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Effect of apigenin on whole transcriptome profile of TNFα-activated MDA-MB-468 triple negative breast cancer cells

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Abstract. The lack of hormone receptors in triple negative breast cancer (TNBC) is associated with the inefficacy of anti-estrogen chemotherapies, leaving fewer options for patient treatment and higher mortality rates. Additionally, as with numerous types of inflammatory breast cancer, infiltration of tumor associated macrophages and other leukocyte sub-populations within the tumor inevitably lead to aggressive, chemo-resistant, metastatic and invasive types of cancer which escape immune surveillance. These processes are orchestrated by the release of potent cytokines, including TNFα, IL-6 and CCL2 from the stroma, tumor and immune cells within the tumor microenvironment. The present study evaluated apigenin modulating effects on the pro-inflammatory activating action of TNFα in TNBC MDA-MB-468 cells, derived from an African American woman. Initially, cell viability was determined to establish an optimal sub-lethal dose of TNFα and apigenin in MDA-MB-468 cells. Subsequently, various treatments effects were evaluated using whole transcriptomic analysis of mRNA and long intergenic non-coding RNA with Affymetrix HuGene-2.1-st human microarrays. Gene level differential expression analysis was conducted on 48,226 genes where TNFα caused significant upregulation of 53 transcripts and downregulation of 11 transcripts. The largest upward differential shift was for CCL2 [+61.86 fold change (FC); false discovery rate (FDR), P<0.0001]; which was down regulated by apigenin (to +10.71 FC vs. Control; FDR P-value <0.001), equivalent to an 83% reduction. Several TNFα deferentially upregulated transcripts were reduced by apigenin, including CXCL10, C3, PGLYRP4, IL22RA2, KMO, IL7R, ROS1, CFB, IKBKe, SLITRK6 (a checkpoint target) and MMP13. Confirmation of CCL2 experimentally induced transcript alterations was corroborated at the protein level by ELISA assays. The high level of CCL2 transcript in the cell line was comparable to that in our previous studies in MDA-MB-231 cells. The differential effects of TNFα were corroborated by ELISA, where the data revealed a >10-fold higher releasing rate of CCL2 in MDA-MB-468 cells compared with in MDA-MB-231 cells, both of which were attenuated by apigenin. The data obtained in the present study demonstrated a high level of CCL2 in MDA-MB-468 cells and a possible therapeutic role for apigenin in downregulating TNFα-mediated processes in these TNBC cells.

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Key words: whole transcriptome, triple negative breast cancer, apigenin, MDA-MB-468

Introduction

Within the past several decades, we have seen an increase in research on the infiltration of leukocyte sub-populations (LSPs), being drawn, sequestered and embedded within solid tumor tissue corresponding to elevated concentrations of chemokines such as CCL2 (1). Both animal and *in vitro* studies have shown CCL2 can sequester macrophages and other immune components such as myeloid-derived suppressor cells or regulatory T cells all of which promote immune evasion, epithelial-to-mesenchymal transition, tumor growth, metastasis, and immune evasion. High concentrations of pro-inflammatory proteins such as CCL2, TNFα, matrix metalloproteinase 9, interleukin-6 (IL-6), chemokine (C-X-C motif) ligands (e.g., CXCL) (1-4), granulocyte-macrophage colony-stimulating factor and other chemokine ligands (e.g., CCLs) (5-9) are commonly reported as tumor promoting proteins in diverse cancers such as thyroid, brain, gastric, lung, glioblastoma multiforme and breast (2-7,10-15).

What is evidently a critical situation is that these inflammatory proteins, in particular, the CCL2 and IL-6 are brought about by the actual cancer treatments themselves (e.g., radiotherapy (16) chemotherapy (8), which in turn are then associated with tumor recurrence (17) and chemo-resistance (18,19), Inflammatory events in general, whether it be from other parts of the body such as the liver (9,20) adipose tissue in obesity or arising from viral origin tend to elevate TNF-a, IL -6 and CCL2 then becoming risk factors for the development of diverse cancers (21) aggressive tumors with advanced stage tumor grade and greater rates of mortality (22,23). Meanwhile, it is believed that drugs or natural compounds that can attenuate CCL2 and IL-6 would slow the aggressive

nature of advanced cancers (24-26) to the inclusion of triple negative breast cancer (TNBC) and hormone positive breast cancers (27,28). It is believed that utilizing synthetic or natural small molecules as CCR2 inhibitors (CCR2i) can increase overall survival odds (29,30).

In our previous work, we found that apigenin, a pigment naturally found in parsley, can modulate TNFα triggered release of chemokines in a TNBC model using MDA-MB-231 cells (31). In the present study, we carried out a similar experiment using a TNBC cell line derived from an African American woman (MDA-MB-468, MDA-MB-468 cells), which express enormously high levels of CCL2 upon impact by TNFα as demonstrated by the current work.

Materials and methods

Triple-negative human breast tumor (MDA-MB-468) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were all obtained from Invitrogen. Recombinant human TNFα and CCL2 ELISA kits were purchased from RayBiotech (RayBiotech Inc.).

Cell culture. MDA-MB-468 cells were grown in high-glucose DMEM (w/phenol red and glutamine) supplemented with 10% FBS and 1% [10,000 U/ml] penicillin G sodium + [10,000 μg/ml] streptomycin sulfate. Cells were grown at 37˚C with humidified 95% air and 5% CO_2 and sub-cultured every 3-5 days.

Cell viability assay. Viable cell count was determined by Alamar blue. Briefly, 96-well plates were seeded with MDA-MB-468 cells at a density of $5x10^4$ cells/100 μl/well with various treatments. After 24 h, Alamar blue (0.1 mg/ml in HBSS) was added at 15% v/v to each well and incubated for 6-8 h. Quantitative analysis of dye conversion was measured using a Biotek Synergy multi-mode detection reader equipped with Gen5 software 550/580 (excitation/emission). Data were expressed as a percentage of the untreated control groups.

CCL2 detection by ELISA. Supernatants from experimental treatments were collected, centrifuged at 1,000 x g for 5 min at 4˚C and evaluated for MCP-1/CCL2 using Human MCP1 ELISA from RaybioRayBiotech Life, following the manufacturer's instructions. Briefly, a dilution from 10-50% of supernatants was made with assay buffer (final working volume = 100 μl), and standards was added to 96-well plates pre-coated with the capture antibody. Samples were washed 4x between steps, and after adding the HRP-conjugate, the substrate/stopping solutions were added, and plates were read at 450 nm using a Biotek Synergy multi-mode detection reader equipped with Gen5 software. All data were expressed as concentration derived from a standard curve in pg/ml.

Microarray WT 2.1 human datasets. Cells were collected by a 3X wash in ice-cold HBSS, then a rapid freeze with storage at -80˚C. Total RNA was isolated and purified using the TRIzol/chloroform method, the quality was assessed, and concentration was equalized to 82 ng/μl in nuclease-free water. Whole transcriptome analysis was conducted according to the GeneChipTM WT PLUS Reagent Manual for Whole Transcript (WT) Expression Arrays for human 2.1 Array Strips (32). Briefly, RNA was synthesized to first strand cDNA, second-strand cDNA and followed by transcription to cRNA. cRNA was purified and assessed for yield, before 2nd cycle single-stranded cDNA synthesis, hydrolysis of RNA and purification of 2nd cycle single-stranded cDNA. cDNA was then quantified for yield and equalized to 176 ng/ml. Subsequently, cDNA was fragmented, labeled and hybridized on to the arrays before being subject to fluidics and imaging using the Gene Atlas (Affymetrix- Thermo Fisher Scientific, Inc.).

Statistical analysis. A Kruskal-Wallis test, followed by a Dunn's multiple comparison test was used to evaluate statistical differences from controls and a one-way ANOVA followed by a Tukey's multiple comparisons test to evaluate statistical differences between two cell lines both using GraphPad prism software (GraphPad Software). The array data quality control and initial processing from CEL to CHP files were conducted using expression console, followed by data analysis using the Affymetrix transcriptome analysis console (Affymetrix-Thermo Fisher Scientific, Inc.). The data have been deposited into the Gene Expression Omnibus for public analysis at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE133968.

Results

A non-lethal working concentration was established in MDA-MB-468 cells for TNFα and apigenin (Fig. 1) to where sub-lethal values were determined by a dose response using apigenin [40 uM], and TNFα [40 ng/ml]. Whole transcriptomic differential changes between untreated controls, TNFα (40 ng/ml), apigenin (40 uM) and co-treatment (CoTx) [TNFα (40 ng/ml) + apigenin (40 uM)] were acquired and the summary by a number of deferentially expressed genes shown in Fig. 2. Comparing the Control vs. TNFα only, we provide a fold change (FC) scatter plot (Fig. 3) corresponding to signal and processed data presented in Table I. Gene level differential expression analysis was conducted on 48,226 genes where TNFα caused significant up-regulation of 53 transcripts and down-regulation of 11 transcripts.

The effects of apigenin on modifying TNFα induced genes are presented in Table II. The table provides data on averaged signals, processed data, and percent reduction of TNFα treated cells. The largest upward differential shift was for CCL2 (+61.86-FC, false discovery rate (FDR) P-value <0.0001); which was down regulated by apigenin (to +10.71 FC vs. Control, FDR P-value <0.001), equivalent to an 83% reduction. The TNFα deferentially up-regulated transcripts were reduced by apigenin included; CXCL10, C3, PGLYRP4, IL22RA2, KMO, IL7R, ROS1,CFB, IKBKe, SLITRK6 (a checkpoint target) and MMP13 Our previous studies in MDA-MB-231 cells according to both mRNA and protein levels for CCL2 was meager in comparison. In order to confirm a heightened level in this particular cell line, and ELISA was conducted on both cell lines for all four groups (Fig. 4). These findings match the current microarray data, where CCL2 release in MDA-MB-468 cells was extremely high in concentration in comparison to MDA-MB-231 cells.

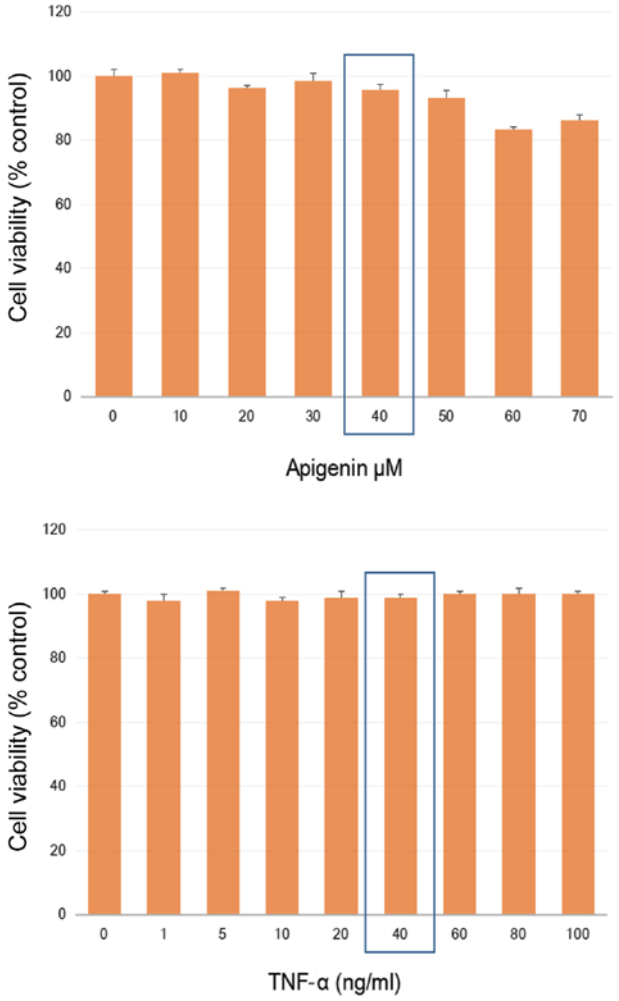

Figure 1. Preliminary determination of sub-lethal working concentrations. The effect of TNFα and apigenin on cell viability of MDA-MB-468 cells at 5% CO_2/Atm for 24 h. The data are presented as viability (% Ctrl), mean ± SEM (n=4). The significance of differences from the Ctrl were determined by a Kruskal-Wallis test, followed by a Dunn's multiple comparison test. N.S. TNF-, tumor necrosis factor α; Ctrl, control.

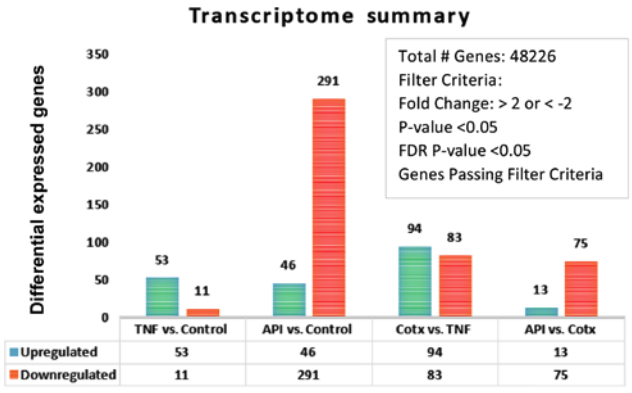

Figure 2. Transcriptome summary. Whole transcriptomic differential changes between untreated controls, TNFα (40 ng/ml), apigenin (40 μM) and CoTx [TNFα (40 ng/ml) + apigenin (40 μM)] after 24 h treatment in MDA-MB-468 cells. A total of 48,226 gene transcripts were analyzed for each group. Diferentially expressed genes (upregulated and downregulated) that passed the filter criteria (FDR P-value and P-values <0.05) are presented by number. FDR, false discovery rate; TNF, tumor necrosis factor; Cotx, co-treatment; API, apigenin.

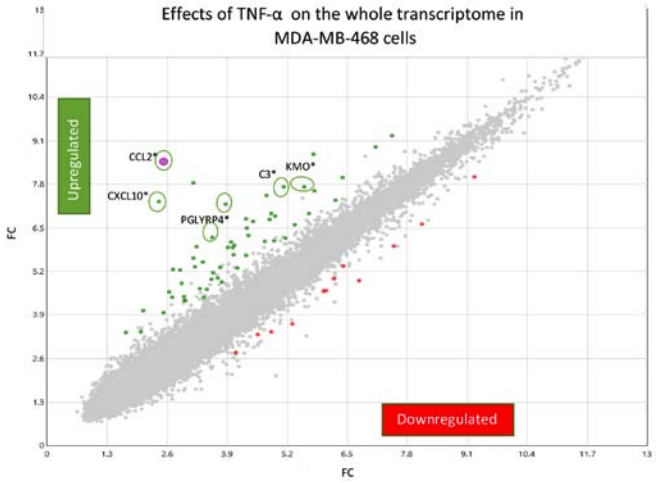

Figure 3. TNF-α (40 ng/ml) induces alterations in MDA-MB-468 cells. The data are presented by a scatterplot showing differentially expressed genes meeting the filter criteria (FDR P-value and P-values <0.05) and FC <-2 and >2 are presented (full description in Table I). Green represents upregulated transcripts, gray represents genes omitted as significant falling below a 2-fold change in either direction, red represents downregulated transcripts and pink is the highest upregulated transcript. Genes relevant to the findings of the present study are circled and presented along with their corresponding official gene symbol. *attenuated by apigenin. FC, fold change; TNF-α, tumor necrosis factor-α; FDR, false discovery rate; CCL2, C-C motif chemokine ligand 2; CXCL10, C-X-C motif chemokine ligand 10; PGLYRP4, peptidoglycan recognition protein 4; C3, complement C3; KMO, kynurenine 3-monooxygenase.

Discussion

Limited therapeutic options are available for TNBC patients and consequently can result in aggressive metastatic disease, with greater mortality rates in African American (AA) women, relative to Caucasian-American (33,34). This health disparity may arise due to diagnosis at later stages of the disease (35) or a predisposed racially distinct genetic or epigenetic profile (36,37) with a propensity toward an overactive oncogenic p38 MAPK, Wnt/β-catenin, IGF2/ERbeta signaling axis (38-40). Additional factors to a health disparity arising in AA women regarding TNBC include vitamin D deficiencies (41) socioeconomic factors, later stage diagnosis, obesity, or even breast feeding patterns (42-44).

As with all human cancers, late stage diagnosis is associated with greater mortality rates to which the immune system can play a critical role. In the case with solid tumors such as breast cancer, inflammatory like secretion of cytokines to the tumor microenvironment can drive infiltration of tumor-associated macrophages (TAMs) and neutrophils (TANs) which promote tumor survival, metastasis, invasion, angiogenesis, resistance and turn off host immune surveillance, all equating to poor survival rates (2,45-47). It is believed that use of drugs or natural compounds that can suppress oncogenic cytokines (e.g., CXCL1, CCL18, CCL8, CCL2, IL-4, IL-8, IL-6, etc.) (17,48-53) such as apigenin, EGCG or butein can curtail these biochemical driven events and provide therapeutic advantages against aggressive inflammatory breast cancers (54,55).

In the present study, an inflammatory profile was evoked by TNFα, where the highest induced transcript in MDA-MB-468 cells was CCL2, confirmed at both the mRNA and protein level. The rise in CCL2 is reported throughout the literature, where it

Table I. Differential whole transcriptome pattern induced by TNFα (40 ng/ml) relative to untreated controls in MM-468 cells.

Control (Avg log_2)	TNFα (Avg log_2)	Fold change	P-value	FDR P-value	Gene symbol	Description
2.52	8.47	61.86	$2.37x10^{-10}$	$3.82x10^{-6}$	CCL2	Chemokine (C-C motif) ligand 2
2.41	7.29	29.40	$1.80x10^{-12}$	$8.70x10^{-8}$	C xCL10	Chemokine (C- x-C motif) ligand 10
3.17	7.84	25.63	$2.28x10^{-10}$	$3.82x10^{-6}$	C xCL8	Chemokine (C- x-C motif) ligand 8
3.86	7.20	10.13	$3.89x10^{-10}$	$4.69x10^{-6}$	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
5.78	8.69	7.56	$6.07x10^{-9}$	$4.18x10^{-5}$	BIRC3	Baculoviral IAP repeat containing 3
3.23	5.94	6.55	$2.62x10^{-7}$	$6.00x10^{-4}$	IGFL1	IGF like family member 1
4.75	7.45	6.50	$4.83x10^{-9}$	$4.18x10^{-5}$	C3	Complement component 3
3.57	6.20	6.18	$1.10x10^{-7}$	$4.00x10^{-4}$	PGLYRP4	Peptidoglycan recognition protein 4
5.12	7.71	6.02	$7.79x10^{-7}$	$1.10x10^{-3}$	KMO	Kynurenine 3-monoo xygenase
2.72	5.27	5.86	$2.80x10^{-7}$	$6.00x10^{-4}$	AMY1B	Amylase, alpha 1B
4.3	6.78	5.57	$7.89x10^{-7}$	$1.10x10^{-3}$	IL22RA2	Interleukin 22 receptor, alpha 2
3.16	5.60	5.42	$8.11x10^{-9}$	$4.89x10^{-5}$	SAA24	Serum amyloid A2
2.87	5.23	5.13	$1.85x10^{-5}$	$1.44x10^{-2}$	CCL20	Chemokine (C-C motif) ligand 20
4.46	6.69	4.69	$1.09x10^{-7}$	$4.00x10^{-4}$	CFB	Complement factor B
4.32	6.48	4.48	$5.63x10^{-9}$	$4.18x10^{-5}$	ABCC4	ATP binding cassette subfamily C member 4
5.56	7.71	4.43	$2.25x10^{-7}$	$6.00x10^{-4}$	TNFAIP2	Tumor necrosis factor, alpha-induced protein 2
3.37	5.49	4.33	$3.71x10^{-7}$	$8.00x10^{-4}$	IL7R	Interleukin 7 receptor
3.98	6.07	4.27	$8.37x10^{-7}$	$1.20x10^{-3}$	ROS1	ROS proto-oncogene 1 , receptor tyrosine kinase
4.85	6.92	4.19	$1.85x10^{-7}$	$6.00x10^{-4}$	OLR1	Oxidized low density lipoprotein receptor 1
3.91	5.90	3.99	$6.35x10^{-7}$	$1.00x10^{-3}$	TNF	Tumor necrosis factor
2.07	4.04	3.91	$4.86x10^{-6}$	$4.60x10^{-3}$	INHBA	Inhibin beta A
2.64	4.58	3.84	$6.31x10^{-6}$	$5.80x10^{-3}$	AMY1B	Amylase, alpha 1B
4.81	6.74	3.81	$1.27x10^{-7}$	$5.00x10^{-4}$	AKR1B1	Aldo-keto reductase family 1, B1
2.91	4.83	3.79	$4.31x10^{-6}$	$4.30x10^{-3}$	AMY1B	Amylase, alpha 1B
4.93	6.85	3.79	$2.41x10^{-6}$	$2.80x10^{-3}$	SAA1	Serum amyloid A1
4.05	5.97	3.78	$2.71x10^{-7}$	$6.00x10^{-4}$	IKBKE	Inhibitor of kappa LPGEK B-cells epsilon
4.36	6.27	3.77	$1.01x10^{-6}$	$1.30x10^{-3}$	TNC	Tenascin C
4.02	5.92	3.72	$4.15x10^{-7}$	$9.00x10^{-4}$	C1QTNF1	C1q and TNF related protein 1
5.78	7.61	3.54	$1.04x10^{-7}$	$4.00x10^{-4}$	MMP7	Matri x metallopeptidase 7
7.11	8.91	3.48	$2.62x10^{-6}$	$2.90x10^{-3}$	EDN1	Endothelin 1
7.47	9.24	3.41	$2.37x10^{-6}$	$2.80x10^{-3}$	ICAM1	Intercellular adhesion molecule 1
1.71	3.38	3.17	$1.86x10^{-7}$	$6.00x10^{-4}$	MMP13	Matri x metallopeptidase 13
4.04	5.69	3.15	$1.00x10^{-6}$	$1.30x10^{-3}$	SGPP2	Sphingosin x 10-1-phosphate phosphatase 2
6.40	7.99	3.01	$7.30x10^{-7}$	$1.10x10^{-3}$	SEMA3C	Semaphorin 3C
3.75	5.33	2.98	$2.03x10^{-5}$	$1.51x10^{-2}$	CHI3L2	Chitinase 3-like 2; DENN/MADD domain containing 2D
3.6	5.17	2.97	$1.45x10^{-5}$	$1.18x10^{-2}$	SOD2	Supero xide dismutase 2, mitochondrial
2.96	4.44	2.80	$5.71x10^{-8}$	$3.00x10^{-4}$	KCCAT211	Renal clear cell carcinoma-associated transcript 211
2.52	3.98	2.74	$2.52x10^{-6}$	$2.90x10^{-3}$	LRRC55	Leucine rich repeat containing 55
4.71	6.10	2.62	$3.87x10^{-5}$	$2.59x10^{-2}$	SLITRK6	SLIT and NTRK-like family, member 6
2.03	3.40	2.59	$1.52x10^{-5}$	$1.21x10^{-2}$	KLHL38	Kelch-like family member 38
4.30	5.68	2.59	$5.30x10^{-7}$	$1.00x10^{-3}$	SLC2A12	Solute carrier family 2 M, 12
2.97	4.32	2.54	$2.82x10^{-5}$	$1.97x10^{-2}$	BBO x1	Gamma-butyrobetaine hydro xylase
2.99	4.32	2.51	$1.41x10^{-5}$	$1.17x10^{-2}$	TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15
3.70	5.00	2.46	$3.69x10^{-5}$	$2.51x10^{-2}$	GBP1	Guanylate binding protein 1, interferon-inducible
5.67	6.92	2.37	$1.26x10^{-5}$	$1.11x10^{-2}$	NFKBIA	NFK light polypeptide GE in B-cells inhibitor, alpha
5.37	6.60	2.35	$4.67x10^{-6}$	$4.50x10^{-3}$	CTSS	Cathepsin S
3.45	4.66	2.31	$1.40x10^{-5}$	$1.17x10^{-2}$	FIBIN	Fin bud initiation factor homolog (zebrafish)
4.92	6.10	2.27	$7.08x10^{-5}$	$4.27x10^{-2}$	NFE2L3	Nuclear factor, erythroid 2-like 3

Table I. Continued.

Control (Avg log_2)	TNFα (Avg log_2)	Fold change	P-value	FDR P-value	Gene symbol	Description
4.12	5.29	2.25	$2.48x10^{-5}$	$1.76x10^{-2}$	KRT6B	Keratin 6B, type II
3.78	4.89	2.17	$6.63x10^{-5}$	$4.05x10^{-2}$	ATP6V1C2	ATPase, H+ transporting, lysosomal 42kDa V1 sub C2
6.24	7.32	2.12	$2.89x10^{-6}$	$3.20x10^{-3}$	IFNGR1	Interferon gamma receptor 1
3.38	4.42	2.05	$3.43x10^{-5}$	$2.36x10^{-2}$	CYP7B1	Cytochrome P450, family 7, sub B, polypeptide 1
5.16	6.19	2.04	$1.99x10^{-5}$	$1.51x10^{-2}$	LACC1	Laccase domain containing 1
6.41	5.35	-2.08	$4.62x10^{-5}$	$2.97x10^{-2}$	CD14	CD14 molecule
6.21	4.98	-2.35	$7.93x10^{-5}$	$4.56x10^{-2}$	P2RY2	Purinergic receptor P2Y, G-protein coupled, 2
9.25	8.01	-2.36	$1.22x10^{-5}$	$1.09x10^{-2}$	GLYATL2	Glycin x 10-N-acyltransferas x 10-like 2
4.08	2.78	-2.46	$1.98x10^{-7}$	$6.00x10^{-4}$	STAC2	SH3 and cysteine rich domain 2
5.98	4.61	-2.59	$1.48x10^{-5}$	$1.19x10^{-2}$	SLC15A2	Solute carrier family 15, member 2
6.04	4.62	-2.69	$3.30x10^{-6}$	$3.50x10^{-3}$	TF	Transferrin
4.85	3.4	-2.74	$4.86x10^{-5}$	$3.08x10^{-2}$	KRT4	Keratin 4, type II
8.11	6.61	-2.83	$2.08x10^{-5}$	$1.52x10^{-2}$	CRISP3	Cystein x 10-rich secretory protein 3
7.51	5.96	-2.93	$2.20x10^{-6}$	$2.70x10^{-3}$	SPDEF	SAM pointed DC ETS transcription factor
5.31	3.63	-3.20	$6.33x10^{-6}$	$5.80x10^{-3}$	SCGB2A2	Secretoglobin, family 2A, member 2
6.76	4.92	-3.59	$1.48x10^{-7}$	$5.00x10^{-4}$	CLCA2	Chloride channel accessory 2

The data are presented as official gene symbol, gene description, bi-weighted averages (n=3), fold change P-value and FDR P-value. Avg, average; FDR, false discovery rate.

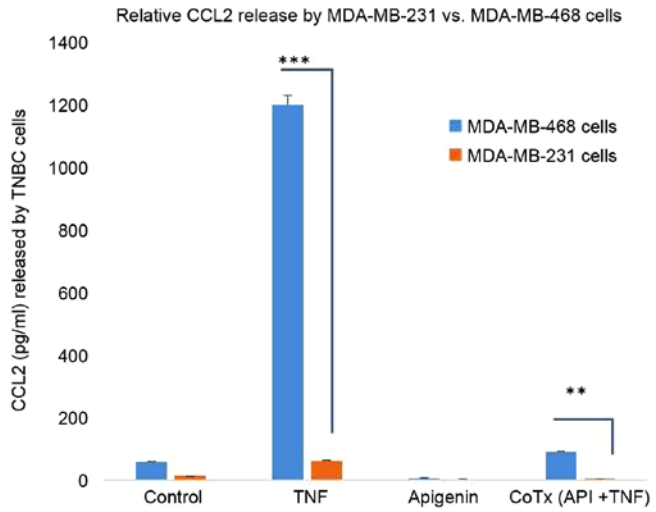

Figure 4. CCL2 released by untreated controls, TNFα (40 ng/ml), apigenin (40 μM) and CoTx [TNFα (40 ng/ml) + apigenin (40 μM)] after 24 h treatment in MDA-MB-231 and MDA-MB-468 cells at equal plating density. The data are expressed as CCL2 (pg/ml), and statistical differences between the same treatments on different cell lines were determined by one-way ANOVA followed by a Tukey's multiple comparisons test. A significant difference was found in the TNF treatment group and the CoTx (API+TNF) treatment group. **P<0.01; ***P<0.0001. TNF, tumor necrosis factor; CCL2, C-C motif chemokine ligand 2; API, apigenin; CoTx, co-treatment.

serves to drive tumor invasion, metastasis, and recurrence (2,17). CCl2 expression is also fairly consistent among breast cancer subcategories: (luminal: ER+ and/or PR+) (56), HER2+ (27) or basal like TNBC cell lines (6,57,58). Given that our studies suggest a possible disparity with higher levels of CCL2 in the African American cell line MDA-MB-468 vs. MM-231, we reviewed oncomine.org Oncomine™ for CCL2 difference among races, finding no obvious difference between African American vs. Caucasian in this aspect. Similarly, in our work-we find no difference in baseline CCL2 levels in the two cell lines, with the disparity arising only with the treatment of TNFα which is an experimental model of inflammatory breast cancer. Future studies will be required to evaluate the inflammatory response across racially divergent breast cancer cell lines or tissues.

What we do know, however, is that compounds like apigenin that attenuate the CCL2/CCR2 axis would slow the aggressive nature of TNBC and hormone positive breast cancers (27,28) by attenuating invasion, metastasis, EMT and the development of drug resistance (59-62). CCL2 inhibitors have been tested in various tumors, tumor cells and xenograft models with CCL2 lowering effects brought about by losartan (63) anlotinib (64) imatinib (65) zoledronic acid (66) oroxylin A (67) aspirin (68) natural compounds in coffee (kahweol acetate, cafestol) (69) or conophylline from Ervatamia microphylla (70) which can reduce invasive inflammatory tumor infiltration. The mechanism of action for CCL2 reducing agents may center around the modification of upstream or downstream targets such as PLEK2/EGRF (71) HER2-EGF/HRG, PI3K-NF-kB axis (27) SRC, PKC (58) the neddylation pathway (72) or the well-known mitogen-activated protein kinases and phosphatidylinositol 3-kinase/Akt cell signaling pathways (73). While others have reported apigenin to have an effect on NF-kappaB/Snail pathway (74), pSTAT3, pERK or PI3K/pAkt (75), our previous studies suggest the effects of TNFα in TNBC cell lines, as it relates to CCL2 are driven through the higher expression of IKBK epsilon (31).

Table II. Differential whole transcriptome changes upregulated by TNF-α (40 ng/ml) and downregulated by apigenin (40 μM) in MM-468 cells.

TNF vs. Apig +TNF (% reduction)	Symbol	Control signal Avg (log2)	TNF signal Avg (log2)	Apigenin+ TNF signal Avg (log2)	Dir	Ctrl vs. TNF (differential) FC	P-value	FDR P-value	Dir	TNF vs. Apig +TNF (differential) FC	P-value	FDR P-value
-83	CCL2	2.52	8.47	5.94	↑	61.86	2.37×10^{-10}	3.82×10^{-6}	↓	-5.78	3.33×10^{-6}	5.70×10^{-3}
-74	CXCL10	2.41	7.29	5.32	↑	29.40	1.80×10^{-12}	8.70×10^{-8}	↓	-3.90	5.29×10^{-8}	5.00×10^{-4}
-76	C3	4.75	7.45	5.40	↑	6.50	4.83×10^{-9}	4.18×10^{-5}	↓	-4.16	6.13×10^{-8}	5.00×10^{-4}
-67	PGLYRP4	3.57	6.20	4.60	↑	6.18	1.10×10^{-7}	4.00×10^{-4}	↓	-3.04	6.08×10^{-5}	2.50×10^{-2}
-82	KMO	5.12	7.71	5.28	↑	6.02	7.79×10^{-7}	1.10×10^{-3}	↓	-5.40	6.09×10^{-6}	8.60×10^{-3}
-69	AMY1B	2.72	5.27	3.56	↑	5.86	2.80×10^{-7}	6.00×10^{-4}	↓	-3.26	2.74×10^{-5}	1.57×10^{-2}
-76	IL22RA2	4.30	6.78	4.70	↑	5.57	7.89×10^{-7}	$1.1.0\times10^{-3}$	↓	-4.23	8.92×10^{-6}	1.02×10^{-2}
-68	CFB	4.46	6.69	5.06	↑	4.69	1.09×10^{-7}	4.00×10^{-4}	↓	-3.09	1.96×10^{-6}	4.30×10^{-3}
-56	ABCC4	4.32	6.48	5.29	↑	4.48	5.63×10^{-9}	4.18×10^{-5}	↓	-2.29	1.70×10^{-5}	1.25×10^{-2}
-76	IL7R	3.37	5.49	3.45	↑	4.33	3.71×10^{-7}	8.00×10^{-4}	↓	-4.10	9.06×10^{-7}	2.90×10^{-3}
-74	ROS1	3.98	6.07	4.15	↑	4.27	8.37×10^{-7}	1.20×10^{-3}	↓	-3.79	1.00×10^{-5}	1.02×10^{-2}
-69	AMY1B	2.64	4.58	2.93	↑	3.84	6.31×10^{-6}	5.80×10^{-3}	↓	-3.13	4.55×10^{-5}	2.07×10^{-2}
-50	AKR1B1	4.81	6.74	5.74	↑	3.81	1.27×10^{-7}	5.00×10^{-4}	↓	-2.00	1.00×10^{-4}	3.46×10^{-2}
-68	AMY1B	2.91	4.83	3.14	↑	3.79	4.31×10^{-6}	4.30×10^{-3}	↓	-3.23	9.18×10^{-5}	3.05×10^{-2}
-59	SAA1	4.93	6.85	5.56	↑	3.79	2.41×10^{-6}	2.80×10^{-3}	↓	-2.45	7.36×10^{-5}	2.75×10^{-2}
-51	IKBKE	4.05	5.97	4.94	↑	3.78	2.71×10^{-7}	6.00×10^{-4}	↓	-2.05	2.00×10^{-4}	4.09×10^{-2}
-64	TNC	4.36	6.27	4.82	↑	3.77	1.01×10^{-6}	1.30×10^{-3}	↓	-2.74	6.37×10^{-6}	8.60×10^{-3}
-133	MMP13	1.71	3.38	1.64	↑	3.17	1.86×10^{-7}	6.00×10^{-4}	↓	-3.32	1.54×10^{-7}	9.00×10^{-4}
-64	SEMA3C	6.40	7.99	6.51	↑	3.01	7.30×10^{-7}	1.10×10^{-3}	↓	-2.79	2.38×10^{-6}	4.90×10^{-3}
-135	CHI3L2	3.75	5.33	3.71	↑	2.98	2.03×10^{-5}	1.51×10^{-2}	↓	-3.07	1.32×10^{-5}	1.16×10^{-2}
-50	KCCAT211	2.96	4.44	3.44	↑	2.80	5.71×10^{-8}	3.00×10^{-4}	↓	-2.01	1.56×10^{-6}	4.00×10^{-3}
-188	SLITRK6	4.71	6.10	3.51	↑	2.62	3.87×10^{-5}	2.59×10^{-2}	↓	-6.01	2.24×10^{-8}	5.00×10^{-4}
-142	BBOX1	2.97	4.32	2.88	↑	2.54	2.82×10^{-5}	1.97×10^{-2}	↓	-2.71	1.04×10^{-5}	1.02×10^{-2}
-147	CTSS	5.37	6.60	5.21	↑	2.35	4.67×10^{-6}	4.50×10^{-2}	↓	-2.62	1.41×10^{-5}	1.17×10^{-2}

Differential whole transcriptome changes upregulated by TNF α (40 ng/ml) and downregulated by apigenin (40 μM) in MM-468 cells. Up and down arrows represent upregulation and downregulation, respectively. The data are presented as official gene symbol, bi-weighted averages (n=3), fold change, ANOVA P-value and FDR P-value. % Reduction in column 1 is derived from % FC Control vs. TNFα and Control vs. co-treatment. AMY1B, AMY1A amylase, alpha 1B (salivary); amylase, alpha 1C (salivary); amylase, alpha 1A (salivary); ABCC4, ATP binding cassette subfamily C member 4; BBOX1, butyrobetaine (gamma), 2-oxoglutarate dioxygenase (gamma-butyrobetaine hydroxylase) 1; CTSS, cathepsin S; CCL2, chemokine (C-C motif) ligand 2; CXCL10, chemokine (C-X-C motif) ligand 10; CHI3L2, DENND2D chitinase 3-like 2; DENN/MADD, domain containing 2D; C3, complement component 3; CFB, complement factor B; IKBKE, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon; IL22RA2, interleukin 22 receptor, alpha 2; IL7R, interleukin 7 receptor; KMO, kynurenine 3-monooxygenase (kynurenine 3-hydroxylase); MMP13, matrix metallopeptidase 13; PGLYRP4, peptidoglycan recognition protein 4; ROS1, ROS proto-oncogene 1, receptor tyrosine kinase; SEMA3C, sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C; SAA1, serum amyloid A1; SLITRK6, SLIT and NTRK-like family, member 6; TNC, tenascin C; FDR, false discovery rate; FC, fold change; Ctrl, control; Dir, direction.

It is important to note that when studying the effects of natural compounds such as apigenin on the entire transcriptome of cancer cells, there will most always likely be changes in both directions for oncogenes and tumor suppressors, some of these changes would not be advantageous. In this work, for example, we show that apigenin suppressed the TNFα mediated rise in a potent tumor suppressor: CXCL10. While previous studies consistently that CXCL10 is up-regulated in normal vs. tumor tissue (76,77) this particular protein acts as the major tumor suppressor, evoked by IFN-γ treatment and somehow plays a role in the re-expression of MHC-1, PD-L1, the infiltration of anti-tumoral CD4(+) and CD8(+) T cells (78,79), NK cells, cytotoxic lymphocytes (CTLs) to the tumor to turn on immune surveillance and heighted survival odds in diverse human cancers (80-82). While the beneficial effects of apigenin in cancer are consistently reported, any compound that would turn off the CXCL9, -10, -11/CXCR3 axis could harm the host immunes system to destroy self-malignant tumor tissue (83).

In contrast, the current study shows that apigenin turns on host immune surveillance by its effect on reducing-TNFα induced SLITRK6. SKITRK 6 is a membrane receptor, which is elevated in many cancers [e.g., epithelial tumors, bladder, lung, breast, and glioblastoma (84,85)] and has been deemed an immune checkpoint for target amongst a relatively new class of drugs approved by the FDA (86). SLITRK6 is the target of an antibody drug conjugate AGS15E currently in phase I clinical trials, believed to reactivate the hosts immune surveillance against self-malignant cells (87). While it is outside the scope of discussion to elaborate on every transcript change, this work serves as a general framework for public genomic data evaluation. Re: Gene Expression Omnibus for public analysis at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE133968.

Previously reported data clearly indicate the existence of disparity in the mortality rates associated with TNBC in African Americans, and there a need for initiatives to establish novel and effective therapies to target aggressive tumors marked by a propelling inflammatory component. Overall, we believe there is enough support to warrant clinical trials for the use of apigenin, as there is a growing body of researching showing its antitumor effects from multiple stand points from blocking mutagenic induced cancers [e.g., methyl-nitrosourea, methyl-n-nitro-N-nitrosoguanidine, benzo(a)pyrene or 2-aminoanthracene] (88) to inhibition of ornithine decarboxylase (89) and its overall antioxidant, anti-inflammatory effects (90,91). Data on the clinical efficacy of substances like apigenin for human use to reduce CLL2 will also need to be confirmed, as well as establishing its bioavailability, absorption, therapeutic concentration and application (prevention, treatment or for chemotherapy drug augmentation) (55,91-94).

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE133968).

Authors' contributions

DB was involved in the conceptualization of the manuscript, conducted the primary research, methodology and data analysis. EM carried out the transcriptomic microarray study, data analysis, and wrote and critically edited the manuscript. KFAS was involved in the conceptualization, experimental design and data analysis, and manuscript preparation and critical revision of the manuscript. In addition, KFAS provided oversight management of the project, including consultation, research direction and was responsible for funds acquisition, and the provision of the resources. AH was involved in the conceptualization of the manuscript, the methodology, and involved in writing and critically editing the manuscript. ETO was involved in the conceptualization of the manuscript, involved in the methodology, data analysis, and was involved in writing and critically editing the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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