Expression profiling of genes modulated by estrogen, EGCG or both in MCF-7 breast cancer cells

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ABSTRACT

(-)-Epigallocatechin-3-gallate (EGCG) is one of the most potent and the most studied green tea catechin. Reports on mechanisms of EGCG action and its cellular targets are plenty. Compelling evidences in the literature in favor of ER being one of its targets suggest that EGCG may have a significant impact on estrogen regulated gene expression. Despite the possible implications on breast cancer chemoprevention or therapy, this aspect of EGCG action has not been adequately investigated. In order to address this issue, we have obtained gene expression profiles of MCF-7 breast cancer cells treated with ethanol (vehicle control) and those treated with estrogen, EGCG or both, using microarrays. Here, we have presented in detail the design and execution of the microarray experiment, quality control checks and analysis of microarray data. The utility and importance of the data generated in this work have been discussed in the context of the background literature. Our data is available in the Gene Expression Omnibus (GEO) database with the identifier GSE56245.

Keywords: EGCG, Estrogen, MCF-7, Microarray

2. Experimental design, materials and methods

2.1. Cell culture

The ER-positive human breast cancer cell line, MCF-7, was routinely cultured in 25 cm² flasks (Greiner Bio-One, GmbH, Germany) under standard culture conditions (37 °C, 5% CO2), under standard culture conditions (37 °C, 5% CO2); in phenol red containing DMEM-F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml of penicillin and 100 µg/ml of streptomycin (M1). Once confluent, the cells were trypsinized and split into fresh 25 cm² flasks in a ratio of 1:3 for expansion. Else, the cells were seeded in 35 mm dishes (2 × 10⁵ cells per dish) using M1 for experimentation.

2.2. Experimental protocol

Once the 35 mm dishes were 60–70% confluent, M1 was replaced with phenol red-free DMEM-F12 supplemented with 10% charcoal stripped heat-inactivated FBS, 100 units/ml of penicillin and 100 µg/ml of streptomycin (M2), and allowed to grow for 4 h. Spent M2 was then replaced with fresh M2 containing ethanol (vehicle), 10 nM of 17β-estradiol (E2), 40 µM of EGCG, or both and incubated further for a period of 24 h before harvesting the cells for total RNA extraction. Thus, the experiment comprised of four treatment groups including the vehicle control. Two dishes (biological replicates for the microarray experiment).
analysis) were assigned for each of the treatment groups. The concentrations of E2 and EGCG were optimized earlier [1]. The effectiveness of 10 nM of E2 was confirmed based on the induction of steady state mRNA levels of two estrogen induced genes, namely trefoil factor-1 (pS2) and progesterone receptor (PR). EGCG at 40 µM concentration was ideally suited for this experiment, since it caused only a modest (20%) reduction in viability of MCF-7 cells. Under this condition the modulation in gene expression by EGCG could safely be interpreted as primary, and not as a collateral effect of cytotoxicity observed at high concentrations [1].

At the end of the experiment, the cells were lysed in RLT buffer (RNeasy kit, Qiagen, GmbH, Germany). Lysates were sent to Genotypic Technology (P) Ltd., Bangalore, India for total RNA extraction, labeling, hybridization, image acquisition, quality control and primary analysis of raw intensity data.

2.3. RNA isolation, labeling, hybridization and image acquisition

RNeasy Mini Kit (Qiagen, GmbH, Germany) was used to extract total RNA. RNA concentrations were determined based on absorbance at 260 nm wavelength of light. The quality of RNA was assessed on 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). RNA samples were considered to be of good quality if they satisfied the following criteria — a) A260/A280 ratio > 1.8, b) A260/A230 ratio ≥ 1.5, and c) 28S/18S intensity ratio > 1.5. All our total RNA samples satisfied these criteria. The RNA quality control data are provided in Table 1.

For each sample, 500 ng of total RNA was labeled (one color with Cy3) using Agilent’s Low Input RNA linear amplification kit (Cat No. 5188–5339). The labeled complementary RNAs were purified using RNeasy Mini Kit, checked for quality (Table 2) and hybridized to Agilent’s human gene expression study 8 × 60 K microarray slides (AMADID:27,114), using the Agilent’s in situ hybridization kit (Cat No. 5184–3568). Following hybridization and washes, the images were scanned in microarray scanner (Model G2565BA, Agilent). The raw intensity data was extracted using Agilent’s Feature Extraction Software.

2.4. Assessment of image quality

The images were manually checked for uneven hybridizations, streaks, blobs and other artifacts. Images were found to be clean with low background noise. Microarray images and signal statistics for each array are provided as supplementary information in Appendices A and B, respectively.

2.5. Microarray data analysis

For data analysis we used LIMMA package from Bioconductor [2]. Background correction was performed using “normexp” method in LIMMA with an offset of 16 [3]. The “quantile” method was used for normalization of the data between arrays. Further, the “avepairs” function was used to average replicate spots. We applied “limfit” (linear model) and “eBayes” (Empirical Bayes method) for determination of differentially regulated genes. The R code used for processing of the data is as follows:

```
library(limma)

Targets = readTargets("targets.txt")
x = read.maimages(targets, source="agilent", green.only=TRUE)
y = backgroundCorrect(x, method="normexp", offset=16)
y = normalizeBetweenArrays(y, method="quantile")
y.ave = avepairs(y, ID=y$genes$ProbeName)
f = factor(targets$Condition, levels=unique(targets$Condition))
design = model.matrix(~0+f)
colnames(design) = levels(f)
fit = limFit(y.ave, design)
contrast.matrix = makeContrasts("E-C", "X-C", "EX-C", levels = design)
fit2 = contrasts.fit(fit, contrast.matrix)
fit2 = eBayes(fit2)
output1 = topTable(fit2, adjust="BH", coef="E-C", genelist=y.ave$gene, number=Inf)
write.table(output1, file="E-C.txt", sep="\t", quote=FALSE)
output2 = topTable(fit2, adjust="BH", coef="X-C", genelist=y.ave$gene, number=Inf)
write.table(output2, file="X-C.txt", sep="\t", quote=FALSE)
output3 = topTable(fit2, adjust="BH", coef="EX-C", genelist=y.ave$gene, number=Inf)
write.table(output3, file="EX-C.txt", sep="\t", quote=FALSE).
```

3. Discussion

An inverse correlation between green tea consumption and breast cancer risk is apparent from the results of epidemiological studies [4–6]. The widespread interest in the major green tea polyphenol, EGCG, as a chemopreventive and chemotherapeutic agent against breast cancer stems from a substantial amount of data available through experimental studies using cell culture and animal models of breast carcinogenesis [7–10]. Molecular targets of EGCG have also been identified [11]. However, despite the fact that majority of breast tumors at diagnosis is estrogen dependent and ER positive, the impact of EGCG on

Table 2
Quality control of Cy3 incorporation in the labeled RNA.

| Sample codes | Cy3 (pmol/µl) | Concentration of labeled RNA (ng/µl) | Absorbance 260/280 | Specific activity pmol dye/µg cRNA |
|--------------|--------------|-------------------------------------|---------------------|----------------------------------|
| C1           | 1.67         | 167.56                              | 2.26                | 9.97                             |
| C2           | 1.02         | 115.87                              | 2.34                | 8.80                             |
| E1           | 1.80         | 176.40                              | 2.28                | 10.20                            |
| E2           | 1.83         | 165.75                              | 2.28                | 11.04                            |
| X1           | 2.25         | 196.64                              | 2.29                | 11.44                            |
| X2           | 2.16         | 202.70                              | 2.31                | 10.66                            |
| EX1          | 2.01         | 193.02                              | 2.35                | 10.41                            |
| EX2          | 1.08         | 126.66                              | 2.34                | 8.53                             |

* C1 & C2—vehicle treated, E1 & E2—10 nM of 17β-estradiol, X1 & X2—40 µM of EGCG, EX1 & EX2—combined treatment with the 10 nM of 17β-estradiol and 40 µM of EGCG.

Table 1
Total RNA quality control.

| Sample codes | Absorbance value 260/280 | Absorbance value 260/230 | RNA concentration (ng/µl) | Total yield (ng) | QC purity | QC conc./yield | QC integrity |
|--------------|--------------------------|--------------------------|---------------------------|-----------------|-----------|----------------|--------------|
| C1           | 2.1                      | 2.2                      | 324.3                     | 6485.6          | Optimal   | Optimal        | Good         |
| C2           | 2.1                      | 1.5                      | 260.1                     | 5202.6          | Optimal   | Optimal        | Good         |
| E1           | 2.1                      | 2.2                      | 336.3                     | 6726.0          | Optimal   | Optimal        | Good         |
| E2           | 2.1                      | 2.2                      | 337.4                     | 6747.6          | Optimal   | Optimal        | Good         |
| X1           | 2.1                      | 2.2                      | 359.1                     | 7182.6          | Optimal   | Optimal        | Good         |
| X2           | 2.1                      | 2.2                      | 410.2                     | 8204.2          | Optimal   | Optimal        | Good         |
| EX1          | 2.1                      | 1.9                      | 283.6                     | 5671.0          | Optimal   | Optimal        | Good         |
| EX2          | 2.1                      | 2.2                      | 338.7                     | 6773.6          | Optimal   | Optimal        | Good         |

* C1 & C2—vehicle treated, E1 & E2—10 nM of 17β-estradiol, X1 & X2—40 µM of EGCG, EX1 & EX2—combined treatment with the 10 nM of 17β-estradiol and 40 µM of EGCG.
A summary of number of probes modulated by E2, EGCG and both.

| Treatment | Total number of probes showing modulation | Number of probes showing induction | Number of probes showing repression |
|-----------|------------------------------------------|----------------------------------|----------------------------------|
| E2        | 3411                                     | 1689                             | 1722                             |
| EGCG      | 3317                                     | 1844                             | 1473                             |
| E2 + EGCG | 9183                                     | 4998                             | 4185                             |

a The concentrations used are 10 nM for E2 and 40 μM for EGCG.

b The number of probes showing modulation with respect to vehicle treated control.

estrogen mediated cellular processes and global gene expression has not been addressed.

Taking a cue from earlier studies which suggest that EGCG can influence estrogen regulated gene expression [12–15], we also demonstrated EGCG mediated increase in mRNA levels of the two estrogen induced transcripts, namely pS2 and PR [1]. The microarray experiment described in this report seeks to address the impact of EGCG on estrogen regulated gene expression[12].

**Fig. 1.** Venn diagram showing the number of probes regulated by the indicated treatments. The concentrations used are 10 nM for E2 and 40 μM for EGCG.

| Treatment | Total number of probes showing modulation | Number of probes showing induction | Number of probes showing repression |
|-----------|------------------------------------------|----------------------------------|----------------------------------|
| E2        | 3411                                     | 1689                             | 1722                             |
| EGCG      | 3317                                     | 1844                             | 1473                             |
| E2 + EGCG | 9183                                     | 4998                             | 4185                             |

a The concentrations used are 10 nM for E2 and 40 μM for EGCG.

b The number of probes showing modulation with respect to vehicle treated control.

**Fig. 1.** Venn diagram showing the number of probes regulated by the indicated treatments. The concentrations used are 10 nM for E2 and 40 μM for EGCG.

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**Supplementary data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gdata.2015.05.040.

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**Conflict of interest**

The authors declare no conflict of interest.