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Utilization of Methylation Levels Across the IGF2 Gene as a Predictive Marker for Various Types of Cancer

A Major Qualifying Project Report

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ABSTRACT

IGF2 is an imprinted gene found on chromosome 11. Epigenetic gene deregulation is increasingly shown to play a role in many types of cancer. Imprinted genes are especially susceptible to epigenetic regulatory changes because of silencing of one allele and the dependence on proper methylation for normal function. One of the goals in cancer research is to identify molecular markers that will predict the onset of specific cancers. The pattern of methylation across the IGF2 gene can be used to characterize the difference between normal and cancerous tissue, as well as the difference between different tumor tissues. By developing methylation assays that can determine the levels of methylation for all of the CpG sites across genes, we can establish patterns that could be predictive in detecting the onset of specific cancer types. We have developed assays to test the methylation levels at several sites across the IGF2 gene, and have assayed those levels in DNA isolated from breast, cervical, ovarian, and colon cancers and compared it to the corresponding normal tissue DNAs. The data shows that there are unique patterns of methylation between normal and cancerous tissues, and distinct methylation patterns across different types of cancers. The differences in methylation patterns between DNA isolated from tumor and normal adjacent tissue across four different tumor types in these genes indicates the critical influence of epigenetic regulation in this IFG2 region on the progression of cancer.

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BACKGROUND

Cancer

Cancer, put quite simply, is the uncontrolled growth of cells within the body. Known medically as malignant neoplasm, cancer comes in many forms, and can arise from many different types of tissues within the body. Through the process of oncogenesis, certain genes in a cell become altered, and the genes that regulate cell growth and division no longer operate correctly (**Figure-1**). Because the tumorigenic cells arise from normal cells in the body, cancer can be very difficult to detect in the early stages. The origin of the cancerous cells also makes treating the disease difficult because an agent that could damage the cancer cells could also cause harm to normal cells. The likelihood for the onset of oncogenesis may be influenced by many external and internal factors such as tobacco use, chemicals, radiation, inherited mutations, or hormones (National Cancer Institute, 2012). All of these forces may act together, or in sequence, to alter the genes that regulate growth and division.

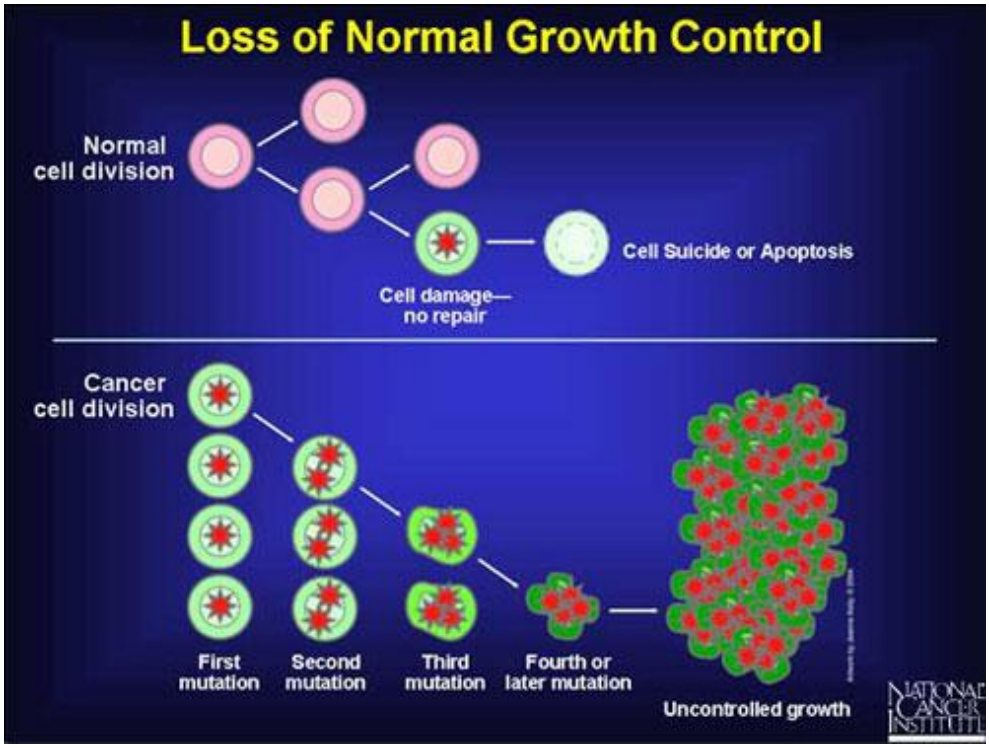
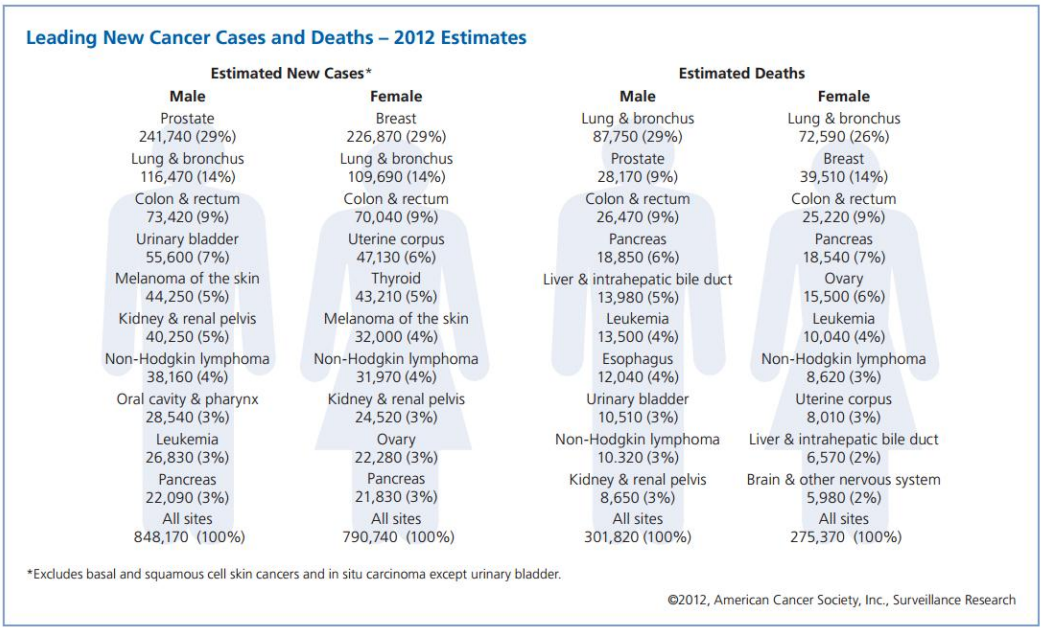


Figure 1: Diagram of Cancer Pathology. Normal cell division is shown in the upper panel, and cancer cell division in the lower panel. Note that a leading theory about cancer formation is the accumulation of mutations to the genes controlling cell growth and division that leads to tumors that can spread throughout the body. (National Cancer Institute, 2012)

The genes that promote growth, are called oncogenes when their expression can cause cancer. These powerful growth genes become overexpressed in cancer, while tumor suppressor genes meant to act as checkpoints in cell division become silenced. The cells then grow and divide rapidly, forming malignant tumors that can invade other parts of the body via the blood stream and lymphatic system to other organs disrupting necessary bodily functions. The mortality of the disease varies greatly depending on the type and location of the tumors as well as how much it has spread. Treatment is usually

in the form of surgery, radiation therapy, and/or chemotherapy, all of which take a heavy toll on the patient. The diagnosis of cancer is often only made after the tumors are large enough to cause symptoms, but regular screenings have increased the chances of catching it in the earlier stages, so the prevalence of some types of cancers has fallen in the last few years. Early detection greatly increases the chance of successful treatment. It is estimated that nearly 12 million Americans today have been affected by cancer, with an expected 1.64 million new cases to be added this year (**Figure-2**) (Cancer Facts, 2012). Cancer is the number two killer of Americans, at a rate of more than 1,500 deaths a day (Cancer Facts, 2012).



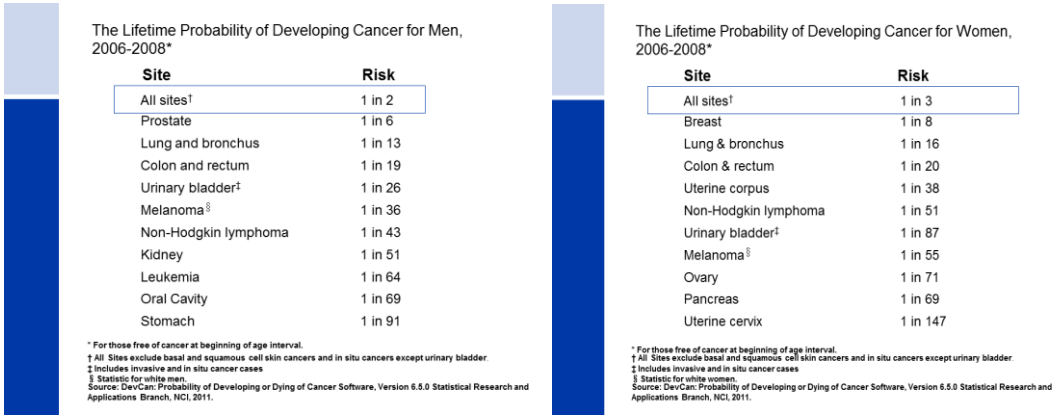
Leading New Cancer Cases and Deaths – 2012 Estimates

Estimated New Cases*		Estimated Deaths	
Male	**Female**	**Male**	**Female**
Prostate 241,740 (29%)	Breast 226,870 (29%)	Lung & bronchus 87,750 (29%)	Lung & bronchus 72,590 (26%)
Lung & bronchus 116,470 (14%)	Lung & bronchus 109,690 (14%)	Prostate 28,170 (9%)	Breast 39,510 (14%)
Colon & rectum 73,420 (9%)	Colon & rectum 70,040 (9%)	Colon & rectum 26,470 (9%)	Colon & rectum 25,220 (9%)
Urinary bladder 55,600 (7%)	Uterine corpus 47,130 (6%)	Pancreas 18,850 (6%)	Pancreas 18,540 (7%)
Melanoma of the skin 44,250 (5%)	Thyroid 43,210 (5%)	Liver & intrahepatic bile duct 13,980 (5%)	Ovary 15,500 (6%)
Kidney & renal pelvis 40,250 (5%)	Melanoma of the skin 32,000 (4%)	Leukemia 13,500 (4%)	Leukemia 10,040 (4%)
Non-Hodgkin lymphoma 38,160 (4%)	Non-Hodgkin lymphoma 31,970 (4%)	Esophagus 12,040 (4%)	Non-Hodgkin lymphoma 8,620 (3%)
Oral cavity & pharynx 28,540 (3%)	Kidney & renal pelvis 24,520 (3%)	Urinary bladder 10,510 (3%)	Uterine corpus 8,010 (3%)
Leukemia 26,830 (3%)	Ovary 22,280 (3%)	Non-Hodgkin lymphoma 10.320 (3%)	Liver & intrahepatic bile duct 6,570 (2%)
Pancreas 22,090 (3%)	Pancreas 21,830 (3%)	Kidney & renal pelvis 8,650 (3%)	Brain & other nervous system 5,980 (2%)
All sites 848,170 (100%)	All sites 790,740 (100%)	All sites 301,820 (100%)	All sites 275,370 (100%)

*Excludes basal and squamous cell skin cancers and in situ carcinoma except urinary bladder.

Figure 2: Leading Causes of Cancer and 2012 Estimates. Shown are the estimated incidence for new cases for various types of cancer in 2012, and the estimated deaths from various types of cancer (Cancer Facts, 2012).

The incidence of cancer has increased steadily over the years due to longer life spans and other environmental factors. In 2008, cancer is credited for 13% of all deaths worldwide with 7.6 million cases. This number is expected to swell to 13.1 million by 2030 (World Health Organization, 2012). The data in **Figure-3** shows that nearly one half of men alive today, and one third of women, are likely to be affected by cancer in their lifetime. As a disease that affects such a large percent of the population, a reliable system for detection and treatment would undoubtedly save many lives and raise the quality of life for billions.



The Lifetime Probability of Developing Cancer for Men, 2006-2008*

Site	Risk
All sites†	1 in 2
Prostate	1 in 6
Lung and bronchus	1 in 13
Colon and rectum	1 in 19
Urinary bladder‡	1 in 26
Melanoma§	1 in 36
Non-Hodgkin lymphoma	1 in 43
Kidney	1 in 51
Leukemia	1 in 64
Oral Cavity	1 in 69
Stomach	1 in 91

* For those free of cancer at beginning of age interval.
† All Sites exclude basal and squamous cell skin cancers and in situ cancers except urinary bladder.
‡ Includes invasive and in situ cancer cases
§ Statistic for white men.
Source: DevCan: Probability of Developing or Dying of Cancer Software, Version 6.5.0 Statistical Research and Applications Branch, NCI, 2011.

The Lifetime Probability of Developing Cancer for Women, 2006-2008*

Site	Risk
All sites†	1 in 3
Breast	1 in 8
Lung & bronchus	1 in 16
Colon & rectum	1 in 20
Uterine corpus	1 in 38
Non-Hodgkin lymphoma	1 in 51
Urinary bladder‡	1 in 87
Melanoma§	1 in 55
Ovary	1 in 71
Pancreas	1 in 69
Uterine cervix	1 in 147

* For those free of cancer at beginning of age interval.
† All Sites exclude basal and squamous cell skin cancers and in situ cancers except urinary bladder.
‡ Includes invasive and in situ cancer cases
§ Statistic for white women.
Source: DevCan: Probability of Developing or Dying of Cancer Software, Version 6.5.0 Statistical Research and Applications Branch, NCI, 2011.

Figure 3: Cancer Probabilities. Shown are the likelihood for men (left panel) and women (right panel) for developing various types of cancer over a lifetime (Cancer Facts, 2012).

Screening programs have been encouraged in many countries, and have resulted in increased early detection and a lower mortality rate. A major issue that still exists is that cancerous tissue is difficult to distinguish from normal tissue, especially in the early stages. An accurate and diagnostic epigenetic assay would greatly aid such efforts by not only providing a clinical diagnostic tool but also by assessing the risk that tissues will become cancerous in the future.

Epigenetics

Although most people today are familiar with the basis of DNA and genetics, epigenetics has been of rising prominence and attention. Epigenetics is considered the study of changes in gene expression in the absence of modification of the underlying DNA sequence. The operation of the epigenome is very complex and not fully understood, but it seems that it is partially inherited from the parents, but unlike the genome, it is also subject to change from environmental pressure. Environmental factors such as diet and stress can often affect the expression of certain genes, and since the sequence in the DNA does not change, except in the case of mutagenesis, there must be some other venue for control (Eccleston et al., 2007). A good analogy is that the genome is like the computer hardware, static and unchanging, while the epigenome is the software, directing gene expression in response to environmental influences.

Some of the epigenetic pathways that affect gene expression are histone modification and DNA methylation. DNA methylation occurs when a methyl group attaches to the cytosine base of the DNA double helix, frequently at CG sites (**Figure-4**). Such areas, known as CpG sites, tend to occur in patterns all along the genome, often clumping around genes in areas known as CpG islands. It is believed that when these areas become methylated, the methyl group changes the DNA's affinity for certain transcription factors (Jones, 1980; Phillip, 2008).

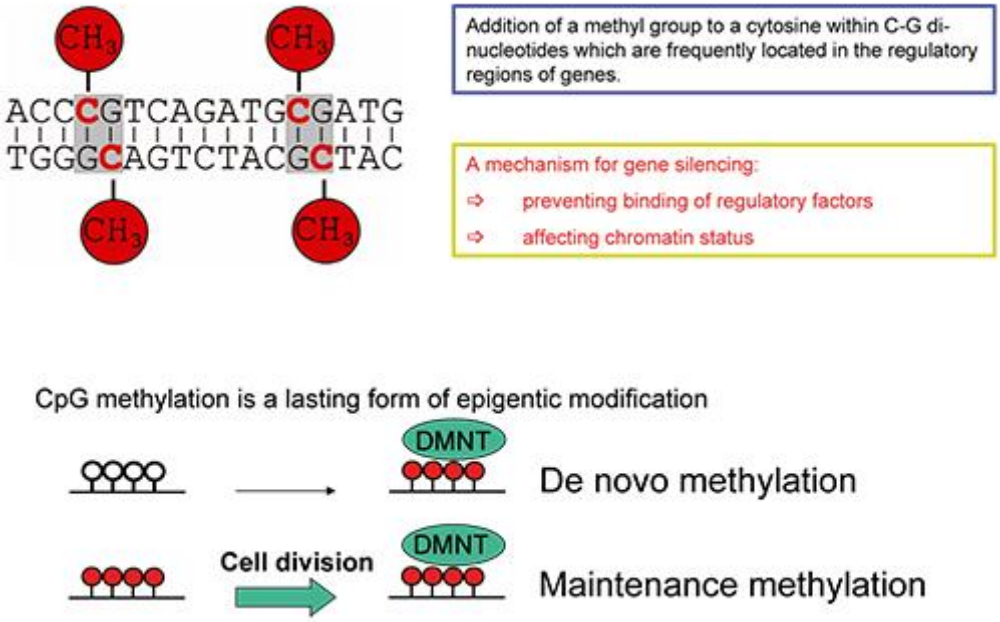


Figure 4: Diagram of DNA Methylation at CpG Islands. DNA methylation occurs when a methyl group composed of a carbon atom and three hydrogen atoms binds onto the cytosine in the DNA double helix. This can cause changes in the expression of the gene including silencing by preventing the binding of regulatory factors. (Max Planck Institute of Psychiatry, 2012)

Contrary to previous conceptions, methylation does not automatically mean gene expression down regulation, instead the effect of methylation depends upon the default state of the region. DNA methylation has been shown to be important in cell differentiation, where it turns certain genes on and off in sequence to guide the cells on their way from a pluripotent stem cell into a fully differentiated cell (Jones, 1980). Aberrant methylation or de-methylation of CpG sites would be able to cause the abnormal expression of the growth regulation genes. DNA methylation's ability to control gene expression made it a suspect in the oncogenic process, and there have been several studies that show a strong correlation between the two (Das, 2004; Momparler and Bovenzi, 2000). As a result, several studies have looked at drugs with the potential

to alter the methylation of DNA to affect gene regulation (Szyf, 1996; Issa and Kantarjian, 2009). An assay designed to reliably and accurately assess gene methylation status coupled with an effective system for altering the methylation states, could lead to a very effective treatment option or in prevention.

Genomic Imprinting

Genomic imprinting is a genetic occurrence where a specific gene is expressed in a parent-of-origin-specific way. Unlike Mendelian genomics, only the non-imprinted gene from one parent is always expressed, while the other is silenced. The mechanism of this gene silencing is through methylation and histone modification (**Figure-5**).

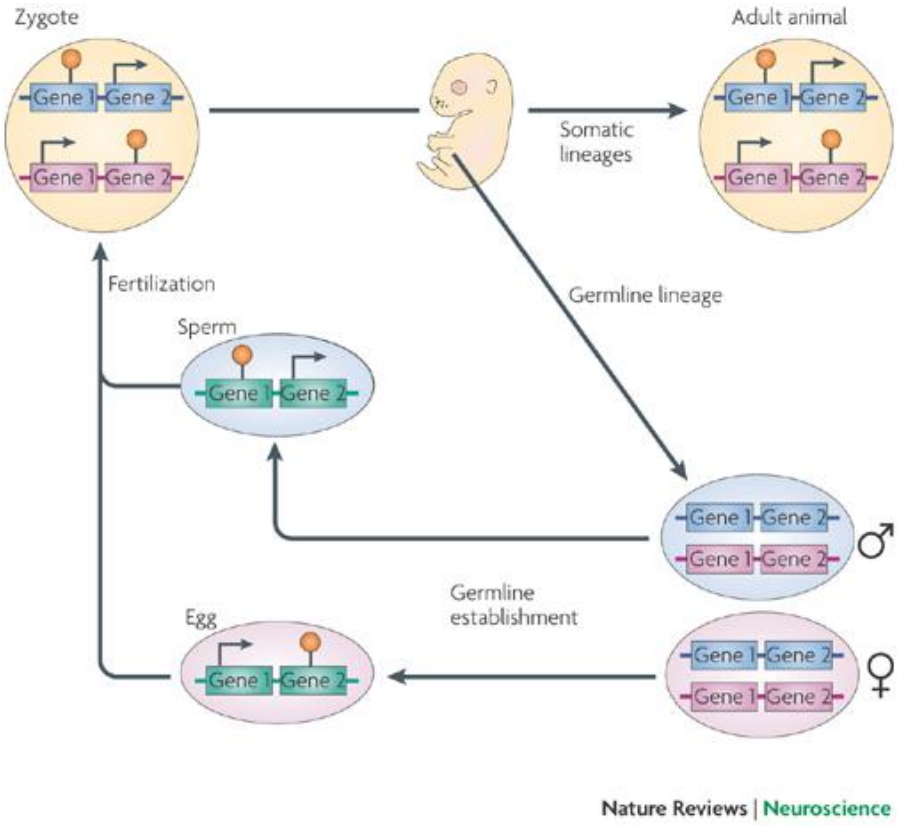


Figure 5: Mechanism of Genomic Imprinting. Shown is the means by which imprinted methylated genes (orange) are inherited. Only one version of each gene is expressed in a parent of origin-dependent way (Wilkinson et al., 2007).

The appropriate suppression of imprinted genes is necessary for normal development. Improper imprinting has been associated with many genetic disorders such as the Beckwith–Wiedemann syndrome, Silver–Russell syndrome, Angelman syndrome and Prader–Willi syndrome (Reik, 1989; Nicholls, 2000). Imprinted genes are especially susceptible to epigenetic regulatory changes because of silencing of one allele and the dependence on proper methylation for normal function. Either loss of imprinting or changes of methylation of the non-imprinted copies could have drastic effects on the expression of the gene. Due to the importance of imprinted genes for normal cell development, and their vulnerability to epigenetic changes, make them likely players in the oncogenic process.

IGF2

IGF2 is a gene located on chromosome 11p15.5 which codes for the insulin-like growth factor 2 protein. IGF2 is a growth and proliferation-promoting gene that is essential in growth and development before birth, but becomes less active in adulthood. IGF2 is an imprinted gene with only the paternal copy being expressed (**Figure-6**). Because of its involvement in cell proliferation and its vulnerability to epigenetic changes due to imprinting, IGF2 is likely to be involved in some forms of oncogenesis. Several studies have been performed in IGF2 and have linked loss of imprinting of the IGF2 gene to cancer (Jirtle, 2004; Sakatani, 2005). For all these reasons, IGF2 was selected as the target for the methylation analysis assays in this project.

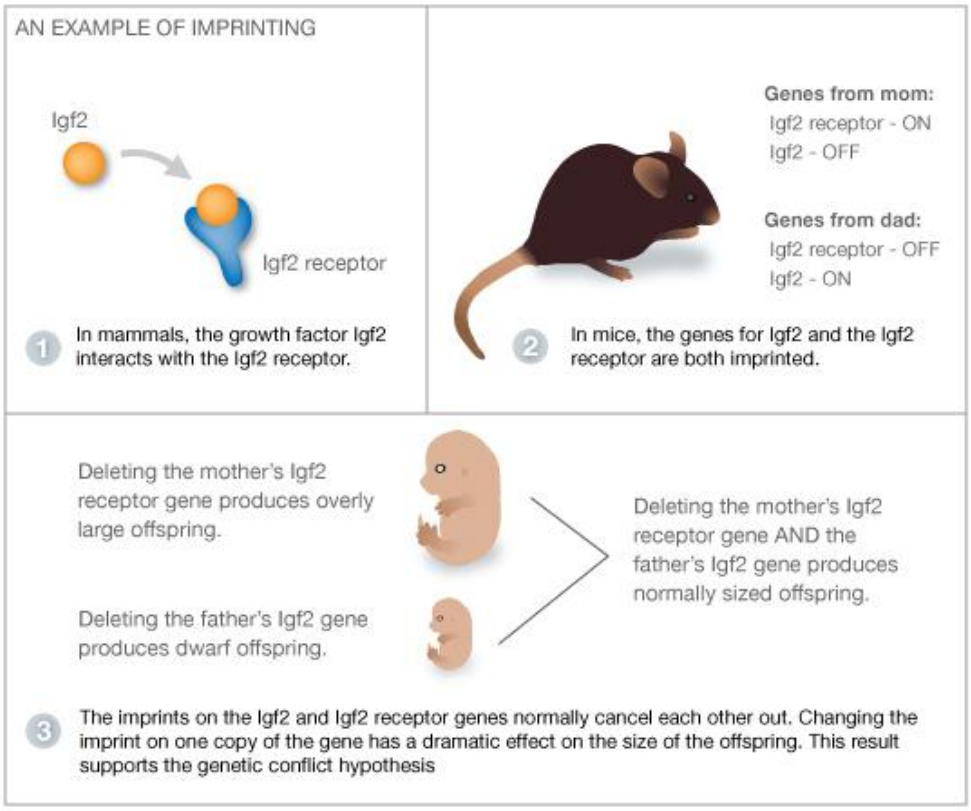


Figure 6: Imprinting and IGF2. Shown is an experiment highlighting the importance of the proper functioning of the IGF2 in normal growth and development. (University of Utah, 2012)

PROJECT PURPOSE

The purpose of this project is to determine the methylation state of the IGF2 gene promoter and selected internal regions, for both normal and cancer tissue for various tissue types. The data will then be analyzed to potentially identify differentially methylated regions between the sample sets to determine specific methylation profiles for the normal and cancerous types. Then the profiles will be assessed to determine the viability of using DNA methylation analysis and known methylation profiles as a diagnostic test for cancer. The hope is that this research will be used as a risk assessment procedure and early detection diagnostic assay which would greatly reduce the mortality of the disease.

METHODS

DNA Extraction

The tissue lines and tumor tissues were acquired from ProteoGenex, Inc. There were 80 samples overall used in this experiment, representing four different tissue types: Breast, Colon, Ovarian, and Cervical; half of which were cancerous and half of which were normal, amounting to 10 samples for each tissue type and cancer state. gDNA (genomic DNA) was extracted from the tissues using a Zymo Research ZR-96 Quick-gDNA^{TM} (Zymo Research). The tissue was first digested in Proteinase K and Genomic Lysis buffer. The samples were then centrifuged, and the supernatants were then added to the Silicon-A Plate. The Silicon-A Plate was then placed on top of a Collection Plate and centrifuged. The aim of this procedure is to collect the DNA in the filter at the bottom of the Silicon-A plate while all the other cell parts flow through into the collection plate. After the centrifuging, the flow through was discarded and some DNA Pre-Wash Buffer was added to the Silicon-A Plate columns and centrifuged over the Collection Plate. The flow through was discarded and another was carried out in the same manner, but this time the g-DNA Wash Buffer was used. The purpose of the repeated washes is to clear out any other cellular debris besides the DNA. After the third wash, DNA Elution Buffer was added to each of the columns of the Silicon-A Plate was placed on top of an Elution plate and centrifuged. The purpose of this step is to remove the DNA from the filter at the bottom of the Silicon-A Plate and collect the extracted gDNA in the Elution Plate for use in the experiment. After the centrifuging, the Elution Plate containing all the gDNA samples was labeled and stored in a -80°C freezer for later use.

DNA Bisulfite Treatment

The extracted gDNA was subject to bisulfite conversion in preparation for PCR using the Zymo Research EZ-96 DNA Methylation™ Kit (Zymo Research). The gDNA was removed from the freezer and thawed before being added to a Conversion Plate. Then 5 ul of M-Dilution Buffer was added to each sample, and the final volume was brought to 50 ul using water. The samples were then allowed to incubate at 37°C for 15 minutes in order to denature the DNA. After the incubation, CT Conversion Reagent was added to each sample and mixed. The Conversion Plate was then put in a thermo cycler and allowed to incubate in the dark overnight. The sodium bisufinate contained in the CT Conversion Reagent will convert all the unmethylated cytosines into uracils by way of the chemical reaction shown in **Figure-7**.

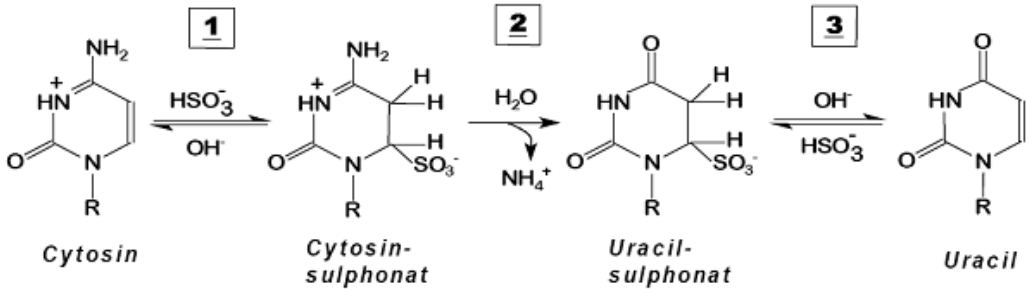


Figure 7: Diagram of the DNA Bisulfite Reaction. In bisulfite conversion, sodium bisulfate converts all unmethylated cytosines into uracils (Schumacher, 2007).

The fact that only the methylated cytosines are converted into uracils will become important in determining the methylation state later on. After the overnight incubation, the bDNA (bisulfite DNA) was incubated at 0- 4°C for 10 minutes. M-Binding Buffer was then added to a Zymo-Spin™ I-96 Binding Plate on top of a Collection Plate. The

bDNA was then added to the spin plate and mixed. The samples were then centrifuged, and the flow through was discarded. Then the samples were washed and centrifuged using M-Wash Buffer, and the flow through was discarded. M-Desuphonation Buffer was then added to each sample and allowed to stand at room temperature. After the incubation, the plate was again centrifuged and the flow through discarded. Two more rounds of washing with M-Wash buffer followed to remove any unwanted material. The Zymo-Spin™ I-96 Binding Plate was then placed on top of An Elution Plate. M-Elution Buffer was then added and the bisulfite converted DNA was collected on the Elution Plate. The plate was then labeled and stored in a -80°C freezer for later use.

Assay Design

The next two procedures, PCR and pyrosequencing rely on DNA replication technology. In order for either of these procedures to work, sequence-specific primers must be designed to ensure the sequence of interest is being replicated. The primers used are short sequences, about 20 bp long, that are complimentary to sequences flanking either side of the area of interest. The regions selected were varied, some were regions known to be differentially methylated while others were promoters or CpG islands. There are several factors that can affect the operation of the primer, so the assays were developed using Qiagen's PyroMark Assay Design Software 2.0. The exact sequences used for the primers and the procedure for deriving them are proprietary property of EpigenDx, Inc. and will not be discussed here. Once the assay had been designed, the primers were ordered from Biomers.net, an oligonucleotide manufacturing service. The areas of the IGF2 gene covered by the assays in this experiment are shown in **Figure-8**.

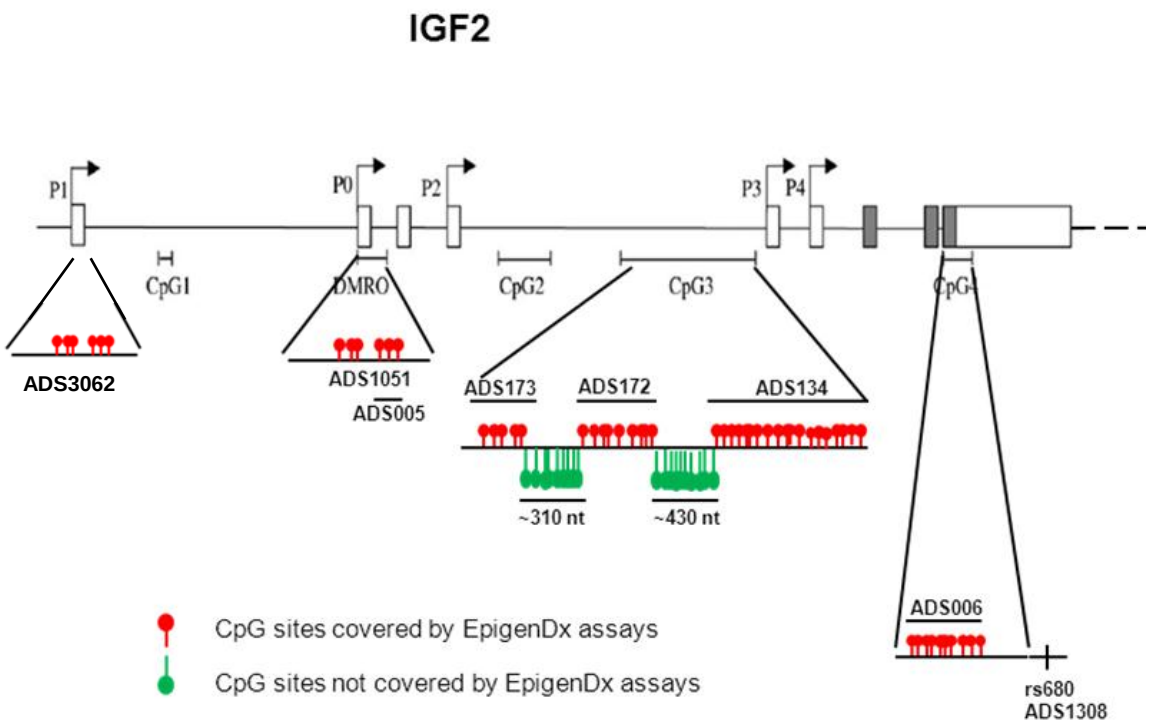


Figure 8: The Location of the IGF2 Methylation Assays. Exons are shown as white boxes, and promoters are labeled as P (P0, P1, P2, etc.). CpG islands are labeled with bars and the assays designed to the specific regions are shown. Red balloons indicate CpG sites covered by EpigenDx assays, while the green balloons show CpG sites not yet covered by EpigenDx assays

PCR

PCR (Polymerase Chain Reaction) is a widely used method of DNA amplification. It works by using the primers and DNA polymerase to build new segments of DNA off the original copies (**Figure-9**). The plate containing the bDNA was thawed along with all the reagents needed. The reagents were then added to the samples, including the primers, DNA polymerase, individual nucleotides and some buffer. The plate was then put into a thermocycler which heats and cools the samples to a specific set of temperatures in repeated cycles. There are three steps to the thermo cycling process, and they are repeated about 40 times, each time making more copies of the DNA. In the

first step, the samples were heated to about 94°C, this is known as the denaturation step during which the DNA unravels and splits off into two single stranded copies. Next is the annealing step, where the samples were brought to about 54°C and the primers that were added bind to the complementary sequence of DNA. The last step is extension, where the samples were heated again to about 72°C, at which time the DNA polymerase binds to the DNA and uses the free nucleotides to build a complimentary strand. Because these steps are repeated, there are more templates to build off of each cycle and the number of copies of the DNA exponentially increases. Also of note is that the new copies of DNA will have thymine in place of the uracils that were inserted during the bisulfite conversion. Using this method allowed the DNA concentration to be increased to a point sufficient for pyrosequencing. Once thermocycling was completed, the samples were kept at 4°C in the thermocycler until pyrosequencing.

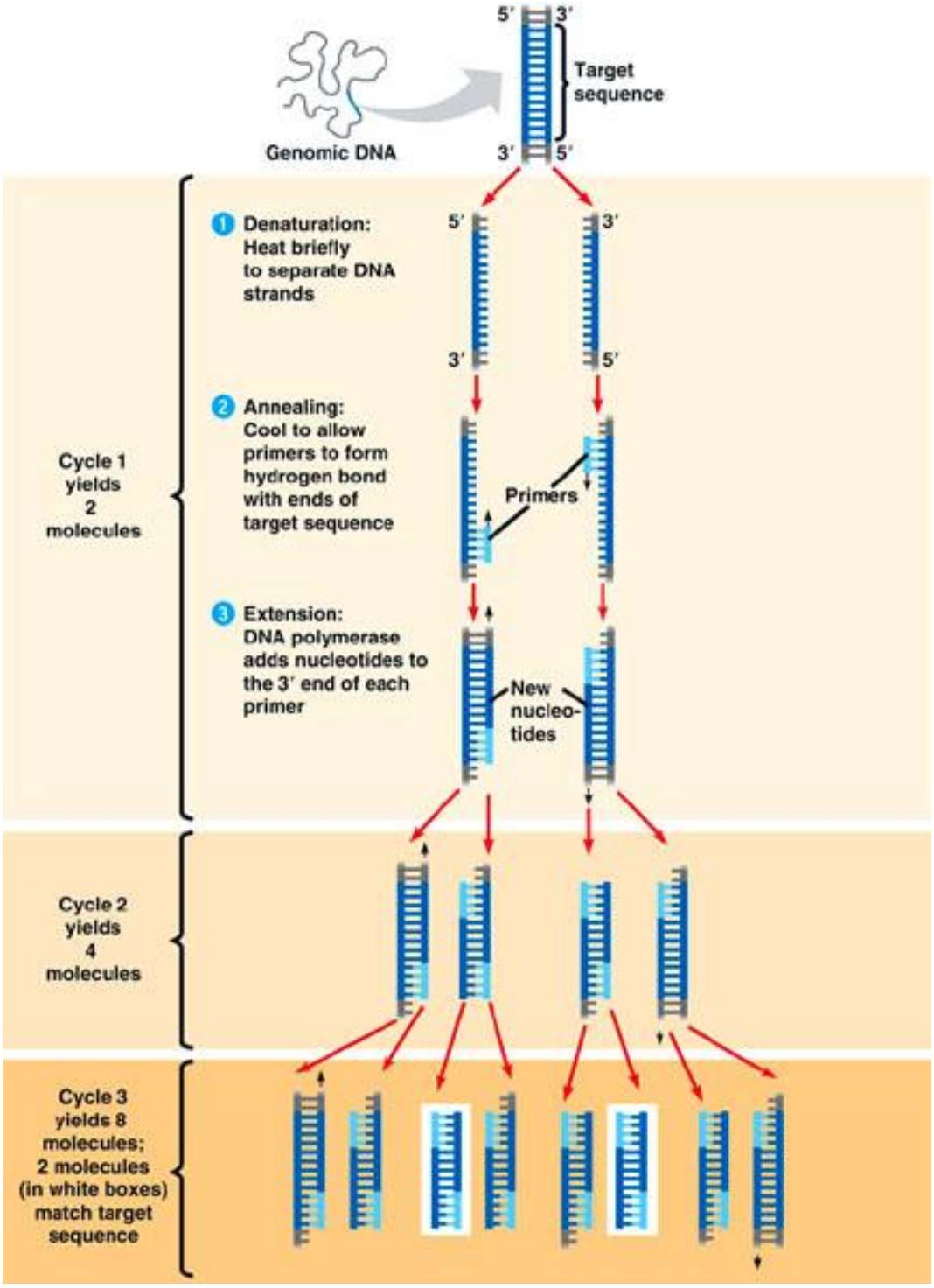


Figure 9: Diagram of the Polymerase Chain Reaction. Through repeated heating and cooling the DNA segments are able to be replicated many times, and amplified. (Orac, 2007)

Pyrosequencing

Pyrosequenceing is a method of DNA sequencing that uses a cascade of enzyme activities to read short sequences of DNA. The idea of it is similar to that of PCR, except that instead of just copying the sequence using DNA polymerase, the activity of DNA polymerase is measured as it adds each individual nucleotide to the sequence. The first step was to aliquot out some streptavidin sepharose beads into a pyrosequencing plate. The DNA binds to these beads very strongly keeping them still while also orienting the strands uniformly. The samples are added to the bead solutions and put on a horizontal shaker to ensure the DNA binds to beads. Once the samples were sufficiently shaken, the beads, along with the DNA attached to them were sucked out of the plate using a PyroMark® Q96 Vacuum Workstation. This consists of a vacuum handle with 96 probes that come down into the wells of the plate. The probes have holes in them but they are just small enough not to let the beads pass through. Suction is kept on to the beads and DNA is stuck as they are subjected to various washes. The first wash is an ethanol bath, which removes any unwanted residues. The next bath was in a Denaturation Solution, which stripped one copy of the DNA off, leaving only single stranded DNA attached to the beads. The beads were then subjected to a wash buffer to remove any remaining ethanol or Denaturation Solution that would affect the pyrosequencing reaction. A pyrosequencing plate was then filled with the sequencing primer complimentary to the area immediately upstream from the target sequence. After the final wash, the beads and DNA were added to the pyrosequencing plate and heated to 80°C to ensure proper annealing. The plate was then loaded into a Qiagen PyroMark Q96 MD for analysis.

Pyrosequencing functions by reading the activity of DNA polymerase as it adds nucleotides to the DNA strand (**Figure-10**). Once the primer and polymerase have bound the sequence in question, the pyrosequencing machine adds more enzymes, then proceeds to sequentially flood each well with one of the the four dNTPs (deoxynucleoside triphosphates) one at a time. If the dNTP just added happens to be complimentary to the next base in the DNA sequence, the polymerase will add it. When this happens, the polymerase releases a pyrophosphate, which is taken up by ATP sulfurylase and converted to ATP. The ATP is then taken up by luciferase which uses it to convert luciferin to oxyluciferin. This process gives off a visible light which can be read by photoreceptors in the pyrosequencer. Unincorporated dNTPs are degraded by the addition of an apyrase enzyme so that they are no longer present during the addition of the next dNTP. By measuring the light given off after the addition of each nucleotide, the sequence of the DNA strand can be read. The light given off is proportional to the number of nucleotides added so if the peak is twice as high, it means two nucleotides were added.

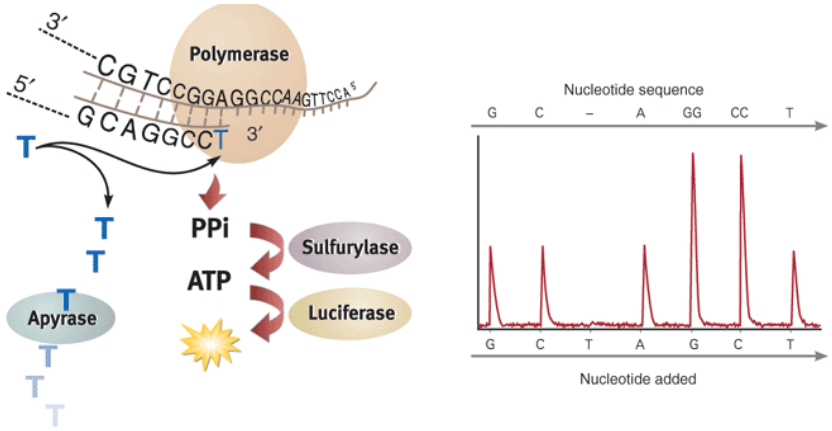


Figure 10: Diagram of Pyrosequencing. This figure diagrams the chemical pathway used by the pyrosequencer to read the sequence. The graph top the left is atypical readout with the nucleotide added and the sequence of the DNA at the top (Nature Methods, 2005).

Because all the unmethylated cytosines were converted to uracils during the bisulfite treatment then to cytosines in the PCR reaction, the only cytosines that remain were the ones that were methylated. By comparing the signals given off during the addition of thymidine and cytosine at a CpG site, the percent methylation of the original site can be deduced (**Figure-11**).

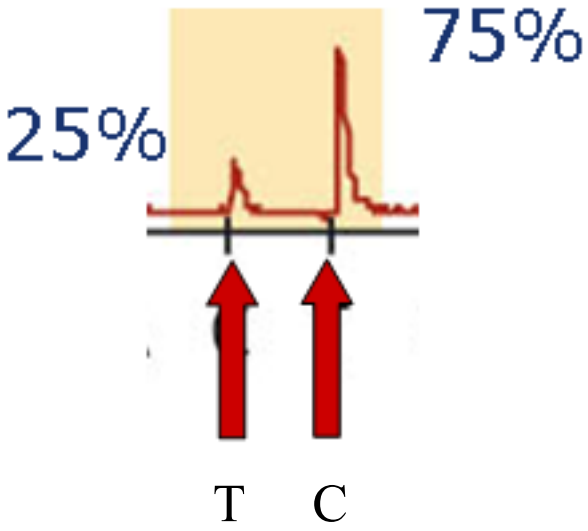


Figure 11: Example Result from a CpG Site. The percent methylation is directly correlated to the ratio of the signals given off during the addition of thiamine and cytosine. (EpigenDx, 2012)

RESULTS

The purpose of this project was to determine the methylation state of the IGF2 gene at multiple locations, for both normal and cancer tissue of various tissue types. The data was then be analyzed to identify differentially methylated regions between the sample sets to determine specific methylation profiles for the various normal and cancerous tissue types. This was accomplished through DNA methylation analysis by pyrosequencing.

The results are very promising, revealing several differentially methylated regions across all the sample sets (**Figure-12**). The cancer tissues seem to be hypomethylated overall, but this is not the case in all regions. ADS 1051 (upper right panel) and ADS006 (lower right panel) both showed hypomethylation of the cancer tissues for all the tissue types compared to the normal, while ADS173 (middle left) and ADS172 (middle right) showed quite the opposite in all tissue types except for ovarian. ADS134 (lower left) showed variation between the tissue types, but there was no variation in this region between the normal and cancerous tissue types. ADS 3602 (upper left) showed hypomethylation of the cancer tissues except for breast, which showed relativity little change.

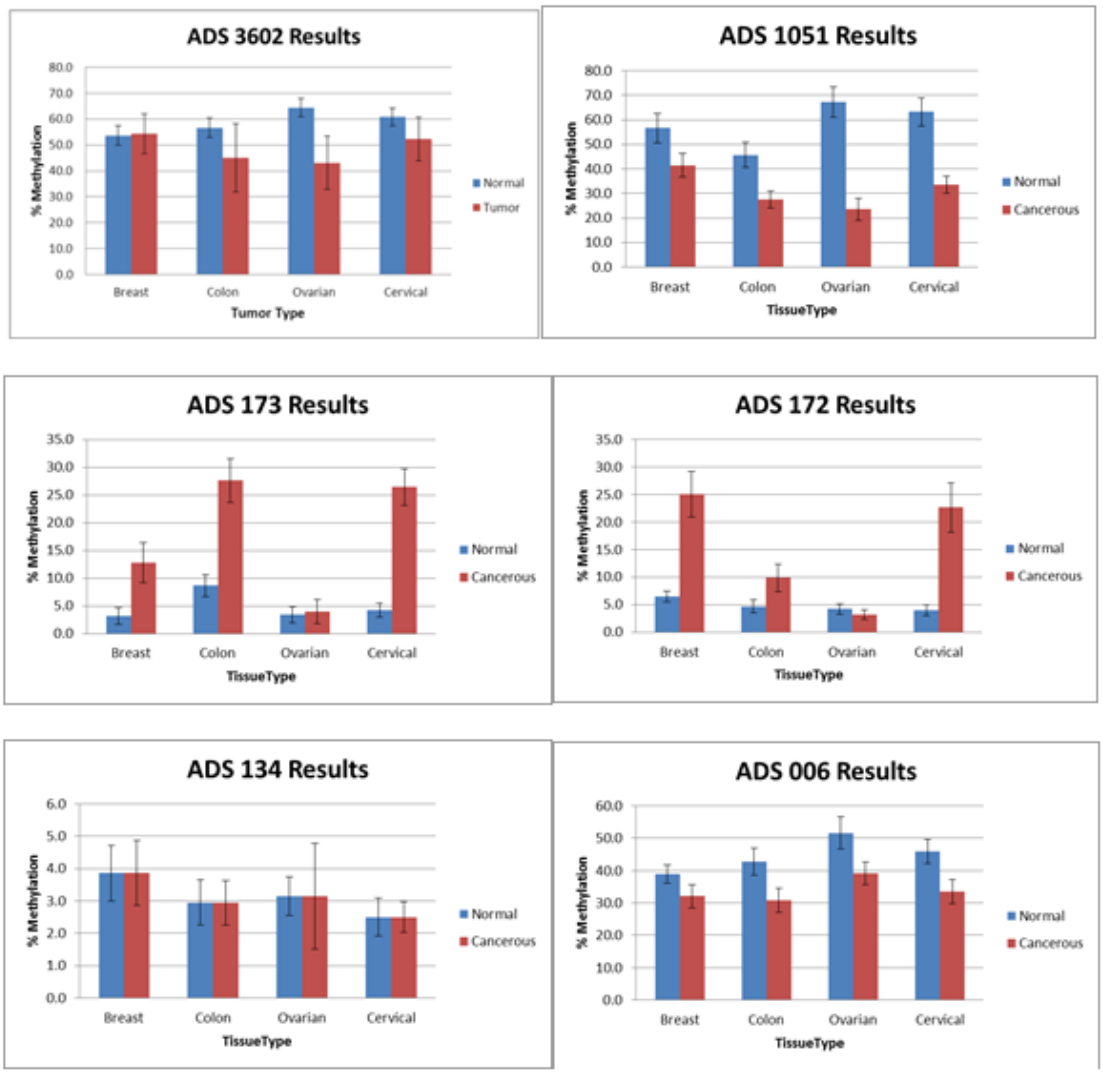
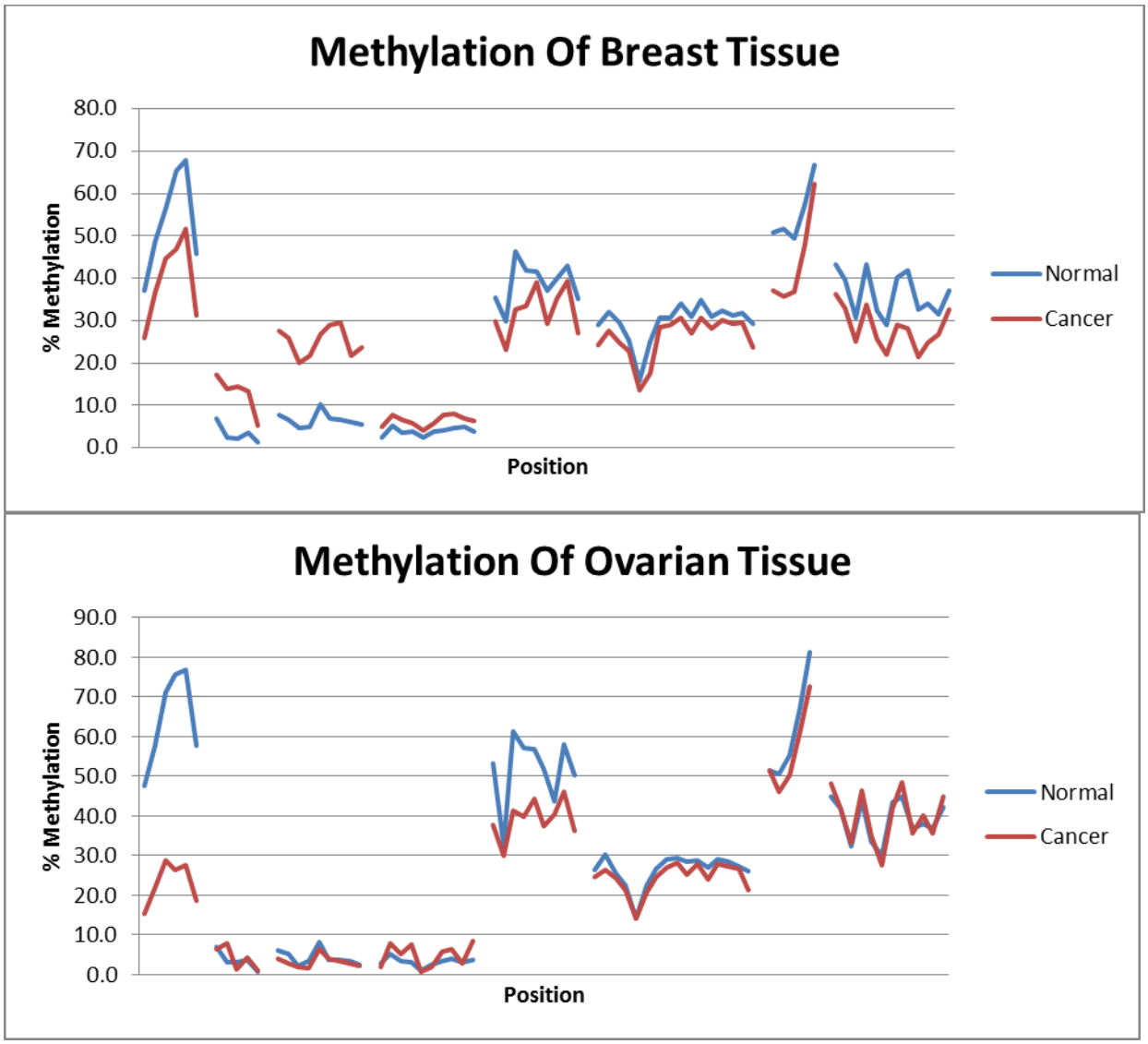


Figure 12: Comparative Results for Cancer and Normal Tissue for all the Cancer Types. The assay ID is located above each result, with the percent methylation on the left.

Although this data may seem rather conflicting, it lends itself to the idea that regulation is not a simple matter of hypo- or hyper-methylation, but rather that location has an effect on the gene regulation. A more complete picture can be gained by plotting the methylation of each location relative to its actual position on the gene (**Figure-13**).

This methylation map or “methylation profile” is unique to each tissue type, both cancerous and normal.



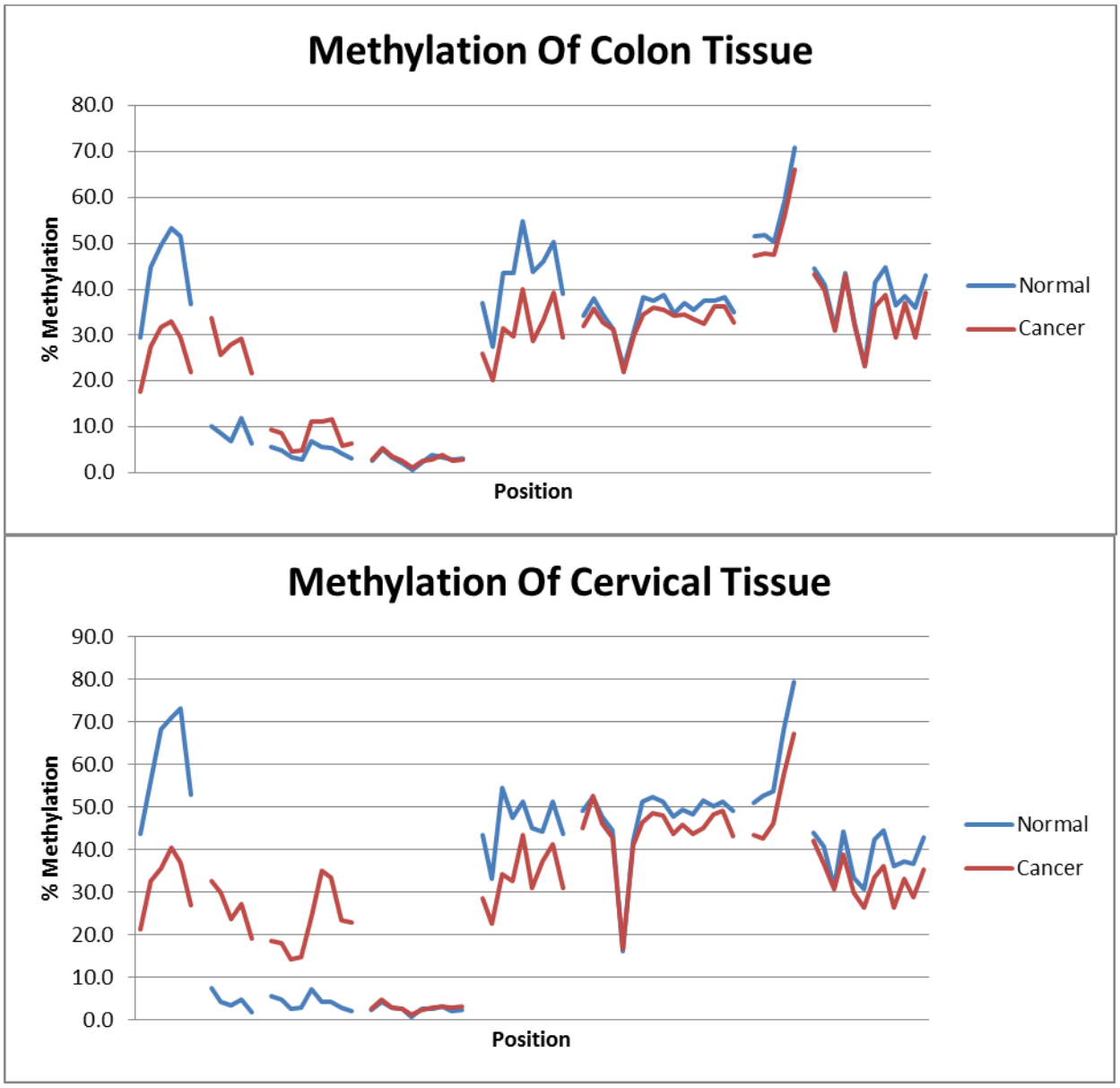


Figure 13: Methylation Profiles for All the Tissue Types. The vertical axis represents the methylation at that specific site, while the horizontal axis denotes the relative position of the CpG site.

DISCUSSION

In this project, an analysis of the methylation levels of different regions of the human IGF2 gene was performed for four tumor types (breast, colon, ovarian and cervical) and their corresponding normal tissue. Several locations within the IGF2 gene were analyzed including the promoter of the P0 exon, the DMR0 region, the CpG island (CpG 3) proximal to the P3 exon, and the CpG island (CpG 4) in the last exon. Within the promoter region of the P0 exon, each tumor type showed a unique pattern of methylation. All tumors were slightly hypomethylated as expected for an over-expressing growth related gene, but the breast tumor least of all. In one region of the promoter there was a large hypomethylation of tumor tissue in ovarian tumors where a more proximal assay showed greater hypomethylation in the cervical tumors. There was significant hypomethylation in all tissue types within the DMR0 region, with ovarian tumors showing the largest difference to normal tissue. The methylation patterns observed in the CpG 3 island show distinct differences within this region. These assays show low levels of methylation in the normal tissues. Assay ADS 134 in the proximal region shows no difference between normal and tumor tissue in any of the different tumor types as seen in the ADS 134 bar graph and its corresponding region on the line graph. However, each of the more distal CpG 3 assays show unique patterns of hypermethylation in tumor tissue. The CpG 4 island assay consistently showed modest hypomethylation in all tumors when compared to their normal counter-parts.

The differences in methylation patterns between DNA isolated from tumor and normal adjacent tissue for 4 different tumor types in the IGF2 genes suggests a critical influence of epigenetic regulation in this region on the progression of cancer. The

methylation changes in the promoter regions could be potential biomarkers for detecting breast cancer, cervical cancer, colon cancer, and ovarian cancer. It appears that methylation analysis as a form of cancer diagnosis is very promising, and further investigation is needed.

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