Y is Male Breast Cancer so Rare?

by
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ABSTRACT

Male breast cancer (MBC) occurs at less than 1% frequency of female breast cancer, and its actual etiology is still unclear. We hypothesize that males are protected from contracting breast cancer because the male-specific region of the human Y chromosome (MSY) has breast tumor suppressive function. Here, we show via fluorescence *in situ* hybridization (FISH) and droplet digital PCR (ddPCR) that Y chromosome was lost clonally at a frequency of ~15% (5/32) in two independently collated cohorts of MBC patients. We also show via FISH that this clonal Y loss occurred since ductal carcinoma *in situ* (DCIS) stage of MBC.

Furthermore, we investigated regional loss of the Y chromosome in MBC patients who retained their Y chromosomes with STS-PCR (sequence-tagged-site PCR), derived from the male specific region of the Y chromosome Breakpoint Mapper (MSY-BPM), and showed nullisomic loss of *TMSB4Y* in a MBC patient. We showed via immunohistochemistry (IHC) that *TMSB4Y* is expressed in normal male breast tissue. Dox-inducible cell lines of *TMSB4Y*, TmY1 and TmY2, were generated in the female breast epithelial cell line MCF-10A background. Dox-induced expression of *TMSB4Y* resulted in altered morphology and reduced cell proliferation. Furthermore, reverse phase protein array (RPPA) analysis on TmY1 showed reduced expression of Syk (spleen tyrosine kinase) after Dox-induced expression of *TMSB4Y*.

Interestingly, though *TMSB4Y* is highly homologous to its homologue *TMSB4X* on the X chromosome, the *TMSB4Y* antibody is solely specific to *TMSB4Y*, which suggests differences between *TMSB4Y* and *TMSB4X*.

Taken together, our results suggest that *in situ* clonal loss of human Y chromosome might contribute to MBC tumorigenesis, and that *TMSB4Y* is tumor suppressive through regulating breast cell morphology and reducing cell proliferation.

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In loving memory of my Vincent Uncle.

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1 Introduction

Male breast cancer (MBC) is a rare disease

Male breast cancer (MBC) is a rare disease that occurs at a frequency of less than 1% of female breast cancers, and MBC makes up less than 1% of all cancers in men [1-3]. In the United States of America, an annual average of about 200,000 females will be diagnosed with FBC, and only about 2000 males will be diagnosed with MBC [4, 5]. Internationally, MBC is proposed to occur at a proposed average incidence rate of <1 per 100,000 men per year [6]. Here, the cancer incidence rate is calculated as the number of new global cases of MBC per 100,000 men per year. After analyzing statistics across the continents of Asia, Europe, America, and Oceania, MBC incidence rate is the highest in Israel (1.24 per 100,000 per year) and the lowest in Thailand (0.16 per 100,000 per year). Furthermore, recent epidemiological MBC studies showed a steady increase in annual occurrence [2, 7, 8].

Interestingly, Klinefelter patients, who are males with an extra X chromosome, are reported to have elevated risks for MBC [9].

This unbalanced statistical occurrence of breast cancer resulted in a female biased availability of breast cancer research resources as well. Based on the established MBC frequency, pathological tissue banks will have 100 times less MBC tumor formalin-fixed paraffin embedded (FFPE) tissue deposits. Such rarity of MBC tissue samples at each individual institution across the world has resulted in very recent consolidation efforts such as the Male Breast Cancer Pooling Project led by the National Cancer Institute [10]. Such collation projects is ongoing and will take a long time due to the complexity of approval protocols of various institutions and countries.

Irrefutably, cancer cell lines are crucial to the progress of cancer research [11]. The first breast cancer cell line established was BT-20 [12], and then widely used breast cancer cell lines include the MDA-MB-coded MD Anderson series and the MCF-7 established by the Michigan Cancer Foundation [13, 14]. To date, the American Type Culture Collection (ATCC) has 58 human mammary gland/breast cell lines available in their comprehensive catalogue, and all of them are of female origin. We managed to acquire one single MBC cell line, COLO-824, available at the Leibniz-Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Germany. However, we were unable to validate its identity from its signature Rb1 deletion, which was documented in the Catalogue of Somatic Mutations in Cancer (COSMIC) when we first acquired the cell line. Therefore, we could not utilize it for further study.

MBC versus female breast cancer

Generally, MBC has a higher age of onset across Western, Middle Eastern, and South Asia compared to female breast cancer [15-17]. Unlike female breast cancer, MBC does not plateau after men become older than 50 years old and ethnically, more blacks than whites in the United States of America [18, 19].

Because of the rarity of MBC, most research studies are based on female breast cancer, and results are generally extrapolated into MBC clinical diagnosis and treatment [15]. However, clinical data from female breast cancer might not be entirely applicable to men since the etiology of MBC is still unclear, and studies have shown clinical and molecular differences in MBC when compared to female breast cancer.

Clinically, surgical management of breast cancer is different in males and females. According to an analysis of 35 years of data (1973 to 2008) queried from the Surveillance, Epidemiology, and End Results (SEER) database, a majority of males with MBC with localized breast cancer (contained in breast) received mastectomy despite equivalent cause specific survival with lumpectomy or radiation therapy. In MBC, post mastectomy radiation therapy is not utilized extensively for patients with regional breast cancer (cancers in chest wall, skin, and regional lymph nodes) [20]. In general, it is noted that a majority of males usually do not opt for breast-conserving surgeries [21, 22].

Molecularly, breast cancers are traditionally classified according to their receptor status, namely Estrogen Receptor (ER), Progesterone Receptor (PR), and Her2 (Epidermal Growth Factor Receptor 2). Females are exposed to hormonal cycles of estrogen and progesterone and it is logical for female breast cells express ER and PR, and

also for the ER+ and/or PR+ female breast cancer cells to maintain their expression. Interestingly, despite the lack of hormonal cycles in males, more than 75% of MBC is ER+ and/or PR+ [21, 22].

The current conceptual molecular subtyping of breast cancer combines information of the receptor status and tumor grade into several classes, namely Basal-like (Triple Negative), Luminal A (low grade and ER+), Luminal B (high grade and ER+), Luminal ER-/AR+, Her2+, and Normal-breast-like [23]. Claudin-low is a recently added molecular subtype that have opposite expression of tight junction genes (low) and epithelial-to-mesenchymal transition genes (high) [24]. These molecular subtypes were established mostly based on female breast cancer studies. Averaging across a few studies that grouped MBC cases accordingly, MBC mostly fall into Luminal A (~80%), some in Luminal B (~15%), and very few that are Basal-like (<5%) or Her2+ (<5%) [25-29]. Female breast cancers mostly fall into Luminal A (71%), some in Basal-like (16%), and very few in Luminal B (6%) and Her2+ (6%) [30]. Interestingly, the percentages of Luminal B and Basal-like seem opposite for male and female breast cancers.

Recent sophisticated studies also highlight global genomic, transcriptomic, and proteomic differences between female and male breast cancers. Global gene expression profiling showed differences in at least 1000 genes between female and male breast cancers [31]. Using a computer framework called COpy Number and EXpression In Cancer (CONEXIC), cancer driver genes were shown to be largely different between male and female breast cancers as well [32].

Physiologically, the human breast tissue lies behind the nipple on the chest wall. In males, it is generally considered non-functional, and also the total number of breast cells compared to females is significantly lower. Male breasts are not exposed to hormonal cycles like in females, but male breast cells do not escape genetic assaults like mutations that can lead to tumor formation. Since cancer is a genetic disease, and the fundamental genetic difference between males and females is the possession of the Y chromosome by males, genetics of the Y chromosome might play a role in why male breast cancer is a rare disease.

The human Y chromosome is a genetic desert

The human Y chromosome is one of the smallest human chromosomes, and it consists of 5% pseudoautosomal region (PAR) homologous to the X chromosome and 95% of the male-specific region of the Y chromosome (MSY) [33]. The MSY is unique to males and only males have this genomic region. The MSY region was sequenced in 2003, and it was revealed to be a mosaic of discrete euchromatic classes, namely X-transposed (99% identity to X chromosome), X-degenerate (ancient autosomal remnant sequences), and ampliconic (repetitive) [34]. Back then, they reported only about 150 transcribed genes, with which only 80 are protein-coding, on the Y chromosome. Recent Y chromosome characterization has increased the total number of annotated genes to about 450 (according to the human genome map, National Center for Biotechnology Information), but the number of protein coding genes remain around 90 [35].

The Y chromosome is best known for its role in sex determination and normal sperm production. SRY is the most well-known gene on the Y chromosome, and is established as the single determining factor for males [36]. Deletions of certain genomic loci on the Y chromosome is reported to cause azoospermic infertility in men [37].

Previous cytogenetic studies of MBC did not show *in situ* Y loss

Previous early studies of MBC involved small case numbers, these include cytogenetic chromosome banding studies [38-40]. Even though the loss of Y chromosome had been reported as a nonrandom chromosome abnormality, results did not show *in situ* loss in tumor tissue. In these reports, the breast tumor tissues were dissociated, put through short term culture, and then underwent cytogenetic analysis.

A recent 2013 study reported that loss of Y chromosome frequency was the same in their MBC patient group relative to their control group, and noted that this loss frequency increases sharply with age [41]. However, this study looked at sex chromosome aneuploidy in the peripheral blood of their human subjects.

To date, there is no report that describes the *in situ* Y chromosome status in MBC.

Hypothesis

Cancer is a genetic disease. Considering the fact that the human Y chromosome is the fundamental genetic difference between males and females, and that MBC is a rare disease, we hypothesize that the human Y chromosome is tumor suppressive in breast cancer. Therefore, we hypothesize that the loss of Y chromosome contributes to the tumorigenesis of MBC. According to our hypothesis, the human Y chromosome should contain breast specific tumor suppressive gene(s), which can be potential target(s) of breast cancer therapy.

2 Materials and Methods

FFPE Tissue of MBC patients

A total of 32 informative MBC patients were collated from 2 independent cohorts. Cohort 1 consisted of 15 MBC patients from the tumor tissue bank of Pathology Department of the Johns Hopkins Medical Institutes. Formalin-fixed paraffin embedded (FFPE) tissue blocks were available and samples were manufactured into a tissue microarray (TMA) for analysis. Cohort 2 consisted of a total of 20 MBC patients; 1 has the same source as cohort 1; the remaining 19 were obtained from the United States Department of Veterans Affairs. For cohort 2, only FFPE tissue slides were available, and 3 out of 20 MBC patients were uninformative due to the quality of FFPE tissue. Thus, cohort 2 consisted of 17 informative MBC patients.

Fluorescence *in situ* hybridization (FISH)

FFPE tissue slides were de-paraffinized by melting paraffin at 65°C for 5min, washed with Xylene for 3x15min, 100% EtOH for 2x5min, and air dried. Briefly, slides were pretreated, hybridized with FISH probes, and then mounted for microscope observation. Pretreatment was performed using Pretreatment Kit I (Abbott Molecular 02J02-032). Slides were submerged for 20min in 0.2N HCl at RT, followed by 30min in Pretreatment Buffer (NaSCN) at 80°C, and then 16min in reconstituted protease solution at 37°C, rinsing and washing with diH2O and 2XSSC in between transitions. Slides were then dehydrated through 70%, 95%, then 100% EtOH, and dried for at least overnight. Probes were mixed with LSI/WCP Hybridization Buffer (Abbott Molecular 06J67-001) and heated to 45°C before being applied to tissue slides, which were denatured for 5min using the Statspin® Thermobrite slide processing system, and incubated at 37°C for 48hrs in a humidity chamber. Slides were then washed in post-hybridization buffer (0.3% IGEPAL CA-630, Sigma 18896, in 2XSSC) for 4min at RT and then 3min at 75°C, counterstained with DAPI, and mounted with ProLong® Gold (Invitrogen, P36930). All fluorescence microscopy photos were imaged with NIS-Elements BR2.30. FISH probes used were all from Abbott Molecular: Vysis CEP X (DXZ1) SO Probe (Centromeric, 05J08-033), Vysis CEP X (DXZ1) SA Probe (Centromeric, 05J09-033), Vysis CEP Y (DYZ1) SGn Probe (q arm, 05J10-034), Vysis CEP Y (DYZ3) SO Probe (Centromeric, 05J08-035), and Vysis LSI SRY SO Probe (p arm, 05J27-089).

Droplet Digital PCR (ddPCR)

Droplet digital PCR (ddPCR) was performed using the QX100™ Droplet Digital PCR System according to the manufacturer's recommendations (BioRad, Hercules, CA), as previously described [42]. TaqMan® Copy Number Assays (Life Technologies) used were Hs00314226_cn (FAM labeled) for XK gene on the X chromosome and Hs04125506_cn (VIC labeled) for EIF1AY gene on the Y chromosome. The positive control gDNA (Promega) used were male human gDNA (G1471) and female human gDNA (G1521). ddPCR was performed with the BioRad's recommended two-step thermo-cycling protocol with a 58°C annealing/extension step. All data analysis was performed using QuantaSoft (BioRad).

gDNA extraction from FFPE tissue slides

Patient tissue slides were first stained with hematoxylin and eosin using standard techniques and our pathologist circumscribed areas of tumor versus normal tissue. Serial tissue slides were de-paraffinized as in our FISH protocol, and pinpoint solution (Zymo Research, D3001-1) was applied specifically onto the tumor versus normal tissue areas. Genomic DNA was then extracted and purified from the dried pinpointed tissue areas using the QIAmp DNA FFPE tissue kit (Qiagen, 56404) as per the manufacturer's protocol.

STS-PCR

Male specific region of the Y chromosome Breakpoint Mapper (MSY-BPM) [43] was used to analyze the sequence-tagged sites (STS) status of gDNA extracted from

patients. 33 sets of STS primers that cover the MSY region (Table 2) and standard PCR conditions described were used to amplify STSs from gDNA extracted from tumor and normal tissue from breast cancer patients. PCR products were then ran on a 1% agarose gel, and the presence and absence of the STS amplicons were compared between the tumor and normal gDNA. For every set of STS-PCR (33 PCR reactions), a positive control (gDNA from a healthy anonymous male) and a negative control (gDNA from a female cell line MCF-10A) were performed synchronously to ensure integrity of PCR reactions. Each set of STS-PCR was repeated 5 times for reproducibility.

Cell culture

TetHyg2.5, derivative of the nontumorigenic human breast epithelial cell line MCF-10A [44] was grown in DMEM/F12 (1:1) supplemented with insulin at 10 µg/mL, hydrocortisone at 0.5 µg/mL, and cholera toxin at 0.1 µg/mL (hereafter denoted as "supplemented DMEM/F12"), 5% horse serum (Gibco), EGF at 20 ng/mL, and hygromycin at 14.3 µg/mL. Dox-inducible TetHyg2.5 derivatives (EV, TmY1, TmY2, and UA3) were grown in supplemented DMEM/F12 with 5% Tet-free FBS (HyClone), EGF at 20 ng/mL, hygromycin at 7.15 µg/mL, and G418 at 120 µg/mL. 293 cells were grown in DMEM media with 5% FBS. All supplements were purchased from Sigma-Aldrich unless otherwise specified. MCF-10A and 293 cells were purchased from American Type Culture Collection (ATCC).

Generation of Dox-inducible clones

TetHyg2.5 cells were seeded at about 50% confluency in a T25 flask in supplemented DMEM/F12 with 5% Tet-free FBS (HyClone), EGF at 20 ng/mL, hygromycin at 14.3 μg/mL on day 0; cotransfected with pBI-EGFP vector (with *TMSB4Y* cloned in) and 6A vector (gives Neomycin resistance) on day 1; changed to selection media (supplemented DMEM/F12 with 5% Tet-free FBS (HyClone), EGF at 20 ng/mL, hygromycin at 7.15 μg/mL, and G418 at 120 μg/mL) on day 2. On day 5, all cells were replated into ten 96-well plates and observed for single-well clones that express GFP upon Dox-induction at 2ug/ml, for 48hrs. The single well clones were then expanded and characterized. EV (empty pBI-EGFP vector) was generated as a negative control cell line, and TmY1 and TmY2 were generated as Dox-inducible cell lines to express *TMSB4Y*.

Immunoblotting

For Dox-induction assays, TetHyg2.5 and its derivatives were seeded in respective media with and without Dox for 48hours before harvesting. For transient expression assays, 293 cells were transfected with expression vectors for *TMSB4Y*, *TMSB4X*, or FLAG-*TMSB4X* , and harvested 48hrs after transfection. Whole-cell protein lysates prepared using Laemmli sample buffer were resolved by SDS-PAGE using NuPAGE gels (Invitrogen), transferred to 0.2 μm pore size Invitrolon polyvinylidene difluoride (PVDF) membranes (Invitrogen), and probed with primary antibody followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The primary antibodies used in this study include anti-*TMSB4Y* (clone 6G4) mouse monoclonal antibody (SAB1403013; Sigma Aldrich), anti-*TMSB4X* mouse antibody (SAB1406539;

Sigma Aldrich), anti-*TMSB4X* (clone 4H7) mouse monoclonal antibody (H00007114-M03; Abnova), anti-FLAG M2 antibody (200472-21; Agilent), anti-rabbit IgG HRP-linked antibody (7074, Cell Signaling Technology), anti-mouse IgG HRP-linked antibody (7076, Cell Signaling Technology), and anti-GAPDH (D16H11) XP rabbit monoclonal antibody (5174; Cell Signaling Technology). Specific RPPA antibodies (according to the RPPA standard antibody list) used include anti-Syk mouse monoclonal antibody (sc1240, Santa Cruz), anti-Rictor (53A2) rabbit monoclonal antibody (2114, Cell Signaling Technology), anti-ATP5H mouse monoclonal antibody (ab110275, Abcam), anti-PDGF Receptor β (28E1) rabbit monoclonal antibody (3169, Cell Signaling Technology), anti-PREX1 rabbit polyclonal antibody (ab102739, Abcam), and anti-PCNA mouse monoclonal antibody (ab29, Abcam).

Cell line tissue block

Cells were trypsinized and fixed in 10% buffered formalin overnight. Thereafter, cells were spun down and resuspended with minimal (1:1) 1X PBS, then mixed via pipetting with 2% agarose solution. Various mixtures solidified after 5minutes forming agar plugs, which were placed in 6-compartment tissue cassettes and processed into paraffin embedded tissue blocks, which were then processed into FFPE cell line tissue slides.

Immunohistochemistry

Immunohistochemistry was performed on de-paraffinized (as in FISH protocol) patient tissue slides and the cell line tissue slides using the PowerVision + Poly-HRP anti-Mouse IHC Dectection System (Immunovision). Briefly, slides were steamed for 40min in EDTA solution (Zymed) and incubated with anti-smooth muscle actin antibody mouse monoclonal (1:800 dilution, DAKO, m0851) or anti-TMSB4Y (clone 6G4) mouse monoclonal antibody (1:200 dilution, SAB1403013; Sigma Aldrich) overnight at 4°C. Poly-HRP anti-mouse IgG antibody was applied for 30min, and then visualized with 3,3'-diaminobenzidine (Sigma) as the chromogen. Finally, slides were counterstained with hematoxylin. TBST rinses were performed in between steps.

Cell proliferation assay

Exponentially growing TetHyg2.5 cells were washed with HBSS twice and seeded in supplemented DMEM/F12 with 1% Tet-free FBS (HyClone), EGF at a physiological dose of 0.2 ng/mL, and hygromycin at 14.3 µg/mL. TetHyg2.5 and Dox-inducible TetHyg2.5 derivatives (EV, TmY1, and TmY2) were seeded in supplemented DMEM/F12 with 1% Tet-free FBS (HyClone), EGF at a physiological dose of 0.2 ng/mL, hygromycin at 7.15 µg/mL, and G418 at 120 µg/mL. Dox induction was done by seeding cells with the respective media with 2µg/mL of Dox. 2 x 10^4 cells per well of a 6-well tissue culture dish on day 0. Medium was changed every third day, and cells were harvested for cell counting on days 3, 6, 7, 13, and 18 using a Beckman Coulter® Vi-CELL™ XR Cell Viability Analyzer. On matched days, cell counts of Dox-induced cells were graphed relative to cell counts of non-induced cells as a percentage of proliferation.

Statistical analysis

All statistical analyses were conducted with GraphPad Prism software. For the t-tests conducted, $P<0.05$ was considered significant.

Reverse phase protein array (RPPA)

Cell lines TeHyg2.5, EV, and TmY1 were grown in 6-wells with and without Dox-induction for 48hrs, and lysates were harvested at a confluency of 80% to 90% using the lysis buffer and 4X SDS sample buffer provided by the RPPA Core Facility – Functional Proteomics of the MD Anderson Cancer Center, University of Texas. Lysates were prepared strictly accordingly to the protocol provided by the RPPA Core Facility. Briefly, cellular proteins were denatured by 1% SDS (with beta-mercaptoethanol) and diluted in five 2-fold serial dilutions in dilution buffer (lysis buffer containing 1% SDS). Serial diluted lysates were arrayed on nitrocellulose-coated slides (Grace Biolab) by Aushon 2470 Arrayer (Aushon BioSystems). Total 5808 array spots were arranged on each slide including the spots corresponding to positive and negative controls prepared from mixed cell lysates or dilution buffer, respectively. Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody. Only antibodies with a Pearson correlation coefficient between RPPA and western blotting of greater than 0.7 were used in reverse phase protein array study. Antibodies with a single or dominant band on western blotting were further assessed by direct comparison to RPPA using cell lines with differential protein expression or modulated with ligands/inhibitors or siRNA for phospho- or structural proteins, respectively. The signal obtained was amplified using a Dako Cytomation–catalyzed system (Dako) and visualized by DAB colorimetric

reaction. The slides were scanned, analyzed, and quantified using a customized-software Microvigene (VigeneTech Inc.) to generate spot intensity. Each dilution curve was fitted with a logistic model ("Supercurve Fitting" developed by the Department of Bioinformatics and Computational Biology in MD Anderson Cancer Center, "http://bioinformatics.mdanderson.org/OOMPA"). This fits a single curve using all the samples (i.e., dilution series) on a slide with the signal intensity as the response variable and the dilution steps are independent variable. The fitted curve is plotted with the signal intensities – both observed and fitted - on the y-axis and the log2-concentration of proteins on the x-axis for diagnostic purposes. The protein concentrations of each set of slides were then normalized by median polish, which was corrected across samples by the linear expression values using the median expression levels of all antibody experiments to calculate a loading correction factor for each sample.

3 Results

Clonal loss of Y chromosome in MBC

To address the hypothesis that Y chromosome might have a protective role in breast cancer, we investigated the presence or absence of Y chromosome in MBC patients. A loss of Y chromosome would support our hypothesis of Y chromosome's protective role in breast cancer. Here, we obtained FFPE tissue blocks of MBC tissue of 15 patients (cohort 1), manufactured them into a tissue microarray (TMA), and analyzed the sex chromosome statuses by FISH (Fig. 1). To survey the entire Y chromosome, we used various combinations of sex chromosome enumeration FISH probes specific for the short arm, centromere, and long arm of the Y chromosome (Fig. 2), and we observed clonal loss of whole Y chromosome in ~13.33% (2 out of 15) MBC patients. Here, we are the first to report *in situ* clonal loss of Y chromosome in MBC, whereas previous cytogenetic reports [38-40, 45] did not show *in situ* clonal of Y loss.

Next, we obtained 20 more MBC patient FFPE slides, but only 17 MBC patients were informative (cohort 2). We were unable to obtain FFPE tissue blocks for these samples, we could not manufacture another TMA. Therefore, we analyzed gDNA extracted from these FFPE tissue serial slides with ddPCR, using taqman probes and primers specific to sex chromosomes (Table 1). The ratio of Y vs X taqman positive droplet signal (ratio Y/X) of our known Y loss patient (Patient 19) was 0.193, the residual Y signal in this sample was attributed to contamination of normal gDNA within the tumor tissue. Subsequently, a ratio Y/X of lower than ~0.2 is considered a most possible Y loss patient; a ratio Y/X of between ~0.2 to ~0.4 is considered a probable Y loss patient; and a ratio Y/X of higher than ~0.4 is considered a Y retention patient. gDNA from one patient (Patient 18) yielded uninformative ddPCR results. Using ddPCR as a first pass analysis, we observed 6 out of 17 analyzable MBC patients who have possible Y loss. FISH was performed successfully in 14 out of these 17 patients to validate the presence or absence of sex chromosomes. Among the 6 patients with the lowest Ratio Y/X (range of 0.0 to 0.4), and it is confirmed that the 2 MBC patients with Ratio Y/X smaller than 0.2 and 1 MBC patient with a Ratio Y/X of ~0.4 are Y loss cases. Therefore, for cohort 2, we observed clonal loss of Y chromosome in ~17.65% (3 out of 17) MBC patients.

Combining results from the two independent cohorts of MBC patients, we observed a Y loss frequency of ~15% (5 out of 32 MBC patients).

Loss of Y chromosome in MBC since DCIS

After observing the loss of whole Y chromosome in MBC tissue, we investigated whether Y was lost in the ductal carcinoma *in situ* (DCIS) stage of MBC. Therefore, we obtained DCIS tissue of one of the Y loss patients (Fig. 3A). An anti-smooth muscle actin stain verified that the tissue was DCIS (Fig. 3B), and we showed via FISH that Y was lost since DCIS, where the DCIS tissue has the absence of the Y chromosome while the adjacent stromal tissue retained the Y chromosome (Fig. 3C).

Nullisomic loss of *TMSB4Y* in male breast cancer patient

In MBC samples that retained the Y chromosome, Y FISH signals were aberrant in tumor cells compared to the surrounding stromal cells in the fibrous tissue (Fig. 1B, Y Probe panel). Thus, we hypothesized that the Y chromosome in MBC tumor cells might be chromosomally unstable, also based on previous studies that microdeletions are common within the Y chromosome [46-50]. To investigate this, we extracted gDNA of five MBC patients who retained the Y chromosome, and amplified 33 Sequence Tagged Sites (STSs) with standard PCR (Table 2, Fig. 4) within the MSY based on the MSY-BM [43]. Paired tumor vs normal gDNA (labeled by pathologist) were extracted from FFPE slides, and only three out of our five patients were analyzable due to the quality of the extracted FFPE gDNA. STS primer set S27 was not amplified from any FFPE gDNA, because the amplicon size is ~1kb and thus cannot be amplified from fragmented FFPE gDNA.

Interestingly, STS primer S17 did not amplify at all for the tumor gDNA for one of the three analyzable MBC patients, but it amplified for his paired normal gDNA

(Fig. 5). STS primer S17 amplifies STS sY1230, which spans exon1 and intron 1 of *TMSB4Y*, the gene coding for an actin sequestering protein, Thymosin Beta 4, Y-linked. Therefore, our STS-PCR data showed the nullisomic loss of *TMSB4Y* in MBC the tumor gDNA of one of our MBC patients who retained the Y chromosome.

TMSB4Y is expressed in normal male breast tissue

We performed immunohistochemistry for *TMSB4Y* on breast tissue of one of our patients who lost the Y chromosome in his breast cancer cells. In this patient, the normal acini were more strongly stained compared to the tumor tissue (Fig. 6), and any faint staining in fibrous tissue and tumor cells was attributed to a low level of background nonspecific staining. This shows that *TMSB4Y* is expressed in normal male breast tissue, and apparently not expressed after the loss of Y chromosome.

TMSB4Y expression alters breast epithelial cell morphology

To investigate the effects of *TMSB4Y* in breast cells, we generated stable Dox-inducible clones in the TetHyg2.5 cell line. TetHyg2.5 is MCF-10A that is engineered to permanently express the reverse Tet repressor; MCF10A is a genetically stable nontumorigenic breast epithelial cell line [44]. *TMSB4Y* was cloned into the bidirectional pBI-EGFP vector, which contains the Tet response element, and expresses GFP simultaneously with *TMSB4Y*. We generated TmY1 and TmY2, two Dox-inducible cell lines that express both GFP and *TMSB4Y* when induced with Dox. EV contains the pBI-EGFP empty vector and expresses only GFP when induced with Dox. Expression of *TMSB4Y* after Dox-induction was verified via western blotting (Fig. 7A) and

immunohistochemistry (IHC) on tissue cell blocks made from the generated cell lines (Fig. 7B). Apparently, TmY1 has stronger induction of *TMSB4Y* compared to TmY2. It is noteworthy that transient expression of GFP in MCF-10A cells is non-toxic (Fig. 8).

Dox-induction of *TMSB4Y* changes the morphology of TmY1 and TmY2 cell lines drastically (Fig. 9A), where cells become enlarged and distorted. Furthermore, F-actin staining after Dox-induction of TmY1 confirms this alteration in cell morphology (Fig. 9B).

TMSB4Y expression reduces cell proliferation

In Fig. 10A, Dox-induction of *TMSB4Y* significantly reduced the growth rates of TmY1 and TmY2 by about 30% when compared to TetHyg2.5 and EV ($P < 0.05$) in a 6-day growth assay, where cell counts of Dox-induced cells were plotted as a percentage of cell counts of non-induced cells. This reduction is proliferation was also observed in a detailed growth assay where cell counts were taken on days 0, 3, 7, 13, and 18 (Fig. 10B).

Syk expression is reduced after Dox-induced *TMSB4Y* expression

In order to find the possible cause of the effects of *TMSB4Y* expression on cell morphology and proliferation, we utilized reverse phase protein array (RPPA) to investigate global protein expression changes in TmY1 after Dox-induction. The change in expression of other proteins might allow us to tease out genetic pathways regulated by *TMSB4Y*. TetHyg2.5 and EV were analyzed with RPPA as negative controls. The RPPA heatmap (Fig. 11A) indicates relative protein expression by a color pattern, where green

equates to low expression, and red equates to high expression. We are interested only in protein candidates that are either noticeably increased or decreased for only TmY1+Dox, and then a similar color pattern for the rest of the samples (TetHyg2.5, TetHyg2.5+Dox, EV, EV+Dox, TmY1). According to this observation pattern, 6 protein candidates were noted. Expression of Rictor was increased, whereas the expression of Syk, ATP5H, PCNA, PDGFRβ, and Prex1 were decreased. Their specific antibodies (according to the RPPA standard list) were utilized to validate the RPPA results. For 5 protein candidates, expression difference was either too subtle, or the antibodies exhibited high non-specificity towards our lysates. Eventually, we were only able to validate the reduced expression ($P<0.05$) of Syk (spleen tyrosine kinase) after Dox-induced expression of *TMSB4Y* in both TmY1 and TmY2 (Fig. 11B).

TMSB4Y **is different from its homologue** ***TMSB4X***

TMSB4Y has an X-linked homologue *TMSB4X*, and they are different only by three out of 44 amino acids. However, their antibodies clearly do not cross-react, which shows that the three amino acid differences cause apparent differences between *TMSB4Y* and *TMSB4X*. We transiently expressed *TMSB4X* in 293 cells, and we did not observe any bands on western blots with our specific anti-*TMSB4Y* and also our two anti-*TMSB4X* antibodies (results not shown). Therefore, we expressed FLAG-*TMSB4X* in 293 cells, confirmed the expression of *TMSB4X* by blotting for FLAG, and then confirmed that our specific *TMSB4Y* antibody cannot detect FLAG-*TMSB4X* (Fig. 12).

X Probe
Y Probe
DAPI
Tri
A
Somatic Control
B
Y Retention
C
Clonal Y Loss

Figure 1. Clonal loss of Y chromosome in MBC

A, Normal somatic male breast acinus has both X and Y sex chromosomes.

B, Male breast cancer tissue that retained both X and Y sex chromosomes.

C, Male breast cancer tissue that retained X chromosomes, lost the Y chromosome clonally in cancer tissue, but retained Y chromosome in surrounding somatic tissue.

Red, X chromosome FISH Probe; Green, Y chromosome FISH Probe; Blue, DAPI.

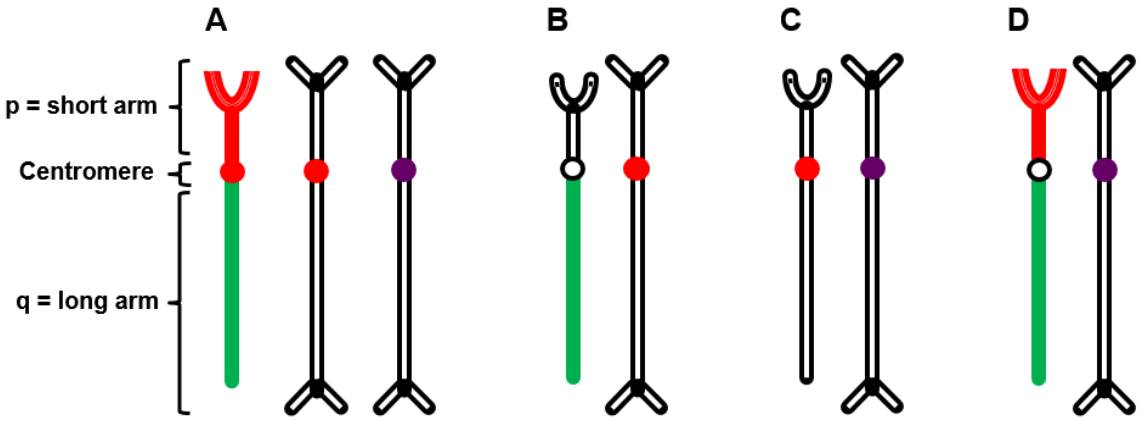


Figure 2. FISH probes for sex chromosomes

In the schematic of the sex chromosomes, the Y-shaped figure represents the Y chromosome and the X-shaped figure represents the X chromosome.

A, Various probes we used for both X and Y sex chromosomes. We have red probes for p-arm and centromere of Y chromosome; green probe for q-arm of Y chromosome; red and violet probes for centromere of X chromosome.

B, **C**, and **D**, three combinations of FISH probes we used for FISH on patient tissues.

S/No	Patient ID	Positive ddPCR Droplets		Ratio Y/X	Observation *
		Y Chr Taqman	X Chr Taqman		
01	0751724 3J 5	7	524	0.013	(F) Y Loss
02	0251354 A 5	6	114	0.053	(F) Y Loss
03	1154102 4A 5	357	1009	0.354	(F) Y Retention **
04	995209 C 5	217	566	0.383	(F) Y Retention **
05	0251134 A 5	84	205	0.410	(F) Y Loss
06	0051749 P 5	54	116	0.466	Y Retention
07	0752630 1D 5	47	73	0.644	(F) Y Retention
08	0252481 B 5	17	24	0.708	(F) Y retention
09	0251600 M 5	158	196	0.806	(F) Y Retention
10	0453365 C 5	57	52	1.096	(F) Y retention
11	9952907 H 5	134	117	1.145	(F) Y retention
12	972005 L 5	334	214	1.561	(F) Y retention
13	0854095 2F 5	482	293	1.645	(F) Y retention
14	0953927 1d 5	156	91	1.714	Y retention
15	1251936 4E 5	2067	1188	1.740	(F) Y retention
16	115759 1T 5	53	23	2.304	Y retention
17	S13 1824	252	86	2.930	(F) Y retention
18	9651911 B 5	3	1	3.000	Uninformative
19	06-70864	101	523	0.193	(F) Y Loss Patient
20	Male gDNA	2628	2720	0.966	Male Control
21	Female gDNA	0	3451	0.000	Female Control

*(F) refers to FISH verified patients.

** Denotes MBC patients who have duplication of X chromosome.

Table 1. Y Loss in MBC Patients Shown via droplet digital PCR (ddPCR)

Tumor gDNA was extracted from FFPE tissue slides of MBC patients and analyzed with ddPCR Taqman probes specific for X and Y chromosomes. Promega Male and Female gDNA were used as control for probe specificity; Patient 19 (from cohort 1) showed Y Loss and is as a gauge of the possible base level gDNA contamination from normal tissue during FFPE extraction. 14 out of 17 ddPCR-analyzable patients were validated with FISH; 3 patients had FFPE tissue that cannot be validated with FISH.

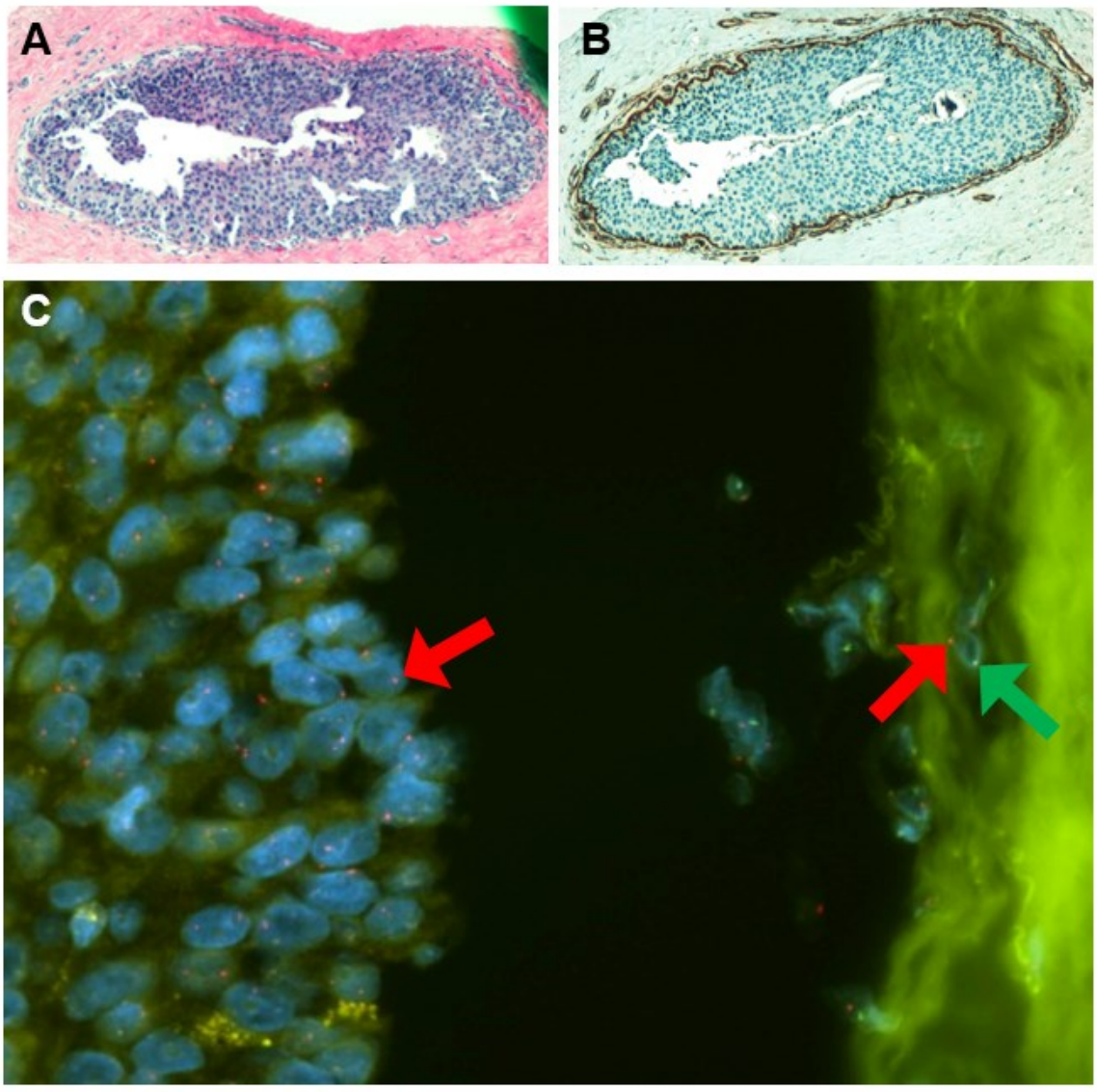

A
B
C

Figure 3. Clonal loss of Y chromosome in MBC since DCIS

A, H&E Stain of a DCIS lesion of a male breast cancer patient who lost the Y chromosome in his cancer tissue.

B, Anti-smooth muscle actin stain confirms that the well- circumscribed lesion is a DCIS lesion.

C, DCIS lesion (left) retained X chromosomes, lost the Y chromosome clonally, but retained Y chromosome in adjacent somatic tissue (right). Red arrows point to the X-probe signal and green arrow points to the Y-probe signal.

Red, X chromosome FISH Probe; Green, Y chromosome FISH Probe; Blue, DAPI.

S/No.	STS Identifier	Primer 1	Primer 2
S01	sY1247	GAACTCTGCAAACCTCCTGG	TTTTGAGGCGGAGTCTCG
S02	sY14	GAATATTCCCGCTCTCCGGA	GCTGGTGCTCCATTCTTGAG
S03	sY274	TTAAGGGGACAGTATTTCAACTTC	CCACATTTAAACTGAGTACAGTCC
S04	sY238	AACAAGTGAGTTCCACAGGG	GCAAAGCAGCATTCAAAACA
S05	sY1254	GACCAATTTGTCTTTGTTGCG	GCTGCTGAAGTCGGCGTA
S06	sY1240	GGGTCCTAGATAGGCTCCAAG	TTCATGTTGGCAGTGATTGG
S07	sY276	CCTACCGCATCAGTGAATTTC	TCTGTATGTGGAGTACACATGG
S08	sY1238	GGTGTGCTAACATTGCATGG	TTTGTTCCATTTCAGAGCGA
S09	sY637	CCTGCCTTTTTTAGTTTCCAGCA	TACTGTGATAGGTAGAATAATGGC
S10	sY1319	ACCTGTCTGGGAAACACCTC	GAGCCCTACAACCAGCTTCA
S11	sY1250	TTTTTCTAACCTTGCCTGCG	TGCAGAGAAGCAGCCTACAA
S12	sY1251	GACTGGAGTGGAACGGTCTC	TCACTTCCCTCCGATTTTCT
S13	sY1317	GAGATTACAGGCATGCACCA	CCACACTTAGCCCACAGTCA
S14	sY1316	AAGGCAGGTCTGATGCATGT	AAAGAAAGCTGCCTCATAGCA
S15	sY1234	TTACCCCTTTCACCCACTGA	CCATAAACTACACAAGGACGAACT
S16	sY1231	TTGCACCCGTAGTCAAATGT	ACCCACAACTCAAATCGTCT
S17	sY1230	CTCTTCCAAGCCAGCCTTTA	AACCTTTGCAAGCCACATTC
S18	sY90	CAGTGCCCCATAACACTTTC	ATGGTAATACAGCAGCTCGC
S19	sY1239	CCTAGCTCTCTTTTTCTTGCAG	CAAATATCGCCAGTGAGGCT
S20	sY210	ATCACTTGGCAGCTTTTCC	GCACTGCAACTTTTATGCCT
S21	sY121	AGTTCACAGAATGGAGCCTG	CCTGTGACTCCAGTTTGGTC
S22	sY1322	TGGAAACATTCTCAACAGGGA	GGCATTTCTCGCATGAGTTT
S23	sY280	AACTGTACTCCTGGGTAGCCTG	CTCCCGTGGGGATGAAGATAATA
S24	sY1233	TCTCCGGTATCCTGATGGAG	AAATAGGGCATTCCCAGCTC
S25	sY1682	GGTTGCACCGTAAAAGGAGA	GTCTGTCAAGACAGCGTCCA
S26	sY142	AGCTTCTATTCGAGGGCTTC	CTCTCTGCAATCCCTGACAT
S27	sY1258	AACCCCATCTCTAGCAAAAATATG	TAGGTGACAGGGCAGGATTC
S28	sY1197	TCATTTGTGTCCTTCTCTTGGA	CTAAGCCAGGAACTTGCCAC
S29	sY1191	CCAGACGTTCTACCCTTTCG	GAGCCGAGATCCAGTTACCA
S30	sY1291	TAAAAGGCAGAACTGCCAGG	GGGAGAAAAGTTCTGCAACG
S31	sY1201	CCGACTTCCACAATGGCT	GGGAGAAAAGTTCTGCAACG
S32	sY1166	AGTCGGAGTCGGAGTGTGAT	ATTCCATTGCTTTCCATTGC
S33	sY1273	GAGCTGCAACATAACAGGCA	AGGGGAACATCACACTCTGG

Table 2. 33 Pairs of Landmark STS Primers within the MSY

33 STSs spanning the male specific region of the human Y chromosome (MSY), as shown in the MSY Breakpoint Mapper [43].

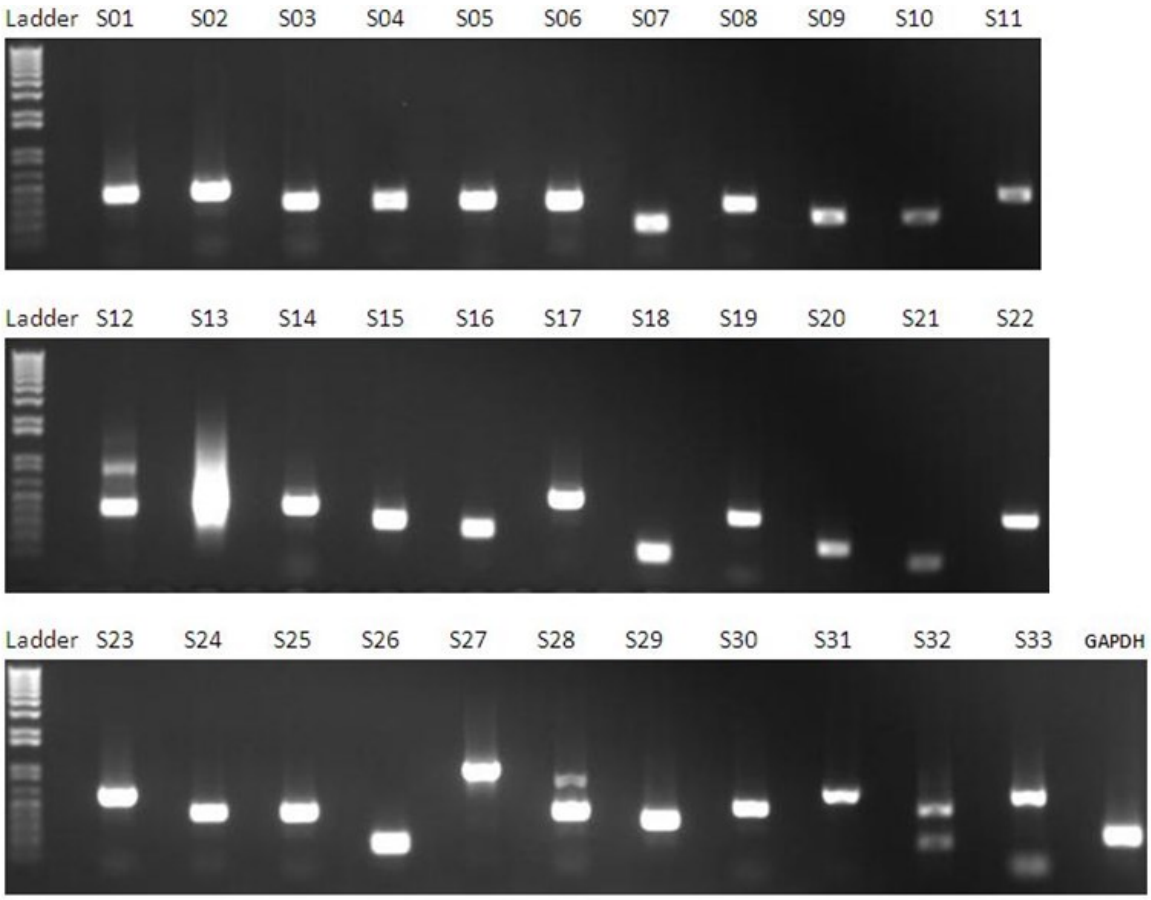


Figure 4. Amplicons of STS-PCR (Sequence-Tagged Sites PCR)

The 33 pairs of MSY Breakpoint Mapper (MSY-BP) primers, S01 to S33, amplify sequence-tagged sites spread across the Male Specific Region of the Y chromosome (MSY). PCR was performed on gDNA extracted from a normal human male. The amplicon sizes range from 176bp to 968bp. S13 is uninformative; S12, S28, S32, and S33 each have one extra nonspecific amplicon. GAPDH is the positive control.

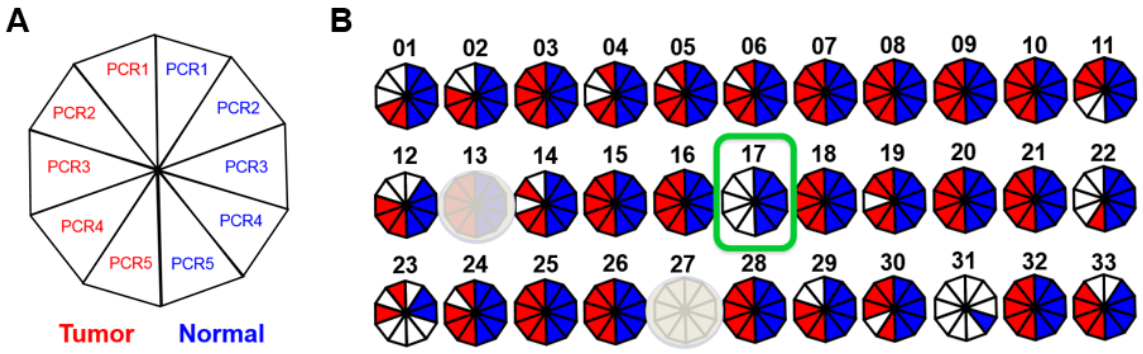


Figure 5. STS-PCR results of MBC patient

A, Each STS-PCR is repeated 5 times on the matched tumor and normal FFPE gDNA from the patient. Each of the 33 sets of tumor vs normal STS-PCR is presented in a decagon. The relative segment of the decagon is filled only if the PCR reaction fired; tumor STS-PCR is filled with blue, represented by the five decagon segments on the left; normal STS-PCR is filled with red, represented by the five decagon segments on the right.

B, Primer sets S13 and S27 are uninformative. A majority of the decagons are completely filled, which signifies that both tumor and normal gDNA retained that respective STS. Only S17 is lost in tumor gDNA, but retained in normal gDNA.

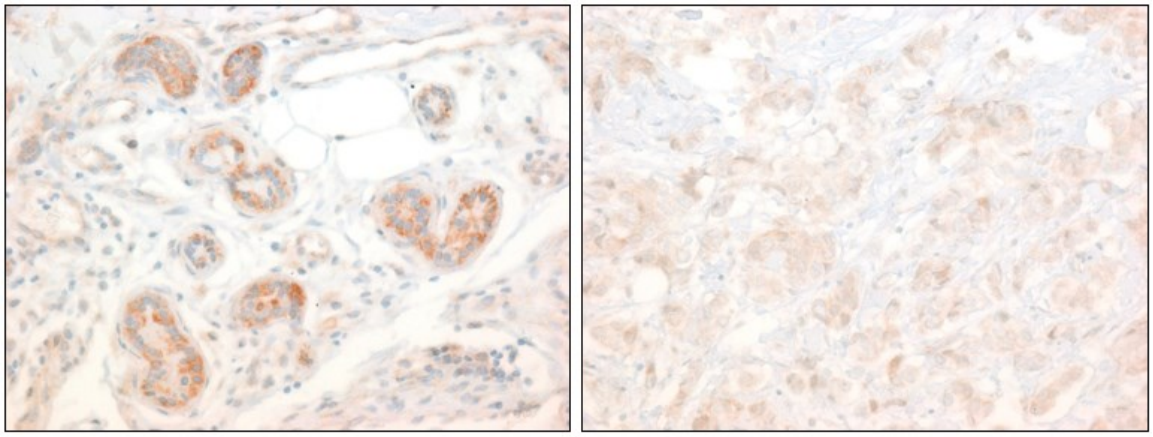


Figure 6. *TMSB4Y* is expressed in normal male breast tissue

Immunohistochemistry (IHC) for *TMSB4Y* was performed on breast tissue from a male breast cancer patient who lost his Y chromosome (shown previously via FISH). Normal acini are more strongly stained compared to tumor tissue, which has no staining above a low level of background nonspecific staining.

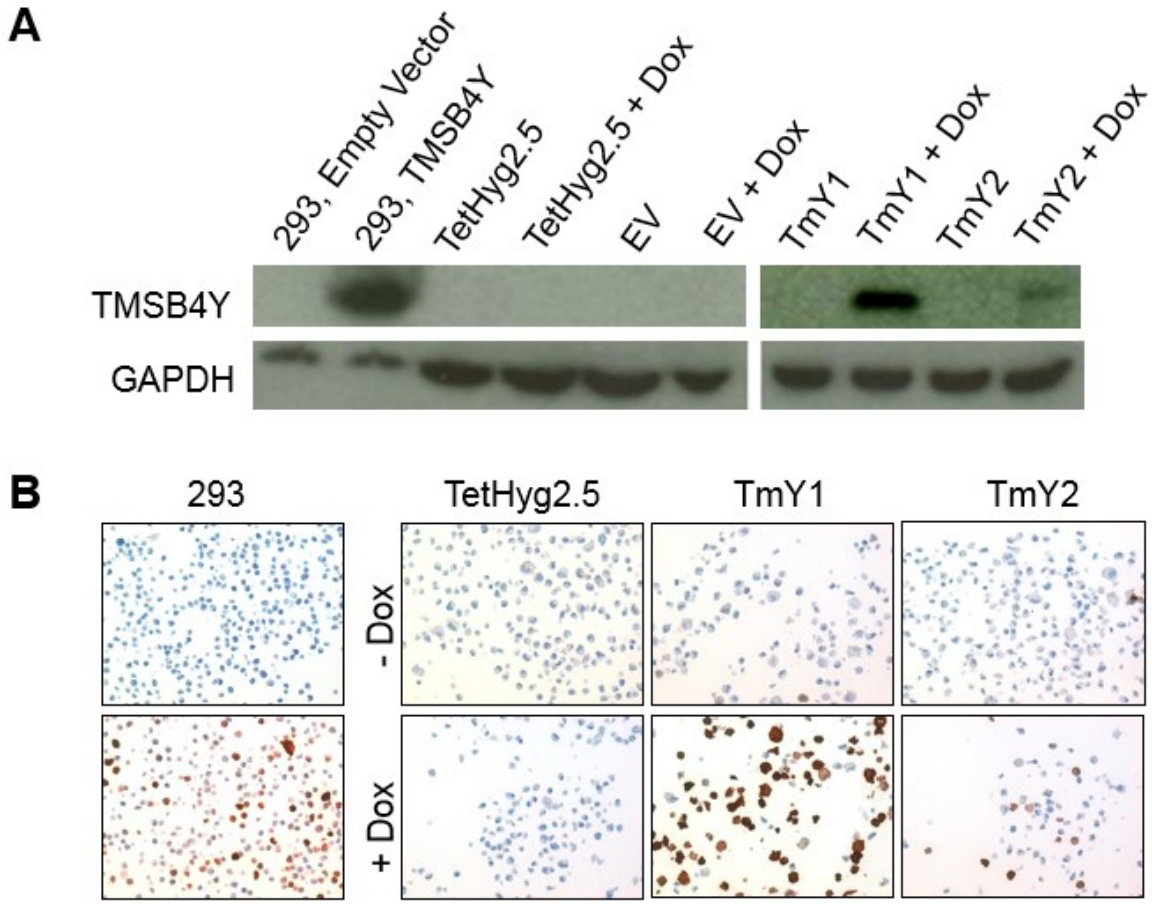


Figure 7. Verification of *TMSB4Y* expression in Dox-inducible clones

TMSB4Y is cloned into the pBI-EGFP vector and transfected into the TetHyg2.5 to make Dox-inducible clones. TmY1 and TmY2 express *TMSB4Y* upon Dox induction, shown via western blotting (**A**) and immunohistochemistry on tissue cell blocks (**B**).

293 transient transfection is the positive control; TetHyg2.5 expresses the reverse Tet repressor and is derived from MCF-10A; EV is generated with the empty pBI-EGFP vector.

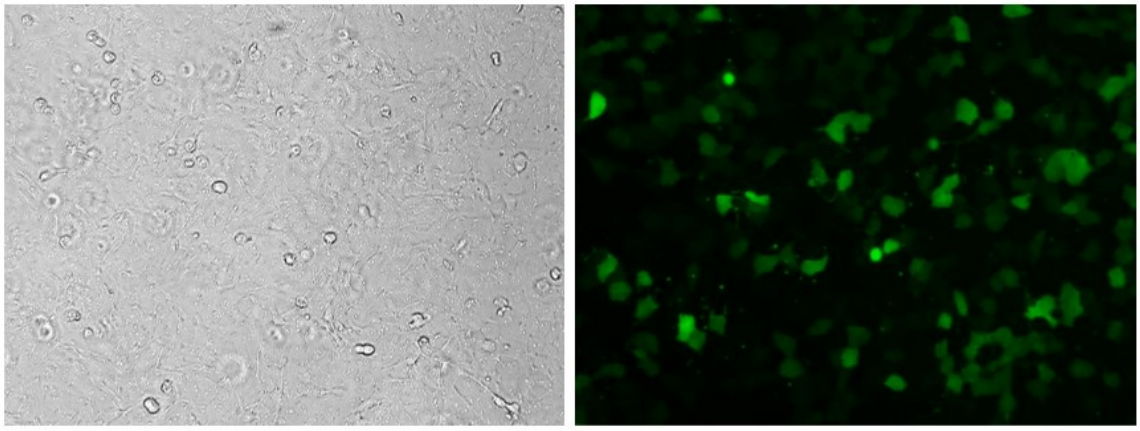


Figure 8. Transient expression of GFP in MCF-10A cells is non-toxic

Transient expression of GFP in MCF-10A cells has no noticeable effect, as shown in phase contrast microscopy (left) and GFP fluorescent signals (right).

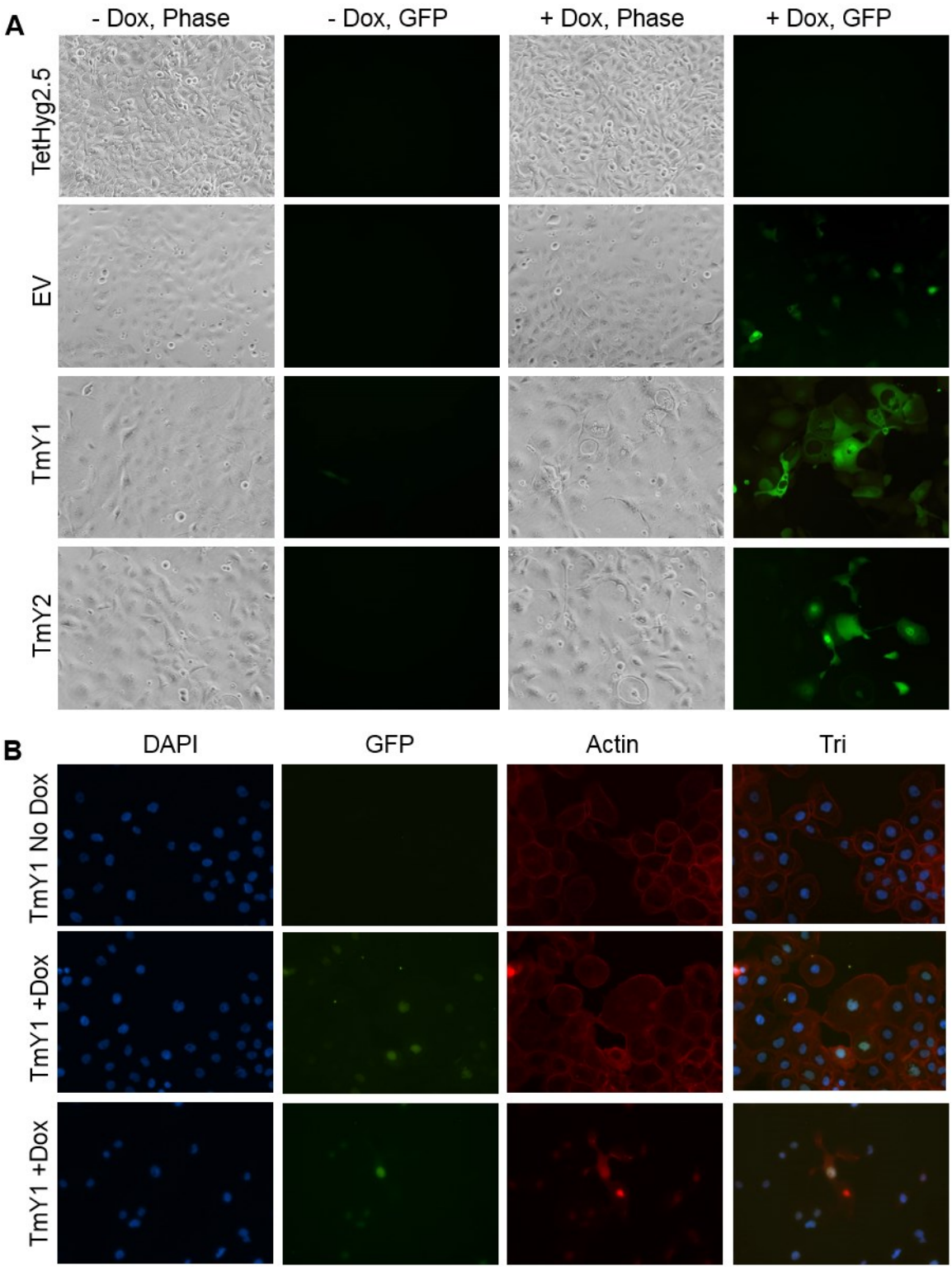

A
- Dox, Phase
- Dox, GFP
+ Dox, Phase
+ Dox, GFP
TetHyg2.5
EV
TmY1
TmY2
B
DAPI
GFP
Actin
Tri
TmY1 No Dox
TmY1 +Dox
TmY1 +Dox

Figure 9. *TMSB4Y* expression induces morphological changes

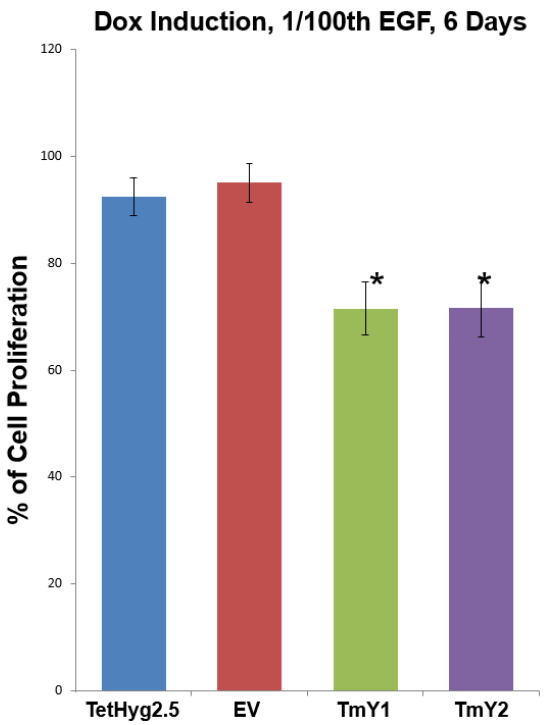
Dox-induction expresses *TMSB4Y* in Dox-inducible clones, and results in aberrant morphological changes.

A, Phase contrast and GFP images of TmY1 and TmY2 show enlargement and distortion of cells after Dox induction.

B, F-actin staining of TmY1 confirms the aberrant morphological changes after Dox-induction.

TetHyg2.5 expresses the reverse Tet repressor and is derived from MCF-10A; EV is generated with the empty pBI-EGFP vector.

A


Dox Induction, 1/100th EGF, 6 Days
% of Cell Proliferation
120
100
80
60
40
20
0
*
*
TetHyg2.5
EV
TmY1
TmY2

B

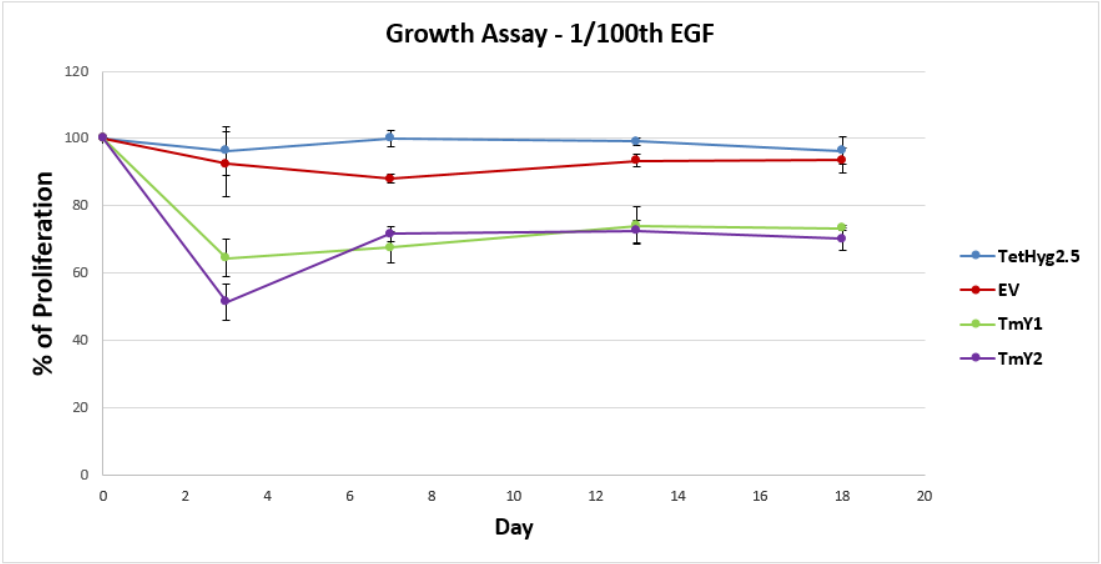

Growth Assay - 1/100th EGF
% of Proliferation
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
Day
TetHyg2.5
EV
TmY1
TmY2

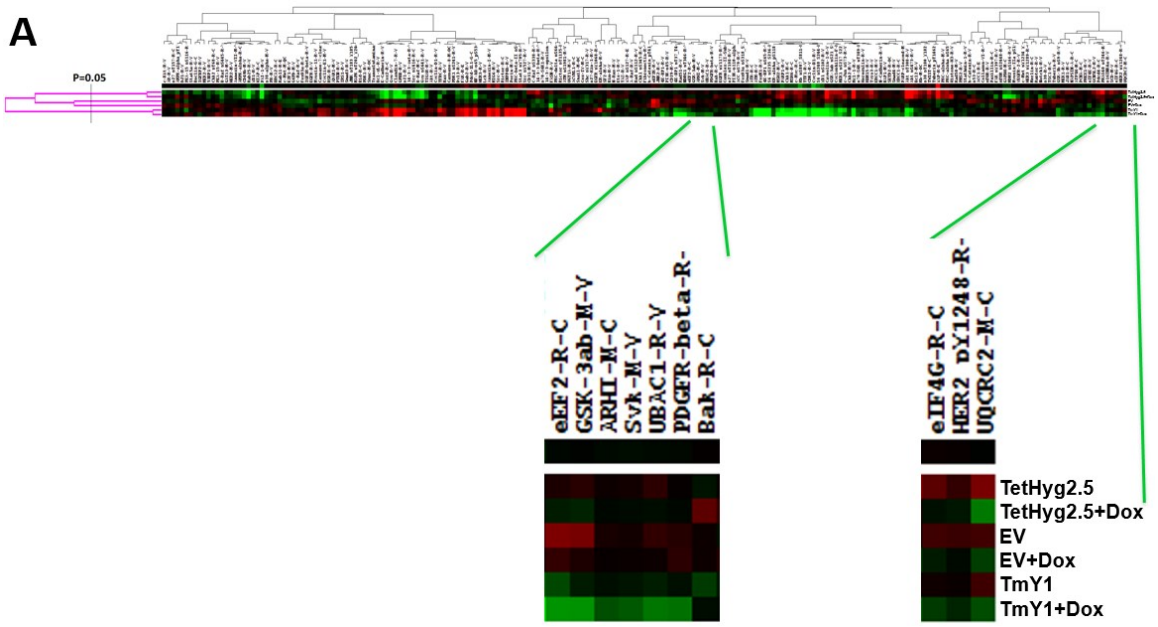
Figure 10. *TMSB4Y* expression reduces cell proliferation

Dox-induction expresses *TMSB4Y* in Dox-inducible clones, and results in reduction in proliferation.

A, TmY1 and TmY2 exhibited ~30% of reduced proliferation upon Dox induction ($P < 0.05$), after 6 days of growth.

B, A persistent ~30% of reduced proliferation after Dox-induction in both TmY1 and TmY2 was observed in a detailed growth assay, with cell counts at Days 3, 7, 13, and 18.

TetHyg2.5 expresses the reverse Tet repressor and is derived from MCF-10A; EV is generated with the empty pBI-EGFP vector.


A
P=0.05
eEF2-R-C
GSK-3ab-M-V
ARHI-M-C
Syk-M-V
UBAC1-R-V
PDGFR-beta-R-
Bak-R-C
eIF4G-R-C
HER2 pY1248-R-
UQCRC2-M-C
TetHyg2.5
TetHyg2.5+Dox
EV
EV+Dox
TmY1
TmY1+Dox

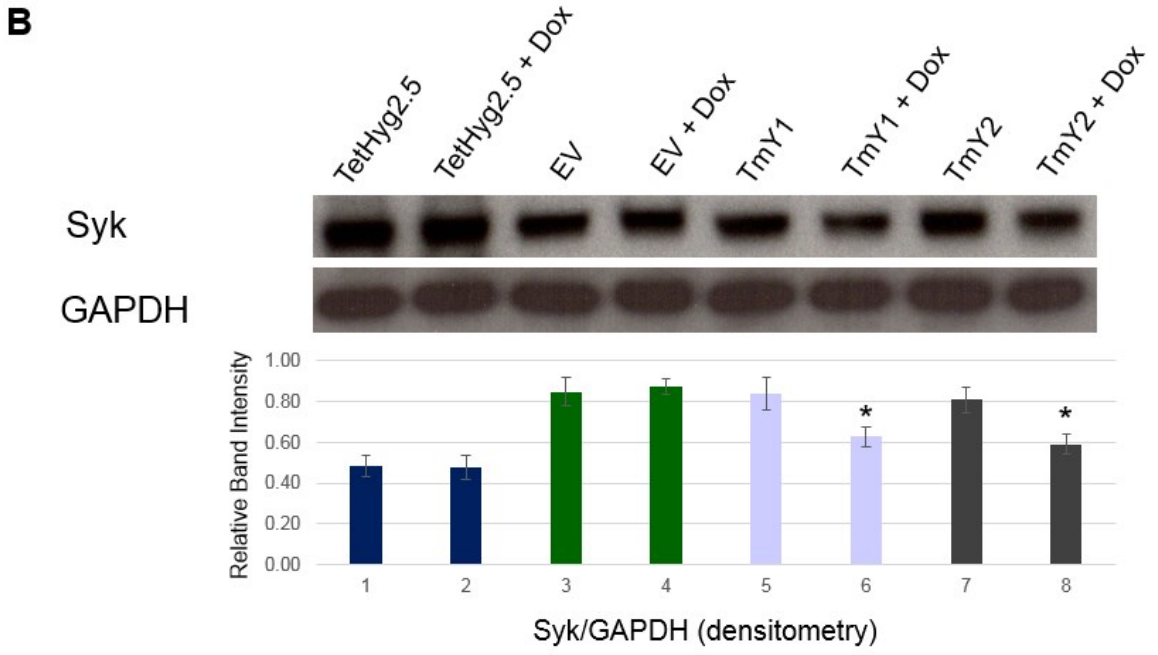

B
TetHyg2.5
TetHyg2.5 + Dox
EV
EV + Dox
TmY1
TmY1 + Dox
TmY2
TmY2 + Dox
Syk
GAPDH
Relative Band Intensity
1.00
0.80
0.60
0.40
0.20
0.00
1
2
3
4
5
6
7
8
*
*
Syk/GAPDH (densitometry)

Figure 11. Syk expression is reduced after Dox-induced *TMSB4Y* expression

Reverse phase protein array (RPPA) was utilized to investigate global protein expression patterns in TetHyg2.5, EV, and TmY1 before and after Dox-induction.

A, RPPA heatmap. Green means low protein expression, and red means high protein expression. Syk expression is green only for TmY1+Dox. Therefore, Syk expression in TmY1 is reduced when *TMSB4Y* is expressed.

B, Syk expression is reduced for both TmY1 and TmY2 after Dox-induced expression of *TMSB4Y*. Densitometry of the western blot shows that this reduction is significant ($P<0.05$).

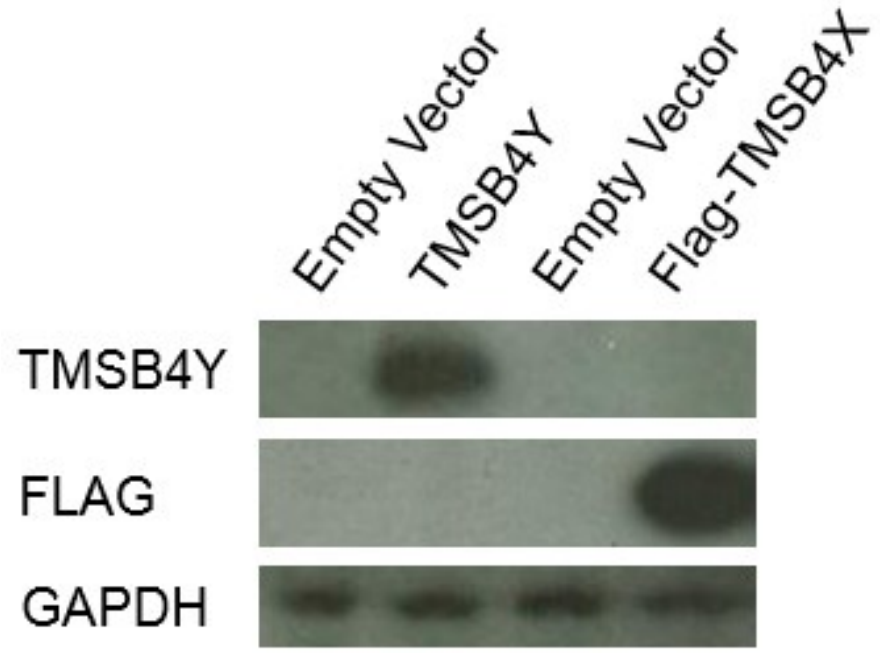


Figure 12. *TMSB4Y* is different from its homologue *TMSB4X*

TMSB4X is a gene on the X chromosome, and it only differs from *TMSB4Y* by three out of 44 amino acids. Here, we transiently expressed *TMSB4Y* and FLAG-*TMSB4X* in 293 cells, and despite the close homology, our specific *TMSB4Y* antibody did not detect *TMSB4X*. *TMSB4X* expression was confirmed via blotting for FLAG.

4 Discussion

Cancer is a disease fundamentally caused by a successive series of genetic alterations that lead to selective proliferative advantages in a single starting cell, which expands into a tumorigenic clone. This classic "Vogelgram" model of sequential accumulation of carcinogenic mutations was first described in colorectal cancer, where progressive accumulation of mutations in *APC* (from normal to early adenoma), *KRAS* (from early to intermediate adenoma), *SMAD4* (from intermediate to late adenoma), and then *p53* which eventually leads to colorectal carcinoma [51]. All the cells that originate from this clone will retain specific genetic signatures acquired in this tumorigenic process. Therefore, our novel FISH report of *in situ* clonal loss of Y chromosome in MBC increases the weight of evidence that Y loss contributes to the tumorigenesis of MBC.

Even though our total MBC sample size of 32 patients is small, it is noteworthy that the Y loss frequency is consistently ~15% in these 2 independent cohort of MBC

patients. Because there is an international effort to consolidate MBC samples that our research group is involved in, we anticipate to greatly expand our sample size to confirm this average frequency of ~15% clonal Y loss in MBC.

Interestingly, we also saw the loss of Y chromosome in the DCIS tissue of one of our MBC patients who has clonal Y loss in his tumor tissue (Fig 3). DCIS slides were only available for 1 out of 5 of our Y loss patients. In consonant with the concept of progressive accumulation of genetic mutations in tumorigenesis, Y loss seems to have already happened since DCIS, which suggests that Y loss might be an early contributive oncogenic factor. However, since we only showed this in one MBC patient, this aspect of our study needs to be expanded after collation of more MBC samples with available DCIS tissue.

FISH presents clonal Y loss clearly *in situ*, however, the success of FISH is dependent on the quality of the FFPE tissue. Therefore, we established a much more sensitive technique of droplet digital PCR (ddPCR) to analyze Y loss in MBC, as shown in our results in patients of cohort 2. However, we have to be cautious when analyzing ddPCR results. Here, we utilized the Y/X fluorescent ddPCR readout ratio to evaluate Y loss status in ddPCR. The loss of Y will skew the Y/X ratio towards zero. ddPCR is a very sensitive technique, and because there is always normal stromal cells and tissue around the tumor tissue, genomic DNA of these normal cells amongst tumor cells serves as contaminating ddPCR readouts. Our previous case study, where we established ddPCR as a tool to analyze loss of heterozygosity of *BRCA2* alleles, also shows a basal level of contamination from surrounding normal stromal cells [52]. Therefore, when analyzing ddPCR results, we have to take this basal level of contamination from normal gDNA into

account, which is shown in our Y/X ratio. According to our Y/X ratios consolidated in Table 1, our three Y loss patients from cohort 2 have Y/X ratios of 0.013 (1.3%), 0.053 (5.3%), and 0.41 (41%); our control Y loss patient has a Y/X ratio of 0.193 (19.3%). The high level of 41% contamination from normal stromal cells was unexpected, and when we relooked at the H&E section of the tumor area we selected for extraction, there was a high level of normal stromal cells, which explains this high level of contaminating Y readouts in a Y loss patient (validated by FISH). Therefore, caution has to be taken while selecting tissue areas on the FFPE slides for gDNA extraction. Targeted tumor areas for gDNA extraction should have a tumor cell population as pure as possible. A strict cutoff for Y/X ratio of below 0.010 (10%) should be established if only looking at ddPCR results. Any Y/X ratios between 0.010 (10%) to 0.040 (40%) should definitely be validated with FISH, otherwise, their Y loss status (based on ddPCR) is debatable. Here, we established ddPCR as a novel tool to evaluate Y loss in MBC, which should be utilized especially when MBC tissue is very limited, and a TMA (for FISH) of MBC samples is not available.

Our ddPCR assay is able to indicate duplication of X chromosome in male breast tumor cells as well. In Table 1, two patients (S/No. 03 and 04) show duplicated X in tumor cells according to FISH, and their Y/X ratios from ddPCR are 0.354 (35.4%) and 0.383 (38.3%), which seems to have almost double the X ddPCR readout compared to the Y ddPCR readout. Thus, our ddPCR assay indicates duplication of X in tumor cells when the Y/X ratio is around 0.35 (35%). Gaining an extra X chromosome might be a causative tumorigenesis factor too, which is in consonant with the concept of the report that Klinefelter patients (XXY) have a higher risk for MBC [9].

Interestingly, recent studies have strongly suggested that the human Y chromosome has tumor suppressive functions that are still not elucidated. Mosaic loss of Y chromosome is now associated with a higher risk of non-hematologic cancers in men [53]. It is shown that men with non-hematologic cancers (such as throat, prostate, stomach), have a loss of Y chromosome in their peripheral blood. Furthermore, this loss of Y in peripheral blood in men is associated with smoking in a dose-dependent manner [54]. It has been well established for more than half a century that cigarette smoking causes many human diseases, which includes cancer, especially lung cancer [55].

Furthermore, male microchimerism in women was shown to reduce the risk of breast cancer to one third [56]. In fact, this male chimerism observed in the peripheral blood of women generally increases the survival of women affected by cancers and cardiovascular diseases [57]. Male microchimerism is a natural phenomenon that occurs in females who are or have been pregnant with a male fetus, and it happens when the male fetus exchanges cells with his mother via the placenta. Some of these male fetal cells presumably persist throughout the mother's lifetime, for as long as almost three decades [58]. Very interestingly, these studies suggest that the presence of the Y chromosome in females is tumor suppressive without any known mechanism.

In order to investigate putative tumor suppressive function of the human Y chromosome, the full Y chromosome was transferred into PC-3, a prostate cancer cell line that has Y loss, via microcell-mediated chromosome transfer (MMCT). This MMCT of the Y chromosome into PC-3 suppressed the formation of xenografts in 59 out of 60 athymic nude mice. This further suggests that the Y chromosome contains some tumor suppressive element.

Taken together, previously described reports and our own report of clonal Y loss in MBC strongly suggest a tumor suppressive role of the human Y chromosome in human cancer.

After seeing whole Y loss in 5 out of 32 (~15%) of our MBC patients, we proceeded to investigate regional loss of the Y chromosome in MBC patients who retained Y. The FISH signals for the Y chromosome are aberrant in the tumor cells of some of our MBC patients who retained Y, compared to the corresponding Y signals in the surrounding normal stromal cells (Fig. 1B). This led us to think that the retained Y chromosome underwent some form of structural rearrangement or deletions, and thus indicative of regional loss of genomic regions on the retained Y chromosome. Therefore, we proceeded to utilize STS-PCR (STS primer sets derived from MSY-BPM) to investigate regional Y loss in MBC patients who retained their Y chromosome.

Using STS-PCR, we were able to show that STS17 was lost in the tumor gDNA in one of our MBC patients who retained Y. Because of the quality of the extracted FFPE gDNA, we were only able to analyze 3 out of 5 of the patients we extracted FFPE gDNA from. Our STS-PCR has certain limitations. Firstly, the quality of FFPE limits the success of our STS-PCR reactions. Secondly, the total amount of gDNA available from our FFPE extractions were also limited. Thirdly, formalin fixing and paraffin embedding degraded the tissue gDNA and thus, the amplicon sizes of our STS-PCRs have to be relatively short, leading to a couple of STS primer sets with amplicon sizes of about 1kb to become uninformative. In future, we look forward to optimize the STS primer sets that cover the MSY region for the highly sensitive ddPCR, and develop STS-PCR into STS-ddPCR.

Eventually, using STS-PCR, we showed focal nullisomic loss of STS17 in one of our MBC patients. STS17 is located within the gene *TMSB4Y*. To increase the argument that *TMSB4Y* is a deleted tumor suppressor gene, we surveyed public databases of sequenced tumors via cBioPortal, a web-based tool that allows analysis of publicly available next-generation sequencing data of tumors, and discovered that *TMSB4Y* was reportedly deleted in 10 out of 61 prostate cancer samples with known copy number alteration profiles [59]. Furthermore, a previous study has shown via array comparative genomic hybridization (aCGH) a 40% (10/25 patients) deletion of Yq11.1-q11.221in MBC, and this Yq region contains TMSB4Y [60].

So far, all these *TMSB4Y* deletion data is shown at the DNA level, which is provocative, because if *TMSB4Y* is a genuine tumor suppressor gene, it should be expressed at the protein level in normal male breast tissue. And we managed to show that *TMSB4Y* is expressed in normal male breast tissue via IHC (Fig. 6), which suggests that *TMSB4Y* has a normal functional role in normal male breast epithelial cells.

Not much is known about *TMSB4Y* itself, except that based on gene sequence, it codes for the Y homologue of an actin monomer sequesterer called Thymosin Beta 4 [61]. Interestingly, *TMSB4Y* was reported to have an unconventional open reading frame that codes for a novel minor histocompatibility antigen [62]. Thymosin Beta 4 is also reported to enhance natural killer cell cytotoxicity [63]. Interestingly, *TMSB4Y* has a homologue on the X chromosome, *TMSB4X*, which is known as a major monomer actin sequesterer, which is the main focus of RegeneRx Biopharmaceuticals. They have shown that *TMSB4X* promotes seemingly oncogenic cellular effects, which includes increased

cell migration, blood vessel formation, cell survival, stem cell differentiation. Therefore, it is currently on clinical trials to be for dermal and corneal repair.

On the contrary, our functional analysis of *TMSB4Y* is consistent with tumor suppressive effects. When we overexpressed *TMSB4Y* in our Dox-inducible system in MCF-10A background, we observed altered cellular morphology, where cells become enlarged with aberrant morphology (Fig. 9), and also persistent reduced cell proliferation (Fig. 10). Because we saw a persistent reduction in cell proliferation after Dox-induction, we are currently looking at cell cycle analysis of the stable Dox-induced clones.

TMSB4Y is highly homologous to *TMSB4X*, with a difference of only three out of 44 amino acids. However, we show here that these X-Y homologues have fundamental differences, especially because the specific antibody for *TMSB4Y* does not recognize *TMSB4X* (Fig. 12) despite their high extent of homology.

Interestingly, mammalian studies introduced a rising concept of antagonism between X-Y homologues, generally seen as genomic battles between the sex chromosomes [64, 65]. These studies infer that X homologues oppose Y homologues functionally, which is in consonance with our functional observations of *TMSB4Y* and functional reports of *TMSB4X*. To date, there is no report of direct interaction between *TMSB4Y* and monomeric actin. Therefore, we are currently trying to investigate this protein-protein interaction via immunoprecipitation assays and also via proximity ligation assay. We believe that *TMSB4Y* and *TMSB4X* might interact differently with actin. More comparative functional studies are required to explore and confirm this proposed opposing functional difference between *TMSB4Y* and *TMSB4X*.

Our functional analysis of *TMSB4Y* in the Dox-inducible system shows that *TMSB4Y* causes aberrant cellular morphology and reduced cell proliferation. In order to find the mechanism behind these effects, we performed RPPA on these Dox-inducible clones to investigate the global protein expression changes caused by the overexpression of *TMSB4Y*. Here, we have to note that caution has to be taken when analyzing RPPA data, because the specificity of the antibody optimized by the RPPA core facility at MD Anderson might be different towards different kinds of cell lines. Overall, subtle protein level changes were observed after Dox-induction in TmY1, and amongst several candidates that we are currently validating, we observed here that the Syk protein expression was modestly reduced after Dox-induction (Fig. 11). Syk is the spleen tyrosine kinase, which is upregulated in T cell lymphoma, suggesting an oncogenic role in tumorigenesis, and various Syk inhibitors are currently available [66]. Our RPPA data suggests that overexpression of our proposed tumor suppressor candidate *TMSB4Y* led to reduced expression of the oncogenic Syk, further suggesting possible inter-regulatory effects between opposing oncogenic and tumor suppressive genes. These are initial results from our RPPA experiment and further analysis is needed to elucidate mechanistic details of our observations.

Because our functional analysis of *TMSB4Y* suggests that it is a breast specific tumor suppressor, it will be informative to be able to knockout *TMSB4Y* in a normal male breast epithelial cell line, which is basically the male equivalent of the female MCF-10A nontumorigenic breast epithelial cell line. In future, perhaps this male equivalent of MCF-10A can also be created via spontaneously immortalizing normal male breast epithelial cells in long-term tissue cultures. It took about 800 days for MCF-10A to be

spontaneously formed and therefore, it might take about the same, a shorter, or perhaps a longer duration for male breast epithelial cells to become spontaneously immortalized [44]. Furthermore, we are also currently trying to establish stable expression clones of *TMSB4Y* in some female breast cancer cell lines to further validate its tumor suppressive role in breast cancer.

In conclusion, our observation of ~15% *in situ* clonal loss of Y chromosome in MBC supports our hypothesis that the human Y chromosome is tumor suppressive in breast cancer. Furthermore, *TMSB4Y* might be a breast specific tumor suppressor gene on the human Y chromosome, and *TMSB4Y* might confer its tumor suppressive effects via regulation of cell morphology and reduction of cell proliferation.

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URECA (NTU President Research Scholar Program), NTU	2005 - 2007
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