Gene Expression Analysis of Resveratrol Treated vs. Media Murine Melanoma Cells

Introduction

Cancer is a group of diseases that involves abnormal cell growth that can invade vital organs of the human body. Cancer has many forms of treatment. Some can help eliminate the cancer or tumors itself, while some treatments can just lessen the effects of it. Chemotherapy is the most common cancer treatment available for patients. However, there are some new experiments and drugs being tested often for cancer research. One of them is resveratrol. Resveratrol is a phytoalexin found in grapes, red wine, and other foods that has been shown to have antioxidant, anti-inflammatory, and anti-cancer properties (Platella et al. 2017). It has also been found that resveratrol can inhibit cellular events associated with tumor initiation, promotion, and progression (Jang et al. 1997). A previous experiment done showed how resveratrol decreases the regulation of androgen receptors through a post-translational mechanism. This decrease slows the development and regulation of prostate cancer (Harada et al. 2007). Another study with prostate cancer showed that resveratrol along with low dosages of docetaxel, a chemotherapy drug, could lead to positive results in treatment (Singh et al. 2017).

Resveratrol also inhibits some signal transduction pathways that control proliferation, apoptosis, metastasis, and angiogenesis. The main focus for this experiment was focused on apoptosis and how resveratrol ties in with that mechanism. Apoptosis is the distinct mode of cellular death that is responsible for cell deletion in normal tissues (Kerr et al. 1994). The genes of interest in this experiment are Rb, p21, and p53. Rb (retinoblastoma protein) is a tumor suppressor protein that is usually dysfunctional in most cancers. p21 also has a similar function of suppressing tumors and plays a role in apoptosis. p53 plays a role in growth arrest which stops the process of the cell cycle and can prevent replication of damaged DNA (Shen and White 2001). In an article by García-Tuñón et al., the authors explained how certain fluctuations in Rb, p21, and p53 can hinder cell proliferation, more specifically growth factors for benign and carcinomatous tumors. This is also true for canine melanoma cells, a common tumor in dogs (Koenig et al. 2002).

The purpose of the lab was to measure gene expression of Rb, p21, and p53 in cancer cells treated with resveratrol and compare it to media melanoma cells. The scientific question in this lab poses the question about how gene expression is altered in Rb, p21, and p53 after murine melanoma cells are injected with resveratrol. The hypothesis was that gene expression in Rb, p21, and p53 will all increase in treated cancer cells.

Materials and Methods

*RNA isolation*

The goal of this method was to isolate RNA from B16 murine melanoma cells both from untreated and treated with resveratrol. This was completed through the spin protocol. 175 μl of RNA Lysis Buffer was added to two tubes, one with media melanoma cells and one with treated cells. 350 μl of RNA Dilution Buffer was added to the tubes and inverted a couple of times. The tubes were then centrifuged for 10 minutes and then transferred to fresh tubes. 200 μl of 95% ethanol was added to the tubes and mixed well. The tubes were then transferred to spin basket assembly tubes, centrifuged for 1 minute, and the eluate was discarded. 600 μl of RNA Wash Solution was added to the tubes, centrifuged for 1 minute, and the eluate was discarded. Then the DNase incubation mix was prepared and 50 μl of it was added to the tubes. The tubes incubated at room temperature for 15 minutes. 200 μl of DNase Stop Solution was added to the tubes and centrifuged for 1 minute. 600 μl of RNA Wash Solution was added to the tubes and centrifuged for 1 minute. Then 250 μl of RNA Wash Solution was added to the tubes, centrifuged for 2 minutes, and transferred to spin basket elution tubes. Lastly, 100 μl of Nuclease-Free Water was added to the tubes and centrifuged for 1 minute.

*cDNA synthesis*

The isolated RNA (9 μl) and reverse transcription primer (1 μl) were mixed in reaction tubes. The GoScript Reaction Mix (1.5 μl of nuclease-free water, 4 μl of GoScript Reaction Buffer, 2 μl of Magnesium Chloride, 1 μl of PCR Nucleotide Mix, 0.5 μl of Recombinant RNasin Ribonuclease Inhibitor, and 1 μl of GoScript Reverse Transcriptase) was prepared and then combined with the RNA and reverse transcription primer in reaction tubes. Then the cDNA was synthesized in the thermocycler using the following reaction: Anneal at 25 degrees Celsius for 5 minutes, extend at 42 degrees Celsius for 1 hour, and inactivate at 70 degrees Celsius for 15 minutes. Then the tubes were stored at -20 degrees Celsius for a week.

*RT-PCR*

20 μl of cDNA of both treated and media cells were diluted with 180 μl of nuclease-free water. 2 μl of the cDNA for media was put into 3 wells in the A through D rows in the well holder. 2 μl of cDNA for treated cells was put into 3 wells in the E through H rows in the well holder. The GoTaq qPCR Reaction Mixes (Master Mix, nuclease-free water, forward and reverse qPCR primers) were prepared at room temperature. A total of 4 mixes were used in this experiment. One contained B-actin primers. The second one contained Rb. The third one contained p21. The fourth one contained p53. Table 1 has the full primers listed. The primer containing B-actin was put into 3 wells in rows A and E. The primer containing Rb was put into 3 wells in rows B and F. The primer containing p21 was put into 3 wells in rows G and C. Lastly, the primer containing p53 was put into 3 wells in rows D and H. The well holder was placed into the thermocycler and underwent the following cycles: 1 cycle of GoTaq Hot Start Polymerase activation at 95 degrees Celsius for 2 minutes, 40 cycles of Denaturation at 95 degrees Celsius for 15 seconds and Annealing/Extension at 60 degrees Celsius for 1 minute, and finally 1 cycle of Dissociation at 60-95 degrees Celsius.

Table 1. Primers used in the PCR Reaction Mixes.

|  |  |
| --- | --- |
| **Gene** | **Primers** |
| p21 | Forward: AGAGAAAAGCCCGTACTTTCAG  Reverse: GGGCAGCCTGTGATTCCAT |
| Rb | Forward: TTCCAGCCTTACCTCGACTCC  Reverse: TCGGCTCCAGATGAACTTCCA |
| p53 | Forward: TCACAGCGTCTGTTGACATTT  Reverse: ACCAAGCTCATTACCCTGACA |
| Β-actin | Forward: GTGTGATGGTGGGAATGGGTCAGA  Reverse: TACGACCAGAGGCATACAGGGACA |

*Data analysis*

For data analysis, Ct values were found by RT-PCR and then fold change expression was quantitated for Rb, p21, and p53 using the “delta delta Ct” method. The genes of interest were normalized to B-actin (the constant or the housekeeping gene in this experiment), then the normalized values were used to compare the media and resveratrol samples. Bar graphs of these values were then constructed.

Results

After the completion of RNA isolation, cDNA synthesis, and RT-PCR, Ct values were found for the media and treated cells. Table 2 has all the values listed below. A through D are the media cells with B-actin, Rb, p21, and p53. The treated cells are rows E through H.

Table 2. RT-PCR Ct Values.

|  |  |  |  |
| --- | --- | --- | --- |
|  | 1 | 2 | 3 |
| A | 18.23 | 18.25 | 18.34 |
| B | 32.53 | 32.62 | 32.64 |
| C | 35.62 | 35.24 | 35.26 |
| D | 30.26 | 30.24 | 30.21 |
| E | 18.56 | 18.57 | 18.53 |
| F | 28.65 | 28.57 | 28.59 |
| G | 29.68 | 29.67 | 29.33 |
| H | 28.65 | 28.67 | 28.34 |

The numbers in the rows are the same but just a little off by decimal points. That shows consistency in micropipetting the cDNA and primers into the wells.

Table 3. Delta Ct Values.

|  |  |
| --- | --- |
| Rb (Media) | 14.32334 |
| p21 (Media) | 17.1 |
| p53 (Media) | 11.96334 |
| Rb (Resveratrol) | 10.05 |
| p21 (Resveratrol) | 11.09667 |
| p53 (Resveratrol) | 10 |

In Table 3, delta Ct values were found by subtracting the Ct value of the gene of interest from the B-actin Ct value.

Table 4. Delta Delta Ct Values.

|  |  |
| --- | --- |
| Rb | 4.27334 |
| p21 | 6.0033 |
| p53 | 1.9633 |

In Table 4, delta delta Ct values were found by subtracting the treated gene of interest from the media one. Then, those values were put into the equation 2X to compared the change in gene expression to the media cells. The Rb value was 19.33764226. The p21 value was 64.14789414. The p53 value was 3.899637443. These numbers indicate a positive increase in gene expression of these genes of interest in the resveratrol treated cells. These trends are shown in the Figure 1,2, and 3 below.

Figure 1. Fold changes in media and treated B16 murine melanoma cells with Rb. The increase in gene expression the resveratrol treated cells is significantly higher than the media cells.

Figure 2. Fold changes in media and treated B16 murine melanoma cells with p21. The increase in gene expression the resveratrol treated cells is significantly higher than the media cells.

Figure 3. Fold changes in media and treated B16 murine melanoma cells with p53. The increase in gene expression the resveratrol treated cells is significantly higher than the media cells.

T-tests were done to show whether or not there were significant differences between the media and treated genes of interest. The three values were below 0.001, so there were significant differences between the media and resveratrol treated melanoma cells.

Discussion

Overall, the goal of this lab was completed successfully. We are able to measure gene expression of Rb, p21, and p53 in cancer cells treated with resveratrol We also were able to compare it to media melanoma cells. The hypothesis of the experiment was that gene expression in Rb, p21, and p53 will all increase in treated cancer cells and that was proven to be true for all three genes. The results showed that there was an increase in gene expression in Rb, p21, and p53 in the cells with resveratrol compared to media cells. The increase in the gene expression for these specific genes indicates that apoptosis is induced often in cancer cells that are injected with resveratrol. This pattern indicts that resveratrol is a source of a decrease in cancer cells in the body and that is can be a good preventative of deadly cancers that can potential form in a person’s body.

If this experiment were to be repeated again, I would pick out three different genes to study in melanoma cells, possibly ones that are related to a different process of the cell cycle. However, I think the results that are presented here are very hopefully for future cancer research and the creation of new treatments. Seeing how resveratrol increases gene expression in the apoptosis mechanisms is promising for cancer treatment. Maybe a new drug can be created from these results and further testing can be done also.

Works Cited

García-Tuñón I, Ricote M, Ruiz A, Fraile B, Paniagua R, Royuela M. Cell Cycle Control Related Proteins (p53, p21, and Rb) and Transforming Growth Factorβ(TGFβ) in Benign and Carcinomatous (In Situ and Infiltrating) Human Breast: Implications in Malignant Transformations. Cancer Investigation. 2006;24(2):119–125.

Harada N, Murata Y, Yamaji R, Miura T, Inui H, Nakano Y. Resveratrol Down-Regulates the Androgen Receptor at the Post-Translational Level in Prostate Cancer Cells. Journal of Nutritional Science and Vitaminology. 2007;53(6):556–560.

Jang M. Cancer Chemopreventive Activity of Resveratrol, a Natural Product Derived from Grapes. Science. 1997;275(5297):218–220.

Kerr JFR, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer Therapy. Cancer. 1994;73(8):2013–2026.

Koenig A, Bianco SR, Fosmire S, Wojcieszyn J, Modiano JF. Expression and Significance of p53, Rb, p21/waf-1, p16/ink-4a, and PTEN Tumor Suppressors in Canine Melanoma. Veterinary Pathology. 2002;39(4):458–472.

Platella C, Guida S, Bonmassar L, Aquino A, Bonmassar E, Ravagnan G, Montesarchio D, Roviello GN, Musumeci D, Fuggetta MP. Antitumour activity of resveratrol on human melanoma cells: A possible mechanism related to its interaction with malignant cell telomerase. Biochimica et Biophysica Acta (BBA) - General Subjects. 2017;1861(11):2843–2851.

Shen Y, White E. p53-Dependent apoptosis pathways. Advances in Cancer Research. 2001:55–84.

Singh SK, Banerjee S, Acosta EP, Lillard JW, Singh R. Resveratrol induces cell cycle arrest and apoptosis with docetaxel in prostate cancer cells via a p53/ p21 and p27 pathway. Oncotarget. 2017 Jun.