute, Kolkata, India and stored at –80°C. Informed consent from patients and Research Ethics Committee of the institute were obtained. All tumors were graded and staged according to International Union Against Cancer (UICC) tumor size, lymph node, metastasis (TNM) classification.<sup>(30)</sup> Detailed clinico-pathological histories of the patients are presented in Table 1. Part of the freshly operated tissues was directly collected in TRIzol reagent (Invitrogen, USA) for RNA isolation and the rest for DNA extraction. The BC cell line MCF-7 was obtained from American Type Culture Collection (ATCC) and grown according to ATCC instructions.

**Microdissection and DNA extraction.** Cryosections (5 µm) were microdissected under a dissecting microscope (Leica MZ 16) using surgical blades to remove contaminant normal cells. Samples containing at least 70–80% tumor cells were taken for DNA isolation via proteinase-K digestion, followed by phenol/chloroform extraction procedure.<sup>(31)</sup>

**Deletion analysis of the chr.3p22.3 region.** Standard polymerase chain reaction (PCR) analysis with (γ-p32) adenosine triphosphate

(ATP) end-labeled forward primer was done using microsatellite markers at chr.3p22.3 intragenic to different candidate genes.<sup>(31,32)</sup> The markers D3S1611 and D3S1007 located intragenic to *hMLH1*, D3S3623 at *APRG1* intron 1–2, D3S3880 at *ITGA9* 3' untranslated region (UTR) region and D3S4237 at *RBSP3/HYA22* 3'UTR region (telomeric to centromeric) were taken for analysis. Primers are listed in Supplement Table 1. All markers were selected on the basis of their map positions and heterozygosity (Ensembl release 44; Genome Database). PCR conditions include a 30 s denaturing step at 95°C, 30 s at appropriate annealing temperature (55–60°C) and 1 min at 72°C followed by final extension at 72°C for 7 min. PCR products were electrophoresed in 8 M urea containing 7% denaturing polyacrylamide sequencing gel and autoradiographed.

Multiplex PCR was done to see homozygous/hemizygous (HD/HED) deletion in the non-informative markers (D3S3880, D3S4237).<sup>(31,32)</sup> The SST (Somatostatin precursor; UniSTS\_NUM 49790) microsatellite marker at chr.3q27.3 was used as control (CL) owing to low frequency of alterations seen in BC (data not shown). Primer sequences are in Supplement Table 1. The PCR products were run on 2% agarose gel, stained with ethidium bromide and visualized under UV transilluminator.

Using a densitometric scanner (Bio-Rad GS-800, USA) the number of alleles at LOI (locus of interest) was determined from the signal intensities according to the following formula:

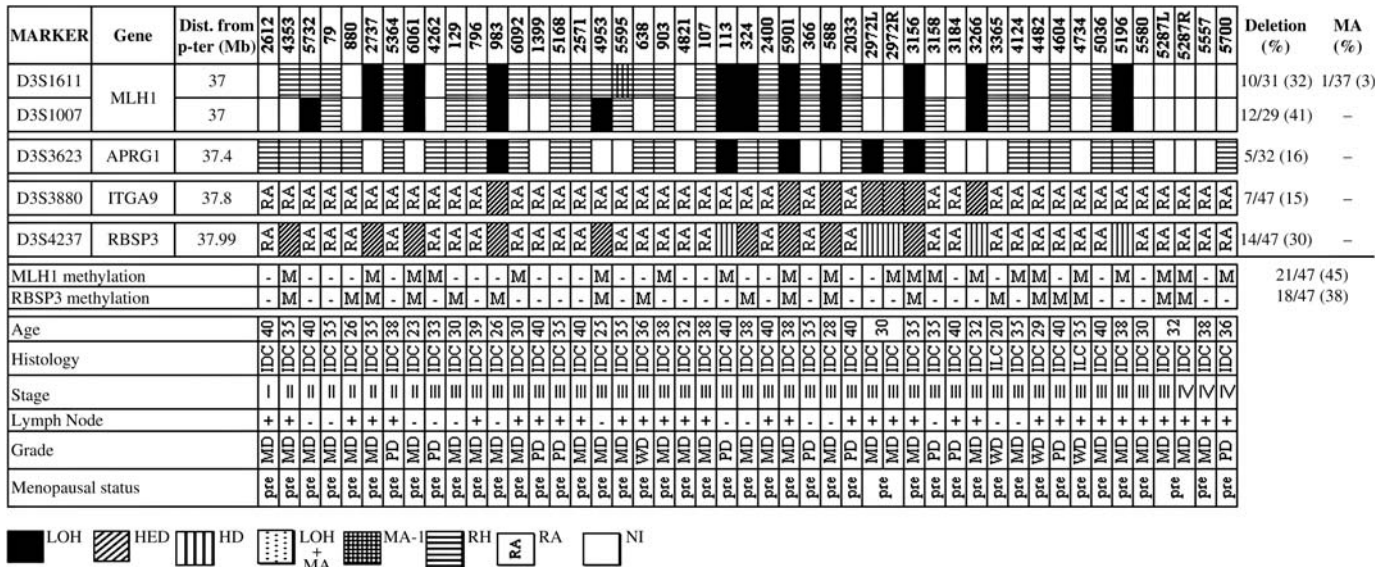
$$\text{Allele Number} = 2 \times \frac{\text{LOI(T)/CT(T)}}{\text{LOI(N)/CL(N)}}$$

where T = tumor DNA, N = normal DNA, CL = control and LOI = locus of interest.

Allelic values of ≤0.5, 0.9–1.7, ≥1.8 to <5.0 and >5.0 will be considered as homozygous deletion (HD), hemizygous deletion (HED), retention of both alleles (RA) and amplification of the alleles respectively.<sup>(33)</sup>

**PCR based methylation sensitive restriction analysis (MSRA).** The methylation analysis of *hMLH1* and *RBSP3/HYA22* gene promoters was carried out by a methylation-sensitive restriction analysis (MSRA).<sup>(34)</sup> Approximately, 100 ng of paired DNA samples were individually digested overnight with a methyl-sensitive restriction enzyme HpaII and its methylation insensitive isoschizomer MspI separately (Roche Diagnostics, Germany). The same digestion mixture was used for PCR analysis of all the genes and controls. Furthermore, mock digestion was carried out with each sample without any restriction enzymes. Primer sequences were: *hMLH1*, –670 FP: 5'-CGCTCGTAGTATTTCGTGC-3' to –61 RP: 5'-ACCTCAGTGCCCTCGTGCTCAC-3'; *RBSP3/HYA22*, –682 FP: 5'-ACTCCTCCCCACATCTCTCA-3' to –490 RP: 5'-CGACACAGGGTGAGTGGAG-3' in respect to transcription start site. Promoter region of *hMLH1* gene harbored four CCGG-recognition sites for the above restriction enzymes, whereas there is only one CCGG site in the *RBSP3/HYA22* promoter. To avoid false positivity, MSRA was done taking two controls: (i) 445-bp fragment of the β-3A adaptin gene (K1: has restriction site both HpaII and MspI enzymes but is never methylated) at chr.5q14.1 was used as digestion control; and (ii) a 229-bp fragment of the RARβ2 exon-1 (K2) at chr.3p24.2 was used as integrity control and contains no recognition sites for the above enzymes.<sup>(35)</sup>

**Quantitative real-time PCR (Q-PCR).** The mRNA expression of *hMLH1* and *RBSP3/HYA22* genes in normal breast tissue (*n* = 6) and primary BC (*n* = 14) and one breast cancer cell line (MCF-7) was analyzed by quantitative real-time (Q-PCR). Total RNA was isolated from samples using TRIzol reagent according to the manufacturer's protocol (Ambion, USA). Complementary-DNA (cDNA) was synthesized from 1 µg total RNA using Random hexamer (Invitrogen, USA) and Superscript III (Invitrogen, USA) according to manufacturer's protocol. PCR was performed in 40



**Fig. 1.** Schematic representation of the pattern of alterations in chromosome 3p22.3 in Early-Onset ( $\leq 40$  years) primary breast cancer (BC). LOH, loss of heterozygosity; HED, hemizygous deletion; HD, homozygous deletion; MA-1, microsatellite size alteration of one allele; LOH + MA, loss of one allele and size alteration of the other; RH, retention of heterozygosity (for informative markers); RA, retention of both alleles (for non-informative markers); NI, non informative; M, presence of methylation; -, absence of methylation; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated tumor grades; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; pre, pre-menopausal; post, post-menopausal; na, not applicable.

cycles on an ABI Prism 7500 using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) in a final volume of 15  $\mu$ L. Each sample was run in triplicates and the average value was considered for analysis. Relative quantities ( $\Delta\Delta C_t$ ) were obtained by normalization against human  $\beta 2$ -microglobulin gene.<sup>(36)</sup> Primers for Q-PCR were: *hMLH1*, F.P. 5'-ttctcaggttatcgagccagcac-3'; R.P. 5'-cttcgtcccaattcactcagtg-3' and *RBSP3/HYA22*, F.P. 5'-aggtgaccaacccaagga-3'; and R.P. 5'-tcacggaagcagcagaaga-3'.

**Immunohistochemistry.** Immunostaining of *hMLH1* was done in 20 primary BC samples using the avidin-biotin-peroxidase complex (ABC) staining kit according to manufacturers protocol (Santa Cruz Biotechnology, CA, USA). About 3–5  $\mu$ m paraffin sections were de-paraffinized, rehydrated and reacted overnight with primary antibodies (rabbit polyclonal immunoglobulin G [IgG] sc-581 for *hMLH1*; Santa Cruz, CA, USA) at a dilution of 1:100 at 4°C. Horseradish-peroxidase (HRP)-conjugated goat antirabbit secondary antibody (sc-2004) was added at 1:500 dilutions. The slides were developed using 3–3' diaminobenzidine (DAB) as the chromogen and counterstained with hematoxylin. The staining intensity (– = negative, + = weak, 2+ = moderate and 3+ = strong) and the percentage of positive cells (– = 0%, + = 1–24%, 2+ = 25–49% and 3+ = 50–100%) were evaluated by two observers independently.<sup>(19)</sup>

**Statistical analysis of clinical data.** Fisher's exact test was used to determine the association between genetic profile of tumors and different clinico-pathological features. Survival analysis was performed according to Kaplan–Meier method. Post-operative overall survival was measured from the date of surgery to the date of last follow-up or death (up to 5 years). Probability value (*P*-value)  $\leq 0.05$  was considered statistically significant. All statistical analyses were performed using statistical programs EpiInfo 6.04b and SPSS 10.0 (SPSS, Chicago, IL, USA).

## Results

**Deletion analysis of chr.3p22.3 region.** Overall deletion noted in the entire 1 Mb analyzed region of chr.3p22.3 was seen to be 32% (15/47) in group-A and 37% (22/59) in group-B samples.

Microsatellite analysis identified deletion in *hMLH1*, *APRG1*, *ITGA9* and *RBSP3/HYA22* genes in both age groups of BC. In group-A, *hMLH1* showed highest 38% (12/32) deletion followed by *RBSP3/HYA22* (30%: 14/47), *APRG1* (16%: 5/32) and *ITGA9* (15%: 7/47). Likewise, in group-B, 32% (12/38) samples showed deletion in *hMLH1* followed by *RBSP3/HYA22* (24%: 14/59), *ITGA9* (14%: 8/59) and *APRG1* (13%: 4/32). Seven BC samples in group-A (#2737, 983, 5901, 588, 2972 L, 3156, 3266) and six in group-B (#2587, 148, 1186, 5213, 2766, 563) showed deletion or non-informativeness (homozygous condition) of the entire studied region indicating loss of the entire region. Five samples in both groups showed homozygous deletion (HD) in *RBSP3/HYA22* (Figs 1, 2 and 3a,d). Microsatellite size alteration (MA) was observed only in 3 out of 106 samples (#5595, 3990, 1273) (Fig. 3b), whereas another sample (#7) showed loss in one allele and microsatellite size alteration of the other allele (LMA) (Fig. 3c). Higher frequency of deletion in *hMLH1* and *RBSP3/HYA22* in both the age groups compared to *APRG1* and *ITGA9*, indicating that they were the candidate TSG associated with pathogenesis in both age groups of BC.

In both groups, deletion in *APRG1*, *ITGA9* and *RBSP3/HYA22* showed significant association (Table 2), whereas *hMLH1* deletion showed significant association with other genes in group-A and with *ITGA9* in group-B.

**Methylation analysis of *hMLH1* and *RBSP3/HYA22* gene promoters.** Methylation analysis of *hMLH1* and *RBSP3/HYA22* were performed owing to their high frequency of deletion in both age groups. Higher frequency of methylation in *hMLH1* promoter was seen in group-A (45%: 21/47) than group-B (34%: 20/59). However, comparable frequencies of methylation in *RBSP3/HYA22* promoter was observed in both group-A (38%: 18/47) and group-B (32%: 19/59) (Figs 1, 2 and 4a,b). Concomitant methylation in *hMLH1* and *RBSP3/HYA22* promoters were seen in 11 group-A and 7 group-B samples. However, no association was seen between methylation of these two genes in either group.

**Association between overall alterations of *hMLH1* and *RBSP3/HYA22*.** Either deletion or methylation in any one of these genes were seen in about 64% group-A and 61% group-B samples,

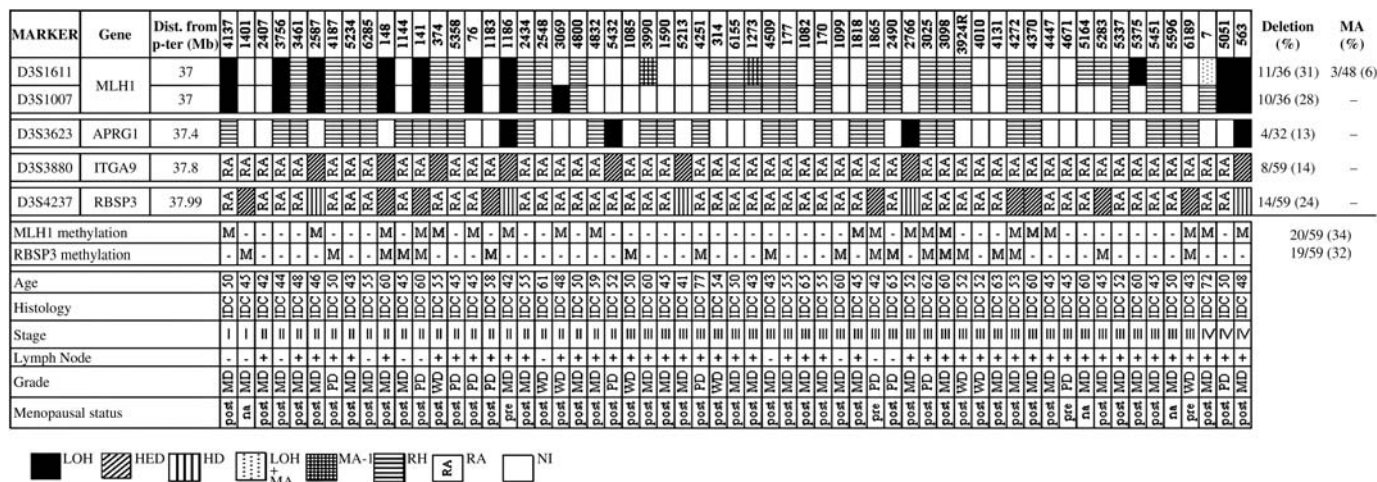


Fig. 2. Schematic representation of the pattern of alterations in chromosome 3p22.3 in late-onset (>40 years) primary breast cancer.

Table 2. Association between 4 candidate tumor suppressor genes loci at chr.3p22.3 in early and late-onset breast cancer

	Group-A								Group-B								
	<i>hMLH1</i>		<i>APRG1</i>		<i>ITGA9</i>		<i>RBP3</i>		<i>hMLH1</i>		<i>APRG1</i>		<i>ITGA9</i>		<i>RBP3</i>		
	D+	D-	D+	D-	D+	D-	D+	D-	D+	D-	D+	D-	D+	D-	D+	D-	
<i>hMLH1</i>	D+	-	-	4	8	6	6	11	1	-	-	2	10	4	8	5	7
	D-			1	34	1	34	3	32			2	45	4	43	9	38
<i>P</i> -value		na		0.003*		0.0001*		0.0000001*		na		0.13		0.02*		0.1	
<i>APRG1</i>	D+	-	-	-	-	4	1	5	0	-	-	-	-	4	0	3	1
	D-					3	39	9	33					4	51	11	44
<i>P</i> -value		na		na		0.00001*		0.0002*		na		na		0.0000002*		0.01*	
<i>ITGA9</i>	D+	-	-	-	-	-	-	7	0	-	-	-	-	-	-	6	2
	D-							7	33							8	43
<i>P</i> -value		na		na		na		0.00001*		na		na		na		0.0002*	

Abbreviations used are: D+: Deletion positive; D-: Deletion negative; \*indicates *P*-value significance.

indicating the importance of chr.3p22.3 in the development of BC. Overall alterations (deletion/methylation) of *hMLH1* and *RBP3/HYA22* in group-A were 51% and 49%, respectively, which was comparatively higher than that seen in group-B (39% each) (Fig. 5). Significant association was seen between overall alterations of *hMLH1* and *RBP3/HYA22* in group-A ( $P = 0.002$ ). Deletion and methylation in *hMLH1* and *RBP3/HYA22* were significantly associated in both group-A ( $P = 0.01$  and  $0.0007$ ) and group-B ( $P = 0.01$  and  $0.02$ ) (Table 3).

**Expression analysis.** Q-PCR analysis of *hMLH1* and *RBP3/HYA22* mRNA revealed reduced expression in both the genes. With respect to non-tumor control, fold reduction in expression in tumors was seen in the following order: *hMLH1* ( $10.4 \pm 13.9$ ) and *RBP3/HYA22* ( $28.9 \pm 39.1$ ). Reduced expression was noted in about 33–40% of the tumors, more than the mean fold reduction of the respective genes (Fig. 6). Immunohistochemical analysis revealed intense nuclear localization of *hMLH1* protein in samples without any alteration (deletion and/or methylation), whereas moderate to negative expression was observed in samples harboring genetic and/or epigenetic alterations. Expression of *hMLH1* protein showed significant concordance with its alteration (deletion/methylation) status ( $P = 0.0004$ ) (Table 4, Fig. 7). The protein expression of *RBP3/HYA22* was not checked owing to the lack of commercially available antibody.

**Association with other candidate genes.** Among the 106 BC samples, a subset of 29 group-A and 33 group-B samples were

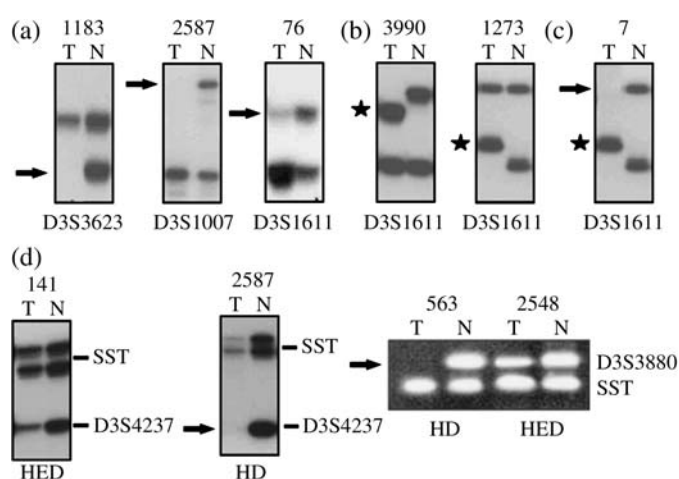
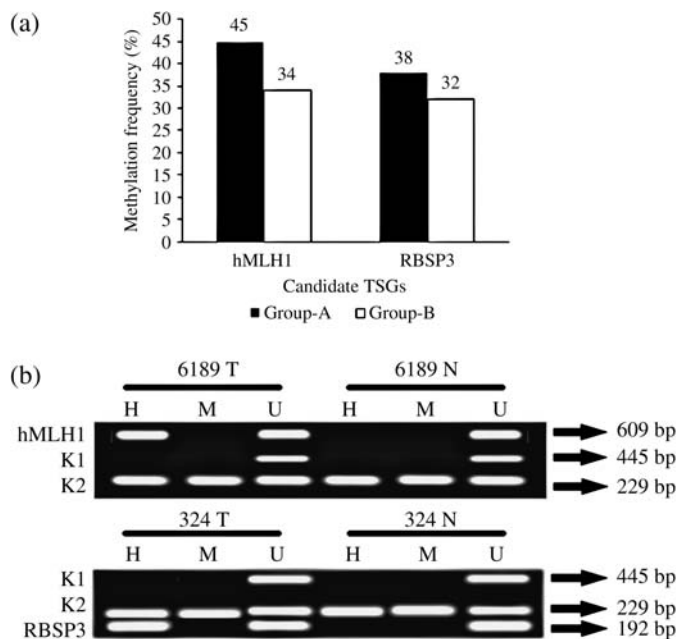


Fig. 3. Representative samples showing: (a) LOH, loss of heterozygosity; (b) MA-1, microsatellite size alteration of one allele; (c) LOH + MA, loss of one allele and size alteration of other; and (d) homozygous/hemizygous deletion (HD/HED). Arrowhead (→) indicates loss of corresponding allele and star (★) indicates size alteration of one or both alleles.

**Table 3. Association between deletion and methylation of *hMLH1* and *HYA22* in two age groups of breast cancer**

		Group-A								Group-B							
		<i>hMLH1</i>		<i>hMLH1</i>		<i>RBSP3</i>		<i>RBSP3</i>		<i>hMLH1</i>		<i>hMLH1</i>		<i>RBSP3</i>		<i>RBSP3</i>	
		D+	D-	M+	M-	D+	D-	M+	M-	D+	D-	M+	M-	D+	D-	M+	M-
<i>hMLH1</i>	D+	-	-	9	3	11	1	8	4	-	-	9	3	5	7	8	4
	D-			12	23	3	32	10	25			11	36	9	38	11	36
<i>P</i> -value		na		0.01*		0.0000001*		0.02*		na		0.0007*		0.1		0.004*	
<i>hMLH1</i>	M+	-	-	-	-	11	10	11	10	-	-	-	-	10	10	7	13
	M-					3	23	7	19					4	35	12	27
<i>P</i> -value		na		na		0.002*		0.07		na		na		0.0006*		0.74	
<i>RBSP3</i>	D+	-	-	-	-	-	-	9	5	-	-	-	-	-	-	8	6
	D-							9	24							11	34
<i>P</i> -value		na		na		na		0.01*		na		na		na		0.02*	

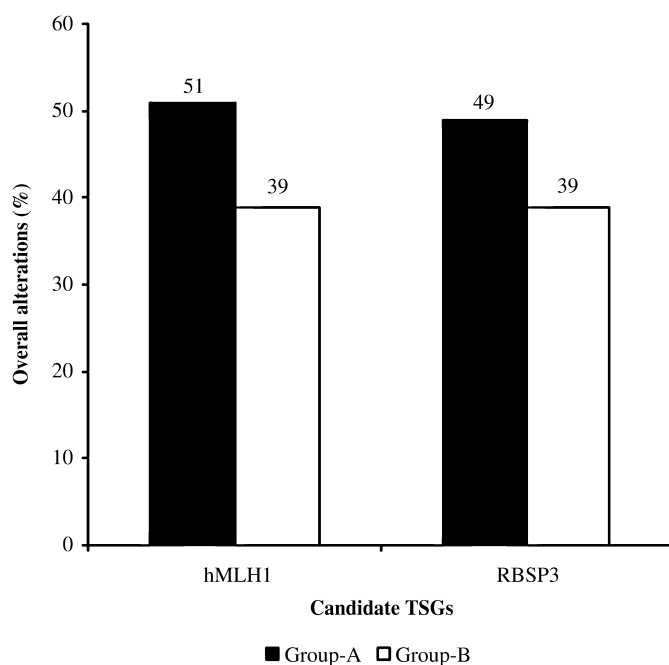
Abbreviations used are: D+: Deletion positive; D-: Deletion negative; M+: Methylation positive; M-: Methylation negative. \*indicates p value significance.



**Fig. 4.** (a) Histogram showing pattern of methylation in *hMLH1* and *RBSP3/HYA22* in two age groups of breast cancer (BC). (b) Representative agarose gels showing methylation in *hMLH1* and *RBSP3/HYA22* done by MSRA. H, HpaI digested DNA; M, MspI digested DNA; U, undigested DNA. Controls: A 445-bp fragment of  $\beta$ 3A adaptin gene (K1) and 229-bp fragment of RAR $\beta$ 2 exon-1 (K2).

further studied to find out the association of *hMLH1* with other DNA repair genes *BRCA1*, *BRCA2*, *TP53* and *RBSP3/HYA22* with *Rb*. Deletion status of these genes was published in our previous report.<sup>(9)</sup> A majority (64–90%) of the samples in either group showed alterations in any one of the DNA repair genes (data not shown). Coincidental alterations of other genes along with *hMLH1* alterations were as follows: group-A: *BRCA1* (52%: 15/29); *BRCA2* (45%: 13/29); *TP53* (28%: 9/29) and group-B: *BRCA1* (27%: 9/33); *BRCA2* (21%: 7/33); *TP53* (27%: 9/33), indicating involvement of multiple DNA repair pathways. Similar to DNA repair genes, 67–79% samples showed alterations of *RBSP3/HYA22* and/or *Rb* in both age groups, suggesting the importance of this pathway in the development of BC. However, the coincidental alteration of *RBSP3/HYA22* and *Rb* was less frequent (21–27%) in both groups.

**Clinico-pathological association and patient survival.** In group-A, overall alterations of both *hMLH1* and *RBSP3/HYA22* were



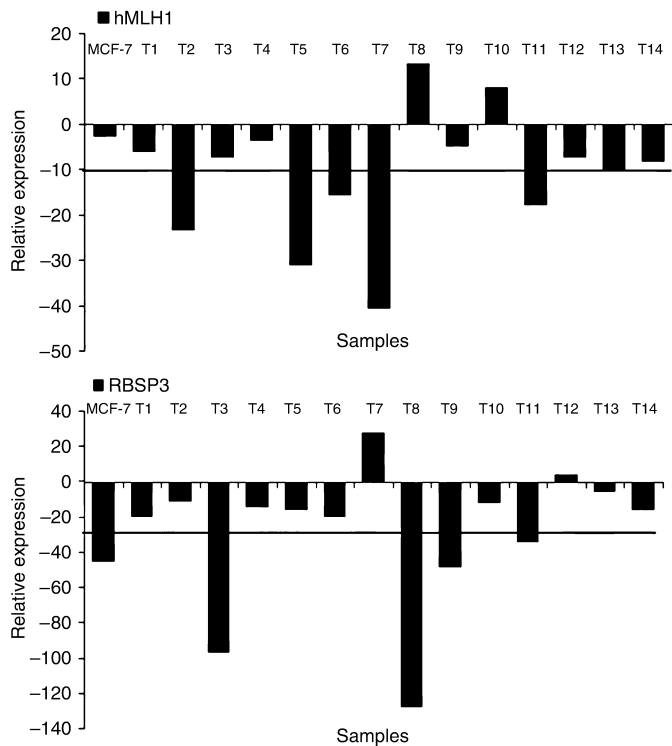
**Fig. 5.** Pattern of overall alterations in *hMLH1* and *RBSP3/HYA22* at chr3p22.3.

significantly associated with higher histological grades ( $P = 0.03$  and  $0.004$ ). Moreover, *hMLH1* deletion showed significant association with lymph node invasion in group-A ( $P = 0.01$ ) and with more advanced stages in group-B ( $P = 0.03$ ). On the other hand, methylation of *RBSP3/HYA22* showed significant association with higher tumor grades in group-A ( $P = 0.002$ ) and lymph node metastasis in group-B ( $P = 0.04$ ).

In both age groups of BC, presence of alterations (deletion and/or methylation) in *hMLH1* showed significantly poor patient survival as revealed by Kaplan–Meier analysis. Contrastingly, only group-A patients with alterations in *RBSP3/HYA22* had poor disease outcome (Fig. 8).

## Discussion

Differential pattern of alterations of different chromosomal regions in early and late-onset BC has been reported in our previous studies.<sup>(6,7,9,10)</sup> Further study of molecular alterations (deletion/methylation) of the candidate genes at chr.3p22.3



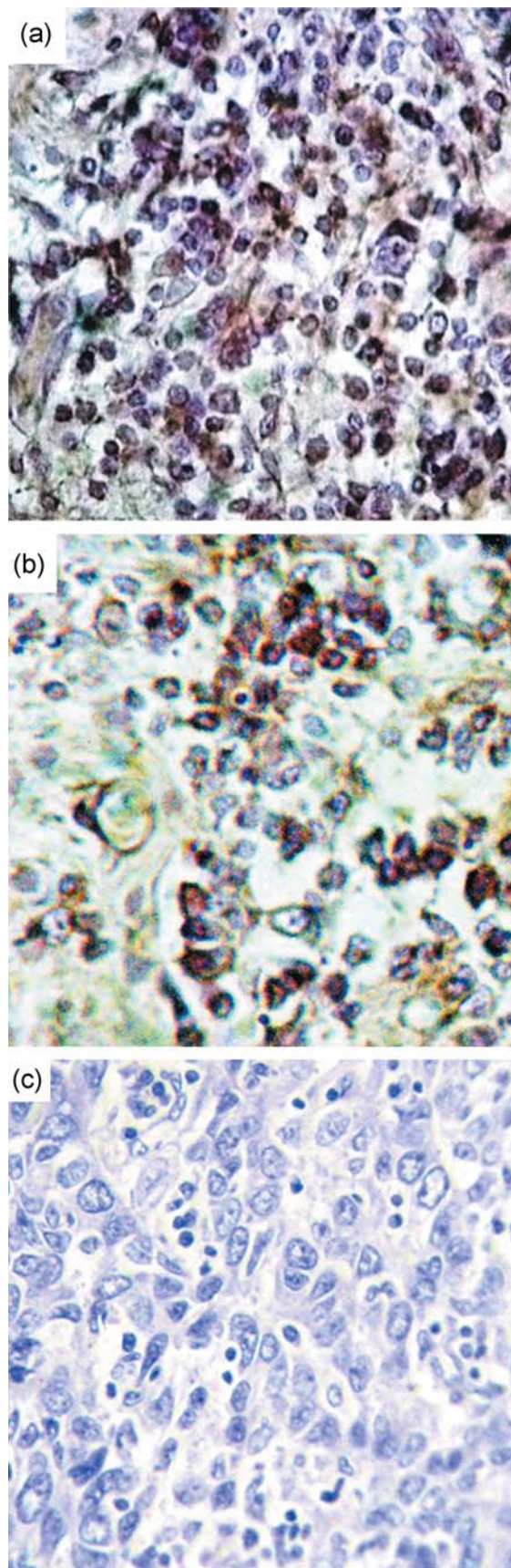
**Fig. 6.** Quantitative real-time-polymerase chain reaction (RT-PCR) analysis showing *hMLH1* and *RBSP3/HYA22* mRNA expression in breast cancer (BC) samples ( $n = 15$ ). Bars represented the gene expression normalized to  $\beta 2$ -microglobulin and relative to normal counterpart using the  $2^{-\Delta\Delta Ct}$  method as described in Materials and Methods. Bar 1 represents the BC cell line MCF-7 and bars 2–15 represents breast tumors. Horizontal line indicates the mean fold of reduction of the respective genes.

**Table 4.** Immunohistochemical expression status of *hMLH1* with respect to alteration (deletion and/or methylation)

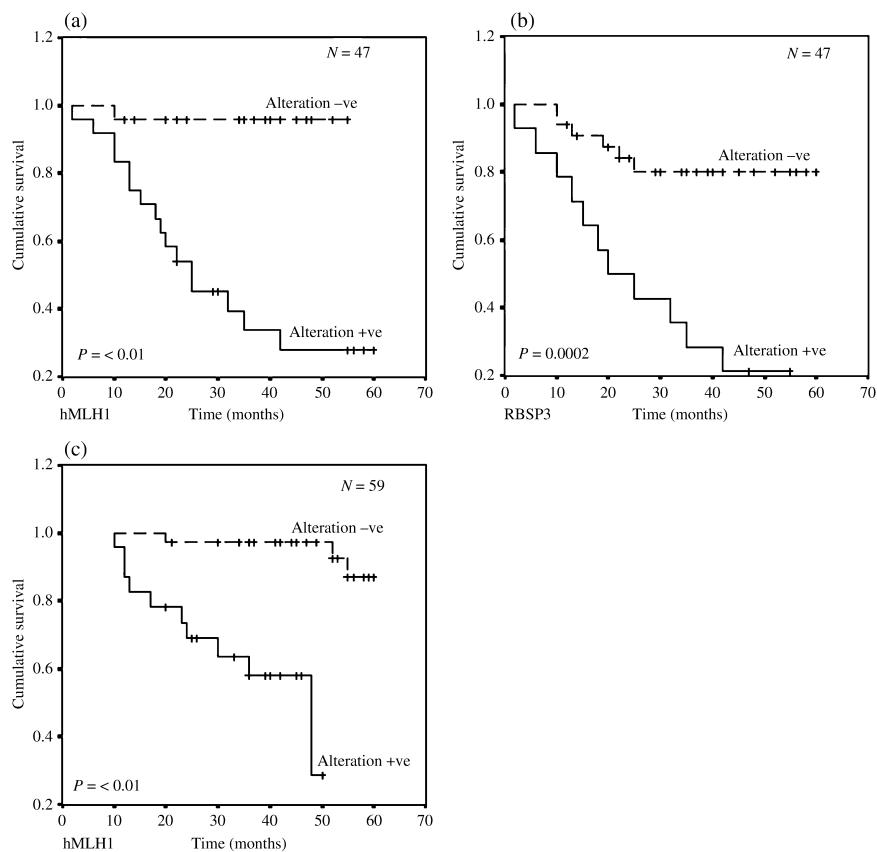
Sample No.	<i>hMLH1</i> alteration Deletion/Methylation	Expression
1186	+/+	-
374	-/+	-
4187	-/-	+++
3266	+/+	-
5451	-/-	+++
5596	-/-	+++
3156	+/+	-
324	+/-	++
4131	+/+	-
5051	+/-	-
4124	-/+	-
1818	-/+	++
113	+/+	-
314	-/-	+++
5732	+/-	-
1865	-/+	-
4671	-/-	+++
3025	-/+	-
6155	+/+	-
5375	+/-	-

*P*-value = 0.0004\*

Sample no is patient's registration number.



**Fig. 7.** Immunohistochemistry of *hMLH1* gene. Breast cancer samples showing high (a) or moderate (b) or low/negative (c) expression of *hMLH1* gene. All magnifications are at 20 $\times$ .



**Fig. 8.** Kaplan-Meier 5-year survival probability curves with cumulative survival of breast cancer (BC) patients by overall alteration status of (a) *hMLH1* and (b) *RBSP3/HYA22* in early-onset BC and (c) only *hMLH1* in late-onset BC. Survival time was defined as the time from surgery to the patient's death, known recurrence or the last time the patient was known to be alive. The log rank test was used to assess the differences in the patient survival between cases with plot for the differences in overall survival among cases with any alteration (Alteration +ve) and no alteration (Alteration -ve) of the analyzed genes. The dotted line represents survival probability without alteration and smooth line represents with alteration probability. **N** total number of early-onset BC.

would provide an in-depth analysis of their association in pathogenesis of the two separate age groups of BC. Overall alterations noted in the entire studied locus were comparable in both age group-A (64%) and group-B (61%), indicating the importance of chr.3p22.3 in the development of BC. Among the four candidate TSG studied, deletion frequency of *hMLH1* and *RBSP3/HYA22* were significantly higher than *APRG1* and *ITGA9*, indicating the importance of the former two genes in the development of BC. In group-A, both *hMLH1* and *RBSP3/HYA22* showed higher frequency of overall alterations than in group-B (Fig. 5). This is the first report of its kind to show differential patterns of alteration in *hMLH1* and *RBSP3/HYA22* genes in the two age groups of BC.

Significant association between deletion and methylation of *hMLH1* and *RBSP3/HYA22* in both groups suggested them to be candidate TSG in BC. Concordant down-regulation of mRNA and protein as a consequence of alterations (deletion/methylation) in these genes further confirmed this contention. Sporadic BC samples having MSI in other chromosomal arms have been reported to show frequent mutation and methylation of the DNA mismatch repair gene *hMLH1*.<sup>(19,37)</sup> On the contrary, we have seen infrequent MSI in different chromosomal arms in primary BC.<sup>(6,7,9,10,38)</sup> This might be due to differences in etiology, ethnicity and probably methods used in the analysis. Hypermethylation of *hMLH1* promoter accounted for the majority of the cases with reduced expression of *hMLH1*.<sup>(19,20)</sup> A similar result was also observed in the present study. The clinico-pathological association of *hMLH1* with progression and prognosis of BC has not yet been reported. Significant association of *hMLH1* deletions with lymph node invasion in group-A and with advanced stages in group-B as well as adverse patient outcome with *hMLH1* alterations in both age groups indicate *hMLH1* to be a good diagnostic and prognostic marker. Moreover, high incidence

*hMLH1* alterations along with other DNA repair genes like *BRCA1/BRCA2* in group-A suggested altered pathogenesis in the two age groups of BC.

Alterations of another candidate TSG, *RBSP3/HYA22* also localized in chr.3p22.3, have been implicated in a number of epithelial malignancies. The primary function of *RBSP3/HYA22* is dephosphorylation of phosphorylated retinoblastoma gene (ppRb) thus preventing E2F-mediated downstream transcriptional signaling, leading to a block of the cell cycle at the G<sub>1</sub>-S boundary.<sup>(27-29)</sup> Frequent homozygous (HD) and hemizygous deletion (HED) of this locus have been reported in BC.<sup>(39)</sup> In acute lymphoid leukemias (ALL) frequent methylation of *RBSP3/HYA22* promoter have been reported that correlated with decreased gene expression.<sup>(40)</sup> However, very few reports exist regarding molecular alterations of *RBSP3/HYA22* and its association with BC. We, for the first time, have showed that *RBSP3/HYA22* indeed undergoes frequent and differential pattern of alterations in both early and late-onset BC. Clinico-pathological studies showed methylation of *RBSP3/HYA22* to be significantly associated with higher tumor grades in group-A and with lymph node invasion in group-B. The association noted between *RBSP3/HYA22* alterations and adverse patient survival in group-A suggested its usefulness as a prognostic tool in this age group. Moreover, frequent alterations of *RBSP3/HYA22* and/or *Rb* indicated ablation of the *RBSP3/HYA22-Rb-E2F* associated cell cycle pathway in BC.

It is evident from the present study that *hMLH1* and *RBSP3/HYA22* are frequently altered (deletion/methylation) in primary BC, but are differentially associated in the two age groups. Thus, it is suggested that the dysregulation of the DNA repair pathways and G<sub>1</sub>-S cell cycle checkpoint might give some selective growth advantage for progression and adverse prognosis of the disease.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1** Rate of mortality in breast cancer patients showing inactivation of either or both the *hMLH1* and *RBSP3/HYA22* genes compared with normal (none of the genes are altered).

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