Green tea catechins and the levels of *miR-125b-5p* in MCF-7 breast cancer cells

Noshin Siddiq[1], Michael Nath[1], Kausar Alkaderi[1], John A. François[2], Lissette Delgado-Cruzata[2] PhD

[1] Stuyvesant High School
[2] John Jay College of Criminal Justice

ABSTRACT

Epicatechin (EC) or catechin hydrate (CH), epigallocatechin gallate (EGCG), and catechin gallate (CG) are organic compounds found in green tea known as polyphenolic catechins that have proapoptotic effects on breast cancer cells. Certain microRNAs (miRNAs), small non coding RNA molecules responsible for regulating gene expression, such as *miR-125b-5p,* have also been shown to promote apoptosis in breast cancer cells. Resveratrol, another polyphenolic compound, promoted apoptosis by downregulating expression levels of *miR-125b-5p* in MCF-7 breast cancer cells in previous research. Our study tested the hypothesis that exposure to 25μM and 50μM EC, CG and EGCG will also decrease *miR-125b-5p* levels in MCF-7 breast cancer cells. We used reverse transcription and relative qPCR for our experiment. Our results suggested that EC and EGCG downregulate the expression of *miR-125b-5p* while CG upregulates its expression. As a result, we concluded that EC and EGCG are most likely to promote apoptosis by downregulating expression levels of *miR-125b-5p*. Overall, this study presented innovative research on the relationship between green tea catechins and the levels of *miR-125b-5p* in MCF-7 breast cancer cells.

Key Words: Epicatechin, Epigallocatechin Gallate, Catechin Gallate, Catechins, miRNA, Breast Cancer

INTRODUCTION

Breast cancer is the leading cause of death for women worldwide [1]. According to the GLOBOCAN 2018 statistics of cancer incidence and mortality produced by the International Agency for Research on Cancer, the incidence rate of female breast cancer was 11.6% with 2,088,849 new cases and the mortality rate was 6.6% with 626,679 resulting deaths [1]. However, these rates vary significantly across countries. Most notably, several epidemiological studies have suggested that the risk of breast cancer is low among Asian-Americans, and this has been attributed to their consumption of green tea [2,3]. Some studies have even confirmed that the increased intake of green tea is related to improved prognosis of human breast cancer [4].

Major components of green tea are polyphenolic catechins that account for 30–42% of the dry weight of solids in brewed green tea including epicatechin (EC) or catechin hydrate (CH), catechin gallate (CG), and epigallocatechin gallate (EGCG) [10]. EC and CH have similar structures so they are used interchangeably in this study. All three are classified as flavonoids because they consist of 15 carbon atoms with 2 aromatic rings (A- and B-rings) connected by a 3-carbon bridge that binds with 1 oxygen and 2 carbons of the A-ring, forming a third 6-carbon ring (C-ring) (Figure 1) [5,6]. They display anticancer properties by acting as a form of antioxidant and inducing the body's detoxification system which, in turn, prevents the formation of cancerous cells [7]. Catechins are also present in dark chocolate, broad beans, black grapes, blackberries, cherries, apricots, and apples with EC being the most abundant in all of these foods, and EGCG not commonly present in these food types [8]. In green tea, however, EGCG is the most abundant catechin, and has been considered the most effective polyphenol with cancer chemopreventive properties [9,10].

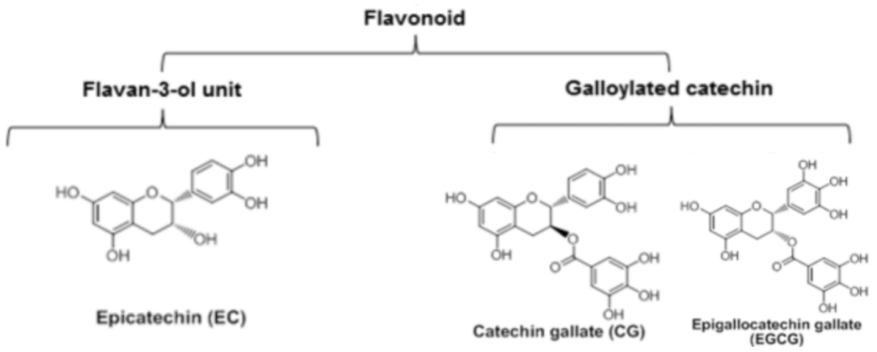

Figure 1. Chemical structures and classifications of EC, CG, and EGCG. EC, CG and EGCG are tea polyphenols that are classified as flavonoids. Flavonoids are further classified into two subgroups: the flavan-3-ol unit group and the galloylated catechin group. EC falls under the flavan-3-ol unit because, unlike CG and EGCG, it lacks a gallic acid group. [8]

Chemicals can promote apoptosis, the programmed process in which a cell is unable to maintain viability and dies, directly or indirectly by inhibiting the mechanisms that would allow cancerous cells to rapidly proliferate [11]. Studies have established that CH, CG, and EGCG induce apoptosis in breast cancer cells in a time and dose-dependent manner [12-15]. These pro-apoptotic effects often take place at the checkpoints of the cell cycle, and affect the initiation, promotion, and progression of carcinogenesis [12]. A concentration of 20μM EGCG inhibits the growth of MCF-7 cells by 75% and induces apoptosis by 43% [13]. Similarly, it has been shown that CH promotes apoptosis and inhibits growth in MCF-7 cells at higher concentrations [14]. Research determined that at 24 hours, 40.7% of 150 μM CH-treated MCF-7 cells undergo apoptosis, whereas almost 100% of them would lose their membrane integrity and viability after 72 hours under the same concentration [14]. Effects of CG on the MCF-7 cell line are very similar. At 48 hours of 25μM CG exposure, 20% of cells had their growth inhibited. At 50μM in the same amount of time, cell growth inhibition increased to about 40% [15]. In these studies that involved MCF-7 cells, the induction of apoptosis and reduced cell proliferation was

accompanied with the increase in expression of similar functioning proteins for each chemical. Most of the EGCG-treated cells remained at the G2/M phase of the cell cycle and interacted with proteins Caspase-9, Caspase-3, and PARP-1, significantly increasing their expression levels [13]. Furthermore, treatment with CH also increased mRNA levels of Caspase-3, Caspase-9, and in the addition of Caspase-8, and TP53. At 24 hours and under a concentration of 150μg/mL, Caspase-3 expression went up 5.81-fold, Caspase-8 1.42-fold, Caspase-9 3.29-fold, and TP53 2.68-fold, all with respect to the control group [14]. This implies that CH is inhibiting a regulatory element that would otherwise promote the expression of these pro-apoptotic proteins. In other research, it has been shown that CG inhibits expression of anti-apoptotic proteins JAK2α and CK2α [15]. Overall, catechins have the ability to promote apoptosis and inhibit cell proliferation in MCF-7 breast cancer cells.

MicroRNAs (miRNA) are small non coding RNA molecules, approximately 20-25 nucleotides in length, that are responsible for regulating gene expression [16]. They are a relatively new discovery in the field of molecular biology and scientists have just recently begun to understand exactly what they are and what functions they have. miRNAs are able to control whether or not the messenger RNA (mRNA) will be translated into proteins by binding to specific sections of the mRNA which would then target them for degradation [17]. In addition, one miRNA can regulate multiple mRNAs and therefore have an effect on a wide variety of biological pathways [16]. It has been shown that in diseases, miRNA can become deregulated and change the way that they function and affect multiple biological processes [18]. Studies have shown that miRNA expression becomes deregulated in breast cancer cell lines and tissues, and researchers were able to identify that 29 miRNAs that had significant changes in their expression including *miR-10b, miR-125b, miR145, miR-21*, and *miR-155* [19].

Studies have shown that polyphenols, including catechins, have the potential to change miRNA expression. Polyphenols such as resveratrol, EGCG, genistein, curcumin, quercetin and camptothecin have been shown to alter miRNA gene expression in pancreatic, lung, colon, prostate, breast and bladder cancer cell lines [20]. Studies with catechins have found that EGCG can up-regulate 13 miRNAs and it can down-regulate 48 miRNAs in hepatocellular carcinoma cells [21]. Other studies found specific effects for EGCG in human nasopharyngeal carcinoma cells and hepatic cells [22,20]. Exposure of nasopharyngeal carcinoma cells to 40 μM of EGCG up-regulated *miR-1246*, and exposure of hepatic cells to 50 μM of EGCG decreased expression of *miR-33a* and *miR-122* levels [22,20]. This last study also found that EC exposure could lead to changes in the expression of *miR-122*, suggesting that multiple catechins can impact miRNA expression [22,20]. No study to date has investigated the expression of miRNA in breast cancer cells. However, exposure of the MCF-7 cell line to 200 μM of resveratrol led to changes in the expression of 36 miRNAs involved in the regulation of apoptosis. The most affected miRNAs were *miR-542-3p* and *miR-125b-5p*, both downregulated after resveratrol exposure [23]. The study also showed that proteins that initiate apoptosis such as Caspase-8 and -9 were activated [23]. This research suggests that the pro-apoptotic effect observed in breast cancer cells may be the result of the deregulated miRNA expression. Here, we investigate whether catechins such as EGCG, EC, and CG alter the expression of *miR-125b-5p* and hypothesize that the polyphenolic catechins EGCG, EC, and CG will decrease the expression of *miR-125b-5p*.

MATERIALS & METHODS

Treatment of MCF-7 cells with catechins

MCF-7 cells were grown in Dulbecco's Modified Eagle Media (DMEM) supplemented with 15% Fetal CBovine Serum (FBS) and Penicillin-Streptomycin, at 5% carbon dioxide and 37 °C. Cells were seeded in a 6-well cell culture plate at 37°C and 5% CO2 overnight. Cells were treated with 25µM and 50µM of EGCG, CG and CH. Duplicate treatment were done for each catechin, and untreated cells were used as the control. The cells were incubated at 37°C for 48 hours. At the end of the exposure period, cells were washed with Phosphate Buffered Saline (PBS) and trypsinized to detach adherent cells from the bottom of the culture dish. Cells were then spun down in a microcentrifuge. Total RNA was extracted from the cells using Direct-zol RNA extraction kit (Zymo Research).

Reverse Transcription

RNA extracted from cells was used to carry out a reverse transcription reaction to make cDNA. The miScript II RT Kit from QIAGEN was used. Each reaction was prepared using 4 µL of 5x miScript HiSpec Buffer, 2 µL of miScript Nucleics Mix, 2 µL of miScript Reverse Transcriptase, 3 µL of the RNA extracted from cells and 9 µL of RNase-free water. Each tube was then gently mixed, centrifuged, incubated at 37°C for 60 minutes, and then incubated again at 95°C for 5 minutes.

Quantitative Polymerase Chain Reaction (qPCR)

After incubation, 2.5 µL of cDNA from each sample was dispensed in a 96-well plate to carry out qPCR for the quantification of *miRNA-125b-5p*. A reaction mixture for

miRNA-125b-5p was prepared using 12.5 µL of 2x QuantiTect SYBR Green PCR Master Mix, 2.5 µL 10x miScript Universal Primer, 5 µL 10x miScript Primer Assay, and 2.5 µL RNase-free water. Another reaction mixture for RNU-6 was prepared using 12.5 µL 2x QuantiTect SYBR Green PCR Master Mix, 2.5 µL 10x miScript Universal Primer, 2.5 µL 10x miScript Primer Assay, and 5 µL RNase-free water. 22.5 µL of both mixtures were added to each well containing cDNA. The plate was then tightly sealed and centrifuged. Lastly, the plate was placed in a real-time cycler that was programmed according to the following conditions for 40 cycles: initial PCR activation step set at 95ºC for 15 min; denaturation at 94ºC for 15 seconds; annealing at 55ºC for 30 seconds; extension at 70ºC for 30 seconds.

Analysis of qPCR by Relative Quantification

We analyzed relative *miR-125b-5p* expression using the $2^{-\Delta\Delta CT}$ method [24]. This method allows for the analysis of the expression of a target miRNA to be compared between various samples. We used *RNU6* as our target miRNA. To calculate the relative expression we used the equation:

$$\Delta\Delta CT = (C_{T,RNU6} - C_{T,miR-125b-5p})_{\ Exposure\ 25\mu M\ or\ 50\mu M} - (C_{T,RNU6} - C_{T,miR-125b-5p})_{\ Exposure\ 0} \quad [24]$$

The results were expressed as mean and standard deviation.

RESULTS

The purpose of our research was to test whether treating MCF-7 breast cancer cells with EC, CG and EGCG will alter the expression of *miR-125b-5p*. qPCR amplification was performed to quantify how *miR-125b-5p* expression levels were affected by the three different catechins.

Relative qPCR amplification showed that for each cell treated with a catechin, the relative expression of *miR-125b-5p* differed from that of the untreated cells.

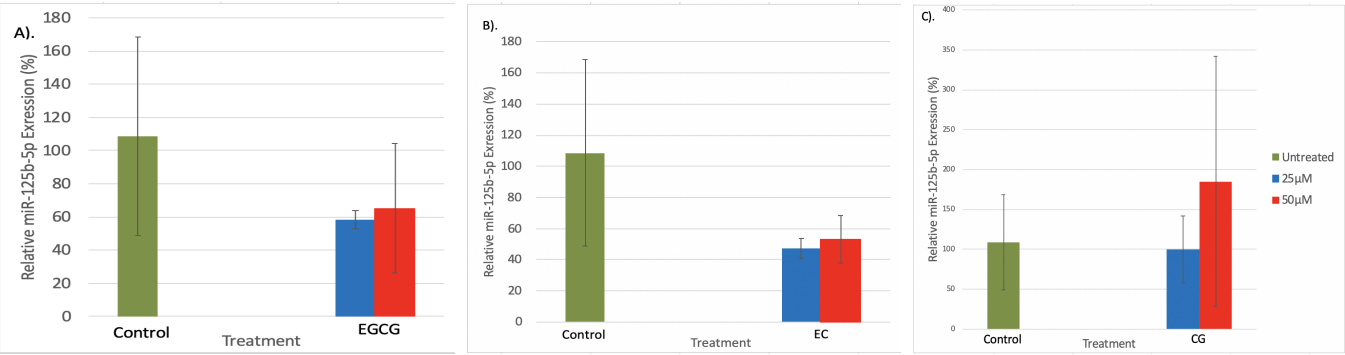

Figure 2. Relative expression of *miR-125b-5p* after 48-hour exposure to EGCG, EC, and CG in MCF-7 breast cancer cells with RNU6 as reference gene. (A) EGCG-treated MCF-7 cells at 25µM and 50µM and compared to untreated cells. (B) EC-treated MCF-7 cells at 25µM and 50µM and compared to untreated cells. (C) CG-treated MCF-7 cells at 25µM and 50µM and compared to untreated cells.

Table 1. Relative expression of *miR-125b-5p* after 48-hour exposure to EGCG, EC, and CG.

Treatment	(Control) Percentage	25µM	50µM
Control	108.57 ± 59.79 %		
CG		99.85 ± 41.75 %	184.92 ± 156.86 %
EC		47.15 ± 6.54 %	52.84 ± 15.29 %
EGCG		58.32 ± 5.63 %	65.10 ± 38.93 %

At 25µM and 50µM, EGCG decreased the amount of *miR-125b-5p*. At 25µM, expression was 58.32 ± 5.63% whereas at 50µM, the relative expression increases to 65.10 ± 38.9%. A similar alteration is shown in EC, where both concentrations lead to a downregulation of miRNA expression. At 25µM, the relative expression was 47.15 ± 6.54%, whereas the expression was about 52.8 ± 15.3% at 50µM. Unlike EGCG and EC, the CG-treated MCF-7 cells had an expression closer to that of the control. 25µM of CG resulted in a relative expression of 99.85 ± 41.75%. Furthermore, 50µM of CG treatment had a considerably upregulated expression of

miR-125b-5p, with expression levels being approximately 184.92 ± 156.86 % (Figure 2 and Table 1).

DISCUSSION & CONCLUSIONS

We hypothesized that *miR-125b-5p* levels would decrease in the presence of EC, CG and EGCG in MCF-7 breast cancer cells because previous published findings have shown that resveratrol, another polyphenol, downregulates the expression of *miR-125b-5p* in the same cell line and, as a result, promotes apoptosis (Figure 3) [25]. In our experiments, exposure to both EC and EGCG at all concentrations resulted in the downregulation of *miR-125b-5p* expression while exposure to 50μM CG resulted in an upregulation of miRNA expression. Other preceding studies have shown that exposure to catechins and loss of *miR-125b-5p* promotes apoptosis [12-15, 19,23]. Therefore, it follows that EC and EGCG, like resveratrol, could possibly induce apoptosis by decreasing levels of *miR-125b-5p* [25].

Past studies also show that EGCG is the most successful catechin in promoting apoptosis by reacting with reactive oxygen species (ROS) to reduce toxic free radicals that lead to cancer [12]. The generation of ROS by cells contributes to host defense mechanisms and tissue damage and is in general associated with the induction of apoptosis [26]. It has also been reported that EC is the least effective in combating cancer since it lacks the ability to inhibit cell growth and endogenous Activator Protein 1 (AP-1) activity [7]. Much of the catechins' anticancer properties come from their structures [5,7-9]. For example, gallic acids

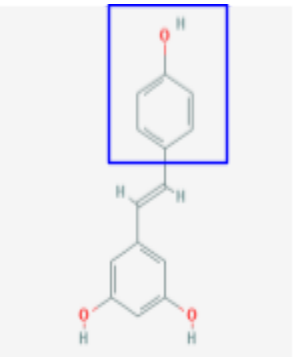

Figure 3. Structure of Resveratrol.
The presence of a phenol functional group (in blue box) makes resveratrol a natural phenol like green tea catechins. [27]

have been shown to increase the antiproliferative effects of compounds significantly and only EC lacks a gallic acid while CG and EGCG are galloylated (Figure 1) [7,8]. Moreover, although CG and EGCG possess similar structures with a gallate group at position three of the C-ring, two hydroxyl groups at the A-ring and two or three hydroxyl groups at the B-ring, only EGCG contains an additional 5'-OH group in the B-ring [5]. Therefore, this suggests that the presence of both gallic acid and 5'-OH makes EGCG more effective than EC and CG. This analysis supports our data because EGCG downregulated the expression of *miR-125b-5p* the most and thus is the catechin most likely to promote apoptosis [12, 26].

If our results are confirmed through future testing of EC, EGCG and CG at higher concentrations, then this would suggest that the moiety shared by EGCG and CG is not involved in the *miR-125b-5p* mediated induction of apoptosis by catechin. It also might suggest that CG uses mechanisms alternate to upregulating *miR-125b-5p* to promote apoptosis. For example, CG might affect the expression of other miRNAs that stimulate apoptosis such as *miR-200c-3p, miR-409-3p, miR-122-5p* and *miR-542-3p* [25].

Limitations of our experiment include that it was only performed twice and high standard deviations suggested that there were large variabilities in miRNA expression levels for all catechins at both concentrations. Carrying out additional experiments would help us understand the strength of the associations observed. In addition, the catechins were tested at only two concentrations of 25μM and 50μM. Higher concentrations could affect miRNA expression levels to a greater extent and thus provide more information on the relationship between exposure to catechins and *miR-125b-5p* expression levels. However, by using the *RNU6* gene as our reference, we normalized our data. *RNU6* has been statistically proven to be a reliable reference RNA target for the standardization of miRNA qPCR data [28]. We also used methods that have

been performed many times before with reliable results. For instance, qPCR has been used frequently to measure levels of expression of miRNAs.

Although many previous experiments have established that catechins have the ability to induce apoptosis in breast cancer cells, the effect of EC, CG and EGCG on *miR-125b-5p* has never been tested before. Our study presents innovative research on the relationship between green tea catechins and the levels of *miR-125b-5p* in MCF-7 breast cancer cells.

REFERENCES

1. Bray, Freddie & Ferlay, Jacques & Soerjomataram, Isabelle & Siegel, Rebecca & Torre, Lindsey & Jemal, Ahmedin. (2018). **Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries: Global Cancer Statistics 2018.** CA: A Cancer Journal for Clinicians.
2. Wu AH, Tseng CC, Van Den B, Yu MC: **Tea intake, COMT genotype, and breast cancer in Asian-American women.** Cancer Research 2003.
3. Wu AH, Yu MC, Tseng CC, Hankin J, Pike MC: **Green tea and risk of breast cancer in Asian Americans.** International Journal of Cancer 2003.
4. Nakachi K, Suemasu K, Suga K, Takeo T, Imai K, Higashi Y: **Influence of drinking green tea on breast cancer malignancy among Japanese patients.** Japanese Journal of Cancer Research 1998.
5. Balentine, D. A., Wiseman, S. A., and Bouwens, L. C. M. **The chemistry of tea flavonoids.** Crit. Rev. Food Sci. Nutr.
6. Fantini, Massimo & Benvenuto, Monica & Masuelli, Laura & Frajese, Giovanni & Tresoldi, Ilaria & Modesti, Andrea & Bei, Roberto. (2015). **In Vitro and in Vivo Antitumoral Effects of Combinations of Polyphenols, or Polyphenols and Anticancer Drugs: Perspectives on Cancer Treatment.** International Journal of Molecular Sciences.
7. Sur, Subhayan & Panda, Chinmay. (2017). **Molecular aspects of cancer chemopreventive and therapeutic efficacies of tea and tea polyphenols.** Nutrition.
8. Arts, Ilja & Van de Vijver, C.A.D.M. & Hollman, Peter. (2000). **Catechin Contents of Foods Commonly Consumed in The Netherlands.** 1. Fruits, Vegetables, Staple Foods, and Processed Foods. Journal of agricultural and food chemistry.
9. Kürbitz, Claudia & Heise, Daniel & Redmer, Torben & Goumas, Freya & Arlt, Alexander & Lemke, Johannes & Rimbach, Gerald & Kalthoff, Holger & Trauzold, Anna. (2011). **Epicatechin gallate and catechin gallate are superior to**

epigallocatechin gallate in growth suppression and anti-inflammatory activities in pancreatic tumor cells. Cancer science.

10. Chung, JY & Huang, Cikun & Meng, X & Dong, Z & Yang, Chwei-Shiun. (1999). **Inhibition of Activator Protein 1 Activity and Cell Growth by Purified Green Tea and Black Tea Polyphenols in H-ras-transformed Cells: Structure-Activity Relationship and Mechanisms Involved** Cancer research.
11. Elmore, S. (2007). **Apoptosis: A Review of Programmed Cell Death**. *Toxicologic Pathology*, *35*(4), 495–516. https://doi.org/10.1080/01926230701320337
12. Ahmad, N., Feyes, D. K., Agarwal, R., Mukhtar, H., & Nieminen, A.-L. (1997). Green Tea Constituent **Epigallocatechin-3-Gallate and Induction of Apoptosis and Cell Cycle Arrest in Human Carcinoma Cells**. JNCI Journal of the National Cancer Institute, 89(24), 1881–1886. doi:10.1093/jnci/89.24.1881
13. Lingling Zan, Qingfeng Chen, Lei Zhang & Xiaona Li (2019) **Epigallocatechin gallate (EGCG) suppresses growth and tumorigenicity in breast cancer cells by downregulation of miR-25**, Bioengineered, 10:1, 374-382, DOI: 10.1080/21655979.2019.1657327
14. Alshatwi AA. **Catechin hydrate suppresses MCF-7 proliferation through TP53/Caspase-mediated apoptosis**. *J Exp Clin Cancer Res*. 2010;29(1):167. Published 2010 Dec 17.
DOI: 10.1186/1756-9966-29-167
15. Afsar T, Trembley JH, Salomon CE, Razak S, Khan MR, Ahmed K. **Growth inhibition and apoptosis in cancer cells induced by polyphenolic compounds of Acacia hydaspica: Involvement of multiple signal transduction pathways**. Sci Rep. 2016;6:23077. Published 2016 Mar 15. doi:10.1038/srep23077
16. Macfarlane LA, Murphy PR. **MicroRNA: Biogenesis, Function and Role in Cancer**. *Curr Genomics*. 2010;11(7):537–561. doi:10.2174/138920210793175895
17. Guo H, Ingolia NT, Weissman JS, Bartel DP. **Mammalian microRNAs predominantly act to decrease target mRNA levels.** Nature. 2010;466(7308):835. doi: 10.1038/nature09267.
18. Iorio, M. V., Ferracin, M., Liu, C.-G., Veronese, A., Spizzo, R., Sabbioni, S., … Croce, C. M. (2005). **MicroRNA Gene Expression Deregulation in Human Breast Cancer**. *Cancer Research, 65(16), 7065–7070.* doi:10.1158/0008-5472.can-05-1783
19. Bartel, D., Chen, C. **Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs**. *Nat Rev Genet* **5,** 396–400 (2004) doi:10.1038/nrg1328
20. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3902894/
21. Tsang, W. P., & Kwok, T. T. (2010). **Epigallocatechin gallate up-regulation of miR-16 and induction of apoptosis in human cancer cells**. *The Journal of Nutritional Biochemistry, 21(2), 140–146.* doi:10.1016/j.jnutbio.2008.12.003

22. Li BB, Huang GL, Li HH, Kong X, He ZW. **Epigallocatechin-3-gallate Modulates MicroRNA Expression Profiles in Human Nasopharyngeal Carcinoma CNE2 Cells**. *Chin Med J (Engl)*. 2017;130(1):93–99. doi:10.4103/0366-6999.196586
23. Venkatadri, R., Muni, T., Iyer, A. *et al.* **Role of apoptosis-related miRNAs in resveratrol-induced breast cancer cell death**. *Cell Death Dis* **7,** e2104 (2016) doi:10.1038/cddis.2016.6
24. Livak, K.J. & Schmittgen, Thomas. (2001). **Analysis of relative gene expression data using real-time quantitative PCR and the 2-DDCt method**. *Methods*. 25. 402–408. doi:10.1006/meth.2001.1262
25. Venkatadri R, Muni T, Iyer AK, Yakisich JS, Azad N. **Role of apoptosis-related miRNAs in resveratrol-induced breast cancer cell death**. *Cell Death Dis.* 2016;7:e2104. doi: 10.1038/cddis.2016.6.
26. Simon HU, Haj-Yehia A, Levi-Schaffer F. **Role of reactive oxygen species (ROS) in apoptosis induction**. *Apoptosis.* 2000;5:415-41811256882
27. National Center for Biotechnology Information. PubChem Database. Resveratrol, CID=445154, https://pubchem.ncbi.nlm.nih.gov/compound/Resveratrol (accessed on Dec. 13, 2019)
28. Peltier, H. J. & Latham, G. J. **Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues**. *RNA* 14, 844–852 (2008).
29. Yang, Chung & Wang, Hong. (2016). **Cancer Preventive Activities of Tea Catechins.** Molecules.
30. Seeram, Navindra & Zhang, Yanjun & Nair, Muraleedharan. (2003). **Inhibition of Proliferation of Human Cancer Cells and Cyclooxygenase Enzymes by Anthocyanidins and Catechins**. Nutrition and cancer. 46. 101-6. 10.1207/S15327914NC4601_13.
31. Lim, L., Lau, N., Garrett-Engele, P. *et al.* **Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs**. *Nature* 433**,** 769–773 (2005) doi:10.1038/nature03315