Role of Slit2 in the Activation of Breast Cancer-Associated Fibroblasts

Research Thesis

Presented in partial fulfillment of the requirements for graduation with research distinction in Molecular Genetics in the undergraduate colleges of the The Ohio State University

by

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Table of Contents

Abstract

Cancer-associated fibroblasts (CAF) are stromal cells that have been shown to regulate invasion and metastasis in various cancers including breast cancer. However, the molecular mechanism in breast cancer is not known. This project aims to analyze the role of tumor suppressor *SLIT2* in regulating CAF differentiation in breast cancer. The level of *SLIT2* mRNA was analyzed in human normal epithelial and breast adenocarcinoma cells by RT-PCR. SLIT2 protein expression in normal breast epithelial cells versus fibroblasts was compared by Western Blot technique. Results showed that *SLIT2* mRNA is downregulated in breast adenocarcinoma cells compared to normal breast epithelial cells. Similarly, SLIT2 is overexpressed in normal fibroblasts versus normal epithelial cells. In addition, fibroblast cells were treated with conditioned media obtained from breast adenocarcinoma cells to induce myofibroblasts differentiation. *SLIT2* and *α-SMA* (myofibroblasts marker) mRNA expression in myofibroblasts and fibroblasts was analyzed by RT-PCR. Results showed that the breast adenocarcinoma cell conditioned media enhanced *α-SMA* and downregulated *SLIT2* in myofibroblasts. SLIT2 expression in patient samples was extracted from publicly available datasets (Oncomine and CBioportal). SLIT2 is significantly downregulated among invasive cancers. *SLIT2* alterations were also investigated and compared to survival outcomes. Alteration(s) in *SLIT2* gene and transcription correlates to lower survival compared to breast cancer patients without *SLIT2* alterations. This study suggests use of CAF-secreted factors

as a tool to develop novel strategy for targeting breast cancer cells. Complete understanding of the cell signaling mechanism between breast adenocarcinoma cells and cancer-associated fibroblasts could be utilized to develop therapeutic agents against breast cancer.

Introduction

Despite being one of the most studied cancers, breast cancer remains a major cause of cancer related deaths and more than 232,000 new cases has been estimated to be reported in 2014 (2). Breast tumor is a complex structure. Tumor cells interact with various cells, growth factors, and extracellular matrix present in microenvironment. All these components together make tumor microenvironment (TME). Figure 1 shows how different stromal cells (endothelial cells and fibroblasts) and immune cells (T-cells, neutrophils, dendritic cell, tumor-associated macrophages, and myeloid-derived suppressor) cell release cytokines and growth factors to promote or inhibit tumor progression.

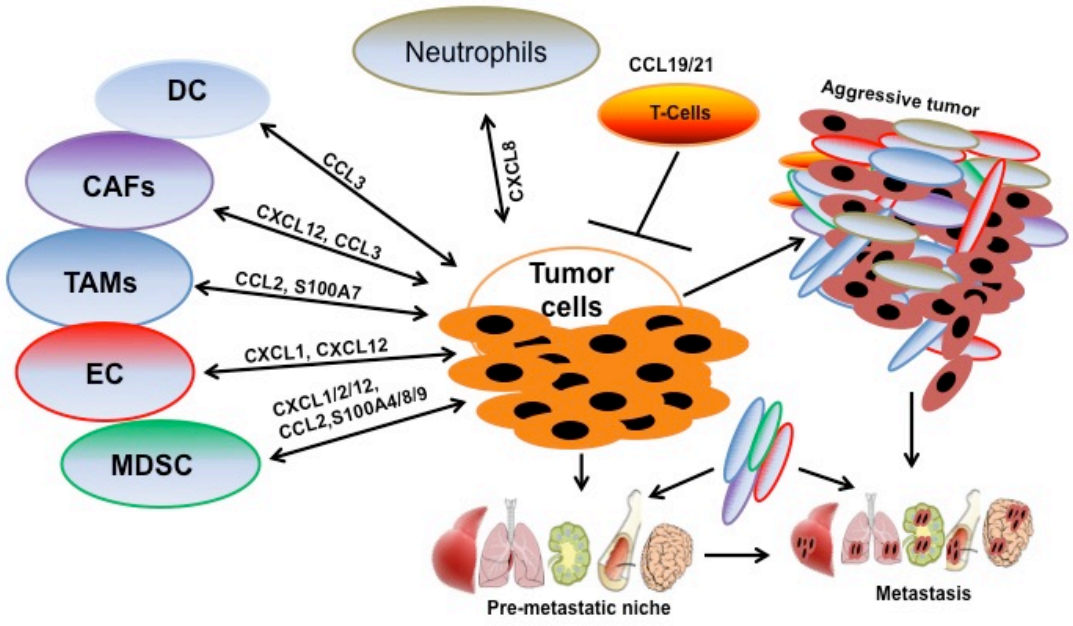


Figure 1. Diagram of cytokines released by T-cells, neutrophils, dendritic cell (DC), cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), endothelial cell (EC), and myeloid-derived suppressor cell (MDSC) that regulate tumor progression and metastasis

Cancer-Associated Fibroblasts (CAFs)

One of the key cell types present in TME is cancer-associated fibroblast (CAF). Cancer-associated fibroblast can be derived from tumor cells and different stromal cells (5). Its key role in different tumours is long recognized. Figure 2 from Cirri and

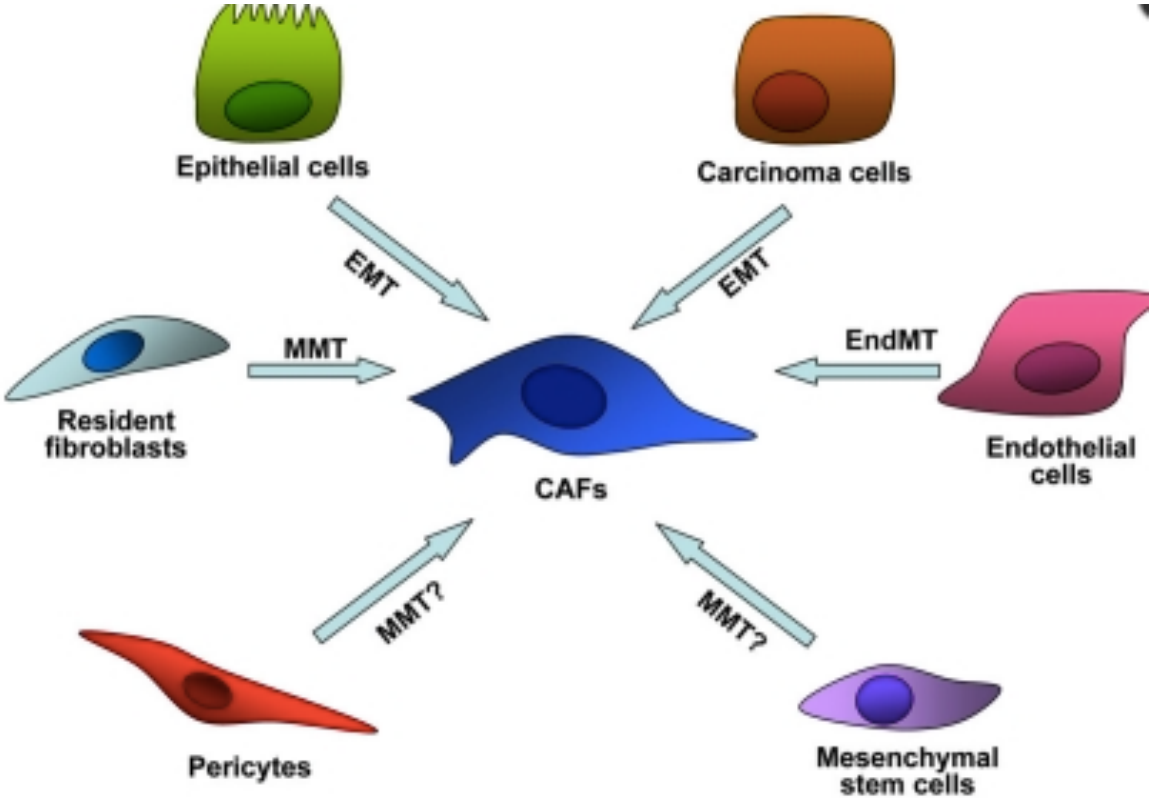


Figure 2. Diagram of different cell types that can differentiate into cancer-associated fibroblasts (CAFs)

Chiarugi's review on cancer-associated fibroblasts illustrates how various types of cells can be activated into cancer-associated fibroblasts through MMT (mesenchymal-mesenchymal transition) and EMT (epithelial-mesenchymal transition. Cirri and Chiarugi also summarized the different factors involved in feedback signaling of CAFs and carcinoma cells (5, *Fig.*3).

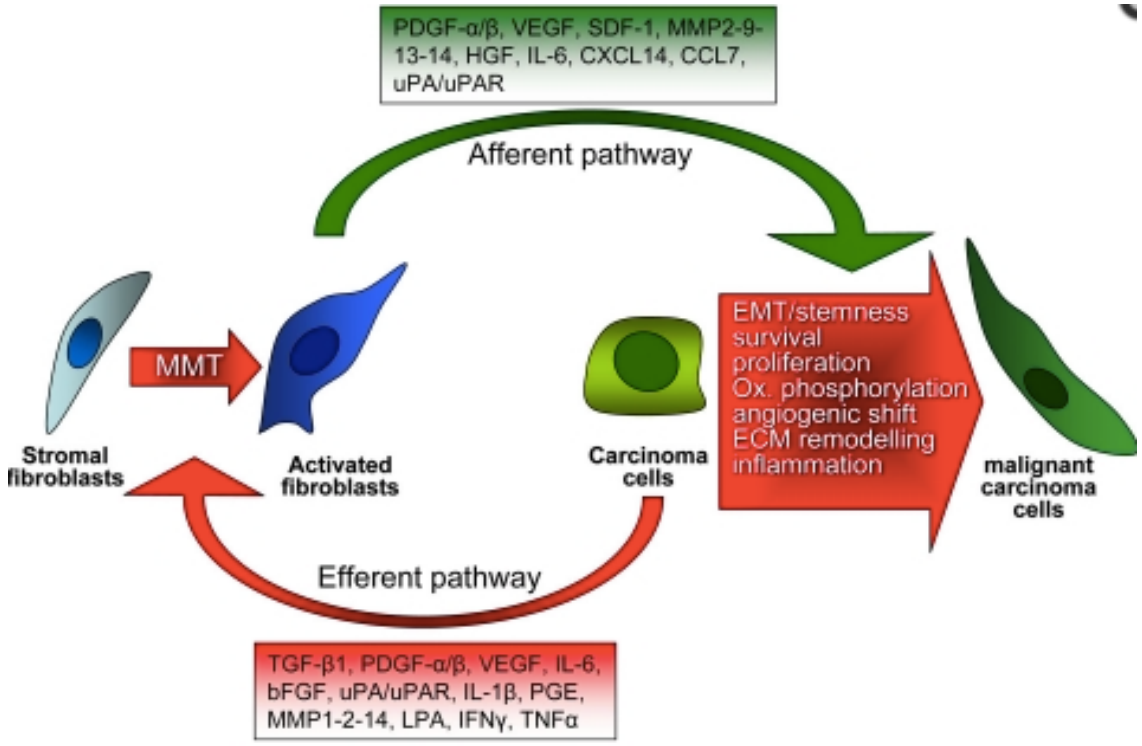


Figure 3. Feedback signaling between fibroblasts and carcinoma cells

More recently, CAFs have been shown to secrete CXCL12, also known as SDF-1 or stromal cell-derived factor 1 to help induce the recruitment of progenitor endothelial cells to tumor cells to initiate angiogenesis **(**16**)**. When bound to its receptor CXCR4, CXCL12 activates a cascade of events resulting to increased tumorigenesis (16)**.** In addition to tumorigenesis, CAFs have also been shown to induce epithelial to mesenchymal transition in breast cancer cells (18).

SLIT2 in Cancer

Contrary to cancer-associated fibroblasts, many studies have demonstrated the inhibitory effect of normal fibroblasts to tumor cells. One of the most well studied factors

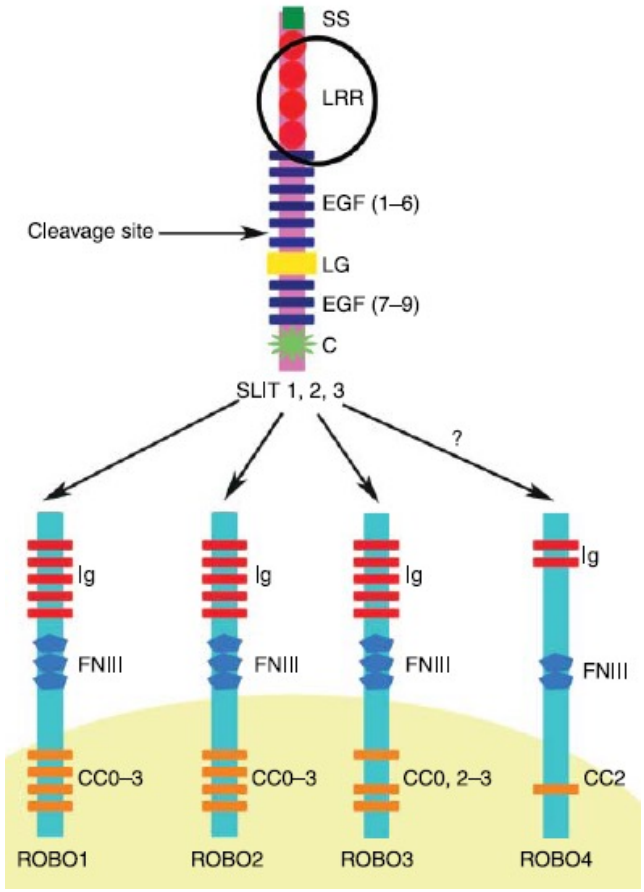


Figure 4. Illustration of SLIT and ROBO

secreted by normal fibroblasts is SLIT2. SLIT2 was initially characterized as an axon guidance cue in *Drosophila* (18). SLIT2 guides cellular migration by interacting with roundabout homolog receptors. SLIT2 binds to the Ig domain of ROBO1 through the second domain of the four leucine-rich tandem repeats of SLIT2 (8, *Fig.4*).

SLIT2, along with SLIT1 seem to be critical in inhibiting axons inappropriately crossing over the midline in the forebrain (20). In addition to repelling axons during neural development, SLIT2 revealed to possess another activity when it inhibited chemotaxis of leukocytes in *Xenopus* (7). Dallol and collegues determined SLIT2 inactivation by promoter hypermethylation and its receptor ROBO1 receptor in lung and breast cancer (7). Conversely, overexpression of SLIT2 inhibited colony growth of several breast cancer cell lines (7). Aside from cell lines and mouse models, analysis of tissue and serum samples from breast cancer patients also indicated significant hypermethylation of SLIT2 promoter (12).

SLIT2 downregulation has also been implicated in other cancers such as head and neck squamous cell carcinoma, esophageal, prostate, and pancreatic cancers (9, 22, 11,

and 24). Nevertheless, it should be noted that SLIT2 can also positively regulate cancer cells by promoting tumor growth in cancers such as intestinal cancers (25). Also, aside from hypermethylation, *SLIT2* can undergo other types of alteration like mutation, deletion, amplification, and combination of these alterations depending on the cancer (3,10). Figure 5 depicts how the *SLIT2* gene is altered in various patient genomic data sets (3,10). *SLIT2* is predominantly mutated in many cancers, which include breast cancer. After mapping the alterations in *SLIT2,* mutations were noticeably dispersed among the exons and introns of the gene (3,10). Only one site, 715th amino acid, accumulated a missense mutation in more than 3 patient data sets (3,10). Figure 6 shows the precise location of mutations acquired by *SLIT2* in breast cancer.

Figure 5. Histogram of frequency of alterations in *SLIT2* among different types of cancer

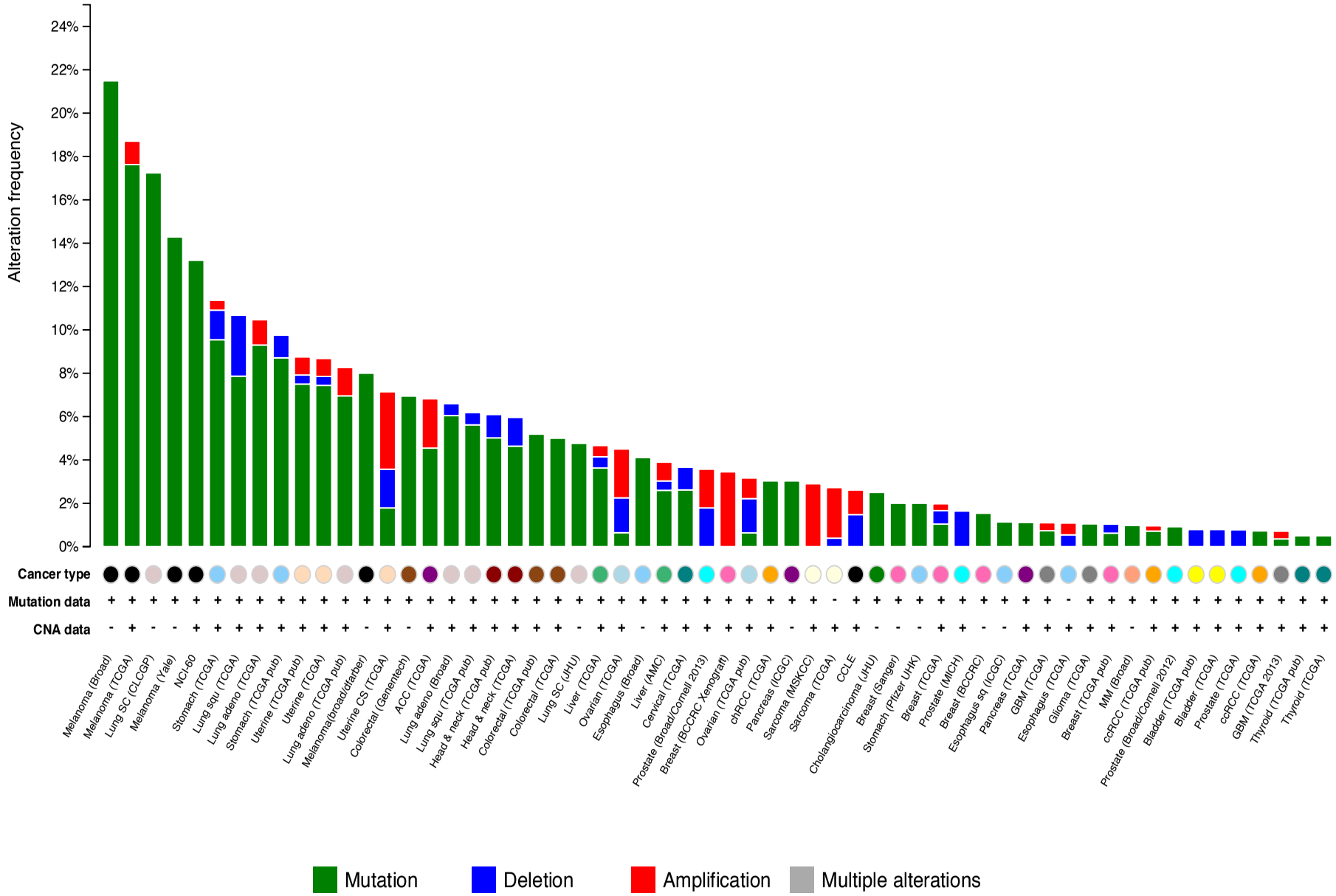
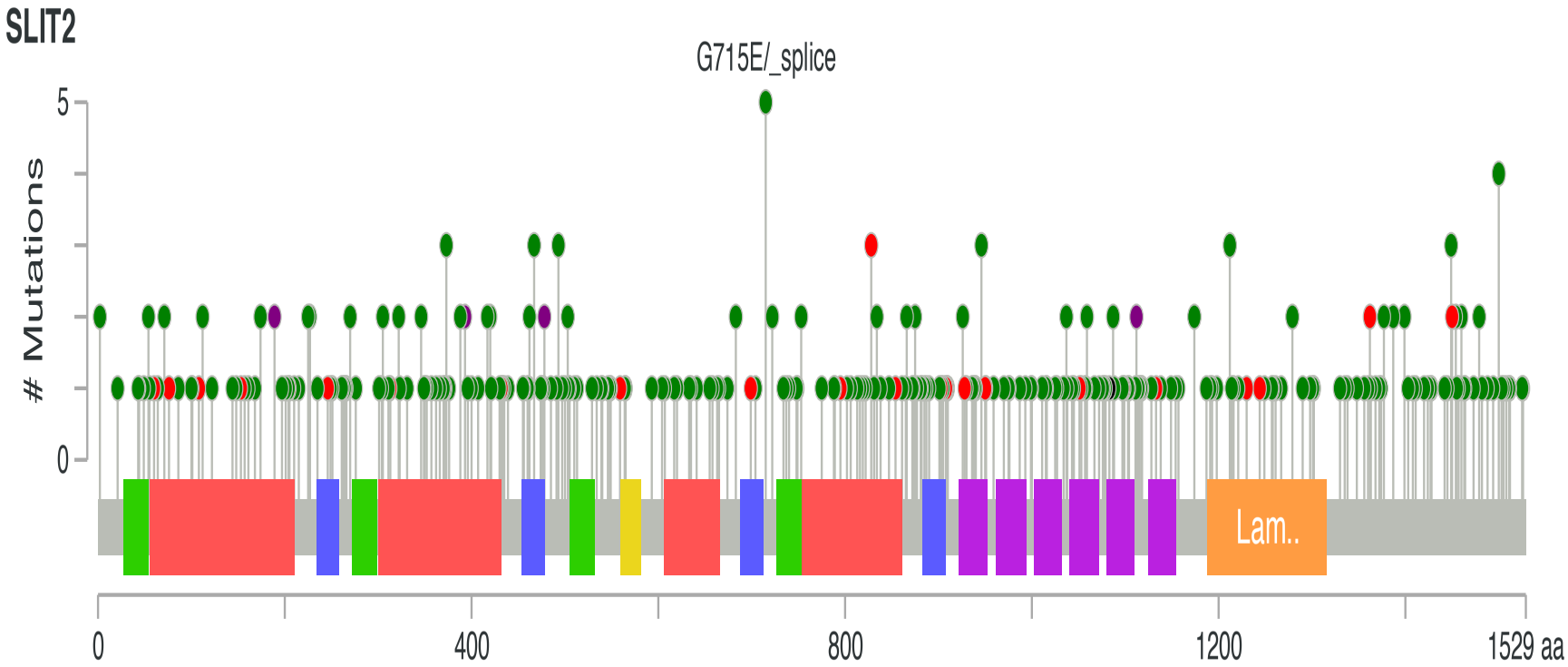


Figure 6. Map of mutations in SLIT*2*, which includes missense mutation in green filled circles and frameshift deletion in red filled circles



SLIT2 Transcription and Signaling

Besides hypermethylation of the promoter, *SLIT2* has been shown to be a direct target of EZH2 in prostate cancer cells (24). EZH2 silences the SLIT2 expression in cancer cells by catalyzing the methylation of lysine 27 of histone H3 (3mH3K27), making it inaccessible for transcription (24). In a separate study, EZH2 was shown to be directly activated by the transcription factor Sox4 in TGF-β-induced EMT in normal and breast cancer cells (23). TGF-β is known to enhance tumor progression and is present abundantly in tumor microenvironment (1,6). TGF-β has also been shown to activate and maintain myofibroblasts in breast cancer (14).

Another mechanism involved in myofibroblasts differentiation is mediated through β-catenin. Studies suggest that fibroblasts are activated into myofibroblasts, which has less Slit2 and higher β-catenin levels (13,14,20). Slit2 downregulates tumor growth by reducing the oncogenic β-catenin protein level in tumor cells by blocking the PI3K/Akt/ β-catenin pathway (4). It is possible that SLIT2 might also suppress β-catenin levels in myofibroblasts and inhibit their activation. Though the regulation of Slit2 has been studied in breast cancer cells, the regulation of SLIT2 in fibroblast is unknown. Based on these observations, perhaps TGF-β from cancer cells suppresses Slit2 via enhancing EZH2 expression. Also, Slit2 and β-catenin levels inversely correlate to each other and extracellular Slit2 may downregulate β-catenin in myofibroblasts and revert them to normal fibroblasts.

In addition to tumorigenesis, SLIT2 was also implicated in migration of colorectal cancer cells (15). SLIT2/ROBO1 interaction causes inactivation of SNAI1, a repressor of E-Cadherin through targeting β-catenin and SNAI1 for degradation (15). SLIT2/ROBO1

signaling also stabilizes β-catenin and E-cadherin interaction, leading to more stabilized cell adhesion (15). The same study also demonstrated how SLIT2/ROBO1 signaling blocks HGF-induced-MET-tumor cell migration by inhibiting Cdc42, which promotes cell motility (15). Figure 4 depicts how SLIT2/ROBO1 signaling is involved in promoting cell adhesion in colorectal cancer cells (15). Perhaps, fibroblasts are induced by cancer cells to secrete less SLIT2 in order to promote cancer invasion and metastasis.

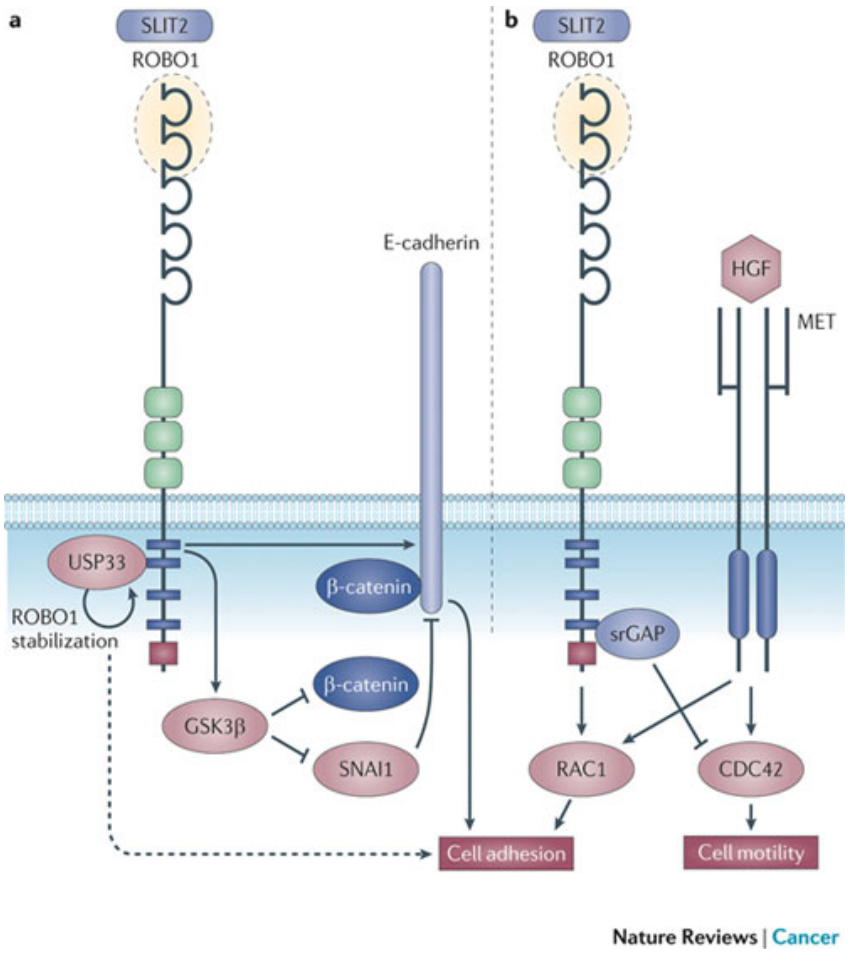


Figure 7. Role of SLIT2/ROBO1 signaling in inhibiting cell motility and promoting cell adhesion

Significance of the Study

Breast cancer is a complex disease. Stromal cells present in tumor microenvironment are getting increased interest as a therapeutic target in personalized medicine (15). Targeting cancer associated fibroblasts to inhibit cancer growth and metastasis has been proposed (19, 15). Complete understanding of the events driving the interactions between tumor cells and their microenvironment is of crucial importance in improving patient outcome. This study will shed light on molecular events involved in transition of normal fibroblasts into myofibroblasts, precursor of cancer-associated fibroblasts by analyzing SLIT2 expression in normal breast epithelial cells, breast adenocarcinoma cells, normal fibroblasts, and myofibroblasts. This study will also show how SLIT2 alterations and downregulation can affect clinical outcomes.

Materials and Methods

Cell Lines

MCF-10A (normal human breast epithelial cells), NIH/3T3 (normal mouse fibroblasts), and NMuMG (normal mouse breast epithelial cells) were purchased from ATCC. Metastatic MVT1 cell line was derived from c-Myc/VEGF tumor explants. DCIS (Ductal Carcinoma In Situ, human) was a generous gift from a former collaborator.

Cell Culture

MCF-10A: Cells were cultured in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum, 5 μg/ml insulin (Sigma), and 5% penicillin (10000U/ml)/streptomycin (10 mg/ml).

DCIS: Cells were cultured in normal breast tissue adherent culture medium: keratinocyte serum-free medium (Gibco) with proper additives such as 10% fetal calf serum, 10 ng/ml human recombinant EGF, 5 μg/ml insulin (Sigma), and 5% penicillin (10000 U/ml)/streptomycin (10 mg/ml).

NIH/3T3: Cells were cultured in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) with 10% bovine calf serum and 5% penicillin (10000 U/ml)/streptomycin (10 mg/ml). Cells were subcultured every two days to avoid the cells reaching 80% confluence.

NMuMG: Cells were cultured in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum, 5 μg/ml insulin (Sigma), and 5% penicillin (10000U/ml)/streptomycin (10 mg/ml).

MVT1: Cells were cultured in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum, and 5% penicillin (10000 U/ml)/streptomycin (10 mg/ml).

All cells were grown at 37°C with 95% air and 5% carbon dioxide.

Breast Cancer Conditioned Media

MVT1 cells were cultured in appropriate medium until confluent. Cells were washed and starved with serum-free media (ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) with 5% penicillin (10000 U/ml)/streptomycin (10 mg/ml)) for 48 hours. The media was collected and separated from the cells through centrifugation. The supernatant was utilized to create a breast cancer conditioned media (70% serum-free media and 30% cell free supernatant). NIH/3T3 cells were treated with the conditioned media (CM), and allowed to grow for 48 hours.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction

Total mRNA was isolated by following the protocol of Qiagen RNeasy Mini Kit. Expression level of *SLIT2* and *ROBO1* mRNA in NMuMG and NIH/3T3 were assayed using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) with *18S* gene as internal control. RT-PCR was also used to measure *SLIT2* mRNA in MCF-10A, DCIS, and CM-treated NIH/3T3 cells. Reverse transcription was carried out using the Applied Biosystems Transcription System. Universal Sybr Green was then utilized for quantitative PCR (5 µl of Sybr Green, 0.5 µl of 10 µM stock forward primer, 0.5 µl of 10 µM stock reverse primer, 3 µl of PCR grade water, and 1 µl of cDNA). Relative

quantification was performed by comparative Ct method. Data was acquired with ABI 7900 HT sequence detection systems and StepOnePlus Real-Time PCR system. The mRNA level was calculated by using $2^{-\Delta\Delta Ct}$ with $\Delta C_t = C_{t,SLIT2} - C_{t,18S.}$ The same formula was used for calculating *ROBO1* mRNA.

Protein Isolation and Western Blot Analysis

Culture media were aspirated and cells were washed with ice-cold PBS twice. The cells were lysed with 200 µl of protein extraction buffer (RIPA buffer). The lysed samples were then incubated at 4°C for one hour before centrifugation. The supernatant was collected for Western Blot analysis (50 µg of protein loaded). Rabbit polyclonal to Slit2 was utilized as primary antibody. Detection of GAPDH was employed as loading control.

Patient Outcome

Oncomine was utilized to analyze if SLIT2 is differentially expressed among different types of breast cancers. The level of expression was also analyzed between breast cancer samples and normal breast samples. Alterations in *SLIT2* were also analyzed using CBioportal to determine if correlation exists between the gene alterations and survival outcome.

Results and Discussions

SLIT2 Downregulation in Human Breast Cancer

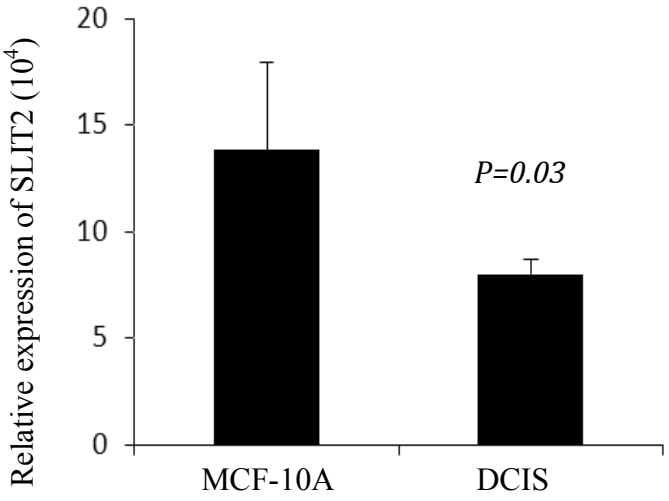


Figure 8. Analysis of Slit2 expression in human normal epithelial cell line MCF10A and transformed derivative breast cancer cell line DCIS

To confirm if SLIT2 is downregulated or upregulated in human breast cancer, *SLIT2* mRNA expression levels were quantified in MCF-10A (normal human breast epithelial cells) and DCIS (ductal carcinoma *in situ*). There is an anomaly in SLIT2 expression levels among different types of cancer. A current study suggests that SLIT2 is upregulated in intestinal cancers while a significant amount of literature suggest otherwise (7,17,22, and 25)). Figure 8 shows that *SLIT2* mRNA is downregulated in DCIS compared to MCF-10A with p=0.03. Based on this data, SLIT2 expression could be hypothesized to be downregulated in breast cancer.

ROBO1 and SLIT2 Expression Patterns in Fibroblast and Breast Epithelial Cells

There is uncertainty exists about the source of Slit2 in breast cancer. Few reports suggest that epithelial cells secrete Slit2, while others state that fibroblasts are major source of Slit2 (14). In order to determine if normal breast epithelial cells or fibroblasts

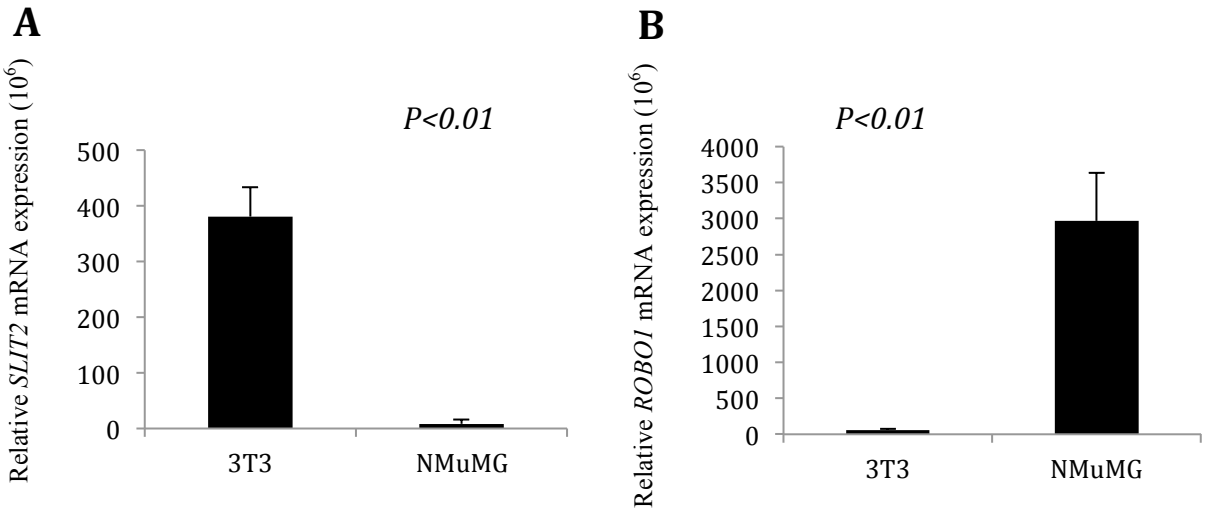


Figure 9. SLIT2 and ROBO1 analysis in NIH-3T3 and NMuMG. (A) *SLIT2* and (B) *ROBO1* mRNA levels in NIH/3T3 and NMuMG cells by RT-qPCR

secrete more SLIT2 or express the ROBO1 receptor, *SLIT2* and *ROBO1* mRNA expression levels were analyzed in NMuMG and NIH/3T3 cell lines. In addition to mRNA levels, SLIT2 protein levels were also investigated using Western Blot technique. Our results show that normal fibroblasts (NIH/3T3) primarily secrete SLIT2 while epithelial cells predominantly express ROBO1.

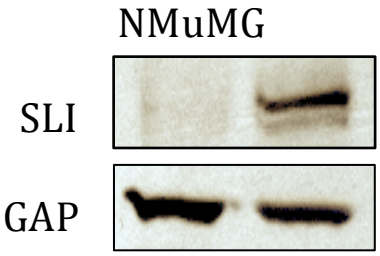


Figure 10. Analysis of SLIT2 expression pattern in NMuMG and NIH/3T3

SLIT2 mRNA is significantly expressed in NIH/3T3 cells, which are normal fibroblasts cells cells ($p<0.01$; Fig. 9A) compared to

NMuMG normal epithelial cells. On expressed in NMuMG cells compared to normal fibroblasts ($p<0.01$; Fig. 9B). In addition to mRNA, SLIT2 protein is also highly expressed in NIH/3T3 cells and not in NMuMG cells (Fig.10).

SLIT2 Downregulation in Cancer-Associated Fibroblasts

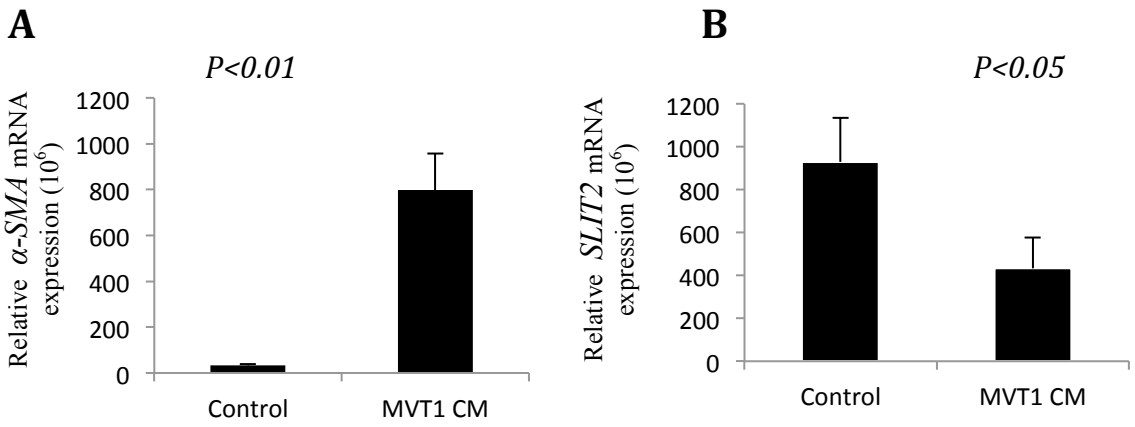


Figure 11. SLIT2 and α-SMA analysis in NIH-3T3. (A) NIH-3T3 cells were treated with 30% MVT1 CM for 48 hrs and analyzed for (A) α-SMA and (B) SLIT2 expression by RT-qPCR

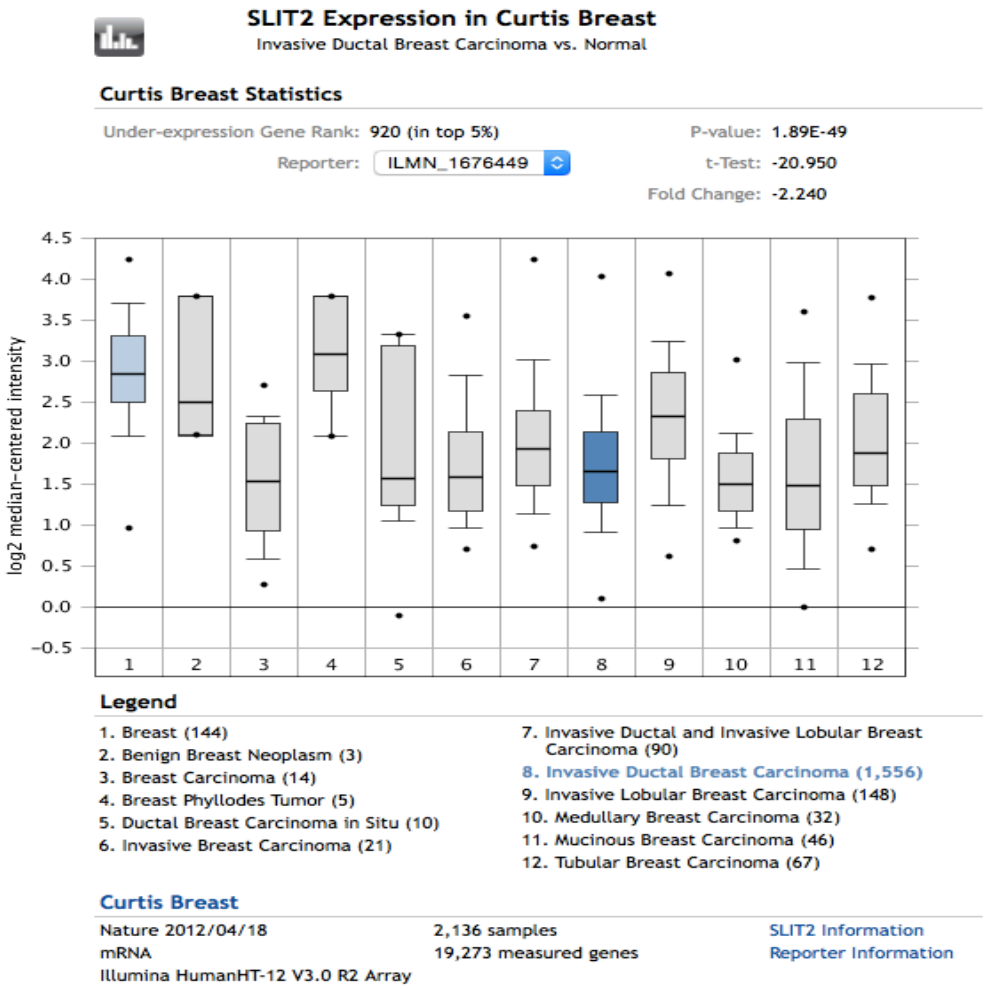
Normal fibroblasts (NIH/3T3) were activated to differentiate into myofibroblasts to mimic cancer-associated fibroblasts by treating the 3T3 cells with MVT1 CM. A myofibroblast marker, α-SMA (alpha smooth muscle actin) was analyzed in terms of mRNA level in order to determine if the normal fibroblasts were successfully activated. The treated cells expressed significantly increase α-SMA ($p<0.01$; Fig. 11A), which indicates that the cells were successfully transformed. *SLIT2* mRNA levels were then compared among normal fibroblasts (control) and treated cells. *SLIT2* mRNA in treated NIH/3T3 cells were significantly reduced compared to the ones untreated

(p<0.05; Fig. 11B). These results suggest that *SLIT2* expression is downregulated in cancer-regulated fibroblasts.

SLIT2 Expression in Patient Samples

Based on analysis of Curtis breast data sets from patients (n=2136 samples) using Oncomine, SLIT2 was found to be in the top 5% of the genes that are under-expressed in various types of breast cancer especially in the more invasive types of cancer (p=1.89E-49; Fig 12A). More specifically, SLIT2 expression was shown to be significantly reduced in invasive breast adenocarcinoma, mucinous breast carcinoma, medullary breast adenocarcinoma, and invasive ductal breast carcinoma (Figs. 12B-E).

A



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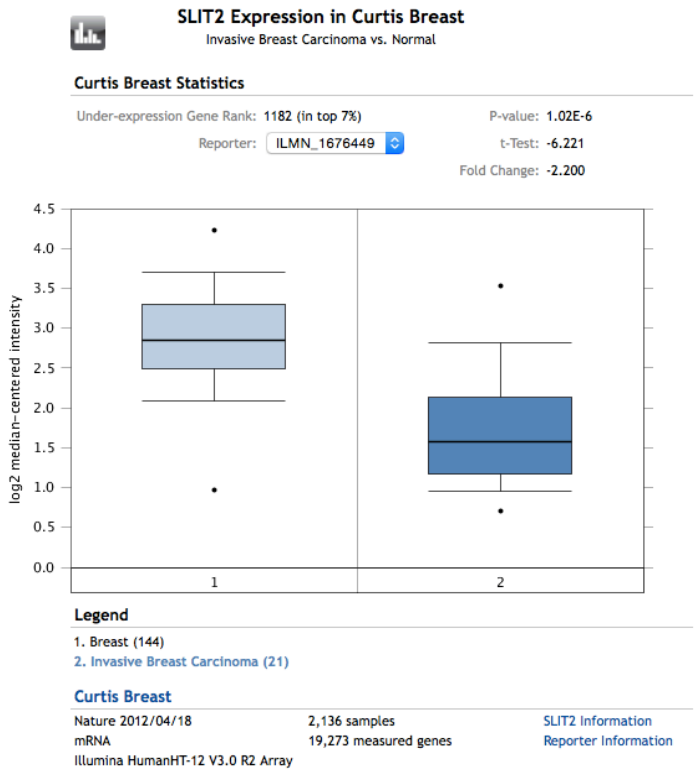

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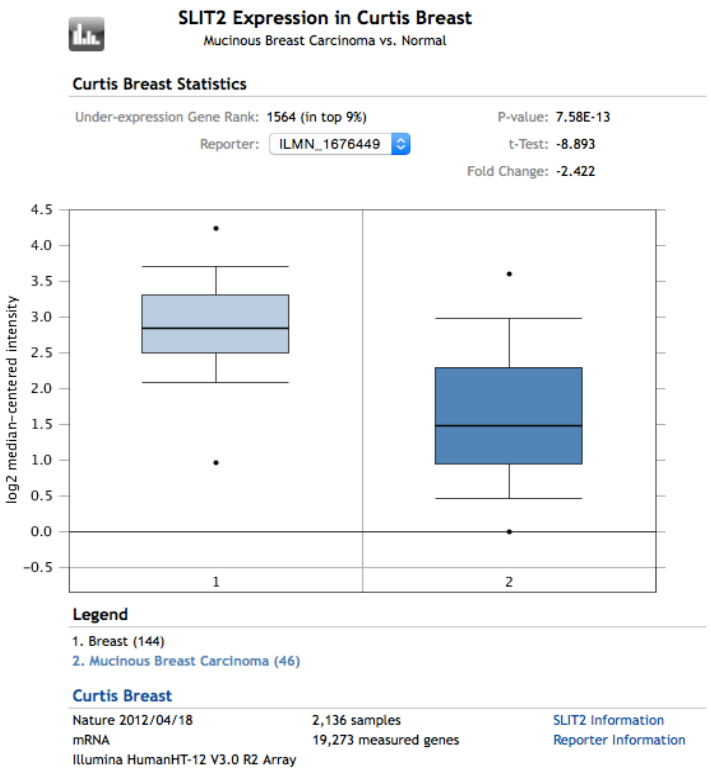

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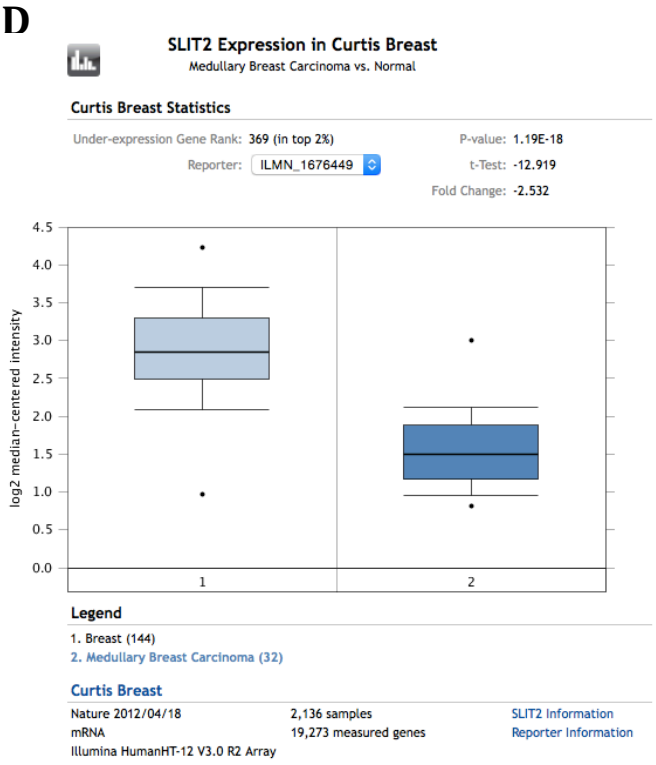

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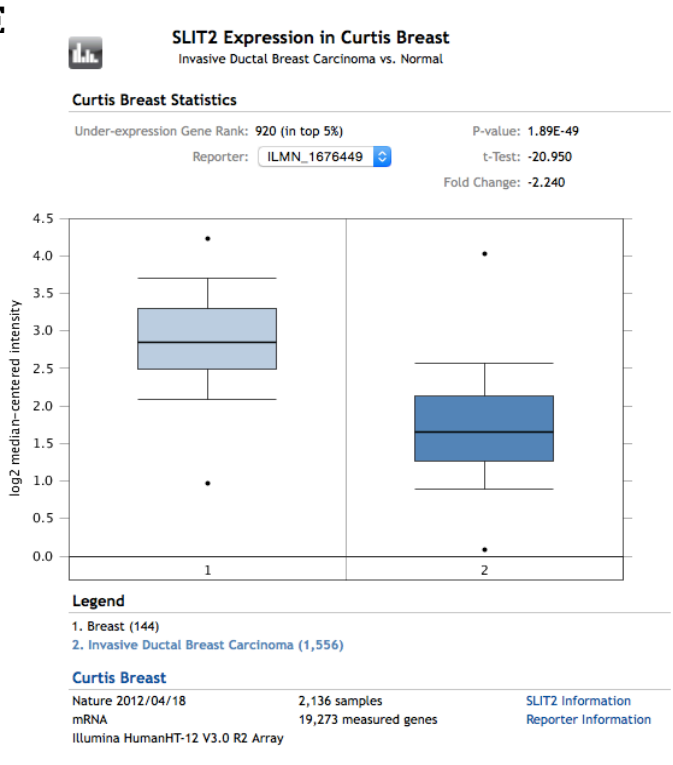

Figure 12. SLIT2 Expression in Curtis Breast Data Sets. (A) SLIT2 expression in different breast tissue samples and specifically in invasive breast adenocarcinoma (B) mucinous breast carcinoma, (C) medullary breast adenocarcinoma (D) and invasive ductal breast carcinoma (E)

SLIT2 Alterations and Survival Outcomes

Based on analysis of TCGA Provisional breast invasive carcinoma samples (n=959 samples) using CBioportal, only 5% of the total samples (n=38 samples) with mRNA, CNA, and sequencing data were found to be altered, with a majority comprised of mRNA downregulation (Fig. 13). Moreover, patients with alterations were found to have lower overall survival rates compared to patients without SLIT2 alteration(s) (Fig. 14).

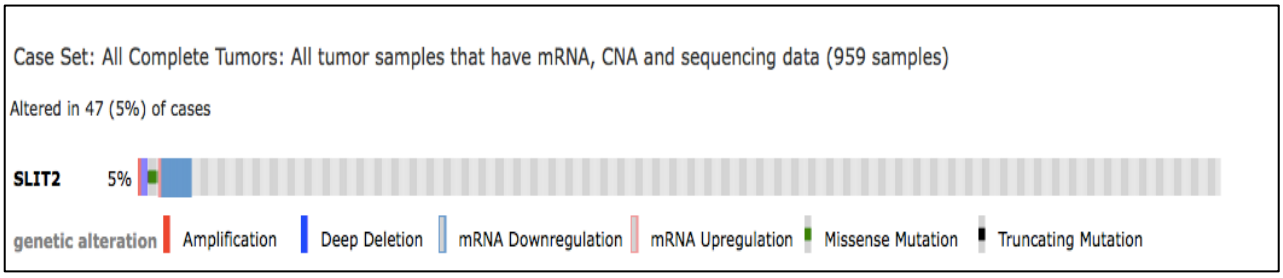

Figure 13. Diagram of the genetic alterations in *SLIT2* in breast invasive carcinoma.

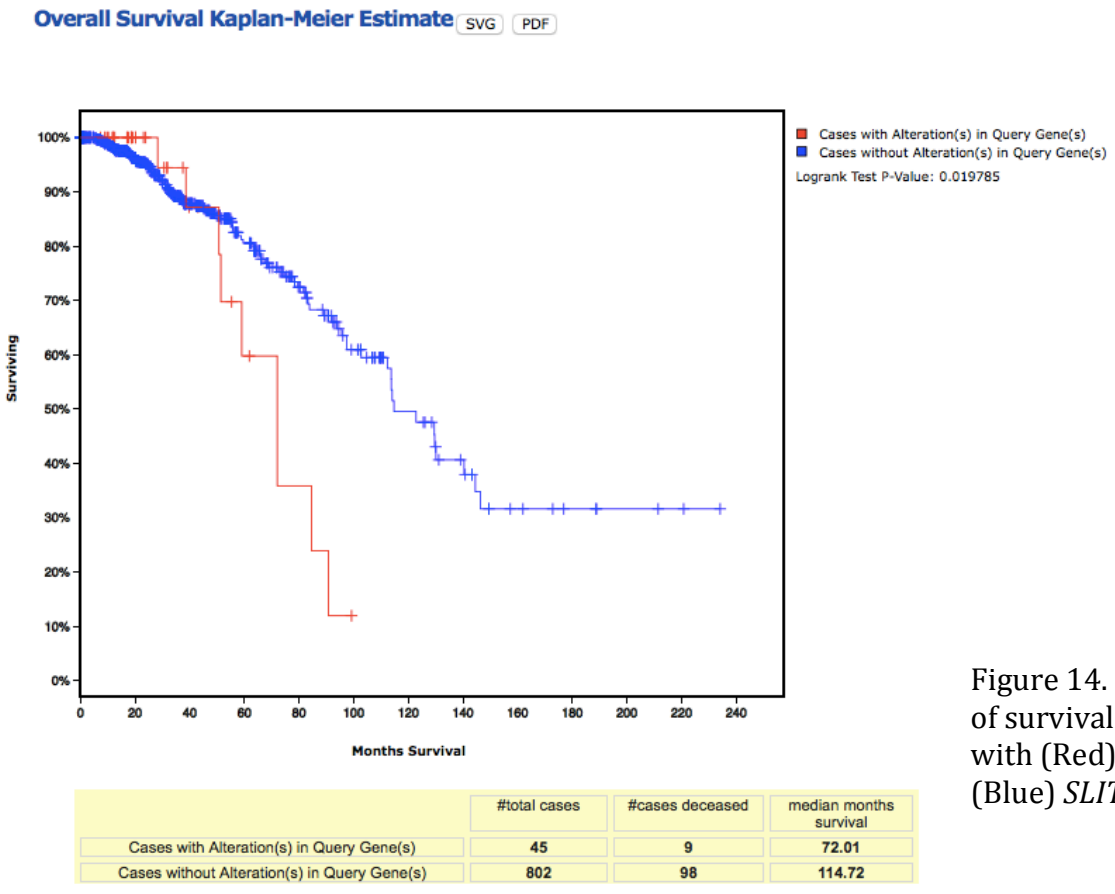

Figure 14. Kaplan-Meier plot of survival rates of patients with (Red) and without (Blue) *SLIT2* alterations.

Conclusion

Breast cells express ROBO1 receptors for SLIT2 that is secreted by fibroblasts. SLIT2 is downregulated in fibroblasts that were induced to differentiate into myofibroblasts. Because myofibroblasts were utilized to mimic CAFs (Cancer-Associated Fibroblasts), this study shed light on the potential effect of CAF differentiation to SLIT2 levels. In addition, SLIT2 is clinically relevant because it was significantly downregulated in breast cancer samples especially in more invasive types (Oncomine analysis). This claim is further supported by the decrease of survival rates among patients with altered downregulated SLIT2 (CBioportal).

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