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Tamoxifen Induces Expression of Immune Response-Related Genes in Cultured Normal Human Mammary Epithelial Cells

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Abstract

Use of tamoxifen (TAM) is associated with a 50% reduction in breast cancer incidence and an increase in endometrial cancer incidence. Here, we documented TAM-induced gene expression changes in cultured normal human mammary epithelial cells (NHMEC strains numbered 5, 16 and 40), established from tissue taken at reduction mammoplasty from 3 individuals. Cells exposed to 0, 10 or 50 μM TAM for 48 hours were evaluated for (*E*)-α-(deoxyguanosin-N^2-yl)-tamoxifen (dG-N^2-TAM) adduct formation by TAM-DNA (DNA modified with dG-N^2-TAM) chemiluminescence immunoassay (CIA), gene expression changes using NCI DNA-oligonucleotide microarray, and real time (RT)-PCR. At 48 hr, cells exposed to 10 μM and 50 μM TAM were 85.6% and 48.4% viable, respectively, and there were no measurable dG-N^2-TAM adducts. For microarray, cells were exposed to 10 μM TAM and genes with expression changes of ≥ 3-fold were as follows: thirteen genes up-regulated and one down-related for strain 16; seventeen genes up-regulated for strain 5; and eleven genes up-regulated for strain 40. Interferon-inducible genes (*IFITM1*, *IFIT1*, *IFNA1*, *MXI* and *GIP3*), and a potassium ion channel (*KCNJ1*) were up-regulated in all 3 strains. No significant expression changes were found for genes related to estrogen or xenobiotic metabolism. RT-PCR revealed up-regulation of interferon α (*IFNA1*) and confirmed the TAM-induced up-regulation of the genes identified by microarray, with the exception of *GIP3* and *MX1*, which were not up-regulated in strain 40. Induction of interferon-related genes in the three NHMEC strains suggests that, in addition to hormonal effects, TAM exposure may enhance immune response in normal breast tissue.

Keywords

microarray; RT-PCR; TAM-DNA chemiluminescence immunoassay; interferon

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Disclosure of Potential Conflicts of Interest
There are no potential conflicts of interest to disclose.

Introduction

In addition to surgery and radiation therapy, estrogen receptor (ER)-positive breast cancer is frequently treated with adjuvant therapy that may include tamoxifen (TAM, Nolvadex®), a TAM analog or an aromatase inhibitor (1-4). TAM therapy reduces the incidence of contralateral breast cancer in breast cancer survivors by 47% (5), and new breast cancers in high-risk women (prophylactic use) by 38% (6). However, increases in endometrial (6,7) and rare uterine cancers (8) in women receiving TAM therapy, raise concern for women receiving TAM for long periods of time. This concern is enhanced by reports of a strong hepatocarcinogenic response in TAM-exposed rats, where both hepatic TAM-DNA adduct formation (9,10) and liver tumor incidence (11) correlated with dose, suggesting that classical genotoxicity may be the predominant mechanism for liver tumor formation in this model (10,12,13). In women, the mechanism underlying TAM-induced endometrial tumor formation is a topic of some controversy, with some studies indicating a genotoxic mechanism and others implying hormonally-controlled events (14-21). A recent population-based case-control study (22), may solve this controversy, but the final report has not been published. The investigators compared endometrial cancer incidence in breast cancer survivors receiving TAM and toremifene (TOR). TOR has been shown to be non-genotoxic in experimental models (23). Preliminary data indicate that TOR and TAM have induced similar incidences of endometrial cancers (K. Holli, personal communication), suggesting that the mechanism may be largely non-genotoxic. However, a report documenting similar frequencies of K-*ras* codon 12 mutations in endometrium from women receiving either TAM or TOR suggests that similar genotoxic events may occur with both treatments (21).

We considered that TAM-induced changes in DNA damage and gene expression may elucidate pathways relevant for molecular mechanisms of drug activity. The current study has focused on normal breast using strains of NHMECs derived from human breast tissue taken at reduction mammoplasty from healthy women. In this study the three different strains, derived from three different individuals, reflect human interindividual variability and similarity with regard to TAM-induced gene expression. Cells were exposed for 48 hr to a TAM concentration similar to that found in human plasma, and there were no measurable TAM-DNA adducts in any NHMEC strain. However, significant changes in gene expression, particularly for immune-response genes, were observed first by microarray and subsequently confirmed by RT-PCR for the genes of interest. This study provides evidence of a non-hormonal mechanism for TAM activity in human breast.

Materials and Methods

Chemicals

TAM and calf thymus DNA were obtained from Sigma (St. Louis, MO). Opaque 96 well high binding plates were purchased from Greiner Labortechnik (PGC Scientific, Frederick, MD). Biotinylated anti-rabbit IgG and streptavidin-alkaline phosphatase were from Applied Biosystems (Foster City, CA). I-Block (Casein) and CDP-Star with Emerald II were from Applied Biosystems. Reacti-Bind DNA coating solution was obtained from Pierce (Rockford, IL). CIA wash buffer was obtained from KD Medical (Columbia, MD). Phosphate buffered saline (PBS) was from GibcoBRL (Grand Island, NY). The Mammary Epithelial Cell Growth Medium (MEGM) Bullet Kit, serum free MEGM and trypsin were purchased from Clonetics™ (Walkersville, MD). TRIzol was purchased from Invitrogen Life Technologies (Carlsbad, CA), cDNA synthesis was performed using the iScript cDNA Synthesis Kit (Bio-Rad Corp., Hercules, CA) and RT-PCR was performed using the SYBR Premix Ex Taq, Perfect Real Time kit (Takara Bio Inc., Shiga, Japan).

NHMEC Culture, Estrogen Receptor (ER) Status, TAM Exposure and Cell Survival

Three primary NHMEC strains, M98040 (strain 40), M98016 (strain 16) and M99005 (strain 5) that were described previously (24), were grown in serum free MEGM (Clonetics™). These strains were characterized for ER by immunohistochemical staining. Briefly, cells that were grown in microscope chamber slides were washed in PBS (KH_2PO_4 [1.06 mM], Na_2HPO_4 [5.6 mM], NaCl [154 mM], pH 7.4) and then fixed with ethanol (5 min, -20 °C). Fixed cells were thawed at 37°C (0.1% Triton X-100 in PBS) and permeabilized in Triton X-100 (ambient temperature, 30 min). The Triton X-100 was then washed out with PBS and the cells were incubated with a primary anti-estrogen receptor rabbit IgG (sc-542, Santa Cruz Biotechnology, Santa Cruz, CA; 4 μg/ml at 4 °C for 16 h). The primary antibody was removed by washing with PBS three times and the cells were incubated in goat serum (37 °C for 20 min) before washing again in PBS. The cells were incubated with fluorescein conjugated goat anti-rabbit (sc-3839, Santa Cruz Biotechnology, diluted 400 fold) at 37 °C for 45 min in the dark, then washed with PBS three times and mounted (M1289, Sigma, St. Louis, MO) for fluorescence microscopy. None of the 3 strains expressed ER_β, but the strain 16 and strain 40 cells were positive for ER_α.

Cells (at passage 7-13) were plated at a density of 1×10^6 cells/15 cm plate or T-175 flask for DNA preparation, and at a density of 1×10^6 cells/6-well plate for RNA preparation and for measuring cell survival. Plated cells were grown for 48 hours prior to treatment with either 10 or 50 μM TAM, or vehicle (dimethylsulfoxide) for an additional 48 hours. After 48 hr, cells were trypsinized and counted using a Coulter Particle Counter (Model Z1, Coulter Electronics, Luton, UK).

For TAM-DNA adduct quantitation, 3 dishes or flasks of cells were exposed under the same conditions on 3 separate occasions. For isolation of DNA, the cells were washed twice in PBS and lysed in cell lysis buffer (50 mM Tris-HCl, 0.1 M EDTA, 0.1 M NaCl, 1.0 % SDS), and incubated first with RNase A for 1 hr at 37°C and then with proteinase K for 1 hr at 70°C. The lysate was then extracted once with phenol:chloroform:isoamyl alcohol, and DNA was precipitated with 1.0 mL of ethanol and subsequently resuspended in water. For some studies, DNA was isolated by non-organic extraction (DNA Extraction Kit, Serologicals Corporation, Norcross, GA). DNA was quantified by ultraviolet spectrophotometry at A_{260}.

For microarray analyses, three replicate exposures were performed for the preliminary study and then confirmed by an independent exposure in duplicate for each cell strain. For isolation of RNA,the cells were lysed with 1.0 mL of TRIzol Reagent (Invitrogen Life Technologies) and RNA was extracted according to manufacturer's protocol. Residual DNA was removed by digestion with DNase I, and the total RNA quantity and purity were assessed by spectrophotometry and gel electrophoresis, respectively.

For RT-PCR experiments, NHMEC strains were subcultured to passage 6 from frozen stocks and exposed to 10 μM TAM for 48 hr on two separate occasions. cDNA was prepared from RNA, and each cDNA sample was assayed 6 times by RT-PCR for *IFIT1, IFITM1, MX1, GIP3* and *KCNJ1*. *IFNA1* was assayed 3 times.

TAM-DNA CIA

Rabbit antiserum, elicited against DNA containing 2.4% modification with dG-N^2-TAM, was employed in the TAM-DNA CIA as previously described (25) with additional specific details below. For the TAM-DNA standard curve we used DNA modified to 4.8 dG-N^2-TAM adducts/10^6 nucleotides, and serial dilutions were carried out to give 6.630 to 0.009 fmol dG-N^2-TAM per well. Competition was achieved by mixing anti-TAM-DNA antiserum with either TAM-DNA standard plus carrier or biological sample DNA in PBS, so that each well contained 5

μg of total DNA. Anti-TAM-DNA was used at a final dilution of 1:1,000,000 in I-Block solution (Applied Biosystems, Foster City, CA). The final light emission was measured at 542 nm using a TR717 Microplate Luminometer (PE Applied Biosystems). For the TAM-DNA standard curve 50% inhibition was at 0.89 ± 0.12 fmol dG-N^2-TAM (mean ± SE, n=5). Since up to 20 μg DNA could be analyzed, the LOD was calculated to be approximately 0.3 dG-N^2-TAM adducts/10^8 nucleotides.

Microarray analysis of gene expression

cDNA, generated from 20 μg RNA by Fairplay Kit (Stratagene, Cedar Grove,TX), was labeled with Cy3 (unexposed control) and Cy5 (TAM-exposed) by indirect coupling, denatured and hybridized to Hs-Operon v2-vB1 oligoarrays containing over 20,000 immobilized human gene elements (Microarray Facility, Advanced Technology Center, NCI). After overnight hybridization at 42°C, microarrays were scanned on a GenePix4000A scanner and analyzed by the NCI MicroArray Database system (mAdb). Genes with ≥ 3-fold color intensity change in >66% of the arrays were considered of interest and subjected to further analysis. For each RNA sample, array data was confirmed once using reciprocal CY3-CY5 labeling. Microarray data has been entered into the GEO system and the MIAME accession number is 2008_279_124158.zip.

RT-PCR

RNA (1.0 μg) was used for cDNA synthesis by iScript cDNA Synthesis Kit (Bio-Rad Corp.). All RT-PCR reactions were performed using the MyIQ Single Color Real Time Detection System (Bio-Rad Corp.), and RT-PCR was performed using the SYBR Premix Ex Taq, Perfect Real Time kit (Takara, Inc.) according to the manufacturer's protocols. The primers used for RT-PCR amplification for gene expression quantification are listed in Table 1 and were purchased from Invitrogen Life Technologies.

Statistical Analysis

Statistical analysis of the microarray data was performed using the NCI MicroArray Database (mAdb) system, with 66% concordance among assays considered significant. RT-PCR data, for the comparison between untreated and TAM-treated cells, was evaluated using Student's *t*-Test.

Results

ER status, cell survival and TAM-DNA adduct formation

The NHMEC strains used in these studies were designated 40, 16 and 5, and were characterized for ER status. None contained ER_β, but the strain 16 and strain 40 cells were positive for ER_α. Unexposed NHMEC strain 40 cells underwent 1.4 population doublings in 48 hr, and by comparison cells exposed to 10 μM TAM and 50 μM TAM had 1.2 population doublings, and 0.67 population doublings. This corresponded to 85.6% and 48.4% survival, respectively. Because the toxicity observed with the higher dose was judged unacceptable, the subsequent microarray and RT-PCR studies employed 10 μM TAM.

DNA extracted from the three NHMEC strains, exposed to 0, 10 and 50 μM TAM for 48 hr, was subjected to TAM-DNA CIA and showed no evidence of measurable dG-N^2-TAM adducts. Using up to 20 μg DNA/well the LOD was 0.3 dG-N^2-TAM adducts/10^8 nucleotides.

Microarray studies in NHMEC strains exposed to 10 μM TAM for 48 hr

Microarray analyses, performed using the NCI microarray system, employed RNA/cDNA samples obtained from three independent exposures for each cell strain. Each RNA/cDNA

sample was assayed on 7-12 microarrays, at least one of which involved reciprocal labeling for microarray confirmation. The data showed primarily up-regulation of genes in TAM-exposed cells compared to unexposed cells. We chose to evaluate only genes that were up-regulated or down-regulated by ≥ 3-fold, and a list of those genes is shown in Table 2. One notable conclusion that can be drawn from Table 2 is that many of the genes that are the most highly up-regulated by TAM appear to be immune response-related genes, associated either with interferon regulation, inflammation, histocompatibility or additional responses to external insult and stress. The specific microarray data for cells altered by ≥ 3-fold are shown in Tables 3, 4 and 5, for strains 16, 5 and 40, respectively. All of the genes altered significantly were up-regulated, with the exception of *SLC7A5*, which was down-regulated. The cell strains can be ranked for magnitude of up-regulation in the following order: strain 16> strain 5 > strain 40. Genes which were up-regulated in all 3 cell strains, by microarray, included *IFIT1, IFITM1, MX1, GIP3* and *KCNJ1*.

RT-PCR of genes up-regulated in all 3 NHMEC strains by exposure to 10 μM TAM for 48 hr

Because micorarray is essentially a screening procedure, it was important to confirm the microarray results with RT-PCR. Primers were designed and RT-PCR was performed for the 5 genes up-regulated in all three NHMEC strains: *IFIT1, IFITM1, MX1, GIP3* and *KCNJ1*. Interferon α (*IFNA1*) was not present on the NCI microarrays used here, but primers were developed and expression of this gene was also assayed by RT-PCR. The results are presented in Tables 3, 4 and 5 (last column).

For the 6 genes examined by RT-PCR there was up-regulation that generally compared well with the Microarray data. Similar to the results of the microarray analysis, strain 16 had the greatest increase in gene expression, followed by strain 5, and strain 40. In addition, in all three NHMEC strains the levels of up-regulation observed with *IFITM1, IFIT1* and *KCNJ1* were greater than those observed with *MX1* and *GIP3* (Tables 3, 4 and 5). When examined by RT-PCR, NHMEC strain 40 showed no up-regulation for *MX1* and *GIP3* (Table 5). In NHMEC strains 16, 40 and 5 the up-regulation observed for *IFNA1* was 7-, 5- and 13-fold, respectively, very much in the same range as the up-regulation of the interferon-inducible genes *IFIT1* and *IFITM1*.

Discussion

In this study we exposed NHMEC strains to 10 and 50 μM TAM for 48 hr to investigate TAM-DNA adduct formation and TAM-induced alterations in gene expression. TAM-DNA adduct formation was not measurable by TAM-DNA immunoassay, but changes in gene expression determined by microarray and confirmed by RT-PCR showed up-regulation of a series of immune response/interferon pathway genes in each of the 3 normal mammary epithelial cell strains.

We used NHMEC strains designated 5, 16 and 40 that were derived from tissue taken at reduction mammoplasty from three different individuals. Strains 16 and 40 were positive for ER_α, and all three strains were negative for ER_β. By microarray we found that, after 48 hr of exposure to a plasma-equivalent TAM dose, 1 gene was down-regulated ≥ 3-fold, and 19 genes were up-regulated ≥ 3-fold. Most of the up-regulated genes were immune-response related genes, and there were no alterations in xenobiotic metabolism or hormone-responsive genes. The most common changes were found in histocompatibility genes and intermediates in the JAK/STAT-interferon signal transduction pathway (26,27), and because all three cell strains showed remarkably similar patterns of gene up-regulation, it appears that these gene expression changes may constitute a relatively-common early stress response to TAM exposure in NHMECs.

The importance of these immune-related pathways was also shown in a murine, human mammary carcinoma xenograft, model by Becker et al (28). The authors cultured human TAM-sensitive MaCa 3366 breast ductal carcinoma cells for 2 years in the presence of TAM, to develop a TAM-resistant version (MaCa 3366/TAM) of this tumor. Both tumor lines were transplanted into nude mice and gene expression was compared in the presence and absence of additional TAM exposure using the Affymetrix microarray. These authors showed up-regulation of 9 interferon-related genes in TAM-resistant human MaCa 3366 cells exposed to TAM; these included, *BST2*, *IFITM2*, *GIP2*, *GIP3*, *IFITM1*, *LGALS3BP*, *IFIT1*, *MX1*, and *IFI27*. Becker et al. (28) also reported differential expression of some estrogen-responsive genes, which was not reproduced in this study.

Several studies using cultured cells have reported TAM-induced alterations in gene expression for the interferon-regulated JAK/STAT pathway. Itoh et al. (29) used ER-positive MCF7 cells that were transfected with the aromatase gene and exposed for 7 days to TAM in the presence of androgen. They reported modest increases in expression for some of the same STAT1 pathway genes seen in our study, including *GIP2*, *IFI27* and *IFIT1*. Perou et al (30) found up-regulation of genes in this pathway, including *STAT1*, *OAS1* and *IFI17*, and postulated that *STAT1* up-regulation was present at all stages of cell growth. Similarly, we found up-regulation of *STAT1*, *OAS1*, *OAS3* and *IFI27*. In a subsequent study, Perou et al. (31) reported substantial variation among primary human breast tumors for genes related to the *STAT1* signal transduction pathway, suggesting that expression of interferon and related events may comprise an important pathway in normal breast tissue with and without TAM, and in breast tumors.

Several studies investigating gene expression patterns in MCF7 breast cancer cells, with or without TAM exposure, did not report alterations in genes related to the JAK/STAT signal transduction pathway. Using MCF7 cells exposed to TAM, Gadal et al. (32) showed altered expression of genes associated with cytoskeletal modeling, DNA repair, active ER formation, growth factor synthesis and mitogenic pathways. Frasor et al. (33) found up-regulation of 50 genes in TAM-exposed ER-positive MCF7 cells, and Hodges et al. (34) found expression changes in cell cycle-related genes in ER-positive MCF7 cells exposed to 4-hydroxy-tamoxifen. It is apparent that different studies queried different numbers of genetic elements, and it is not clear if our genes of interest were always examined.

It is likely that gene expression data obtained from a cancer cell line, such as MCF7 cells, or from human breast cancer tissue (35,36), will have different expression patterns than normal human breast tissue or cultured breast epithelial cells. In performing these experiments with NHMECs we attempted to model events occurring in normal breast tissue in order to focus on TAM-induced alterations in gene expression. One conclusion that can be drawn from these studies is that, whereas ER status is not the same in all 3 of these NHMEC strains, the TAM-induced gene expression patterns were so similar, in cells that were ER_{α} positive and negative, that these particular changes appear to be independent of ER status.

In addition to the classic immune response mediation, interferons have long been known to have static and chemotherapeutic effects on tumor cells (26,37). Interferons, given in conjunction with TAM, enhanced growth inhibition in various human tumor cell lines, including estrogen-dependent and -independent MCF-7 breast cancer xenografts (38,39), and six additional hormone-dependent and -resistant breast cancer cell lines (40). Underlying mechanisms appear to include the induction of apoptosis (41,42) with participation of the interferon regulatory factor-1 and/or thioredoxin reductase. The induction of interferon-associated genes in NHMECs exposed to TAM in this study suggests that, in addition to the known hormonal mechanisms, TAM may act to inhibit the appearance of nascent breast tumors by inducing some interferon-related genes and possibly enhancing apoptosis. Alternatively, the products of interferon-related gene expression in normal tissue may have an inhibitory

effect on the growth of neighboring nascent tumor cells. Current literature supports the contention that TAM induces expression of JAK/STAT pathway intermediates, and available studies suggest that there may be important non-hormonal mechanisms of TAM activity in normal breast tissue.

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Abbreviations

CIA, chemiluminescence immunoassay; ER, estrogen receptor; LOD, limit of detection; NHMEC, normal human mammary epithelial cell; PBS, phosphate buffered saline; RT-PCR, real time-PCR; TAM, tamoxifen, Nolvadex ®; TOR, toremifene; dG-N^2-TAM, (*E*)-α-(deoxyguanosin-N^2-yl)-tamoxifen; TAM-DNA, DNA modified with tamoxifen, with the major adduct being dG-N^2-TAM.

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Table 1

Primer sequences for real time PCR

Gene	Forward Primer	Reverse Primer
KCNJ1	GTGCCAAGACCATTACGTTC	TAGCCACTCGGATTAGGAGG
IFIT1	TTGCCTGGATGTATTACCAC	GCTTCTTGCAAATGTTCTCC
IFITM1	TCTTCTTGAACTGGTGCTGTC	GTCGCGAACCATCTTCCTGT
MX1	AGGACCATCGGAATCTTGAC	TCAGGTGGAACACGAGGTTC
G1P3	CTGATGAGCTGGTCTGCGAT	TAGCTATGACGACGCTGCTG
IFNA1	TCGCCCTTTGCTTTACTGAT	GGGTCTCAGGGAGATCACAG

Table 2

TAM-Induced Gene Expression Changes in NHMEC

Change	Gene	Name	Function
Up	cig5	Viperin	Unknown; Similar to imflammatory response protein 6
Up	BST2	Bone marrow stromal cell antigen 2	Unknown; pre-B-cell growth
Up	TRIM22	Tripartite motif containing 22	Down regulates txc from HIV-1 LTR promoter
Up	SPP1	Secreted phosphoprotein 1 (osteopontin)	Target of p53, role in osteoclast adhesion
Up	OAS3	2′,5′ oligoadenylate synthase 3	Catalyzes 2′-5′ oligomers of dA to bind/activate RNase L; inhibits cell protein synthesis and viral infection resistance
Up	OAS1	2′,5′ oligoadenylate synthase 1	Catalyzes 2′-5′ oligomers of dA to bind/activate RNase L
Up	KCNJ1	Potassium inwardly-rectifying channel	K2+ homeostasis, Bartter Syndrome (salt wasting, low blood pressure)
Up	C1orf29	Chr 1 ORF 29	histocompatibility 28
Up	B2M	beta-2-microglobulin	β-chain of major histo. complex
Up	IFITM1	Interferon induced transmembrane protein 1	Cell growth Control; involved in transduction signaling for antiproliferation and homotypic adhesion
Up	G1P3	Interferon, alpha-inducible protein	Unknown; membrane protein?
Up	WARS	Tryptophanyl- tRNA synthetase	Aminoacyl tRNA catalyze aminoacylation of tRNA with tryptophan
Up	STAT1	Signal transducer and activator of txc	Txc activation; important for cell viability in response to different cell stimuli and pathogens
Up	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	Unknown
Up	MX1	Myxovirus resistance 1	Similar to mouse protein that protects against flu infection
Up	IFIT4	Interferon-induced protein	Unknown
Up	IFI27	Interferon, alpha-inducible protein 27	Unknown
Up	THBS1	Thrombospondin 1	Adhesive glycoprotein; cell/cell or cell/matrix interactions
Up	LGalS3 BP	Lectin, galactoside-binding, soluble	Modulates cell/cell or cell/matrix interactions
Down	SLC7A5	Solute carrier family 7	

Table 3

Gene expression changes (≥ 3-fold) examined by NCI microarray and RT-PCR in NHMEC 16 cells exposed for 48 hr to 10 μM TAM, compared to unexposed NHMEC strain 16 cells

Gene	Microarray Mean Log_2 ± SD	Microarray Mean fold change	Number of Micro-Arrays	RT-PCR Mean fold change[b,c]
IFITM1	4.68 ± 1.30	25.8	9	20.8 ± 3.7[c]
KCNJ1	4.04 ± 2.22	16.4	9	4.4 ± 1.8 [c]
IFIT1	4.01 ± 1.71	16.2	9	15.6 ± 5.0 [c]
IFIT4	3.30 ± 1.80	9.9	7	NA
GIP3	3.03 ± 0.99	8.2	8	3.3±1.1
MX1	2.82 ± 1.18	7.0	9	9.5 ± 2.0 [c]
IFI27	2.57 ± 1.11	5.9	9	NA
STAT1	2.55 ± 0.87	5.7	8	NA
BST2	2.50 ± 0.55	5.7	6	NA
OAS3	2.47 ± 0.90	5.6	6	NA
HLA-C	2.23 ± 0.83	4.7	9	NA
B2M	2.05 ± 0.52	4.1	9	NA
LGALS3BP	1.18 ± 0.72	2.3	9	NA
IFNA1[a]	NA			7.1 ± 2.3 [c]
SLC7A5	-1.75 ± 0.54	0.3	9	NA

[a] NA= no assay. *IFNA1* was not printed on the original microarray.

[b] Each cDNA assayed 6 times by RT-PCR, except *IFNA1*, which was assayed 3 times.

[c] $p<0.05$

Table 4

Gene expression changes (≥ 3-fold) examined by NCI microarray and RT-PCR in NHMEC 5 cells exposed for 48 hr to 10 μM TAM, compared to unexposed NHMEC strain 5 cells

	Microarray Mean Log_2 ± SD	Microarray Mean fold change	Number of Micro-Arrays	RT-PCR Mean fold change[b,c]
cig5	3.72 ± 1.33	13.2	13	NA
IFIT1	3.33 ± 1.17	10.1	14	6.8 ± 2.0[c]
IFITM1	3.19 ± 0.92	9.1	14	7.6 ± 1.1[c]
KCNJ1	3.04 ± 1.48	8.2	14	16.3 ± 12.4[c]
C1orf29	2.41 ± 0.73	5.3	14	NA
GIP3	2.16 ± 0.51	4.5	14	3.0 ± 1.5
MX1	2.12 ± 0.89	4.4	14	6.0 ± 2.4[c]
BST2	2.50 ± 0.55	3.6	14	NA
IFNA1[a]	NA			13.1 ± 6.1[c]

[a] NA= no assay. *IFNA1* was not printed on the original microarray.

[b] Each cDNA assayed 6 times by RT-PCR, except *IFNA1*, which was assayed 3 times.

[c] $p<0.05$

Table 5

Gene expression changes (≥ 3-fold) examined by NCI microarray and RT-PCR in NHMEC strain 40 cells exposed for 48 hr to 10 μM TAM, compared to unexposed NHMEC strain 40 cells

Gene	Microarray Mean Log_2 ± SD	Microarray Mean fold change	Number of Micro-Arrays	RT-PCR Mean fold change[b,c]
cig5	3.11 ± 0.82	8.7	10	NA
IFITM1	3.09 ± 0.49	8.5	10	5.6 ± 2.9 [c]
SPP1	2.90 ± 0.41	7.5	10	NA
KCNJ1	2.49 ± 0.87	5.6	12	3.5 ± 1.5 [c]
IFIT1	2.42 ± 0.65	5.3	10	4.4 ± 1.3 [c]
MX1	2.07 ± 0.65	4.2	12	0.5 ± 0.1
GIP3	2.03 ± 0.73	4.1	12	0.6 ± 0.1
IFNA1 [a]	NA			5.2 ± 1.2 [c]

[a] NA = no assay. *IFNA1* was not printed on the original microarray.

[b] Each cDNA was assayed 6 times by RT-PCR, except *IFNA1*, which was assayed 3 times.

[c] $p<0.05$