The effect of herbicide glyphosate on the hypermethylation and downregulation of tumour suppressor genes and subsequent breast cancer progression

Introduction

In 2016, an estimated 1,685,210 new cases of cancer will be diagnosed and 595,690 patients of those will perish to the disease. Breast cancer will account for 12% of diagnosed cases, defining it as the second most common cancer [1,2]. As one of the leading causes of death, cancer has become a field of research that is vastly growing in its knowledge to try and combat its growing presence in everyday life. Cancer cells demonstrate six key hallmark features that allow for increased proliferation and cell survival, of which includes evading tumour suppressors [3]. Recent advancements have signalled the field of epigenetics plays an integral role within cancer, for both genetic and epigenetic modifications contribute to the modification of cancer progression [4]. Collectively, alterations to one's genome causes for widespread deregulation of genes and disruption in signalling networks, that will subsequently affect proliferation and cellular functions [4]. This deregulation causes the expression of subsequent tumour suppressor genes to be decreased [4]. DNA methylation, addition of methyl groups to DNA, is now known as one of the key factors in regulating gene expression through inactivation of tumour suppressor genes [4]. CpG islands, short interspersed sequences that are GC-rich and CpG-rich, are normally not methylated within normal somatic cell functioning and are associated with transcription initiation [5]. These sites are then equipped to silence promoting regions through methylation or polycomb recruitment [5]. Hypermethylation within CpG islands, both within the promoter region and throughout the genome is firmly established with gene inactivation, and is found within almost every tumour type. [6].

Considering that 24% of diseases are caused by environmental exposure, a correlation between chemical exposure and epigenetic variation has been established [7]. Glyphosate, the most common agricultural herbicide, is a growing contributor of the influence of chemical exposure upon the development of diseases [7]. Through determining the regulatory effects of glyphosate upon methylation of promoter and CpG island sites of tumour suppressor genes within breast cancer, a correlation between breast cancer progression and glyphosate exposure of these sites can be established. Glyphosate is linked to global hypermethylation and to CpG island sites within the human genome [8]. Yet, the direct link between glyphosate and tumour formation is yet to be established. Protein expressional analysis, through real-time PCR (rtPCR) of human tumour samples in comparison to non-diseased samples will determine the downregulation of the tumour suppressor genes. Luminometric Methylation Assay (LUMA) and quantitative multiplex-methylation specific PCR (QM-MSP) will determine the percent methylation extent within different genes' CpG islands sites and promoter regions of the tissue sample. Exposure of glyphosate to rats allows for the direct correlation, in a live animal, between the toxin and tumour formation, with subsequent expression and hypermethylation analysis to expose the molecular level of influence. The exposure of glyphosate to various cells lines will allow for analysis of genomic hypermethylation without external influence from other biological processes occurring within an animal, but still display a direct correlation between exposure and cancer progression.

A link between exposure and cancer progression of cancer is integral in trying to combat the ever-growing disease. We are trying to combat the disease when it has already taken over our body. Through establishing the source of the cancer, this can eliminate pain and suffering of many individuals, as well as money and time. By establishing that glyphosate is linked to cancer and hypermethylation of the genome, this can allow for subsequent regulation of use and decrease in overall cancer cases being diagnosed. This research can also allow for further investigation into various other herbicides and their impact on human health.

Ethical considerations must be considered when obtaining human tumours and non-diseased tissue samples, along with the utilization of rats. Consent with the patient and federal regulations must be obtained, as well as the assurance for well-designed and effective research to be completed with samples [9]. Research conducted upon rats must be approved by the IACUC board and its ethics committee. Pain and suffering levels must be weighed against the beneficence of the study.

If successful, the proposed research will determine of the effects of the herbicide glyphosate on the hypermethylation and downregulation of tumour suppressor genes in the progression of breast cancer.

Hypermethylation, a significant increase in methylation compared to normal basal level, can lead to down regulation due to decrease of expression from the disruption of transcriptional activity when present in CpG islands and promoters [4]. Tumour suppressor genes negatively regulate cell proliferation, protecting cells from multiplying uncontrollably. Furthermore, when genetically or epigenetically modified, they can result in uncontrollable cell growth [3]. Recent studies have shown a link between DNA damage and methylation induced by glyphosate, through determining the global and CpG islands percent methylation within genes p16 and p53 within peripheral blood mononuclear cells [8]. Yet, this study doesn't relate directly to the causation of cancer, for only methylation was investigated. Within breast cancer CpG islands within the p53 promoter are commonly hypermethylated and downregulated, causing for cancer progression [10]. Hundreds of hypermethylated genes have been associated with breast cancer, but APC, RASSF1A, ERα, CDH1 and Cyclin D2, APC, BIN1, BRCA1, CST6, GSTP1, P16, P21 and TIMP3, and P53 are the most common to be hypermethylated and downregulated and have been observed to be significant for the progression of breast cancer [10,11].

By proposing experiments that are able to link the toxin glyphosate directly to the disease, a direct correlation can be established instead of establishing the toxin effect solely on the genome, or how the hypermethylation and expressional levels of tumour suppressor genes allow for cancer progression. Obtaining human tumours and non-diseased tissue allows for the expressional level of tumour suppressor genes to be determined through rtPCR, with Ct value ratios against ß actin determining expressional levels in comparison to non-diseased adjacent tissue. Utilizing rats as a test subject for exposure allows for direct observation and testing of the cancers and diseases that arise when glyphosate is present. Exposure of glyphosate to non-cancerous cell lines investigates the direct effect of the toxin upon cells, rather than being processed through an organism's metabolism. The difference between hypermethylation and expression of tumour suppressor genes between human tissue samples will establish the effect of one's metabolism of the toxin and subsequent reactions to create another chemical form within the body to cause these epigenetic changes.

Expression analysis of tumour suppressor genes will be performed on the human tissue samples, rat tumour sample, and various cell lines. Homogenization of tissue, purification of mRNA and conversion to cDNA and amplification through rtPCR, allows for the Ct values to represent the relative fold decrease ($Ct_{\text{tumour suppressor gene}}/Ct_{\text{ß actin}}$) between both the diseased and non-diseased samples. Percent methylation of DNA will be determined through either quantitative multiplex-methylation specific PCR (QM-MSP) or Luminometric Methylation Assay (LUMA). QM-MSP is a highly sensitive assay specified to determine methylation within breast cancer [12]. It allows for assessment of promoter hypermethylation for many genes simultaneously, with high specificity and detection rates [12]. This allows for a variety of small samples to be analysed from different tissues, co-amplifying and analysing genes within a sample [12]. QM-MSP has a drawback in that the determination is either all or

nothing, due to the inherent nature of a qualitative experiment based on visualization of fluorescent probes signalling methylation. However, it can still detect 1/10,000 specificity [12]. LUMA utilizes a two-restriction enzyme digest, HpaII (CpG specific) and MspI (methylation insensitive) reactions in parallel followed by pyrosequencing [13]. Since it only requires a small amount of DNA, and specificity is high with low variability. This is optimal for human tissue analysis since there is only a small sample of DNA available for analysis [13]. LUMA will be used to determine exact percent methylation if methylation is significantly present within the sample when analysed by QM-MSP. ELISA assays are regularly used, due efficiency and price, within the determination of methylation within the genome, but this technique has high variability, and only gives a rough estimation of the percent methylation [13]. Thus, LUMA will allow for a more accurate and precise determination of percent methylation than previous studies. For proliferation assays CyQUANT® Direct fluorescent molecular probe, that binds to DNA, will be utilized due to the high precision, since DNA replication is highly regulated and such a direct correlation between the number of cells present and fluorescing can give a reading relative to the number of cells present within the well [14].

The proposed research above will be able to tie all three aspects of the toxin, glyphosate, percent methylation and downregulation of tumour suppressor genes, and the progression of breast cancer. Whereas most research has established only the connection between toxin and methylation, or methylation and cancer. This type of experimentation will allow for there to be a direct correlation between the manipulation of toxin administered and the observation and analysis of methylation and expression upon cancer.

Proposed Research

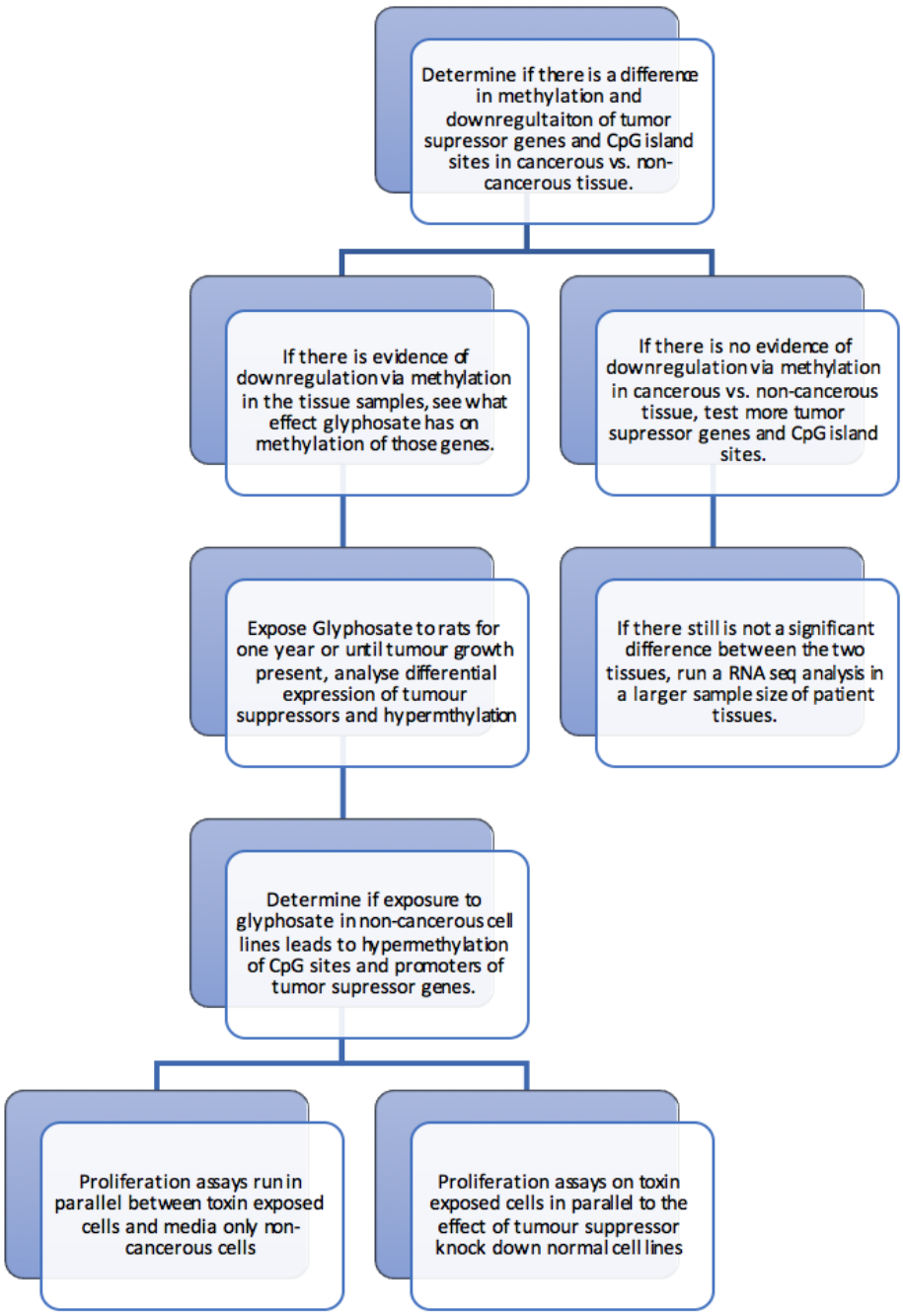

Figure 1. Workflow of the following experiments investigating glyphosate's role in hypermethylation and downregulation in the progression of breast cancer.

In preliminary experiments, tissue of breast cancer and their non-cancerous adjacent tissue pair will be obtained from patients. Breast cancer will be utilized due to the presence of the p53 gene hypermethylation contributing to the formation of breast cancer [10]. Manual homogenization of tissue in liquid nitrogen and RNA purification will be performed upon the various samples and reverse transcribed into cDNA, in order to determine the relative

expression through Ct values under real-time PCR. Real-time PCR allows for the amplification of the respective genes, allowing for only a small sample of cDNA to be utilized in order to determine relative expression. Ratios of the desired gene and ß actin ($Ct_{\text{tumour suppressor gene}}/Ct_{\text{ß actin}}$) will allow for the discrimination of an increase or decrease in comparison to the normal tissue ratios. ß actin is assumed to be moderately expressed under the same level across tissues, encoding for the ubiquitously expressed cytoskeletal protein [15]. With this, the expression of ß actin can be used as an internal control, allowing for a cross comparison of expression between multiple genes and tissue samples. Normal tissues will be established as the control for expression level, and results will be portrayed through fold change difference. It will be expected that the APC, RASSF1A, ERα, CDH1 and Cyclin D2 [11], APC, BIN1, BRCA1, CST6, GSTP1, P16, P21 and TIMP3, and P53 [10] and will be significantly lower than those within the adjacent normal tissue samples. This is due to the assumption that these genes are being hypermethylated, and thus subsequent downregulation of the genes will be observed. If such results are obtained, scrutiny of the percent methylation of the genes that are being downregulated will be conducted through quantitative multiplex-methylation specific PCR (QM-MSP) specific to breast cancer, utilizing methylation and unmethylated sequence specific promoters for each specific gene of interest [8,12]. Multiple runs of QM-MSP will have to be performed on the same tissue sample due to the restriction of only amplifying 5 genes per reaction. LUMA (luminometric methylation assay) will also be performed in order to determine the methylation extent within CpG island sites, due to the specificity of restriction enzyme (HpaII) to CpG island methylation [13]. LUMA has very high precision and low variability, needing only a small sample of DNA in order to determine methylation percent [13]. Parallel reactions will be run with HpaII and MspI (methylation nonspecific) restriction enzymes, with the internal control restriction EcoRi in each condition, followed by pyrosequencing reactions, specific for each gene, in order to allow for light signalling [13]. The ratio of HpaII/MspI will represent the relative methylation of CpG islands within the respective genes [13]. Since CpG island sites are not highly methylated under normal somatic cell conditions, the presence of such methylation will signal such importance in the progression of cancer, due to the disruption of transcriptional mechanisms within the cell [16]. The HpaII/MspI ratio comparisons between the cancerous tissue and normal adjacent tissue will establish the significant difference of fold change in the percent methylation of each CpG island and promoter site in each gene. It is expected that, under both techniques, the ratios would be significantly higher than that of the noncancerous control tissue. If such results are observed, it can be theorized that the hypermethylation of the CpG island sites in the respective genes are causing for there to be less regulation in cell repair mechanisms, thus contributing to the progression of the cancer.

If such genes are not being downregulated and there is no significant difference in the relative expression of each gene, expansion of tumour suppressor genes and CpG island sites will be expanded to investigate if hypermethylation is key to breast cancer development. This will be conducted in the same conditions of the initial experiment for relative expression assessed under rtPCR and relative Ct values. If there is still no significant difference in relative expression of the genes analysis, RNAseq analysis will be conducted in order to determine the relative expression of such genes within a larger sample size of patient tissues, and more tissue samples will be obtained in order in respect to RNAseq results. The same protocol will be followed as above to determine expressional level and methylation. In the case that there is no evidence for hypermethylation of the CpG island sites in tumour suppressor genes, but downregulation is still observed, further investigation will be conducted in order to determine the cause for the downregulation of each gene. New promoter primers will be bought for QM-MSP assays to determine if there is an issue with specificity to the specific methylation sequence.

If subsequent research is successful in determining that downregulation and hypermethylation are present in such genes, the effects that glyphosate, the most widely used herbicide, upon methylation of these genes will be conducted [11]. The pesticide glyphosate is a molecule that affects the methylation of various genes, and is linked to causing hypermethylation in p53, promoter region and global methylation of the genome [8]. Soybeans will be the model crop for the average amount of glyphosate being ingested per day by one person. In genetically modified soybeans an average amount of 3.26mg/kg of glyphosate is present, with a maximum value of 8.8mg/kg of glyphosate present [11]. With the average human consuming 5 servings of vegetables per day the consumption of glyphosate would be 4.375mg through 5 (1 cup) servings of soybeans per day. Sample sizes of 1 mg, 5 mg, and 10 mg of glyphosate will be given to individual populations of rats on identical diets, only varying in amount of glyphosate present, with a control group of zero exposure to glyphosate in food. Food will be tested prior to serving to ensure that there are consistently precise and accurate amounts of glyphosate being administered to the experimental group. For the control group, food will be also tested to ensure that there is no trace of glyphosate. Rats will be given food, with respective amount of glyphosate present, for one year or until tumour growth becomes evident. The manipulation of feeding glyphosate to rats, under control conditions, will be conducted in order to determine the effects of such toxin on the hypermethylation of CpG islands and global-methylation under LUMA and QM-MSP analysis of purified excised tumours. Tumours, and adjacent non-diseased tissue control, will be excised and RNA purified to cDNA, and quantitatively analysed through real time PCR with the same internal control of ß actin to determine the fold change in expression of select tumour suppressor genes. Percent methylation will be obtained from the tissue sample by purifying DNA and using LUMA, HpaII and MspI restriction enzymes with control EcoRi in each condition, followed by pyrosequencing. It is expected that the ratio of HpaII/MspI will be higher in the rat population fed glyphosate, in comparison to that of the no glyphosate control population due to subsequent effect of the toxin upon hypermethylation. For QM-MSP, it is expected that the tumorous tissue will be significantly higher than that of the non-diseased adjacent tissue. If there are tumours present in areas other than breast tissue, they will also be excised and purified for assessment of methylation within tumour suppressor CpG islands and global methylation. If there is a significant difference in the difference between the two samples, cancerous and normal, an increase in methylation for the cancerous sample suggests that the herbicide glyphosate plays a pivotal role in the hyper methylation and subsequent the progression of the respective cancer types. Expressional analysis of the tissue will also be conducted in order to determine the fold difference of presence of tumour suppressors within adjacent non-cancerous tissue and cancerous tissue, in order to support previous research on human tissue. If there is no evidence of tumours forming within the rats, the amount of glyphosate will be increased upon approval of IACUC, and the same experimental procedures will be followed in order to determine its effects upon genome methylation.

Additional research will be conducted within non-cancerous cell lines (Sw527 mammary breast normal, 184a1 mammary gland/breast epithelium, and Hs190t mammary gland, breast) [17]. For the non-cancerous cells, conditions of exposure to glyphosate will allow for direct correlation, observation, and analysis without any internal factors that biological systems present within living organisms could introduce into the experiment. This will determine if glyphosate is the direct correlative to hypermethylation of tumour suppressor genes, or if there is an interaction within the animal body that is causing for it to increase methylation. Non-cancerous cell lines will be treated with 1 mg, 5 mg, and 10 mg of glyphosate, within cell media over conditions of 12-24 hours. A control group will be cultured with no glyphosate given and will follow the identical protocol for analysis of

methylation and expression. Cells will then be homogenized, DNA purified, and run through QM-MSP and LUMA to determine methylation of CpG sites and promoter methylation. It is expected that cells exposed to glyphosate will possess a higher methylation percentage of CpG sites and promoter methylation of tumour suppressor genes. If there is no significant change in methylation, then glyphosate can be increased to see if there is a difference based on the amount of toxin exposed. In addition, various experiments can be conducted in order to see the interaction between glyphosate and bodily mechanisms that cause for there to be a chemical change present upon exposure that subsequently changes the epigenetics of the genome. With the addition of each new chemical, and control with no chemical, there will be DNA purification and LUMA analysis of relative methylation. Expressional analysis, in order to determine the amount of protein present, will also be conducted under the same conditions of RNA purification and rtPCR amplification, with ß actin as an internal control and non-diseased tissue as experimental control for amplification comparison of C_t value ratios.

If it is determined that the exposure of glyphosate to non-cancerous cells causes hypermethylation and downregulation of tumour suppressor genes, then proliferation assays can be conducted on such treated cells in order to demonstrate the increased ability for a cell to multiply, signalling the beginning stages for a cell to become cancerous by evading tumour suppressor genes. Cells will be seeded in a 96 well plate with media that contains the respective amounts of glyphosate under time constraints of 12-24 hours and run for another 24 hours in treated media to determine the rate at which the cells multiply. A control of cells seeded in media with no glyphosate will be run in parallel in 96 well plate to determine the difference in proliferation rate. In cells that are treated it is expected that they will possess higher proliferation rates in comparison to the control, due to the downregulation of tumour suppressor genes allowing for uncontrolled cell growth. The rate of division will be quantified through CyQUANT® Direct, a fluorescent DNA binding dye that allows for direct determination of the number of cells present within a well [14]. If there is an increase in proliferation for the treater cells, then glyphosate can be correlated to the beginning stages of cancer that consist of an increase of uncontrollable cell growth and replication through tumour suppressor evasion. While there is a hypothesized link of glyphosate in the formation of tumours present in rats, this potential finding for a correlation back to the beginnings of cancer formation proves that this toxin, in conjunction with methylation and expressional data, progresses the formation of cancer.

Through the methylation determination of normal cells being treated with glyphosate, knock out cells can be created that mimic those of the toxin treated cells to show the effectiveness of the hypermethylation upon transcriptional activity. Knock outs for desired tumour suppressor gene of the non-cancerous cell lines will be obtained through CRISPR technology, ensuring that the gene is no longer present within the cell. Proliferation assays between non-diseased cells, within glyphosate media, will be run parallel to that of the knockout cell line. This will demonstrate the significance of the hypermethylation of the tumour suppressor gene in comparison to that of the toxin. The difference in proliferation will signal how effective the hypermethylation caused by glyphosate is in preventing transcriptional activity of tumour suppressor genes.

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