

***LARG* at chromosome 11q23 has functional characteristics of a tumor suppressor in human breast cancer**

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Abstract

Deletion of 11q23-q24 is frequent in a diverse variety of malignancies, including breast and colorectal carcinoma, implicating the presence of a tumor suppressor gene at that chromosomal region. We show here that *LARG*, from 11q23, has functional characteristics of a tumor suppressor. We examined a 6-Mb region on 11q23 by high-resolution deletion mapping, utilizing both loss of heterozygosity (LOH) analysis and microarray comparative genomic hybridization (CGH). *LARG* (also called *ARHGEF12*), identified from the analyzed region, was underexpressed in 34% of primary breast carcinomas and 80% of breast cancer cell lines including the MCF-7 line. Multiplex ligation-dependent probe amplification on 30 primary breast cancers and six breast cancer cell lines showed that *LARG* had the highest frequency of deletion compared to the *BCSC-1* and *TSLC1* genes, two known candidate tumor suppressor genes from 11q. *In vitro* analysis of breast cancer cell lines that underexpress *LARG* showed that *LARG* could be reactivated by trichostatin A, a histone deacetylase inhibitor, but not by 5-Aza-2'-deoxycytidine, a demethylating agent. Bisulfite sequencing and quantitative high-throughput analysis of DNA methylation confirmed the lack of CpG island methylation in *LARG* in breast cancer. Restoration of *LARG* expression in MCF-7 cells by stable transfection resulted in reduced proliferation and colony formation, suggesting that *LARG* has functional characteristics of a tumor suppressor gene.

Introduction

Chromosome 11q23-q24 deletion is frequent in a variety of tumor types, including tumors of the breast, colorectum, ovary, stomach, lung, cervix, nasopharynx and malignant melanoma, implicating that this region is important in the tumorigenesis of diverse tumor types (1-9).

Functional evidence suggesting the involvement of this region in tumorigenesis has been demonstrated by microcell-mediated chromosome transfer (10). The MCF-7 cell line transferred with the entire chromosome 11 was nontumorigenic, whereas the MCF-7 line which had the transfer of chromosome 11 lacking the distal portion of 11q maintained the tumorigenic phenotype, suggesting the presence of one or more tumor suppressor gene(s) in the distal region of 11q (10). Furthermore, significant tumor suppression has been demonstrated in fibrosarcoma cells and lung carcinoma cell lines transfected with YACs mapping to the 11q23 region (11, 12).

Two independent regions of LOH at 11q23 have been identified previously in breast cancer (13). The *BCSC-1* candidate tumor suppressor gene is located in the second, more distal region (LOH11CR2), and is implicated as the target of deletion in breast cancer based on LOH analysis, Northern analysis on cell lines (but not primary tumors), suppression of colony formation *in vitro* and tumorigenicity *in vivo* (3).

However, we and others have identified a third region of loss of heterozygosity (LOH) in breast and colorectal cancers, which lies between these two regions and from which a

candidate tumor suppressor gene has yet to be identified (1, 2, 14, 15). In this present study, this third region of LOH was analyzed by high-resolution deletion mapping, and a candidate tumor suppressor gene, *LARG*, was identified. We show here that expression of *LARG* is frequently silenced in primary breast cancer and breast cancer cell lines. Furthermore, tumor suppressive function of *LARG* was demonstrated by both colony formation and cell proliferation assays, and the mechanisms for silencing of *LARG* were elucidated.

Materials and Methods

Samples

Breast tumor tissues were collected from 58 patients at the Singapore General Hospital and were snap-frozen in liquid nitrogen upon resection and stored at -70°C or in liquid nitrogen. Peripheral blood samples were collected in EDTA tubes from each of the patients, and frozen at -70°C . Informed consent from all patients was obtained. The demographic and clinical information on the cases are summarized in **Supplementary Table S1**. The study protocol was approved by the Institutional Review Boards of the Singapore General Hospital and the National Cancer Centre of Singapore.

DNA and RNA extraction

DNA and RNA were isolated from microdissected tumor tissues as described previously (2). Only samples that had at least 70% tumor cells were processed for DNA extraction using DNAzol (GIBCO/BRL) according to the manufacturer's instructions, or RNA extraction using a column-based method (Qiagen, RNeasy; Qiagen, Hilden, Germany) or Trizol (Invitrogen). DNA was extracted from blood samples using sucrose lysis buffer and proteinase K digestion.

Microsatellite analysis

Loss of heterozygosity at the 11q22-23 chromosomal region was assessed using seven microsatellite markers (**Fig. 1**), as previously described (2). Polymerase chain reactions (PCR) were performed with 100-400ng of tumor or normal (blood) DNA. The sense primer was end-labeled with ^{33}P . The PCR reaction was carried out for 1min at 94°C ,

1min at between 60°C and 67°C, and 1min at 72°C for 24 cycles. The PCR products were separated on an 8% polyacrylamide gel and exposed to X-ray film overnight and also exposed to the CS phosphor screens (Biorad, USA) for 4 to 6 hours.

Assessment of LOH

LOH was assessed by densitometry by scanning the CS screens with a Molecular Imager (Biorad, USA). LOH was determined by quantitation of the signal intensity of each allele, and comparing the ratios of the intensity of the alleles from the tumor DNA with that of the constitutional (blood) DNA, using the formula $T1:T2/N1:N2$. Samples with ratios of less than 0.5 or more than 1.5, indicating a reduction of more than 50% of one allele, were deemed to have LOH. All samples with LOH were reanalyzed by repeating the microsatellite analysis, in order to confirm the results.

Array CGH

The BAC arrays were constructed at the University of California San Francisco Cancer Centre, and included 41 BAC clones from 11q23 (**Fig. 1**). Array CGH analysis was done as described (16). In brief, one ug of DNA from primary breast cancer tumors (test) and reference DNAs were digested with *DpnII* and labeled with Cy3-dUTP or Cy5-dUTP respectively (Amersham, UK) by random priming (Invitrogen, Carlsbad, CA), and were cohybridized with tRNA and human Cot-1 DNA (Roche, CA) onto array slides for 48 h. Slides were then washed and TIF images were captured using a GenePix scanner (Axon Inc.). The images were analyzed using the UCSF Spot and Sproc software (17) and an Excel macro developed at UCSF. After normalization, the mean log₂ ratios were plotted

and fluorescent Cy3 (test)/Cy5 (reference) log₂ ratios were classified as genomic gain, if greater than 0.3 or genomic loss, if lower than -0.3. The UCSF SPOT and SPROC software are available at <http://cc.ucsf.edu/jain/public>.

Fluorescence in situ hybridization (FISH)

DNA from the BAC clone RP11-15I6 from chromosome 11q23 was labeled by nick-translation with SpectrumGreen (Vysis, Downers Grove, IL) as described (18). A centromeric chromosome 11 probe to D11Z1 (CEP 11) labeled with SpectrumOrange (Vysis) was used as an internal control. Interphase FISH was done by hybridizing the probes to frozen tumor sections and counterstaining with DAPI. At least 100 cells were examined using a fluorescent imaging workstation by two independent investigators (Applied Imaging, Santa Clara, CA). Samples with signal ratios of test (RP11-15I6) to control (CEP11) probes of <0.8 were deemed as having deletion.

Multiplex ligation-dependent probe amplification (MLPA)

To compare DNA copy number between different genes on chromosome 11q, MLPA using synthetic probes was done. MLPA probes were designed according to guidelines recommended by MRC-Holland (www.mlpa.com) and as previously described (19), except that the minimum length difference between probes was 3 nt and not 4 nt. Two MLPA probes targeting different exons were designed for *LARG* and two other genes on chromosome 11q which are known to be deleted in cancer, *TSLCI* and *BCSC-1*. Probes for two control genes, *GUSB* and *TBP* were included for MLPA analysis. Each complete probe was unique in size and differed by three or more nucleotides in length to avoid

electrophoretic overlap. Details of the probes designed are provided in **Supplementary Table S2**.

The MLPA hybridization, ligation and PCR amplification was done using reagents from MRC-Holland (Amsterdam, The Netherlands) as described (19) with minor modifications: 1ul of MLPA probe mix (4fmol of each probe) was used instead of 1.5ul; and hybridization at 60°C was done for 4 hr. PCR products were analyzed using capillary electrophoresis on the CEQ 8000 Genetic Analysis System (Beckman Coulter) or the 3130xl Genetic Analyzer (Applied Biosystems). The copy number for each probe was expressed as a dosage quotient where 1.0 indicated the presence of two alleles, and a value of <0.8 was scored as a deletion (20). Three control DNA samples from two normal individuals and a commercial normal female human genomic DNA control (Promega, Madison, WI) were included in every experiment. For the five experimental batches, the mean \pm S.D. dosage quotient values for the controls were 1.00 ± 0.012 for *LARG*, 1.00 ± 0.033 for *TSLC1*, and 1.00 ± 0.029 for *BCSC-1*.

Real-Time Quantitative PCR

cDNA was generated from 500 ng of total RNA from each sample by reverse transcription using the iScript cDNA Synthesis kit (Biorad). The expression level of the *LARG* gene was determined using the Assay-on Demand™ Gene Expression product (Applied Biosystems, Foster City, CA), with the *GAPDH* housekeeping gene as the endogenous control. Three normal breast RNAs were included as controls (B7N; commercial controls of breast RNA from Ambion and Stratagene), and a commercial

human breast total RNA (Ambion), was designated as the calibrator. The reactions were performed in triplicate on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems).

Sequencing

The entire coding region and flanking exon-intron boundaries of the *LARG* gene was screened for mutations by direct sequencing on a CEQ 2000 sequencer (Beckman Coulter, Fullerton, CA) as described (21).

DNA methylation analysis

Genomic DNA of ten breast cancer cell lines (MCF-7, BT20, BT549, HCC2218, HCC1937, HCC1500, ZR75.1, T47D, BT474, and MBA MD231) was modified by bisulfite treatment using the MethylSEQRTM Bisulfite Conversion Kit (Applied Biosystems, USA). The bisulfite-treated genomic DNA was PCR amplified (6 overlapping fragments of 356bp, 476bp, 397bp, 420bp, 382bp and 362bp) and directly sequenced (BigDye Terminator v3.1 cycle sequencing kit, Applied Biosystems, USA). A region encompassing 1334bp before the transcription start site and 474bp after the first exon (42bp) was analyzed.

DNA methylation analysis was also performed by Sequenom, Inc., using the MassARRAY system, which utilizes MALDI-TOF mass spectrometry analysis of base-specifically cleaved amplification products (22). Two CpG-rich regions upstream of exon

1 (-12240 to -11474 and -1585 to -197 relative to the transcription start site) were screened for methylation.

Treatment of Cells with 5-Aza-2'deoxyctidine (5Aza-dC) and Trichostatin A (TSA)

1×10^5 breast cancer cells were seeded in 60-mm dishes and treated 24 hours later with $1\mu\text{M}$ 5Aza-dC (dissolved in dimethyl sulfoxide) (Calbiochem) for 96 hours or $1\mu\text{M}$ TSA (dissolved in ethanol) (Sigma) for 48 hours. For co-treatment of cells with 5Aza-dC and TSA, cells were treated initially with 5Aza-dC ($1\mu\text{M}$) for 48 hours, followed by the addition of TSA ($1\mu\text{M}$) and treatment for an additional 48 hours. After treatment, expression of *LARG* was evaluated by real-time quantitative RT-PCR.

Plasmid construction and Gene Transfection

Full-length *LARG* cDNA in pCMV6-XL4 was obtained commercially (Origene Technologies Inc, Rockville, MD). As pCMV6-XL4 lacks an antibiotic resistant gene for selection of stable transfectants, the full length *LARG* cDNA was subcloned into the *NotI* restriction site of pcDNA3.1 (Invitrogen). The pcDNA3.1-LARG construct was transfected into the breast cancer cell line MCF-7 using Lipofectamine 2000 (Invitrogen) and stable transfectants were selected with $400\mu\text{g/ml}$ Geneticin (Invitrogen). The MCF-7 cell line was also transfected with the pcDNA3.1 vector with no insert (empty vector), as controls. The MCF-7 cell line was purchased from the American Type Culture Collection (Manassas, VA) and was maintained in RPMI1640 supplemented with 10% fetal bovine serum. To confirm that selected clones were successfully transfected, clones were

screened with PCR, real-time RT-PCR and western blotting analysis both before and after performing the cell proliferation and colony formation assay.

Screening of transfectants by PCR

The PCR primers to screen empty vector clones were

5' CCACTGCTTACTGGCTTATC 3' (forward) and 5' TAGAAGGCACAGTCGAGG 3' (reverse) generating a PCR product of 202bp. The primers for screening the pcDNA3.1-LARG construct were 5' CAGAATACTCACTCCGATGG 3' (forward, exon 38) and 5' TAGAAGGCACAGTCGAGG 3' (reverse, pcDNA3.1) generating an amplicon of 743bp.

Western Blot Analysis

Whole cell lysates (30µg) were electrophoresed on 6% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Biorad). The membranes were incubated with a customized polyclonal rabbit anti-LARG primary antibody (Zymed) followed by a HRP-conjugated anti-rabbit secondary antibody (Amersham Biosciences). The signals were visualized by enhanced chemiluminescence, using ECL Western Blotting reagents (Amersham Biosciences).

Cell Proliferation Assay

The cell proliferation rate of pcDNA3.1-LARG and pcDNA3.1 (empty vector) transfected MCF-7 cells were compared using the CellTitre 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Cells were

seeded at a density of 5000 cells/well in 100ul of medium in triplicate onto 96-well plates. The estimated number of cells was determined every 48 hours from day 1 for 7 days. In brief, the cells were incubated for 2 hours at 37°C in a humidified, 5% CO₂ environment with 20ul of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) solution. The absorbance of a formazan product, bio-reduced from MTS tetrazolium by dehydrogenase enzymes in metabolically active cells was recorded at 450nm using a microplate reader.

The cell proliferation rate of cells treated with 5Aza-dC and TSA was as described above, but using 24-well rather than 96-well plates to enable more cells to be counted. Each well was seeded with 3×10^4 cells in 300ul of media, and using 60ul of MTS solution. After incubation, 100ul was then transferred to a 96-well plate for reading on the microplate reader.

Colony Formation Assay

Nine hundred cells were seeded onto 10-cm Petri dishes in triplicate and maintained for 14 days in selection media. Cells were fixed with methanol-acetic acid (3:1; vol/vol) for 15min and stained with 1% (wt/vol) crystal violet for 1 hour. Colonies greater than 1mm in diameter were counted.

Results and Discussion

High-resolution deletion mapping

The frequency of LOH and the heterozygosity rate of seven microsatellite markers on chromosome 11q23 in 58 primary breast carcinoma specimens are shown in **Fig. 1**. The demographic and clinical details of these patients are summarized in **Supplementary Table S1**. The frequency of LOH was high for all markers ranging from 45% at D11S4104 to 66 % at D11S29, and heterozygosity rates ranged from 0.586 to 0.877 (**Fig. 2A**). Overall, 41 of 58 (71%) of all tumors showed LOH for at least one of the seven microsatellite markers (**Fig. 2B**). Notably, 16 cases had either LOH and/or homozygosity at all 7 microsatellite markers suggesting that chromosomal non-disjunction may have occurred with loss of the entire chromosomal region (**Fig. 2B**). Representative examples of LOH are shown in **Fig. 2C**.

A customized CGH microarray was constructed to further define the region of deletion. The microarray included 41 BAC clones within a ~6Mb region from 11q23.3 to 11q24.1, and spanned the microsatellite markers D11S29 to D11S1345 (**Fig. 1**). All BAC clones were tested by fluorescence *in situ* hybridization (FISH) on normal metaphase chromosome spreads to verify that the clones were indeed from this chromosomal region. Our FISH analysis revealed that eleven clones either hybridized to other chromosomes (RP11-8K10, RP11-158K18, RP11-271P14) or gave non-specific signals on FISH (RP11-712L22, RP11-630O14, RP11-778017, RP11-640N11, RP11-812L16, RP11-166D19, RP11-811I7, RP11-93E4). These clones were subsequently excluded from array CGH analysis.

The frequency plot of copy number alterations for the remaining 30 BAC clones is shown in **Fig. 3A**. A heat map representing the array CGH copy number alterations for the primary breast cancer tumors analyzed showed a high frequency of copy number loss with RP11-15I6 (**Fig. 3B**). RP11-15I6 was selected for further characterization as several tumors (B4, B12, B16, B20, B28, B45, and B46) had copy number losses at RP11-15I6 but not at adjacent BAC clones, suggesting that a tumor suppressor gene may lie within the genomic region encompassed by RP11-15I6.

Dual color FISH using the BAC clone RP11-15I6 and a chromosome 11 centromeric probe (CEP11) was conducted on frozen sections from six available primary breast tumors to confirm the copy number losses observed from array CGH analysis. Copy number losses were detected in all primary breast tumors and representative examples are shown in **Supplementary Fig. S1**.

The gene *LARG* (leukemia-associated Rho guanine-nucleotide exchange factor) maps within BAC RP11-15I6. *LARG* spans 152.7kb and comprises 40 exons with a transcript length of 9453bp (www.ensembl.org, release 44).

Comparison of frequency of deletion of *LARG* with *BCSC-1* and *TSLC1*

To compare the frequency of deletion of *LARG* with that of other known candidate tumor suppressor genes on 11q23-q24, thirty primary breast cancer samples and six breast cancer cell lines were screened for genomic deletions to *LARG*, *BCSC-1* and *TSLC1* using multiplex ligation-dependent probe amplification (MLPA) with synthetic probes

(**Fig. 4; Supplementary Table S2**). By utilizing a dosage quotient threshold value of 0.8, deletions were observed in 8, 2 and 6 primary breast cancer samples in the *LARG*, *BCSC-1* and *TSLC1* genes respectively (**Fig. 4B**), with the highest frequency of deletion observed in *LARG*. Two of the six cell lines (MCF-7 and HCC1500) had deletions for all three genes (**Fig. 4C**).

Frequent underexpression of *LARG* in primary breast cancer and breast cancer cell lines

Real-time quantitative RT-PCR analysis was done on all available tumor samples ($n=38$) and ten breast cancer cell lines to determine if *LARG* is underexpressed in breast cancer. Reduced mRNA expression of *LARG* of less than 50% relative to human breast RNA (Ambion), the calibrator, was observed in eight of ten breast cancer cell lines and 13 of 38 (34%) of the primary breast tumor samples (**Fig. 5A**).

Mutation analysis of *LARG*

Since inactivating mutations are a known mechanism for gene silencing, the entire coding sequence and the intron-exon boundaries of *LARG* were screened for mutations in the same panel of primary breast cancers, with available genomic DNA ($n=40$). A missense mutation was detected in one case resulting in the amino acid substitution Q2219P (**Supplementary Table S3**). Several polymorphisms were also detected which included previously documented SNPs (www.genecards.org) and novel alterations present in normal controls. All cases with intronic splice site alterations were subjected to RNA analysis and none showed aberrant splicing.

Lack of methylation of the *LARG* promoter

To explore the possibility that silencing of *LARG* expression may be a result of methylation of CpGs within the CpG island upstream of the transcription start site (TSS) of *LARG*, bisulfite sequencing of genomic DNA from ten breast cancer cell lines was done. CpG island methylation was not detected in any of the cell lines. Further evidence for the lack of CpG island methylation in primary breast cancer samples ($n=24$) and breast cancer cell lines ($n=7$) was obtained from qualitative high-throughput analysis of DNA methylation by base-specific cleavage and mass spectrometry using the SEQUENOM MassARRAY System (**Supplementary Fig. S2**).

Restoration of *LARG* expression by a histone deacetylase (HDAC) inhibitor but not a demethylating agent

Treatment of four breast cancer cell lines that underexpress *LARG* with the demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC) did not lead to the reactivation of *LARG*, further suggesting that the silencing of *LARG* was not due to methylation of CpGs (**Fig. 5B**). To determine if inactivation of *LARG* may alternatively have occurred through epigenetic silencing by histone modification, the same cell lines were treated with the histone deacetylase inhibitor, trichostatin A (TSA) (**Fig. 5B**). Reactivation of *LARG* was observed in the breast cancer lines, ZR75.1 and BT20, treated with TSA, and in MCF-7 treated with a combination of TSA and 5-aza-dC. Both ZR75.1 and BT20 are not deleted, as determined by MLPA (**Fig. 4C**), whereas MCF-7 is deleted. In addition, inhibition of

cell proliferation was observed in MCF-7 treated with TSA and 5Aza+TSA (**Fig. 5C**)(23).

Tumor suppressive function of *LARG*

To investigate the tumor suppressive function of *LARG in vitro*, constructs containing *LARG* or empty vector were transfected into the MCF-7 breast cancer cell line, which underexpresses the *LARG* gene (**Fig. 5A**). Growth suppressive activity was assessed using the colony formation assay and the MTS cell proliferation assay. The colony numbers of cells transfected with *LARG* was significantly decreased compared to the empty vector transfected cells ($P= 0.003$, *t*-test) (**Fig. 6A and 6B**). Growth inhibition was also observed in MTS cell proliferation assays of MCF-7 cells transfected with *LARG* (**Fig. 6C**). Thus, *LARG* has functional characteristics of a tumor suppressor gene *in vitro*.

LARG was initially identified as a novel gene, found to be fused with the mixed-lineage leukemia (*MLL*) gene in a patient with primary acute myeloid leukemia (AML)(24). The in-frame *MLL-LARG* fusion is thought to have occurred as a result of an interstitial deletion rather than a balanced translocation, with the breakpoint in *LARG* at its 5' end after nucleotide 931, resulting in the deletion of the amino-terminal end and the region encoding the PDZ domain(24). It is possible that tumorigenesis in AML resulting from the *MLL-LARG* fusion may be due to loss of the N-terminal and PDZ domains, which may possess tumor suppressive function.

The predicted protein of *LARG* is a member of the Dbl family of proteins which function as guanine nucleotide exchange factors (GEFs), most often for the Rho family of GTPases (24). GEFs mediate the activation of Rho proteins, which function as molecular switches by cycling between an active (GTP-bound) and an inactive (GDP-bound) state. Rho GTPases regulate numerous actin-dependent processes including cell migration and adhesion; microtubule cytoskeleton; gene expression and cell cycle progression (25). PDZ domains mediate protein-protein interaction, usually through binding of the C-terminal of the target protein(26). Other domains in *LARG* are the RGS-like domain which functions primarily as a GAP (to accelerate GTPase activity) for G α proteins (27, 28); the Dbl homology (DH) domain, for GEF activity (29); and a pleckstrin homology (PH) domain. The PH domain has been thought to be involved in the subcellular localization of the Rho GEF protein and to directly regulate the DH domain (29, 30).

Conclusions

Our data has shown that inactivation of *LARG* in breast cancer occurs by deletion of one allele and loss of expression of the remaining allele. Inactivating mutations or epigenetic gene silencing by CpG island methylation are mechanisms that account for gene inactivation for many tumor suppressor genes, e.g. *BRCA1*, *BRCA2*, *APC*, and *RUNX3*, but which were not observed for *LARG*. Rather, we report here that epigenetic suppression by histone deacetylation is the likely mechanism for gene silencing of *LARG*. Hence, restoration of *LARG* expression with the therapeutic use of histone deacetylase inhibitors could potentially benefit breast cancer patients with inactivation of *LARG*.

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Figure Legends:

Figure 1. Location of microsatellite markers, BAC clones and cancer related genes across the chromosome 11q23-q24 region based on Ensembl (Release 43).

Figure 2. Loss of heterozygosity analysis in primary breast cancers. **A**, the frequency of LOH and heterozygosity rate at 7 microsatellite markers on chromosome 11q22-q23 in breast carcinoma. **B**, results of microsatellite analyses on 58 breast carcinoma samples. L, LOH; Ho, homozygous or non-informative; He, heterozygosity retained; MI, microsatellite instability; nd, not determined. **C**, examples of LOH on chromosome 11 in representative breast carcinoma samples. *Top*, case numbers. *Left*, microsatellite markers. N, normal; T, tumor. *Arrowheads* indicate the allele lost in tumor DNA.

Figure 3. Array CGH analysis of 40 primary breast tumors and two breast cancer cell lines. **A**, frequency plot of copy number gains or losses for 30 BAC clones on chromosome 11q23. **B**, heat map of DNA copy number ratios for 30 BAC clones on chromosome 11q23. Color codes are indicated below the heat map.

Figure 4. Detection of DNA copy number changes of *LARG*, *BCSC-1* and *TSLC1* by MLPA, using control genes, *GUSB* and *TBP* for normalization. **A**, electropherogram showing peaks of 8 synthetic probes for the control sample in comparison to two breast tumor samples. B1, *BCSC-1* exon 5; C1, *GUSB* exon 8 (control gene); L1, *LARG* exon 10; C2, *TBP* exon 5 (control gene); L2, *LARG* exon 40; B2, *BCSC-1* exon 17; T1, *TSLC1* exon 2; T2, *TSLC1* exon 7. *Red arrows* indicate the fragments deleted in the two breast

tumor samples. DNA size scale is shown below the trace. **B**, average dosage quotient of 30 breast tumor samples. The average dosage quotient is the average of the dosage quotients of each fragment against the 2 control fragments. A deletion is scored if the average dosage quotient is <0.8 , and is indicated by an *asterix*. **C**, average dosage quotient of six breast cancer cell lines.

Figure 5. **A**, the relative mRNA levels of *LARG* was measured in 38 breast tumor samples and 10 breast cell lines by real-time quantitative RT-PCR (mean \pm SD), using *GAPDH* as the endogenous control gene. The level of *LARG* mRNA was expressed relative to a normal human breast RNA (Ambion), the calibrator, shown in black. Two additional normal breast RNA samples were included (Stratagene and B7(N)). **B**, effect of 5-Aza-2'deoxyctidine and TSA on the expression of *LARG* in breast cell lines MCF-7, BT549, ZR75.1 and BT20. Cells were treated with 5-Aza-2'deoxyctidine, TSA or a combination of both. Expression was quantified by real-time quantitative RT-PCR (mean \pm SD) and normalized using *GAPDH*. **C**, cell proliferation assay of MCF-7 cells treated with 5-Aza-2'deoxyctidine and TSA showing slower growth of cells treated with TSA.

Figure 6 Growth suppression of MCF-7 cells transfected with *LARG*. **A**, colony formation assay. Stable lines of MCF-7 that were transfected with pcDNA3.1 (empty vector) or a pcDNA3.1 construct with *LARG* were grown for 14 days and visualized by crystal violet staining; **B**, cell colonies greater than 1mm in diameter were counted. **C**, cell proliferation assay of MCF-7 cells transfected with pcDNA3.1 (empty vector) or *LARG* showed slower growth of MCF-7 cells transfected with *LARG*.

Supplementary Information

Supplementary Table S1. Demographic and clinico-pathological parameters of breast cancer patients ($n=58$).

Supplementary Table S2. Probes used for multiplex ligation-dependent probe amplification.

Supplementary Table S3. Mutation and polymorphisms in *LARG*.

Supplementary Figure S1.

A and *B*, two representative examples of dual color FISH demonstrating deletion in breast cancer cases. Red: chromosome 11 centromeric probe; green: BAC probe RP11-15I6, which spans the genomic region containing *LARG*.

Supplementary Figure S2.

DNA methylation status determined by base-specific cleavage and mass spectrometry. Twenty-four breast tumor samples, seven breast cell lines, one non-tumorigenic breast cell line (MCF10A) and one normal breast tissue sample (B13N) were analyzed. Each circle represents one CpG site. *Red* indicates a low level of methylation; *yellow*, a high level of methylation; and *gray*, missing data. Numbers above arrowheads at the top of the figure indicate the nucleotide number relative to the transcription start site.

References

1. Tomlinson, I. P., Strickland, J. E., Lee, A. S., Bromley, L., Evans, M. F., Morton, J., and McGee, J. O. Loss of heterozygosity on chromosome 11 q in breast cancer. *J Clin Pathol*, *48*: 424-428, 1995.
2. Lee, A. S., Seo, Y. C., Chang, A., Tohari, S., Eu, K. W., Seow-Choen, F., and McGee, J. O. Detailed deletion mapping at chromosome 11q23 in colorectal carcinoma. *Br J Cancer*, *83*: 750-755, 2000.
3. Martin, E. S., Cesari, R., Pentimalli, F., Yoder, K., Fishel, R., Himelstein, A. L., Martin, S. E., Godwin, A. K., Negrini, M., and Croce, C. M. The BCSC-1 locus at chromosome 11q23-q24 is a candidate tumor suppressor gene. *Proc Natl Acad Sci U S A*, *100*: 11517-11522, 2003.
4. Gabra, H., Watson, J. E., Taylor, K. J., Mackay, J., Leonard, R. C., Steel, C. M., Porteous, D. J., and Smyth, J. F. Definition and refinement of a region of loss of heterozygosity at 11q23.3-q24.3 in epithelial ovarian cancer associated with poor prognosis. *Cancer Res*, *56*: 950-954, 1996.
5. Baffa, R., Negrini, M., Mandes, B., Ruge, M., Ranzani, G. N., Hirohashi, S., and Croce, C. M. Loss of heterozygosity for chromosome 11 in adenocarcinoma of the stomach. *Cancer Res*, *56*: 268-272, 1996.
6. Rasio, D., Negrini, M., Manenti, G., Dragani, T. A., and Croce, C. M. Loss of heterozygosity at chromosome 11q in lung adenocarcinoma: identification of three independent regions. *Cancer Res*, *55*: 3988-3991, 1995.
7. Pulido, H. A., Fakruddin, M. J., Chatterjee, A., Esplin, E. D., Beleno, N., Martinez, G., Posso, H., Evans, G. A., and Murty, V. V. Identification of a 6-cM minimal deletion at 11q23.1-23.2 and exclusion of PPP2R1B gene as a deletion target in cervical cancer. *Cancer Res*, *60*: 6677-6682, 2000.
8. Hui, A. B., Lo, K. W., Leung, S. F., Choi, P. H., Fong, Y., Lee, J. C., and Huang, D. P. Loss of heterozygosity on the long arm of chromosome 11 in nasopharyngeal carcinoma. *Cancer Res*, *56*: 3225-3229, 1996.
9. Robertson, G., Coleman, A., and Lugo, T. G. A malignant melanoma tumor suppressor on human chromosome 11. *Cancer Res*, *56*: 4487-4492, 1996.
10. Negrini, M., Sabbioni, S., Possati, L., Rattan, S., Corallini, A., Barbanti-Brodano, G., and Croce, C. M. Suppression of tumorigenicity of breast cancer cells by microcell-mediated chromosome transfer: studies on chromosomes 6 and 11. *Cancer Res*, *54*: 1331-1336, 1994.
11. Koreth, J., Bakkenist, C. J., Larin, Z., Hunt, N. C., James, M. R., and McGee, J. O. 11q23.1 and 11q25-qter YACs suppress tumour growth in vivo. *Oncogene*, *18*: 1157-1164, 1999.
12. Murakami, Y., Nobukuni, T., Tamura, K., Maruyama, T., Sekiya, T., Arai, Y., Gomyou, H., Tanigami, A., Ohki, M., Cabin, D., Frischmeyer, P., Hunt, P., and Reeves, R. H. Localization of tumor suppressor activity important in nonsmall cell lung carcinoma on chromosome 11q. *Proc Natl Acad Sci U S A*, *95*: 8153-8158, 1998.
13. Negrini, M., Rasio, D., Hampton, G. M., Sabbioni, S., Rattan, S., Carter, S. L., Rosenberg, A. L., Schwartz, G. F., Shiloh, Y., Cavenee, W. K., and et al. Definition and refinement of chromosome 11 regions of loss of heterozygosity in

- breast cancer: identification of a new region at 11q23.3. *Cancer Res*, 55: 3003-3007, 1995.
14. Koreth, J., Bakkenist, C. J., and McGee, J. O. Allelic deletions at chromosome 11q22-q23.1 and 11q25-qterm are frequent in sporadic breast but not colorectal cancers. *Oncogene*, 14: 431-437, 1997.
 15. Tomlinson, I. P., Nicolai, H., Solomon, E., and Bodmer, W. F. The frequency and mechanism of loss of heterozygosity on chromosome 11q in breast cancer. *J Pathol*, 180: 38-43, 1996.
 16. Massion, P. P., Kuo, W. L., Stokoe, D., Olshen, A. B., Treseler, P. A., Chin, K., Chen, C., Polikoff, D., Jain, A. N., Pinkel, D., Albertson, D. G., Jablons, D. M., and Gray, J. W. Genomic copy number analysis of non-small cell lung cancer using array comparative genomic hybridization: implications of the phosphatidylinositol 3-kinase pathway. *Cancer Res*, 62: 3636-3640, 2002.
 17. Jain, A. N., Tokuyasu, T. A., Snijders, A. M., Segraves, R., Albertson, D. G., and Pinkel, D. Fully automatic quantification of microarray image data. *Genome Res*, 12: 325-332, 2002.
 18. Lee, A. S., Rudduck-Sivaswaren, C., Khun-Hong Lie, D., Li-Ming Chua, C., Tien, S. L., Morsberger, L., and Griffin, C. A. Overlapping deletion regions at 11q23 in myelodysplastic syndrome and chronic lymphocytic leukemia, characterized by a novel BAC probe set. *Cancer Genet Cytogenet*, 153: 151-157, 2004.
 19. Schouten, J. P., McElgunn, C. J., Waaijer, R., Zwijnenburg, D., Diepvens, F., and Pals, G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res*, 30: e57, 2002.
 20. Bunyan, D. J., Eccles, D. M., Sillibourne, J., Wilkins, E., Thomas, N. S., Shea-Simonds, J., Duncan, P. J., Curtis, C. E., Robinson, D. O., Harvey, J. F., and Cross, N. C. Dosage analysis of cancer predisposition genes by multiplex ligation-dependent probe amplification. *Br J Cancer*, 91: 1155-1159, 2004.
 21. Lee, A. S., Ho, G. H., Oh, P. C., Balram, C., Ooi, L. L., Lim, D. T., Wong, C. Y., and Hong, G. S. Founder mutation in the BRCA1 gene in Malay breast cancer patients from Singapore. *Hum Mutat*, 22: 178, 2003.
 22. Ehrich, M., Nelson, M. R., Stanssens, P., Zabeau, M., Liloglou, T., Xinarianos, G., Cantor, C. R., Field, J. K., and van den Boom, D. Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proc Natl Acad Sci U S A*, 102: 15785-15790, 2005.
 23. Vigushin, D. M., Ali, S., Pace, P. E., Mirsaidi, N., Ito, K., Adcock, I., and Coombes, R. C. Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo. *Clin Cancer Res*, 7: 971-976, 2001.
 24. Kourlas, P. J., Strout, M. P., Becknell, B., Veronese, M. L., Croce, C. M., Theil, K. S., Krahe, R., Ruutu, T., Knuutila, S., Bloomfield, C. D., and Caligiuri, M. A. Identification of a gene at 11q23 encoding a guanine nucleotide exchange factor: evidence for its fusion with MLL in acute myeloid leukemia. *Proc Natl Acad Sci U S A*, 97: 2145-2150, 2000.

25. Rossman, K. L., Der, C. J., and Sondek, J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol*, *6*: 167-180, 2005.
26. Harris, B. Z. and Lim, W. A. Mechanism and role of PDZ domains in signaling complex assembly. *J Cell Sci*, *114*: 3219-3231, 2001.
27. Fukuhara, S., Chikumi, H., and Gutkind, J. S. Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G(12) family to Rho. *FEBS Lett*, *485*: 183-188, 2000.
28. Siderovski, D. P., Strockbine, B., and Behe, C. I. Whither goest the RGS proteins? *Crit Rev Biochem Mol Biol*, *34*: 215-251, 1999.
29. Whitehead, I. P., Campbell, S., Rossman, K. L., and Der, C. J. Dbl family proteins. *Biochim Biophys Acta*, *1332*: F1-23, 1997.
30. Liu, X., Wang, H., Eberstadt, M., Schnuchel, A., Olejniczak, E. T., Meadows, R. P., Schkeryantz, J. M., Janowick, D. A., Harlan, J. E., Harris, E. A., Staunton, D. E., and Fesik, S. W. NMR structure and mutagenesis of the N-terminal Dbl homology domain of the nucleotide exchange factor Trio. *Cell*, *95*: 269-277, 1998.

Figure 1

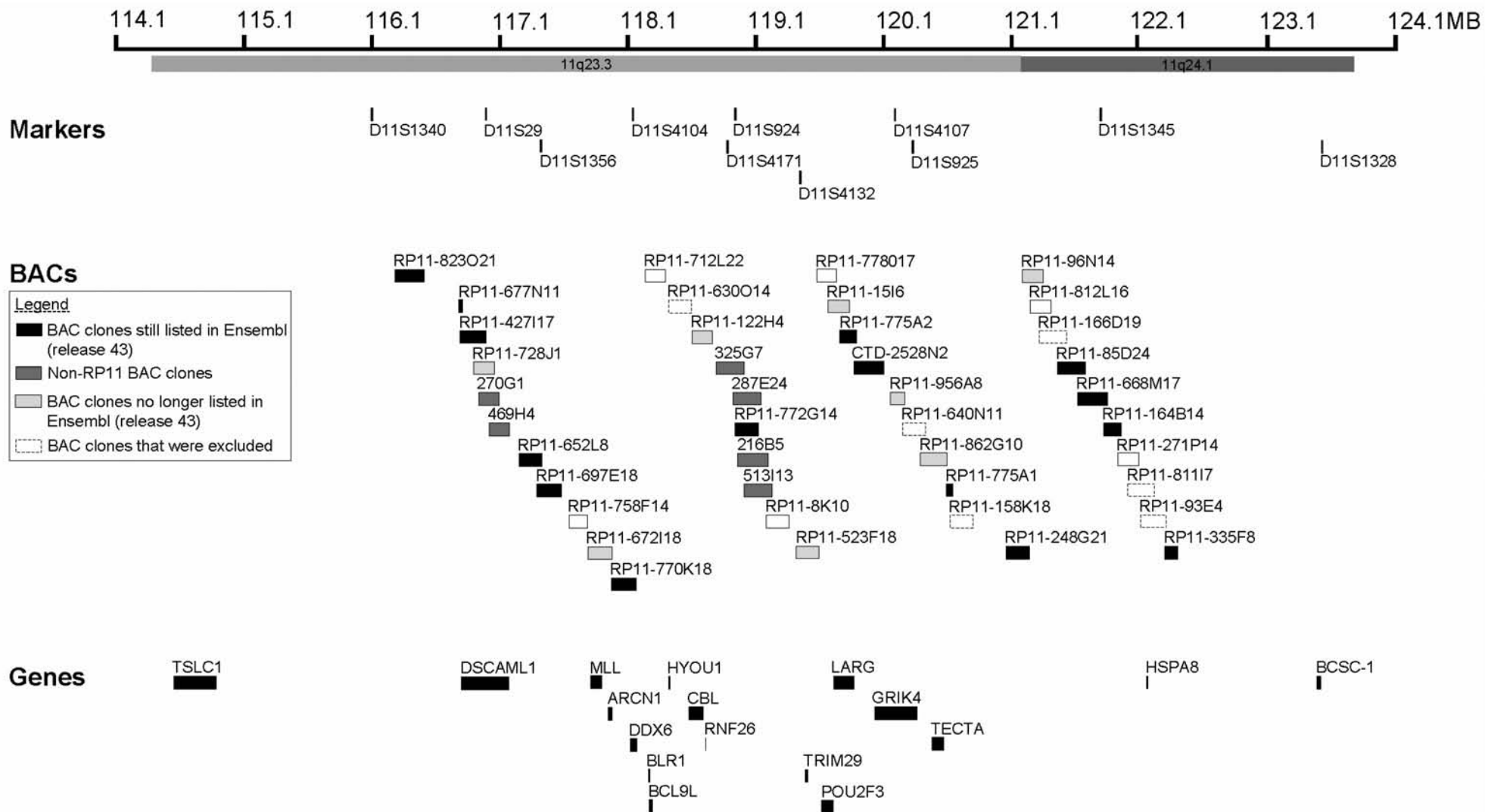


Figure 5

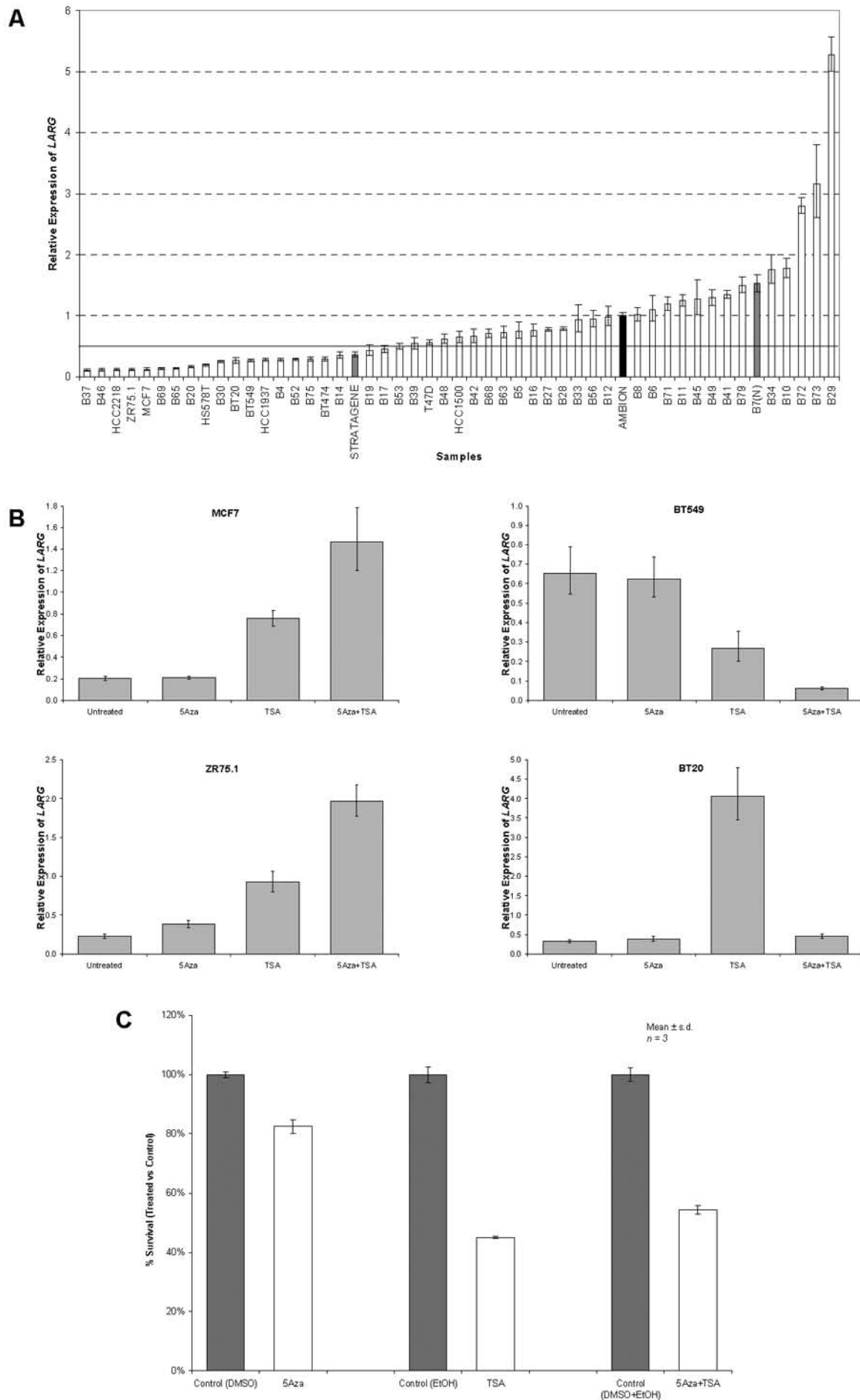


Figure 4

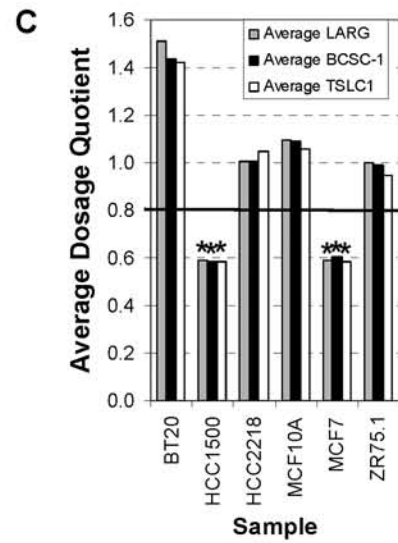
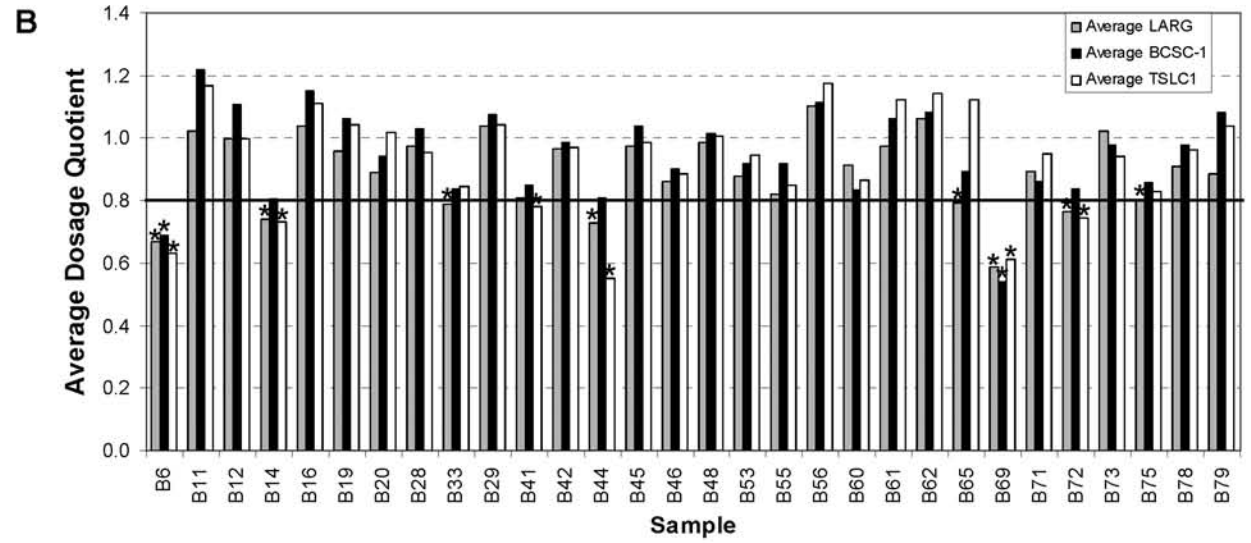
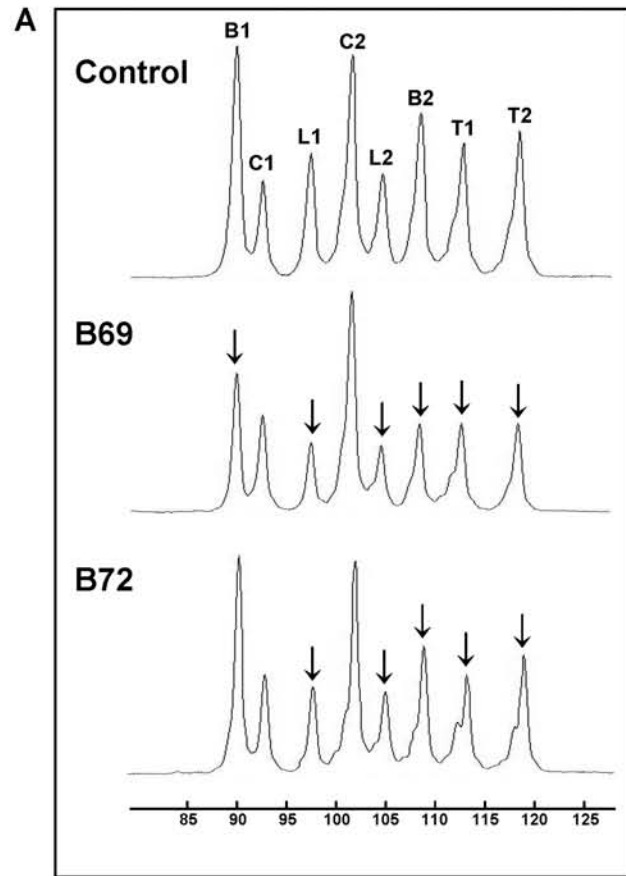
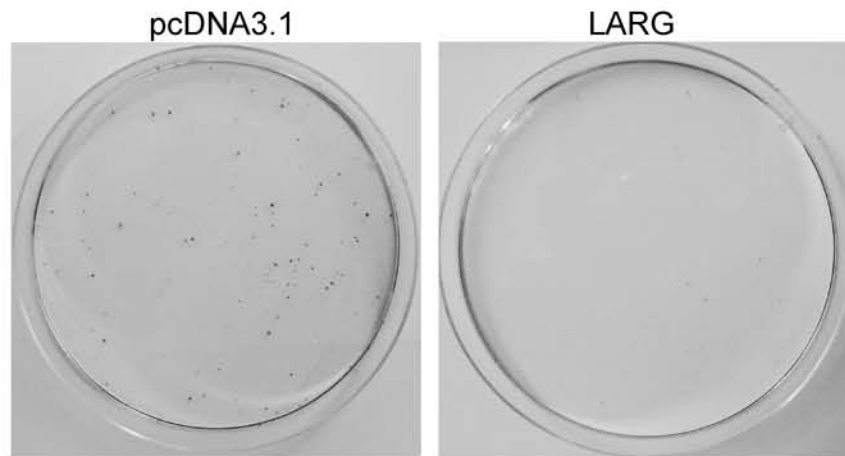
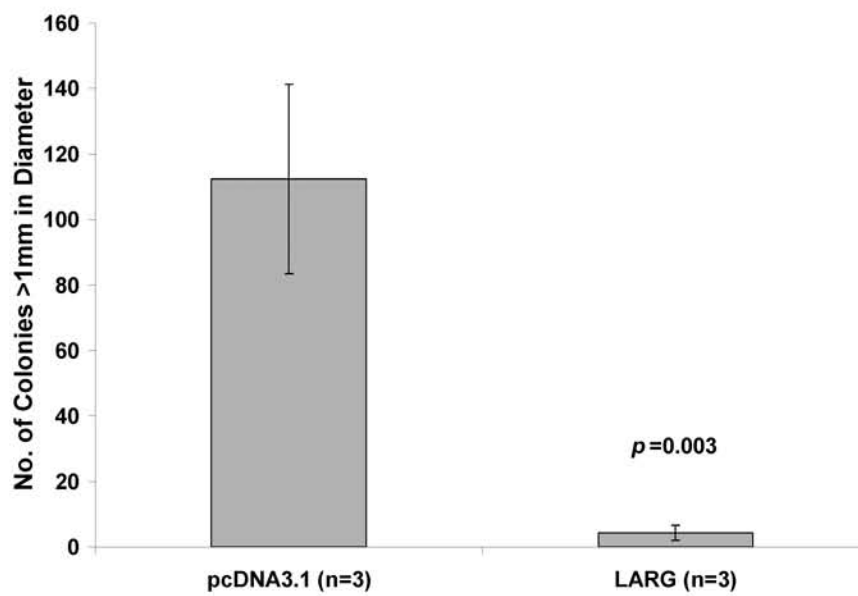


Figure 6

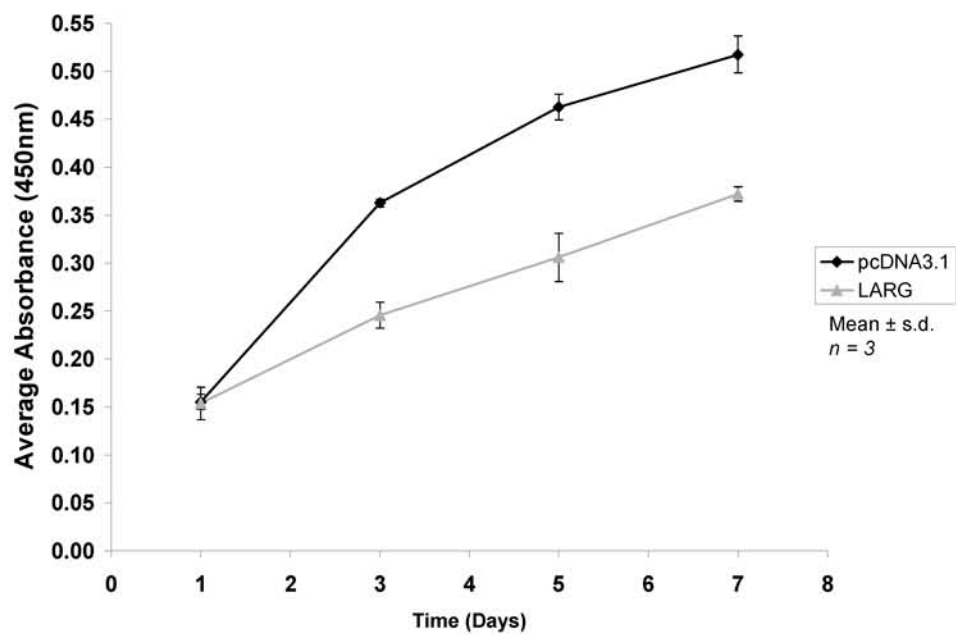
A



B



C



Supplementary Table S1

| | | |
|--------------------------------|--|-------|
| <i>Age</i> | Mean (yrs) | 53.1 |
| | Range (yrs) | 30-82 |
| <i>Race</i> | Chinese | 43 |
| | Malay | 12 |
| | Other | 2 |
| | Unknown | 1 |
| <i>Histology</i> | | |
| | Ductal carcinoma <i>in situ</i> (DCIS) | 3 |
| | Infiltrative ductal carcinoma: | |
| | Grade I | 6 |
| | Grade II | 16 |
| | Grade III | 28 |
| | Other & unknown | 5 |
| <i>Stage</i> | | |
| | I | 3 |
| | IIA | 14 |
| | IIB | 33 |
| | IIIA | 6 |
| | IIIB | 1 |
| | IV | 1 |
| <i>Hormone Receptor Status</i> | | |
| | ER- | 23 |
| | ER+ | 27 |
| | Unknown | 8 |
| | PR- | 24 |
| | PR+ | 24 |
| | Unknown | 10 |

Supplementary Table S2

| Probe Name | 5' Half-Probe Hybridizing Sequence* | 3' Half-Probe Hybridizing Sequence [#] | Exon | Amplification Product Size |
|-------------|--|---|------|----------------------------|
| GUSB_Ex8 | GGTGATGGAAGAAGTGGTGCGTAGGG | ACAAGAACCACCCCGCGGTCGTGAT | 8 | 93 |
| TBP_Ex5 | TAAGAGAGCCACGAACCACGGCACTGATTT | TCAGTTCTGGGAAAATGGTGTGCACAGGAG | 5 | 102 |
| LARG_Ex10 | CTGGAGATGTACTGGGCAGGACTGACTG | TAGCAGTGGAGATGCTTCTCGGCCAG | 10 | 97 |
| LARG_Ex40 | TCACAGAAGGTGGAGGAAAGTTACACCATTCTTTGCC | AAAGGCTGGCTGGATCAGCCCTCACAG | 40 | 106 |
| TSLC1_Ex2 | TGTTTACGAAAGACGTGACAGTGATCGAGGGAGAG | GTTGCGACCATCAGTTGCCAAGTCAATAAGAGTGAC | 2 | 113 |
| TSLC1_Ex7 | CATCAATAACCTAAACAAAACAGATAATGGTACATACCGCTGTGAA | GCTTCAAACATAGTGGGGAAAGCTCACTCGG | 7 | 119 |
| BCSC-1_Ex5 | CCTGAAGTATGTGCAGGAGCTGCCTC | TGGAAGCAGATGGGGCTCTGCG | 5 | 90 |
| BCSC-1_Ex17 | GGAGAGAATCACCTTGTGCAGCTGATTTACCA | CCAAAATGCAAATGGTTCCTGGGATCTGAATGAAG | 17 | 109 |

*The 5' half-probes contain a 5' universal primer tag (5' GGGTTCCCTAAGGGTTGGA 3') upstream of its hybridizing sequence.

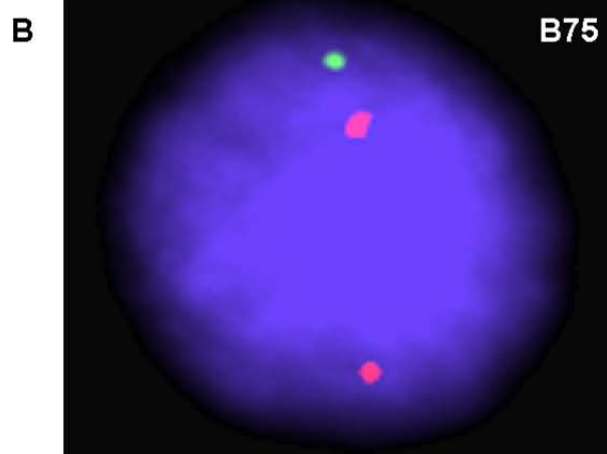
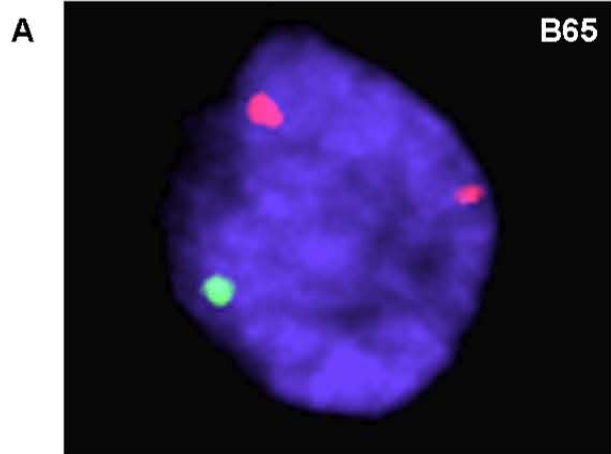
[#]The 3' half-probes contain a 5' phosphate group (required for ligation) upstream and a 3' universal primer tag (5' TCTAGATTGGATCTTGCTGGCGC 3') downstream of its hybridizing sequence. The universal primer tags and criteria for the design of synthetic MLPA probes were obtained from www.mlpa.com.

Supplementary Table 3

| MUTATIONS | | | | | | |
|---------------|--------------------|-------------------|---|-------------------------------|--------------------------------|--|
| Case | Exon | Nucleotide Change | Amino Acid Change | Mutation Type | | |
| B28 | 36 | A3663G | Q1219P | Missense mutation | | |
| POLYMORPHISMS | | | | | | |
| Exon/Intron | Nucleotide Change | Amino Acid Change | Present in SNP Database (www.genecards.org) | Frequency in normals % (n=50) | Frequency in subjects % (n=40) | |
| Intron 4 | IVS4-35 T>C | none | no* | 18.0 | 30.0 | |
| Intron 10 | IVS10+9 T>C | none | yes | | 12.5 | |
| Exon12 | A1091T | H362Y | no* | 4.0 | 2.5 | |
| Intron 12 | IVS12+48 A>C | none | yes | | 12.5 | |
| Intron 13 | IVS13-67 G>R | none | yes | | 55.0 | |
| Intron 16 | IVS16+58-60delAAG | none | no* | 0.0 | 2.5 | |
| Intron 19 | IVS19+7 A>C | none | yes | | 60.0 | |
| Intron 20 | IVS20-45 G>A | none | no* | 22.0 | 27.5 | |
| Intron 22 | IVS22-46 A>T | none | yes | | 7.5 | |
| Exon 25 | T2524G | none | yes | | 15.0 | |
| Intron 28 | IVS28+23 A>G | none | yes | | 7.5 | |
| Exon 29 | A2925T | Y973F | yes | | 27.5 | |
| Intron 33 | IVS33-41-5insGTTGT | none | no* | 44.0 | 55.0 | |
| Intron 36 | IVS36-5 T>G | none | no* | 0.0 | 2.5 | |
| Intron 40 | IVS40+14 A>G | none | yes | | 80.0 | |

*All alterations that were not documented in the SNP database were screened in 50 normal controls.

Supplementary Figure S1



Supplementary Figure S2

