Abstract

A subset of human triple-negative breast cancer cell lines exhibit aberrant DNA hypermethylation that is associated with DNA methyltransferase hyperactivity, overexpression of DNMT3b, and methylation-dependent silencing of numerous genes. These breast cancer cell lines are resistant to standard chemotherapeutic agents, but can be sensitized to chemotherapy with demethylating drugs, such as 5-aza-2'deoxycytidine. In this study, we surveyed expression of 328 genes associated with apoptosis and survival in an index cell line, Hs578T, using BioRad Prime PCR Pathway Plates. This analysis identified several pro-apoptotic genes that are not expressed (undetected) or expressed at negligible levels in Hs578T cells, including FASLG, IGF1, CD27, and BLK. Epigenetic silencing of these genes (directly or indirectly) would convey a survival advantage to the cancer cells through elimination of pro-apoptotic signaling. These pro-apoptotic genes and/or members of their pathways have been shown to be subject to methylation-dependent silencing in cancer cells. In addition, several anti-apoptotic genes were abundantly expressed in Hs578T cells, including HSPB1 and NPM1. Epigenetic mechanisms have not yet been shown to contribute to overexpression of anti-apoptotic genes in breast cancer. The contributions of aberrant DNA hypermethylation to the silencing of pro-apoptotic genes (and/or activation of anti-apoptotic genes) as a mechanism of chemotherapeutic resistance was explored further using a panel of breast cancer cell lines. The results suggest that treatment of triple-negative breast cancer cell lines that display aberrant DNA hypermethylation with demethylating agents (like 5aza) sensitizes them to standard chemotherapeutic drugs (Doxorubicin, Paclitaxel, 5-fluorouracil) by reactivation of pro-apoptotic genes. This experimental observation forms the basis for designing new treatment approaches for patients with triple-negative breast cancer that combine epigenetic and cytotoxic drugs.

Introduction:

Breast cancer is a common malignancy in females in the United States and worldwide, and is associated with a high cancer-related mortality. Breast cancer is a heterogeneous disease that is diverse in natural history, histopathology, gene expression patterns, response to treatment, and patient outcomes. The spectrum of breast cancer includes several distinct biological and morphological subtypes. Clinical classification (based upon immunohistochemistry) and transcription profiling of invasive breast cancers has identified several subtypes with distinct clinical characteristics that are indicative of patient outcome and survival, including luminal A (ER+/PR+/HER2-), luminal B (ER+/PR+/HER2+), HER2+ (ER-/PR-/HER2+), and basal-like (ER-/PR-/HER2-) breast cancers (Sandhu). With the implementation of anti-estrogen therapies in combination with chemotherapy, the outcomes of ER+ breast cancer patients significantly improved. Similarly, improved survival rates in HER2-overexpressing breast cancers reflect the use of various anti-HER2 compounds with chemotherapy in these patients. However, by virtue of lacking a targeted therapeutic approach, basal-like breast cancers represent a significant clinical challenge, requiring additional treatment options and novel treatment strategies to achieve improved results for patients.

We have observed that breast cancer cell lines (in particular triple-negative breast cancer cells) frequently exhibit aberrant DNA hypermethylation associated with hyperactivity of the DNA methylation machinery due to overexpression of DNA methyltransferase 3b (DNMT3b) (Sandhu). We also observed an increase in the effectiveness of chemotherapeutic drugs after inhibition of DNMT3b (either targeted or using epigenetic drugs). These observations suggest that DNMT3b is an excellent target for development of rational therapeutic approaches breast cancers that exhibit aberrant DNA hypermethylation. Whereas we have shown that targeting

DNMT3b in breast cancer cells sensitizes them to cell killing by cytotoxic drugs, the mechanism responsible for improved cell killing after treatment with epigenetic drugs has not been explored. In the current work, we performed a discovery-based study to identify genes associated with apoptosis that might be epigenetically regulated and silenced in breast cancer cells that exhibit aberrant DNA hypermethylation, and investigated the response of the identified genes to epigenetic treatment with 5-aza-2'-deocycytidine.

Methods

Breast Cancer Cell Lines and Cell Culture.

Human breast cancer cell lines BT20 (ATCC# HTB19), MCF7 (HTB22), MDA-MB-453 (HTB131), MDA-MB-231 (HTB26), SKBR3 (HTB30), Hs578T (HTB126) were obtained from the Tissue Culture Core Facility of the UNC Lineberger Comprehensive Cancer Center. Human breast cancer cell line SUM159 was obtained as a kind gift from the laboratory of Dr. Carolyn I. Sartor (Department of Radiation Oncology, UNC School of Medicine). All cell lines were propagated in DMEM/F12 mix medium (GIBCO/Invitrogen Life Technologies) containing 10% fetal calf serum (Hyclone) and 1% Antibiotic-Antimycotic (GIBCO/Invitrogen Life Technologies). For some experiments, cell lines were cultured in DMEM/F12 mix medium, 10% fetal calf serum, 1% Antibiotic-Antimycotic, and 500 nM 5-aza-2'-deoxycytidine (Sigma-Aldrich) for 7 days (Sandhu et al., 2012, Breast Cancer Research and Treatment 131:385-399).

RNA Isolation.

Confluent cell cultures were harvested for RNA preparation according to the method of Chomczynski and Sacchi (1987, Anal. Biochem 162:156-159), utilizing TRIzol Reagent (Invitrogen Life Technologies), according to the manufacturer's protocol. RNA preparations were cleaned using the RNeasy Mini Kit (Qiagen). The respective 260/280 values of each RNA

isolate were measured to ensure that there was no contamination from DNA or preparatory chemicals such as isopropanol using a NanoDrop1000.

Discovery of Candidate Genes Related to Apoptosis and Survival in Human Hs578T Breast Cancer Cells.

To identify candidate epigenetically-regulated genes in breast cancer, RNA isolates from Hs578T were utilized for gene discovery using Bio-Rad PrimePCR Pathway SYBR Green real-time PCR assays – Anti-Apoptotic TNFs/NF-kB/IAP Pathway (#100-25087), Apoptosis and Survival Tier 1 (#100-25097), Apoptosis and Survival Tier 2 (#100-25869), and Apoptosis and Survival Tier 3 (#100-25870). These assays provided information on 328 unique genes that contribute to several apoptosis-related pathways. The resulting gene expression levels were analyzed to identify genes expressed at low levels or not expressed in Hs578T cells, and to identify genes expressed at high levels in these cells.

Gene Expression Changes in Response to 5-aza Treatment.

To examine changes in gene expression following epigenetic treatment, multiple breast cancer cell lines (BT20, MCF7, MDA-MB-453, MDA-MB-231, SKBR3, Hs578T, and SUM159) were cultured in control medium or medium containing 500 nM 5-aza-2'deoxycytidine for 7 days. RNA isolates from control and 5-aza treated cells were utilized for Bio-Rad PrimePCR SYBR Green Assays for selected genes (based upon discovery results in Hs578T cells). Genes examined include FASLG (qHsaCED0003635), IGF1 (qHsaCED0038638), CD27 (qHsaCID0017180), BLK (qHsaCID0014815), HSPB1 (qHsaCED0023813), and NPM1 (qHsaCED0038211). Additional Tumor Necrosis Factor (TNF) and TNF Receptor superfamilies were examined, including TNFSF8 (qHsaCID0013499), TNFSF10 (qHsaCED0036477), TNFSF11 (qHsaCID0015585), TNFSF14 (qHsaCED0036496), TNFSF15 (qHsaCED0004479),

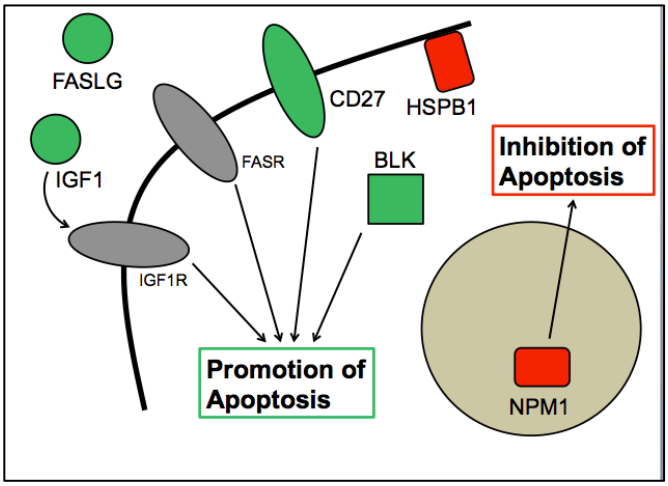
TNFRSF13B (qHsaCED0023685), TNFRSF17 (qHsaCED0020893), and TNFRSF18 (qHsaCID0011446). All real-time PCR assays were controlled using ACTB (qHsaCED0036269).

Results

The expression of 328 genes related to apoptosis was examined in Hs578T cells to identify candidate epigenetically-regulated genes based upon expression pattern. **Table 1** [*Appendix*] summarizes 36 genes that were found to be of interest - 27 genes that were not detected, 4 genes that were expressed at low levels, and 5 genes expressed at high levels. The majority of genes that were not expressed or expressed at low levels are inducers of apoptosis, whereas some of the genes expressed at high levels are inhibitors of apoptosis.

A subset of fourteen genes was selected for further examination in breast cancer cell lines that were treated with 500 nM 5-aza for 7 days. Several of these genes are known to be methylation-sensitive.

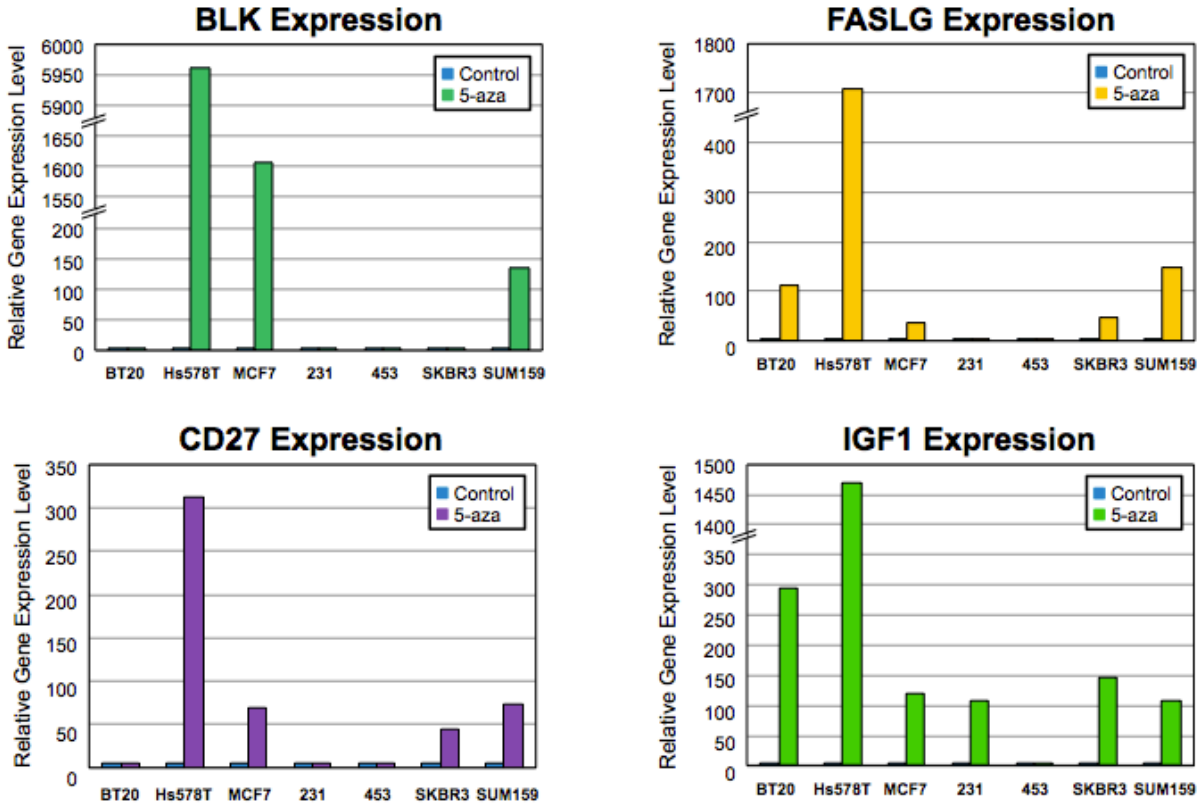
Figure 1: Genes Analyzed Involved in Apoptotic Pathways



Six genes were examined in the current study, as seen in **Figure 1**. Four of these genes are directly involved in pro-apoptotic pathways, including cell surface receptor CD27

(TNFRSF7), ligands for FASR (FASLG) and IGF1R (IGF1), and cytoplasmic tyrosine kinase (BLK). The protein products for these genes are shown in green in the schematic. Two genes that inhibit apoptosis were examined, including a chaperone of the small heat shock protein HSPB1 (HSP27) and a nucleolar phosphoprotein NPM1. The protein products for these genes are shown in red in the schematic.

Figure 2: Expression of Genes Regulating Pro-Apoptotic Pathways in Control and 5-aza Treated Cell Lines

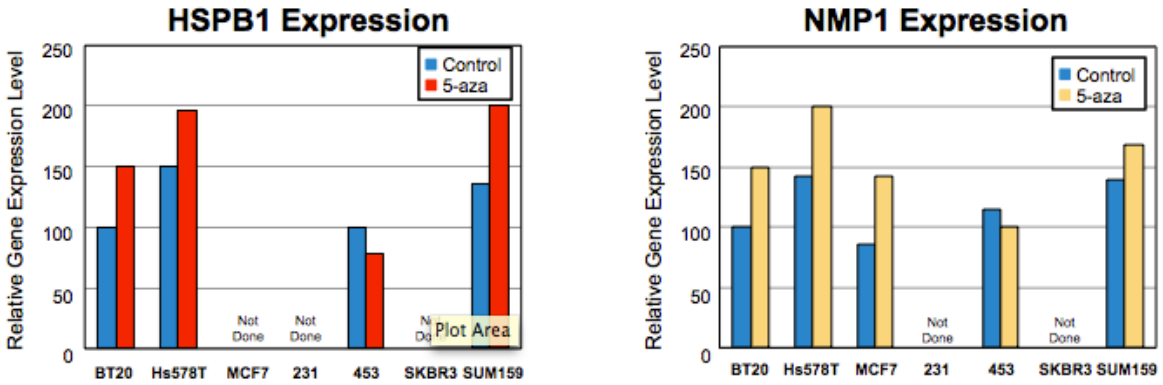


Among the genes examined that regulate pro-apoptotic pathways, lack of expression was a consistent feature among breast cancer cell lines propagated in control medium as seen in **Figure 2**. However, significant induction of gene expression was observed for each of these genes in the index Hs578T cell line, and substantial induction was seen in specific cell lines for

specific genes. BLK is known to be methylation-sensitive, consistent with these responses to 5-aza treatment. These results suggest that FASLG, CD27, and IGF1 may also be subject to methylation-dependent silencing in breast cancer. Further studies are needed to examine promoter methylation events in these genes.

Among the genes examined that regulate anti-apoptotic pathways (and/or inhibit apoptosis), both were found to be expressed abundantly in all breast cancer cell lines investigated as seen in **Figure 3.** In some cases, 5-aza treated cells expressed more of these genes, but the induction of expression in response to 5-aza was substantially less in magnitude (related to the high baseline levels of expression observed).

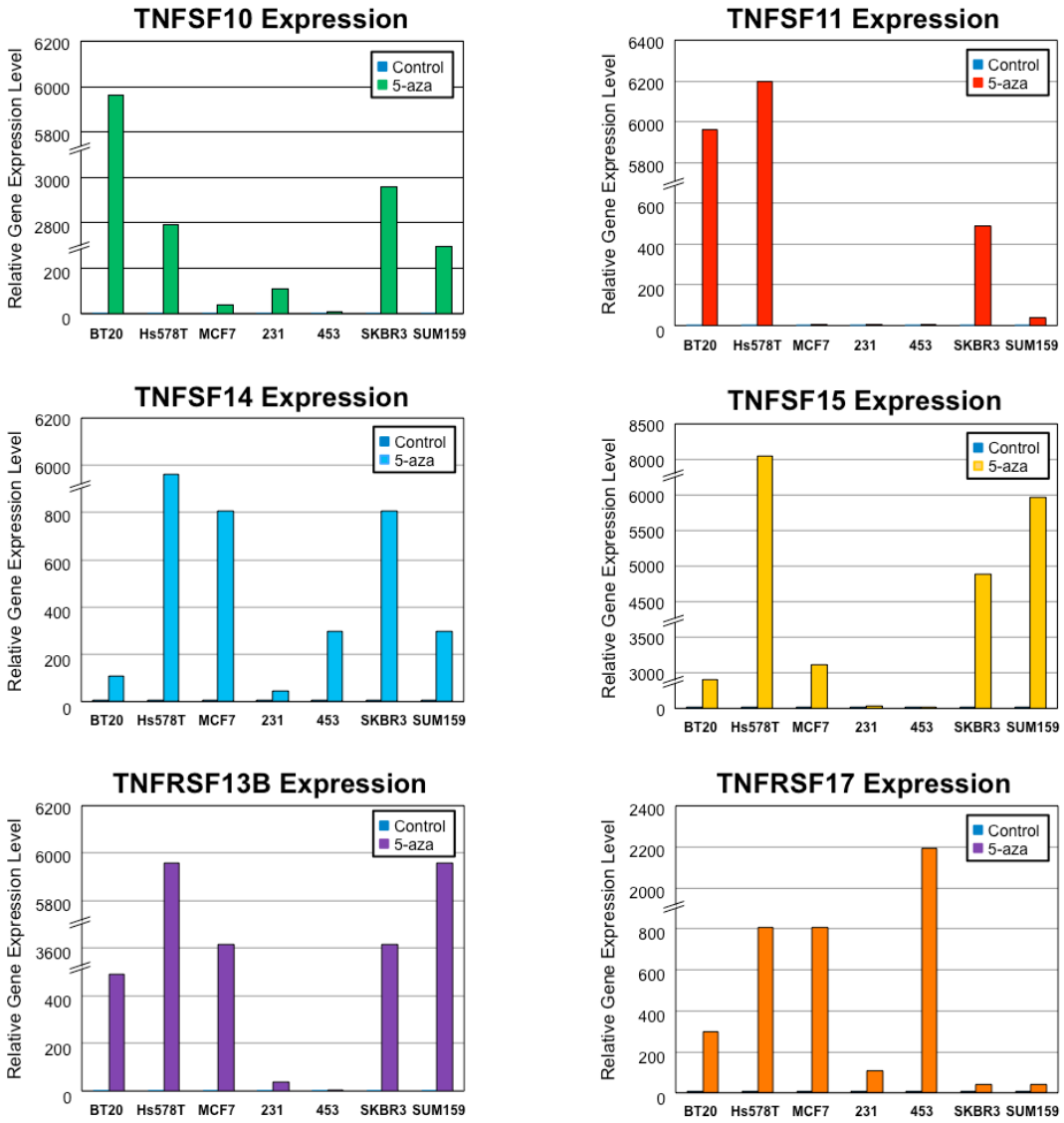
Figure 3: Expression of Genes Regulating Anti-Apoptotic Pathways in Control and 5-aza Treated Cell Lines



A specific subset of genes coding for tumor necrosis factor molecules and their receptors was examined as these gene regulate pro-apoptotic pathways. **Figure 3** illustrates that in conjunction with BLK, FASLG, CD27, and IGF1, TNF and TNFR genes showed lack of expression in the control medium. However, expression was induced upon treatment with 5-aza

in the index Hs578T cell line, and substantial induction was also seen in specific cell lines for specific genes. TNFSF10, TNFRSF1B, and TNFRSF8 are known to be methylation-sensitive, consistent with these responses to 5-aza treatment. These results suggest that TNFSF11, TNFSF14, TNFSF15, TNFRSF13B, and TNFRSF17 may also be subject to methylation-dependent silencing in breast cancer.

Figure 4: Expression of Tumor Necrosis Factor and Receptor Genes Regulating Pro-Apoptotic Pathways in Control and 5-aza Treated Cell Lines



Discussion

Epigenetics is a course of study that is currently under wide investigation as many of these mechanisms that regulate gene expression are still unknown. Here we explored the effect of a demethylating agent on human breast cancer cells lines and the epigenetic mechanism for chemotherapeutic resistance. It has been shown that using 5-aza to inhibit DNMT3b, which encodes a DNA methyltransferase, can increase the efficacy of cytotoxic treatment (Mohandas). While we know that 5-aza can be effective, the mechanism of this drug is still unknown.

By combining epigenetic and cytotoxic treatments, we hope patients with cancer who are resistant to standard treatment may be reached. Epigenetic silencing of pro-apoptotic genes, as seen in the breast cancer cell line Hs578T, would give a survival advantage to cancer cells attempting to metastasize, as these cells would grow infinitely. If we are able to reactivate these apoptotic pathways with a demethylating agent such as 5-aza, then we could sensitize these cells to chemotherapeutic treatments to allow for programmed cell death. These treatments could also be made available as one sensitive cancerous tumor develops a resistance to treatment.

Our results could be improved by using a general control cell line. Our experiment used HS578T as a control but we need an intentional control cell line to compare the treated and non-treated gene expression. Possible error in this investigation could come from DNA or other chemical contamination in the RNA isolate samples. Additionally, we made the assumption that all the down-regulated genes we found are methylation-sensitive or significant in someway while they could just be unexpressed in all breast cells.

Further research may follow by examining additional breast cancer cell lines and a greater variety of genes within these and other specific pathways. We could also attempt promoter methylation analysis to see the direct methylation status of the gene promoter of

interest. This method would assist in focusing on the epigenetic mechanisms of cancer development. Finally, to quantize our results we could use apoptosis detection kits consisting of an APO-tag that labels the cells of nuclei going through apoptosis. With control and 5-aza cell lines, apoptosis would be induced and the nuclei counted.

Possible goals for the future may include using varying dosages of 5-aza to see if any additional genes are affected by a more concentrated treatment. We could also explore other 'triple-negative' breast cancer cell lines along with some of the less aggressive forms of breast cancer. Another possibility would be to perform Western blots to study post-translational modifications of proteins in the breast cancer cells.

Further investigation of the genes assessed could result in a biomarker for breast cancer patients (or any cancer) that can predict their sensitivity to certain drugs. From this biomarker, physicians would be able to determine if a de-methylating agent would be a correct path for treatment, or if an alternate route would be more efficient. As people begin to develop more resistance to chemotherapies, epigenetic modifications may be the direction that medicine will take in order to sensitize these cancer cells. Personalized medicine becomes more efficient as we discover the mechanisms of the pathways involved in various diseases.

References

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Appendix

Discovery of Candidate Epigenetically-regulated Genes Related to Apoptosis and Survival in Human Hs578T Breast Cancer Cells

Genes Not Expressed

ADCY1	Hs.192215	Adenylate cyclase 1
BCL2A1	Hs.227817	BCL2-related protein A1
BLK	Hs.146591	B lymphoid tyrosine kinase
CCL4	Hs.75703	Chemokine (C-C motif) ligand 4
CCL19	Hs.50002	Chemokine (C-C motif) ligand 29
CD27	Hs.355307	CD27 molecule
CR2	Hs.445757	Complement component receptor 2
CXCL13	Hs.100431	Chemokine (C-X-C motif) ligand 13
FASLG	Hs.2007	Fas ligand (TNF superfamily, member 6)
FCER2	Hs.465778	Fc fragment of IgE receptor, low affinity II (CD23)
FLT3	Hs.507590	Fms-related tyrosine kinase 3
IGF1	Hs.160562	Insulin-like growth factor 1
IL-10	Hs.193717	Interleukin 10
IL-2	Hs.89679	Interleukin 2
INPP5D	Hs.262886	Inositol polyphosphate-5-phosphatase
NOS1	Hs.654410	Nitric oxide synthase 1
NOS3	Hs.647092	Nitric oxide synthase 3
PRKACG	Hs.158029	Protein kinase, cAMP-dependent, catalytic, gamma
TNFSF10	Hs.478275	Tumor necrosis factor superfamily, member 10
TNFSF11	Hs.333791	Tumor necrosis factor superfamily, member 11
TNFSF14	Hs.129708	Tumor necrosis factor superfamily, member 14
TNFSF15	Hs.23349	Tumor necrosis factor superfamily, member 15
TNFRSF1B	Hs.256278	Tumor necrosis factor receptor superfamily, member 1B
TNFRSF8	Hs.1314	Tumor necrosis factor receptor superfamily, member 8
TNFRSF13B	Hs.158341	Tumor necrosis factor receptor superfamily, member 13B
TNFRSF17	Hs.2556	Tumor necrosis factor receptor superfamily, member 17
VAV1	Hs.116237	Vav 1 guanine nucleotide exchange factor

Genes Expressed at Low Levels

CCL21	Hs.57907	Chemokine (C-C motif) ligand 21
CD40LG	Hs.592244	CD40 ligand
PRKCZ	Hs.496255	Protein kinase C, zeta
TNFSF8	Hs.494901	Tumor necrosis factor superfamily, member 8

Genes Expressed at High Levels

GNAS	Hs.125898	GNAS complex locus
HSPB1	Hs.520973	Heat shock 27kDa protein 1
NPM1	Hs.557550	Nucleophosmin
PPIB	Hs.434937	Peptidylprolyl isomerase B
SQSTM1	Hs.724025	Sequestosome 1

Table 1: Showing expression levels of genes related to apoptosis and survival in the index breast cancer cell line Hs578T