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Hyperproliferation and Parathyroid Neoplasia due to Genetic Variants

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Hyperproliferation and Parathyroid Neoplasia due to Genetic Variants

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Master of Science Thesis

Hyperproliferation and Parathyroid Neoplasia due to Genetic Variants

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Abstract

Primary Hyperparathyroidism (PHPT) is a common endocrine disorder, which is seen at all ages, but primarily in post-menopausal women. While commonly asymptomatic, clinical manifestations include kidney stones, osteoporosis, and other symptoms. Parathyroid adenomas are the most common cause of PHPT (85%), followed by hyperplasia (15%) and carcinomas (~1%). Established genes associated with sporadic parathyroid adenomas include: proto-oncogene cyclin D1 (*PRAD1, CCND1*), tumor suppressor gene *MEN1*, and several cyclin-dependent kinase inhibitor genes *CDKN2B, CDKN2C, CDKN1A,* and *CDKN1B*. Recently, compelling genetic evidence implicated that the *ZFX* gene, part of the Kruppel C2H2 type zinc finger protein family, is a likely parathyroid adenoma oncogene. It was found to be involved in almost 5% of all cases (Soong & Arnold, 2014).

Genetic analysis is essential to this study, and the quality of the DNA used is crucial in further analyses. Formalin-Fixation and Paraffin-Embedding (FFPE) is commonly practiced preservation method, which unfortunately sacrifices the quality of the DNA obtained from each sample. This is why it is imperative to optimize a protocol that yields the most DNA, without introducing further degradation. We tested multiple protocol modifications and determined that the modified Qiagen Kit protocol, using Qiagen deparaffinization solution, with one-

hour proteinase K incubation yielded optimal DNA concentrations and the least amount of protein degradation.

With the discovery of *ZFX* as a candidate parathyroid oncogene, the potential involvement of the highly homologous gene, *ZFY*, came to light. Since analogous mutations in *ZFY* could have similar tumorigenic effects as those in *ZFX*, we analyzed the entire coding region of *ZFY* in sporadic parathyroid adenomas from male patients. This region was PCR-amplified and Sanger sequenced in 117 adenomas. One novel variant c.1402C>T was found, resulting in a predicted histidine to tyrosine change at position 468 of the ZFY protein. This variant may have a role in the molecular pathogenesis of sporadic parathyroid adenomas. Grasping the full understanding of the biology of *ZFX* and *ZFY*'s mutations in the context of parathyroid tissue will further illuminate the pathogenesis of parathyroid neoplasms.

In addition to involvement in sporadic parathyroid adenomas, recent studies have also suggested that germline mutation in several CDKIs can cause sydromic HPT (Pellegata et al 2006), with phenotypic presentation similar to multiple endocrine neoplasia type 1 (MEN1). A patient diagnosed with prolactinoma and familial hyperparathyroidism was referred for mutational analysis of CDKIs p15, p18, p21, and p27. Mutational analysis of four CDKI genes identified one novel variant, c.34G>A, in *CDKN2C,* encoding p18. We believe this variant may contribute to the molecular pathogenesis of the patient's familial hyperparathyroidism and prolactinoma.

In order to understand hyperproliferation and parathyroid neoplasia, an inclusive study to optimize methodology for DNA extraction from FFPE samples and, mutational analysis of *ZFY, ZFX*, and CDKIs was performed. The overall findings of this project may suggest that *ZFY* or CDKIs mutations contribute to the development of hyperparathyroidism.

I. Calcium Homeostasis

Calcium is essential to most mammalian cellular functions. Serum calcium levels are maintained by the parathyroid gland, which is the master regulator of calcium homeostasis, through regulated secretion of parathyroid hormone (PTH). Under normal physiological conditions, subtle decreases in blood calcium levels stimulate the secretion of PTH, which in turn stimulates the increase of serum calcium through effects on the gastrointestinal, renal, and skeletal systems. This stimulation increases calcium absorption, as well as the mobilization of mineralized calcium in the bone, and decreases calcium excretion. The resulting elevation in serum calcium levels feed back on the parathyroid glands to decrease PTH secretion (Figure 1).

Primary hyperparathyroidism (PHPT) is a common endocrine disorder, with its highest incidence in post-menopausal females (Marcocci and Cetani 2011). Patients with primary hyperparathyroidism exhibit hypercalcemia with an unsuppressed parathyroid hormone level. While primary hyperparathyroidism is generally asymptomatic in the western world, marked hypercalcemia can lead to gastrointestinal symptoms, bone problems such as osteoporosis and osteitis fibrosa cystica, effects on the central nervous system, and kidney stones.

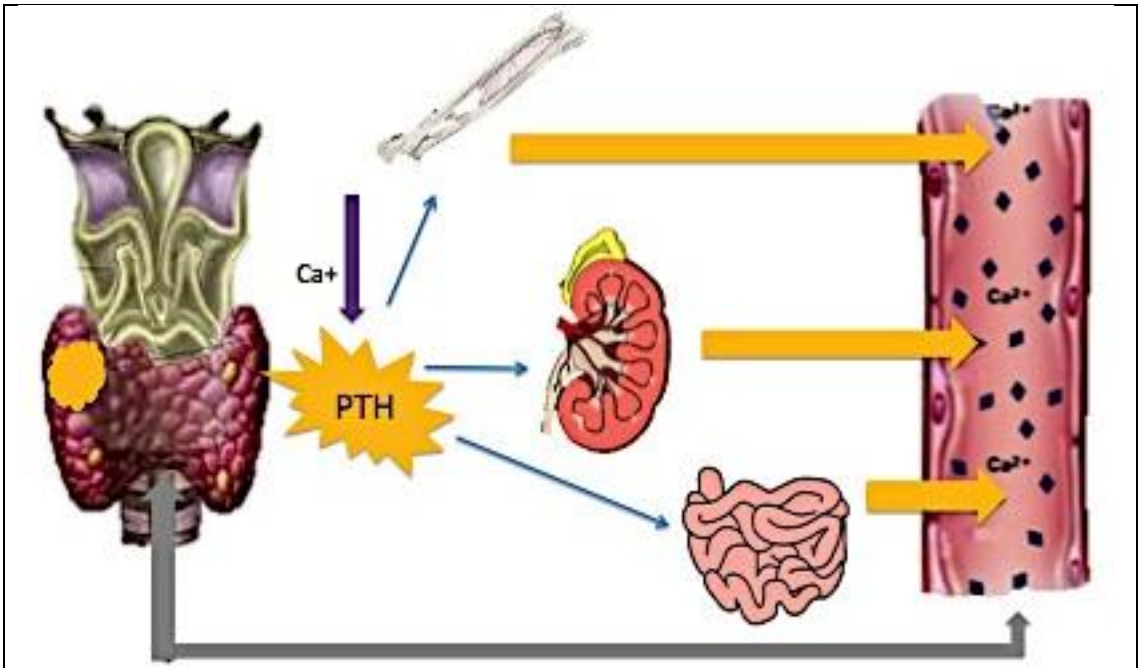


Figure 1. Calcium Homeostasis
The parathyroid glands function to produce PTH in response to low serum calcium. PTH acts on the kidneys, bones and intestines. All three target organs work together to increase serum calcium levels, which then feedback on the parathyroid glands, shutting down PTH production. In primary hyperparathyroidism, this feedback loop is lost, and this leads to uncontrolled PTH secretion and elevated calcium levels.

II. Basic Tumor Biology

A. Tumorigenesis

Tumors are caused by genetic mutations triggering uncontrolled cell proliferation. Gene mutations are typically categorized as either somatic or germline mutations. Somatic mutations can occur in any of the cells of the body except the germ cells (sperm and egg) and therefore are not passed on to children (Rati, 2011). In contrast, germline mutations can be passed on to some or all progeny. Somatic mutations can be represented in a sector of identical cells from a single progenitor cell or phenotypically represented as a dominant

mutation that typically occurs after the organism has fully developed (Li, 2004). Mutations that occur in germline tissue happen at fertilization or during the process of development in sex cells. Familial syndromes stem from germline mutations that are transmitted to offspring and are present in every cell. When a mutation gives a selective advantage to a cell, leading to a clonal outgrowth, it is considered a driver mutation. Alternatively, passenger mutations have no effect on the fitness of a cell but may be associated with a clonal expansion. Neoplasia or abnormal cell growth may be malignant or benign. Malignant tumors are invasive with rapid growth, while benign adenomas are slow growing non-invasive tumors that do not metastasize.

B. Clonality

The term clonality is related to understanding the cell of origin giving rise to daughter cells. Monoclonality refers to daughter cells arising from a single cell, while polyclonality refers to cells proliferating from multiple progenitor cells. Clonality can be assessed with molecular analysis. In female patients, tumor clonality can be evaluated with X chromosome inactivation assays, based on the concept that in every female cell, one X chromosome becomes inactivated and the pattern of which chromosome becomes inactivated is random (Brown CJ et al 2000).

C. Tumor Genes

Mutations in tumor suppressor genes play a role in the pathogenesis of cancer, whether it is complete or partial tumor suppressor inactivation (Berger et al 2011). Oncogenes are activated by mutation, gene amplification, or

chromosome rearrangements that contribute to the development of a neoplastic phenotype. There are two classes of oncogenes one lowers the requirements for growth factors leading to a change in the cells phenotype, while the other class saves cells from apoptosis.

III. Hyperparathyroidism

Hyperparathyroidism is a biochemical diagnosis in which excessive secretion of PTH leads to high blood calcium. Discerning if a patient should be diagnosed with primary or secondary hyperparathyroidism relies on the biochemical differences of high and low calcium levels, respectively. There are two forms of primary hyperparathyroidism, sporadic and familial.

A. Familial Hyperparathyroidism

Familial hyperparathyroidism can be diagnosed based on a family history of PHPT or other features suggestive of a familial multiple endocrine neoplasia syndrome. Inherited (familial) syndromes account for ~5% of Primary Hyperparathyroidism (PHPT) (Pepe J et al 2011).

The multiple endocrine neoplasia term is based on two or more endocrine glands producing tumors. Depending on the pattern of the benign or malignant tumors, it is characterized as one of the several distinct syndromes, which are summarized in Table 1. MEN1 is characterized by tumors in the endocrine pancreas, pituitary, and most commonly in the parathyroid. This syndrome is caused by mutations in the *MEN1* gene (70-95%) of cases or cyclin dependent

kinase inhibitor genes (CDKIs) p15 (1%), p18 (0.5%), p21 (0.5%), p27 (1.5%) (Marini F 2008).

MEN2 is caused by mutations in the *RET* gene and is further divided into two different subtypes: MEN2A and MEN2B. MEN2A causes medullary thyroid cancer, pheochromocytoma, and adenomas in the parathyroid. MEN2B causes medullary thyroid cancer, marfanoid habitus/ mucosal neuroma, and pheochromacytoma. MEN4 shares the same phenotypic presentation as MEN1, however MEN4 is caused by mutations in the *CDKN1B* (p27) gene (Table 1).

Table 1. Multiple Endocrine Neoplasia Syndromes

Syndrome	Gene	Manifestations
MEN1	*MEN1*	Pituitary Adenoma, Parathyroid Neoplasia, and Pancreatic Tumors
MEN2A	*RET*	Parathyroid Neoplasia, Medullary Thyroid Carcinoma, and Pheochromacytoma
MEN2B	*RET*	Mucosal Neuromas Marfanoid Habitus, Medullary Thyroid Carcinoma, and Pheochromacytoma
MEN4 (MENX)	*CDKN1B*	Pituitary Adenoma Parathyroid Neoplasia, and Pancreatic Tumors

B. Sporadic Hyperparathyroidism

There are three ways for sporadic, primary hyperparathyroidism to manifest: parathyroid adenomas are most common (85%), parathyroid hyperplasia is less common (15%), and rarely, parathyroid carcinoma can occur

(<1%) (Loya SolIs 2014). There are a few established tumor genes including cyclin D1 (*CCND1/PRAD1*) and multiple endocrine neoplasia type 1 (*MEN1*) (Costa-Guda,,2014). Candidate oncogenes include β-Catenin (*CTNNB1*), Enhancer Of Zeste 2 Polycomb Repressive Complex 2 subunit (*EZH2*), and Zinc Finger Protein X-linked (*ZFX*) (Duan K 2015). The frequencies of various gene mutations identified in sporadic parathyroid adenoma are illustrated in Figure 2.

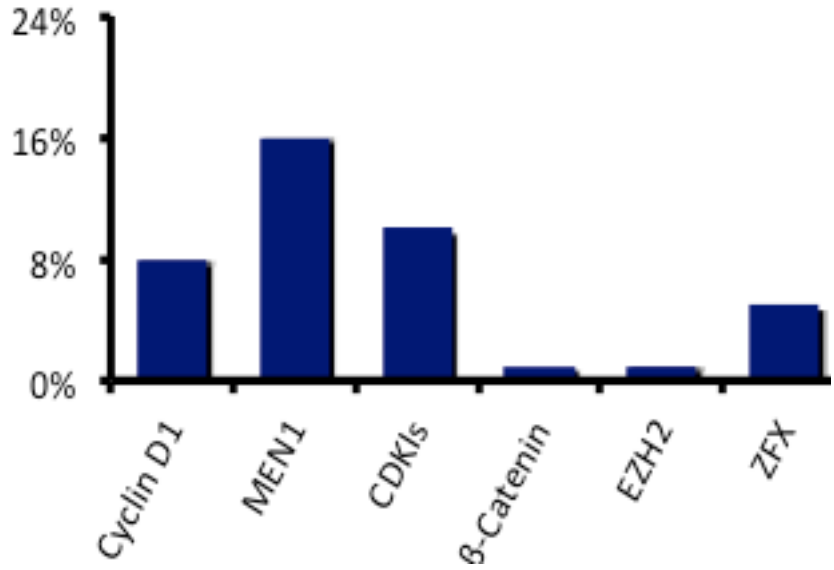


Figure 2. Genetic Abnormalities in Sporadic Parathyroid Adenomas
MEN1 is the most frequently mutated gene in sporadic parathyroid adenomas. Rearrangements of cyclin D1 and intragenic point mutations of CDKI genes have also been identified as genetic drivers. Rarely, mutations occur in other genes, such as β-Catenin (*CTNNB1*) and *EZH2.*

CCND1/PRAD1, which encodes cyclin D1, was first identified as a human oncogene based on its involvement in parathyroid tumors (Motokura T., Arnold, 1991). Overexpression of cyclin D1 is seen up to 40% of sporadic parathyroid tumors. Cyclin D1 gene rearrangements seen in up to 8% of tumors directly causes this overexpression. Cyclin D1 gene rearrangement is a pericentromeric

inversion of chromosome 11 that is well characterized, which places the 5' PTH regulatory region upstream of the cyclin D1 gene, as shown in Figure 3.

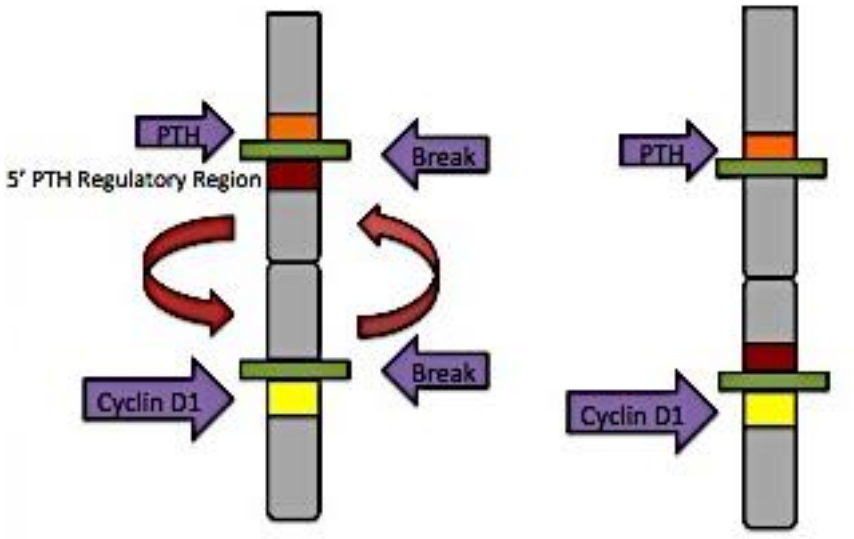
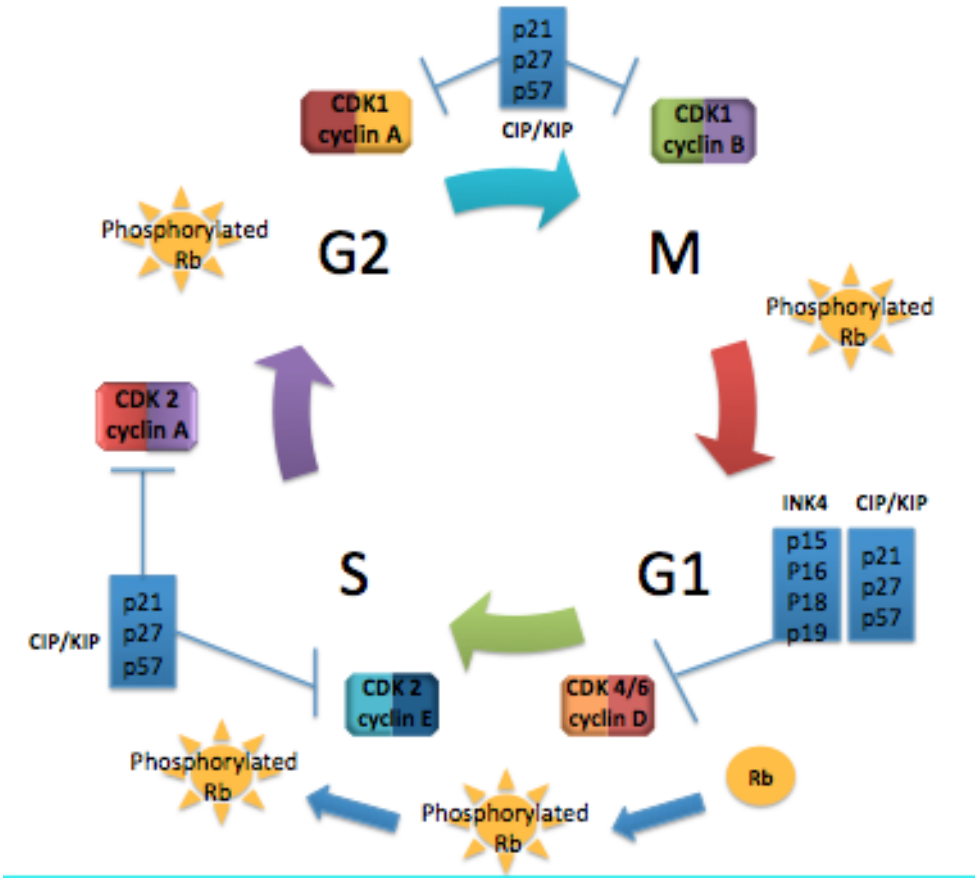


Figure 3. CCND1/PRAD1 gene rearrangement
A schematic of the pericentromeric inversion of chromosome 11. This rearrangement places the 5' PTH regulatory region upstream of the cyclin D1 gene.

Cyclin D1 is a regulator of the subunits CDK4 and CDK6 these complexes are critical for the progression of the cell cycle, specifically during the G1 phase (Figure 4). Cyclin dependent kinases (CDKs) and cyclins bind together to promote the continuation of the cell cycle, while cyclin dependent kinase inhibitors (CDKIs) are put in place to counteract cell proliferation. In the early G1 phase, cyclin dependent kinase 4 and 6 are associated with cyclin D. Later in the G1 phase, after the restriction point, CDK2/ cyclin E complex stimulates the transition of the cell cycle from G1 to S phase, which determines cell division.

The combined CDK 2/ cyclin A complex governs the S phase entry, initiating DNA replication. The cell is then committed to proliferate with the cyclin B and CDK1 complexes activating mitosis.

Mutations in several CDKI genes, including p27, have recently been identified as important contributors to a subset of parathyroid adenomas. Mutations have been identified in a few additional candidate genes, but, to date, their ability to drive parathyroid tumorigenesis experimentally has not been demonstrated. Mutations in β-catenin and *EZH2* appear very rarely in parathyroid adenomas. In contrast, mutations in *ZFX* appear to be more common.



Adapted from Donavan J. et. Al. 2000

Figure 4. Cell Cycle Control
The cell cycle displays the four phases; cell growth (G1), DNA synthesis (S), cell growth (G2), and mitosis (M). Progression through the cell cycle is governed by interactions between Cyclins, CDKs and CDKIs

Aims and Objectives

The overall aim of this dissertation was to further the understanding of genetic events that lead to parathyroid hyperproliferation and neoplasia. This was done through the following specific aims*:*

1. To optimize a protocol for DNA extraction from formalin fixed paraffin embedded tissue.

An optimized protocol for DNA extraction from formalin fixed paraffin embedded (FFPE) tissue is essential for further analysis of genetic events promoting parathyroid neoplasia. To this end, we tested several different protocol modifications that produce the highest DNA yields while minimizing degradation.

2. **To determine the role of *ZFY* in sporadic parathyroid adenomas.**

Recurrent, somatic mutations in the *ZFX* gene have recently been identified in parathyroid adenomas. Since *ZFX* and another gene, *ZFY,* are virtually identical, we hypothesized that parathyroid adenomas may contain mutations in the *ZFY* gene, similar to those observed in *ZFX.* To test our hypothesis, we directly analyzed the entire coding region of *ZFY* in parathyroid adenomas from male patients.

3. To perform CDKI mutational analysis in a patient with unexplained MEN1.

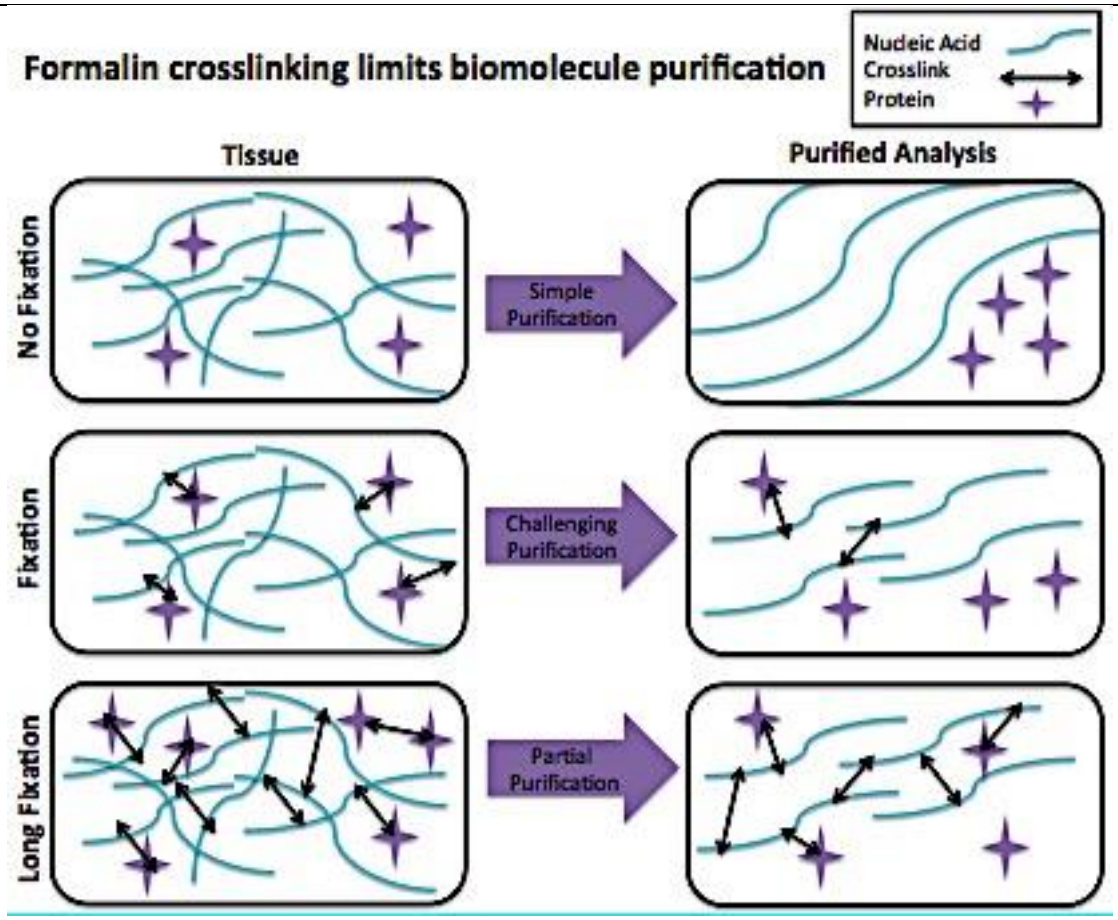
A patient with phenotypic MEN1 (prolactinoma and familial hyperparathyroidism), but negative for *MEN1* gene mutation was referred to us for Cyclin Dependent Kinase Inhibitor gene sequencing, in order to bring clarity for genetic counseling.

Chapter 1. Optimization of a Protocol for DNA Extraction from Formalin Fixed Paraffin Embedded Tissue

Introduction

Tissues are commonly fixed in formalin and embedded in paraffin for histopathological diagnosis and preservation after biopsies or surgeries. Formalin fixation and paraffin embedding is invaluable for the purpose of preserving tissue for long-term storage and later pathological studies. Fixation is one of the most vital steps in the preservation process. As illustrated in Figure 5, formalin cross-links amino acids in proteins and nitrogen molecules in nucleic acids to prevent tissue decay by autolysis or putrefaction. Unfortunately, in comparison to preservation by quickly freezing tissue, the formalin fixation process decreases the quality of the nucleic acids that can be extracted from the tissue. Therefore, optimizing a protocol for DNA extraction from formalin-fixed, paraffin-embedded (FFPE) tissue is crucial for gaining higher DNA yields without increasing degradation.

Extraction of DNA from FFPE tissues requires removal of the paraffin wax (deparaffinization), cell lysis releases proteins and nucleic acids, proteinase K digestion to remove contaminating proteins and inhibit nuclease activity, and purification through a DNA-binding column or other methods. To develop an optimized protocol for DNA extraction from FFPE tissue, we tested two different deparaffinization methods, modifications to the proteinase K digestion time and elution protocol, using column-based DNA purification.



Adapted from Qiagen QIAMP DNA FFPE Tissue Handbook 2012

Figure 5. Formalin Fixation Effects

Crosslinking between proteins and nucleic acids are caused by formalin fixation. This figure represents how prolonged fixation induces crosslinking.

Materials and Methods

Deparaffinization

Extracting DNA from paraffin blocks begins with slicing tissue with a microtome into sections of 20 µM. The deparaffinization procedure in this experiment used two different methods: either xylene-ethanol or Deparaffinization Solution (Qiagen).

Deparaffinization with xylene-ethanol is done under the hood to minimize exposure to hazardous fumes. The tissue section is soaked in 1 mL of xylene for 10 minutes, and then centrifuged for 30 seconds. This step is followed by saturating the tissue in 1 mL of 100% ethanol for 10 minutes and centrifuged again for 30 seconds. This process of xylene and ethanol is repeated in a second cycle. After the cycles are completed, the sample must be dried at room temperature. Tested modifications to the xylene-ethanol deparaffinization protocol include preheating xylene to 65°C, chilling the ethanol to 4°C, and increasing the number of cycles.

Deparaffinization with Qiagen Deparaffinization Solution is done by incubating the tissue section with 320 µL of Deparaffinization Solution at 56°C for either three or eight minutes.

Proteinase K Digestion

Following deparaffinization, the tissue was incubated with proteinase K in the presence of ATL lysis buffer. Incubations were done for either one hour, 90 minutes, or overnight.

Column Purification

DNA was purified using the QIAamp DNA FFPE Kit, following the manufacturer-recommended protocol. Modifications tested included using chilled ethanol or increasing the incubation time with the AE elution buffer from 5 to 10 minutes. A summary of all protocol modifications is shown in Table 2.

Analysis of DNA Quality and Yield

The amount of DNA degradation was analyzed using gel electrophoresis and imaging. DNA quality and yield were further analyzed using a Nanodrop 2000c and Qubit fluorometer. The Nanodrop is used to quantify DNA via spectrophotometry. Nucleic acids absorb light at a wavelength of 260 nm, the 260/280 and 260/230 ratios measure different characteristics. The 260/280-ratio measures purity of DNA and RNA; the optimal range for DNA is 1.7-2.0 ratios above 2 suggest the presence of RNA, while a ratio lower than 1.7 suggest contamination with phenol, protein, or EDTA. The 260/230 ratio represents pure DNA at a score that ranges from 2.0-2.2. A score that is lower than this range may reveal contamination of carbohydrates, phenol, or EDTA.

The Qubit fluorometer can quantify DNA, RNA, and protein using different fluorescent dyes. Each dye is specific for one type of molecule: DNA, RNA or protein. The Qubit is generally more precise in determining the DNA concentration but cannot simultaneously give information about RNA or protein contamination.

Table 2. Formalin Fixed Paraffin Protocol Embedded Protocol

Protocol	Qiagen	Xylene-Ethanol
Deparaffinization Original	Incubate at 56°C-3 min, Cool at (15-25°C)	1 ml 100% EtOH 10 min (2x's) Centrifuge at Max 1 min 1 mL 100% EtOH 10 min (2X's) Dry at room temperature
Deparaffinization Method Modified	Incubate at 56°C-8 min Cool at (15-25°C)	Preheat: Xylenes 65°C Chill: Ethanol 4°C 1 mL 100% EtOH 10 min (5X's) Dry at room temperature
Proteinase K Incubation Original	1 Hour	1 Hour
Proteinase K Incubation Modified	1 Hour 90 Minutes Overnight	1 Hour 90 Minutes Overnight
Column Purification Original	Add AL Buffer 200L → Add EtOH 200 µL Centrifuge QI amp Column →AW1 500 µL → AW2 500 µL → 25 µL AE Buffer 5 min	
Column Purification Modified	Add AL Buffer 200 µL > Add chilled EtOH 200 µL Centrifuge QI amp Column → AW1 500 µL → AW2 500 µL → 25 µL at AE Buffer -10 min	

Results

Five different protocols, include two distinct approaches of deparaffinization were tested. The first comparison explored the two different deparaffinization methods, using an overnight proteinase K digestion and the manufacturer-recommended QIAamp DNA FFPE Kit ("original") protocol (Figure 6). The xylene-ethanol method (designated A) demonstrated consistently high levels of DNA degradation across all four tumors. In comparison, the Qiagen Deparaffinization method (designated B) showed less degradation. However, the xylene-ethanol method gave higher DNA yields.

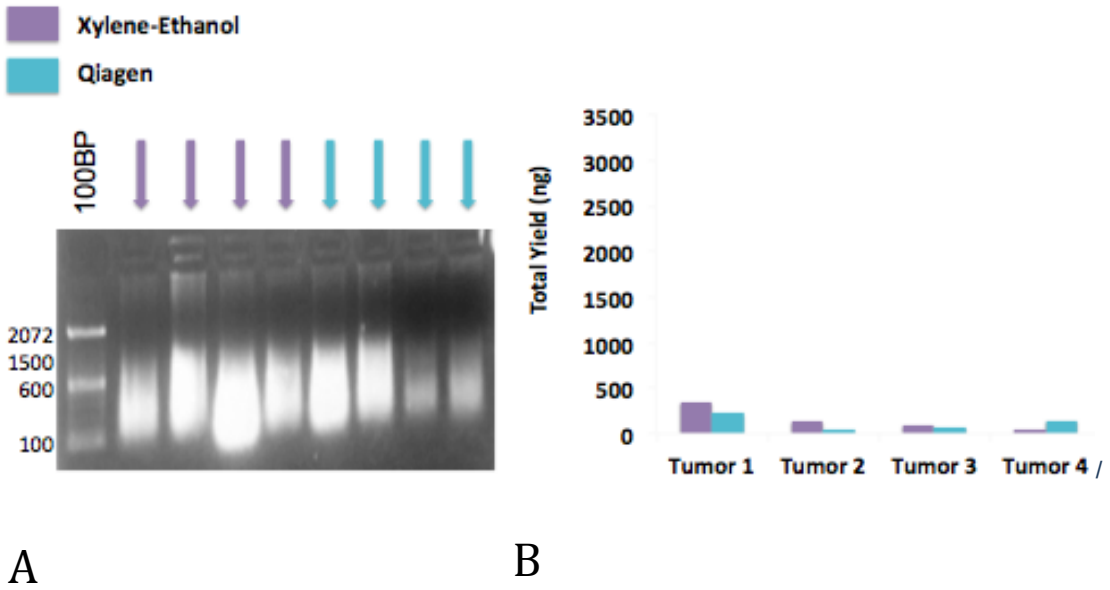


Figure 6. Original Protocol with Overnight Proteinase K Incubation
A. Lower levels of DNA degradation were noted with the xylene-ethanol method as compared to the Qiagen method when using the original protocol with overnight proteinase K digestion.
B. The xylene-ethanol method also produced greater DNA yield.

The second comparison explored the two different deparaffinization methods, using a shorter, one-hour, proteinase K digestion and the manufacturer-recommended QIAamp DNA FFPE Kit ("original") protocol (Figure 7). In this experiment, the Qiagen Deparaffinization method consistently showed less degradation across samples and much higher (up to 3x) DNA yields.

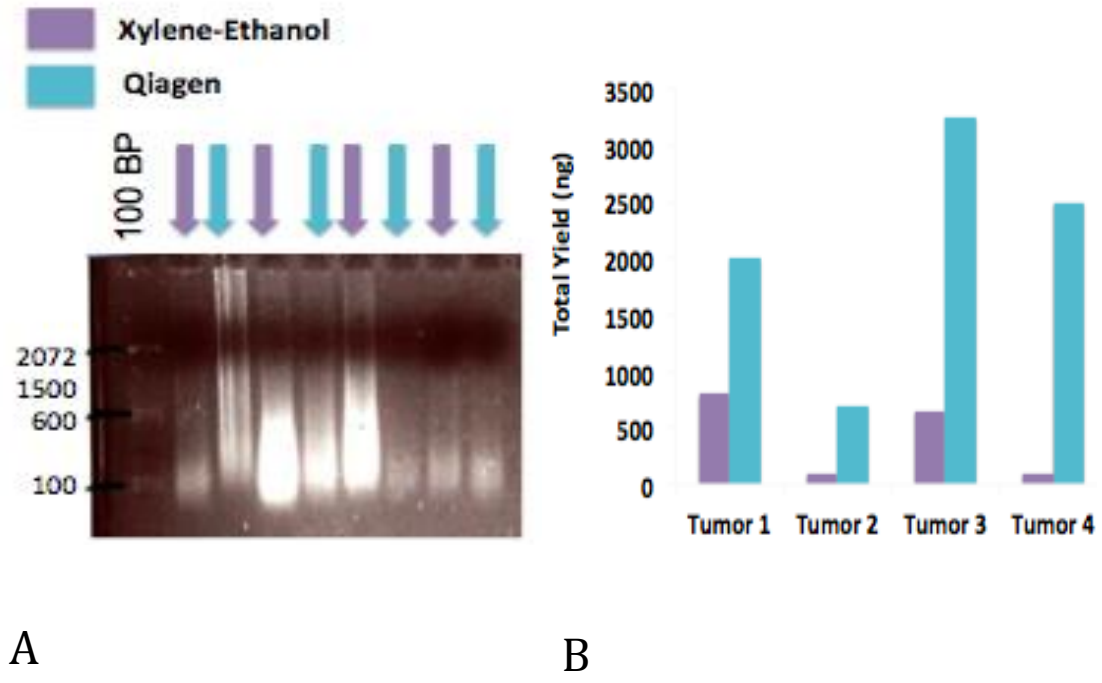


Figure 7. Original Protocol with One Hour Proteinase K Incubation
A. Lower levels of DNA degradation were noted with the Qiagen method as compared to the xylene-ethanol method when using the original protocol with 1-hour proteinase k digestion.
B. The Qiagen method also produced greater DNA yield.

The third comparison explored the two different deparaffinization methods, using a one-hour proteinase K digestion but with modifications to the manufacturer-recommended QIAamp DNA FFPE Kit ("modified") protocol (Figure 8). The modifications included using chilled ethanol and increased incubation time with the elution buffer. For the xylene-ethanol method, the number of cycles was also increased from two to five cycles. In this experiment, the Qiagen deparaffinization method demonstrated significantly lower degradation with higher DNA yields.

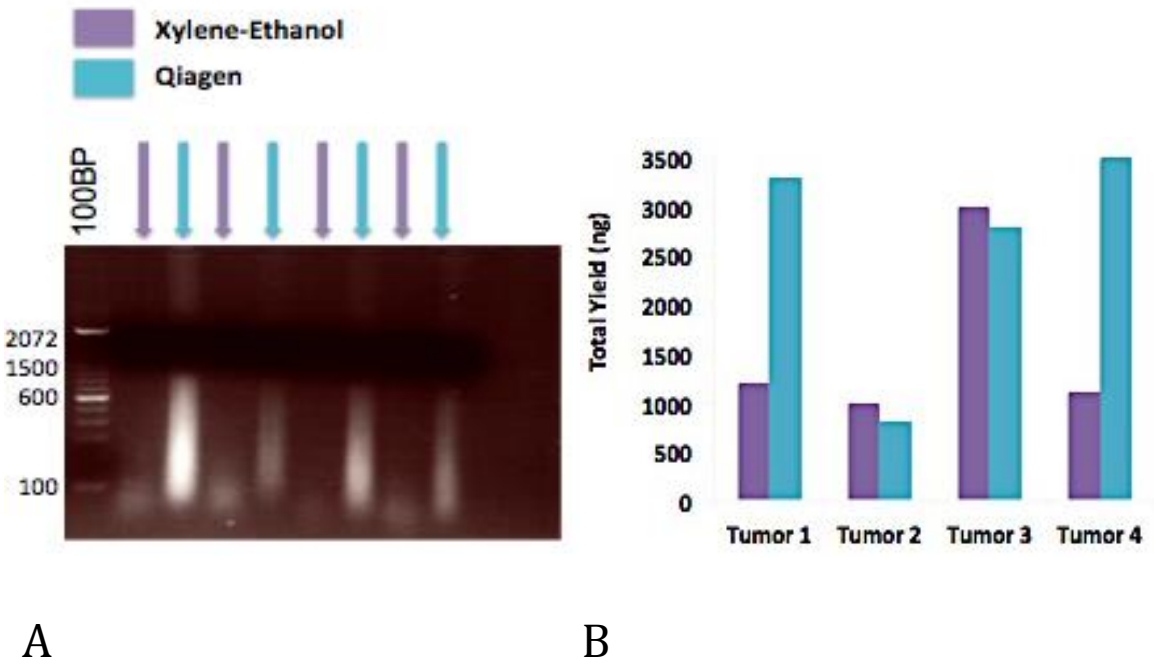


Figure 8. Modified Protocol with One Hour Proteinase K Incubation
A. Lower levels of DNA degradation were noted with the Qiagen method as compared to the xylene-ethanol method when using the modified protocol with 1-hour proteinase K digestion.
B. The Qiagen method also produced greater DNA yields quantified by Nanodrop and Qubit. The modified protocol with 1-hour proteinase K Incubation demonstrated the best DNA yields out of all the observed protocols.

The fourth comparison explored the two different deparaffinization methods, using an overnight proteinase K digestion and with modifications to the manufacturer-recommended QIAamp DNA FFPE Kit ("modified") protocol (Figure 9). Again, the xylene-ethanol method demonstrated consistently higher degradation and consistently lower DNA yields.

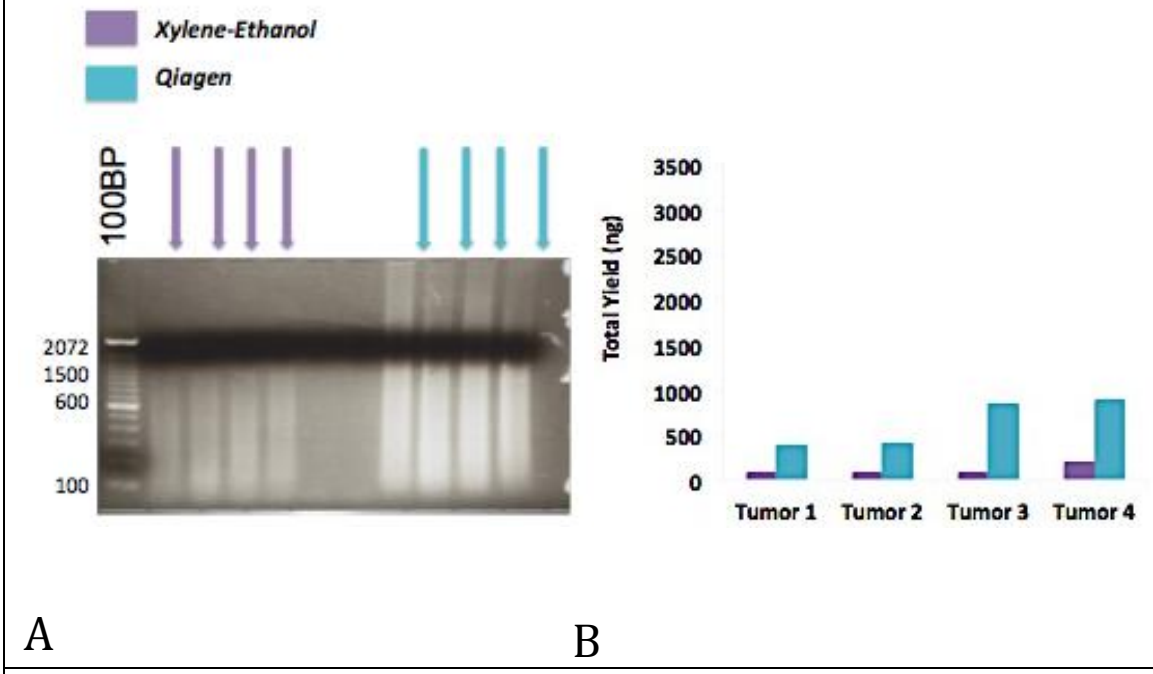


Figure 9. Modified Protocol with Overnight Proteinase K Incubation
A. Lower levels of DNA degradation were noted with the Qiagen method as compared to the xylene-ethanol method when using the modified protocol with overnight proteinase k digestion.
B. The Qiagen method also produced greater DNA yield.

The fifth and final comparison explored the two different deparaffinization methods, using a 90-minute proteinase K digestion and with modifications to the manufacturer-recommended QIAamp DNA FFPE Kit (“modified”) protocol (Figure 10). Again, the Qiagen deparaffinization method demonstrated lower degradation with higher DNA yields. However, the DNA yields in this experiment were lower than in most other experiments.

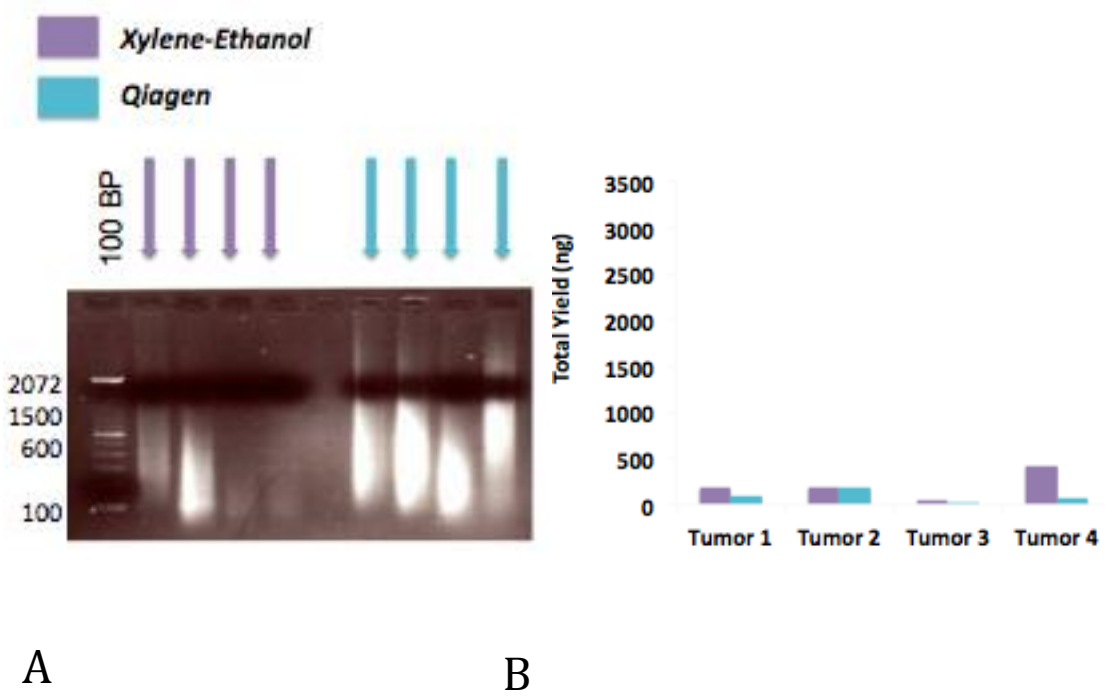


Figure 10. Modified Protocol with 90 Minute Proteinase K Incubation
A. Lower levels of DNA degradation were noted with the Qiagen method as compared to the xylene-ethanol method when using the modified protocol with 90 minute proteinase K digestion.
B. The Qiagen method also produced greater DNA yield.

Overall, the method that appeared to produce the highest DNA yields with the lowest amount of DNA degradation was the Qiagen deparaffinization method, using a one-hour proteinase K digestion and with modifications to the manufacturer-recommended QIAamp DNA FFPE Kit protocol.

Discussion

Narrowing down an optimized protocol that effectively isolates DNA rom FFPE samples is beneficial since this has been a challenge in the research community. There were several considerations to account for when it came to maintaining the integrity and the yields of the sample. After observing different FFPE protocols incorporating modifications to the deparaffinization, proteinase K incubation, and elution stages. It appears that the modified protocol with one-hour proteinase K incubation using the Qiagen kit solution yielded the best results for DNA preservation.

The previous thoughts were that the xylene-ethanol method with modifications and an overnight incubation of proteinase K would work best for producing the best DNA yields. However, it appears that the longer the proteinase K incubation was, the lower the results for the DNA yield appeared. It also seems that the increased cycles of xylene boosts the levels of degradation. Although xylene-ethanol protocols have been frequently practiced, a more bio-friendly solvent for deparaffinization may be preferred, like the Qiagen kit offers.

Chapter 2. Mutational Analysis of ZFY in Sporadic Parathyroid Adenomas

Introduction

Recently, compelling genetic evidence was reported implicating the Zinc Finger protein gene encoded by the X-chromosome (*ZFX)*, a member of the Kruppel C2H2 type zinc finger protein family, as a likely parathyroid adenoma oncogene involved in almost 5% of cases (Soong, Arnold 2014). Recurrent somatic mutations in *ZFX* were strikingly specific, focused on a hotspot of two consecutive highly conserved arginine residues in the most C-terminal zinc finger domain of the protein. *ZFX* is situated on the X-chromosome but escapes X-inactivation and is thus transcribed from both alleles in females. In males, the highly homologous Zinc Finger protein gene encoded by the Y-chromosome (*ZFY)* is expressed and provides dosage compensation. *ZFX* and *ZFY* are highly homologous; in fact overall they are 92% identical, and while the zinc finger domains are 97% identical. *ZFY*, like *ZFX* is a part of the Kruppel C2H2 type zinc finger protein family. *ZFY* is a transcriptional activator that binds to the consensus sequence 5'-AGGCCY-3'. There is very little known about *ZFY,* however it is involved in multicellular organismal development, and has thirteen zinc finger domains involved in nucleic acid binding (Lova-Solis 2014).

Since analogous mutations in *ZFY* could have similar tumorigenic effects as those in *ZFX*, and with one such *ZFY* mutation already noted in a colon carcinoma (COSMIC), we analyzed *ZFY* in a series of typical sporadic parathyroid adenomas from male patients.

Methods

Patients and Samples

Tumor samples were retrieved from patients that received a parathyroidectomy under the circumstances of managing primary hyperparathyroidism. Samples were collected from solely sporadic related cases. This is based on negative family history related to primary hyperparathyroidism, and multiple endocrine neoplasia. Every tumor sample had both the tumor and matched germline control DNA for every designated patient. All samples were obtained with informed consent in accordance with institutional review board-approved protocols.

PCR and Sanger Sequencing

Primers were designed for *ZFY's* seven coding exons (Table 3). Primers were designed so that intron regions at the splice junctions were also examined (Figure 11). The PCR reactions were carried out in 20 *μ*L reaction volumes, containing 25 *ng* of sample DNA, 12 *μL* of deionized H_2O, 2 *μL* of 10X PCR Buffer, 10 *μM* of dNTPs, *50 mM of* $MgCl_{2,,}$ 1 *μL* of forward and reverse primer, and *1 μL* of Taq Gold (Applied Biosystems, Foster City, CA, USA). The thermal cycles began with cycling conditions for exons 2-8. The PCR reactions were performed by incubating at 95°C for 10 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds (60°C for exon7) and 72°C for one minute, and a final elongation step of 72°C for 10 minutes.

Table 3. ZFY Primer Design List

Exon	Forward	Reverse	Expected Product Size (BP)
1	AGAGAAAGGCCGTCCTGCAGC	CTTCGCGGACATTTACATAGC	360
2	CCCATGTATCCCTGGAAATC	CCCATGTATCCCTGGAAATC	446
3(pt.1)	GAGGAACACCAAGGACATAG	TTCTGGCATAGACATTGAGG	400
3(pt.2)	CAAGTGCTGGACTCAGATGT	TGTTAGAAAACACAGGTTTC	295
4	AACAAAGATGACATATGTCC	CATTTGGTTGAAACATTTGG	342
5	ATACACTAAAACGTGTAAGC	TGATGGGTTGATAGGTGCAG	278
6	CTGTGTGATCTCTGTAAACC	AGAAACCACTTTCAACAGTG	297
7	TGTCATTCATGAGTGTCAGG	AATACATGTGGCCTACTAGC	441
8(a)	ATTCATGAGGAGACCAGAAG	GAGGTGGCGATTCAATAACC	493
8(b)	AAAAGGGGCCAACAAAATGC	TTCTGAAGGCCTGTGAAAGC	454
8(c)	CACATCAGTGTTTGCATTGC	TCTGCTTCAAGGCCAACATC	431
8(d)	ACCTTCAGAAAAGAACCAGC	GGCCTGTGAAAGCCTTTCTCG	362

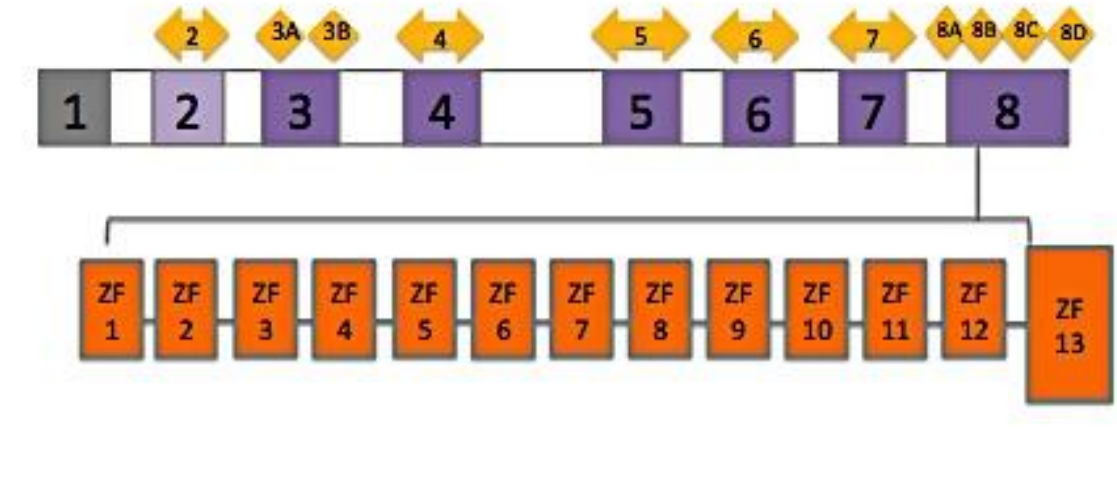


Figure 11. ***ZFY*** **gene Primers**
All the coding regions (exons 2-8) were PCR amplified. The 13 zinc fingers are included in Exon 8.

The PCR products were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA) and were sequenced using standard Sanger sequencing methodology

(GeneWiz Inc., South Plainfield, NJ). Sequence data were analyzed using the Sequencher DNA sequence analysis software to align multiple tumor sample sequences along with reference sequences and to analyze possible variants in the tumor and control samples. Potential mutations were confirmed by a second cycle of sequencing from independent PCR reactions and by sequencing the DNA from the blood sample, to check for germline variants.

Results

A total of 117 parathyroid adenomas were tested for sequence variants in the coding region of *ZFY*. If the true frequency of *ZFY* mutation in parathyroid adenoma was comparable to that of *ZFX* (6/130, 4.6%), this sample size of 117 was sufficiently powered to detect such mutations, with a statistical power of 80% (Table 4).

Table 4. Statistical Power Calculation

Power Calculation	ZFX	ZFY
Sample Size	130	117
Average Value	6	5 (Expected Value)
Statistical Power	80%	

Out of 117 cases, two variants were found (one still unconfirmed), each represented in two different patients. The variants include one single nucleotide

polymorphism and one point mutation. The single nucleotide polymorphism (rs111915341) was found in the intron region preceding exon 5 (Figure 12).

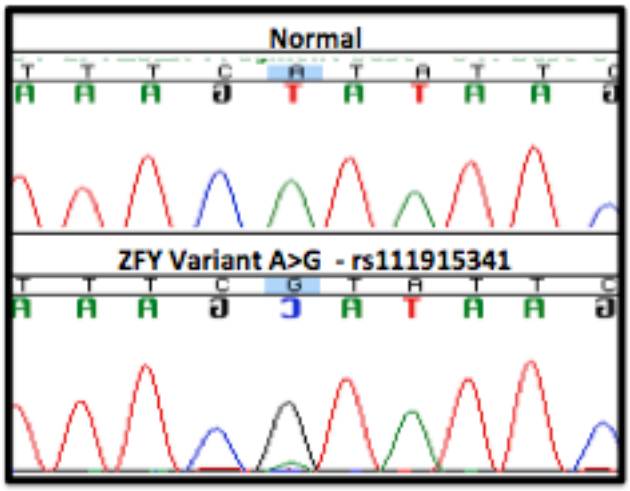


Figure 12. ZFY Direct genomic DNA Sequencing of rs111915341
The single nucleotide polymorphism (rs111915341) was found in the intron region preceding exon 5.

The novel variant, c.1402C>T, affects the first base of the codon, resulting in histidine to tyrosine change at position 468 of the protein, affecting the second zinc finger domain. Analysis of germline DNA suggested the variant was somatic but neither finding has yet been replicated and must be considered preliminary (Figure 13).

Two methods were used to determine any possible changes in functionality due to the variant. The first tool used was a software program called Polymorphism Phenotyping V2 (PolyPhen-2). With this tool, the histidine to tyrosine change at position 468 is predicted to have a probably damaging effect, with a score of 0.998 out of 1.0. This prediction was confirmed using a second

software tool, PROVEAN, which also forecasts the impact of a variant on the biological function of a protein. In this analysis, the default threshold is -2.5; variants with a score equal to or below -2.5 are considered “deleterious”. Variants with a score above -2.5 are considered “neutral”. The PROVEAN score for the H468Y variant calculates it to be -3.068, suggesting it is deleterious.

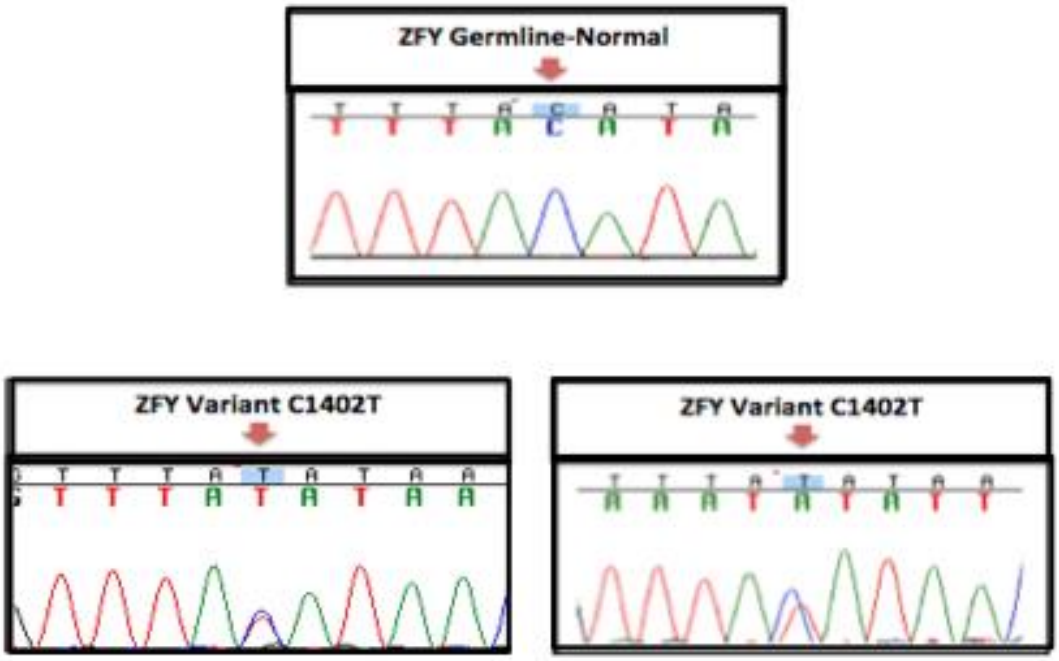


Figure 13. ZFY Direct genomic DNA Sequencing of C1402 mutation.
A novel variant c.1402C>T was found in exon 8, the top chromatogram demonstrates the normal sequence.

Discussion

Interest in sequencing *ZFY* came from the finding of recurrent, somatic mutations of *ZFX* in parathyroid adenomas. While in *ZFX*, identified mutations targeted a hotspot of two consecutive, highly conserved arginine residues at positions 786 and 787 in the last zinc-finger domain. Sequencing the entire

coding region was important to determine if there were any other mutations outside of the hot spot region that could contribute to parathyroid tumorigenesis. While no *ZFY* mutations had previously been identified in other endocrine tumors, COSMIC notes a *ZFY* R782 mutation (corresponding to *ZFX* R787) in colon cancer. With this knowledge in mind, *ZFY* was analyzed in a series of typical, sporadic parathyroid adenomas from male patients.

One novel variant, c.1402C>T that remains to be replicated, was identified in one tumor. The base change results in a histidine into tyrosine change at position 468, altering it from basic to aromatic, yet still remaining polar. This is a non-conservative mutation, which likely has deleterious effects. The variant is found in the N terminal region where there are multiple C2H2 Zinc finger repeats. This region has been shown to activate transcription when fused to a DNA binding domain. This genetic alteration, if confirmed, would support the idea that *ZFY*, like *ZFX*, may function as a direct-acting oncogene in the context of parathyroid adenomas.

Surprisingly, the chromatogram of variant c.1402C>T appears heterozygous, with two peaks representing the mutant thymine and the normal cytosine. This is a noteworthy finding because typically only one peak for a male patient would appear. The observed heterozygosity of the *ZFY* mutation may be due to contamination with non-tumor cells, mosaicism, or tumor heterogeneity.

In mosaicism, subjects have an apparently heterozygous genotype due to the presence of two different cell populations. Mosaicism is due to mitotic errors during embryonic development. Mosaicism is rare, however there has been a

study that provides evidence that mosaicism can account for some sporadic cases of MEN1 (Klein R. 2004).

In tumor heterogeneity, clonal evolution results from distinct genetic events that occur in the pathway from a normal cell into a tumor. In this transformation, a normal cell is hit randomly by a genetic event. This event causes a genetic modification that leads to a clonal, subpopulation of cells. These cells, unlike the normal cells, multiply more and die less often. Those same cells may be hit again and begin to further expand because they have a growth advantage over the previous cells.

The entire coding region of *ZFY* was fully sequenced in 117 of these tumors, with one preliminary variant found with confirmation pending. The highest priority in future observations will incorporate replicating this variant to confirm this mutation. This study suggests experimental evidence for the new role of mutant *ZFY* as a possible parathyroid oncogene. It will also provide a framework for our subsequent studies to address the binding sites and target gene promoters crucial to *ZFY'*s oncogenic functions. Further, these studies of the mechanisms behind parathyroid adenoma development will help address crucial and more general questions on how benign and malignant growths differ and how that information might help in preventing human malignancies in multiple tissue contexts.

Chapter 3: CDKI Mutational Analysis

Introduction

The cyclin dependent kinase inhibitors (CDKIs) function as negative regulators of cell cycle progression. CDKIs are categorized into either the Cip/Kip or INK4 family. The cyclins that are a part of the Cip/Kip family include p21 (encoded by *CDKN1A*), p27 (*CDKN1B*), and p57 (*CDKN1C*). The Cip/Kip family is well characterized for their role as negative regulators of G1 phase cell cycle progression by inhibiting a broad spectrum of cyclin-CDK complexes (Sherr and Roberts 1999). The INK4 family members include p16 and p14, (ARF encoding, *CDKN2A/ARF*), p15 (*CDKN2B*), p18 (*CDKN2C*), and p19 (*CDKN2D*). The INK4 family inhibits the activity of CDK6 and CDK4 complexes.

A germline mutation identified in *CDKN1B*, encoding p27, established the involvement of CDKI gene mutations in a MEN 1-like syndrome (Pellegata et al., 2006). This discovery was later confirmed by other investigators (Malatore S., et. al. 2010, Martucci F, 2012), and led to the identification of variants in other CDKIs (Costa-Guda J., 2013). There are no established genotype-phenotype correlations between patients presenting with MEN1 syndrome due to mutations in the *MEN1* gene or in the *CDKI* genes. For this reason, it is especially important to determine the underlying genetic cause in each patient who presents clinically with MEN1 syndrome features, so that potential genotype-phenotype correlations can be made, which may ultimately help guide treatment decisions.

Methods

One female patient with a diagnosis of phenotypic MEN1, based on a personal history of prolactinoma and familial hyperparathyroidism, but a normal *MEN1* gene status was examined in this study. We received germline DNA and performed PCR and Sanger sequence analysis of four CDKI genes (*CDKN2B*, *CDKN2C*, *CDKN1A*, or *CDKN1B*), as previously described (Costa-Guda J., et. al 2011, 2013).

Results

The four CDKI genes were analyzed and one sequence variant was identified. This novel c.34G>A variant was found in exon 2 of the *CDKN2C* gene. This heterozygous, missense mutation changes the first base of the codon, resulting in an amino acid change at position 12 from an alanine to threonine (Figure 14).

Two methods were used to predict the functional effects of the *CDKN2C* variant. The polymorphism phenotyping V2 (Polyphen-2) software program was used for predicting the impact of an amino acid substitution on the structure and function of a human protein using physical and comparative considerations. The PolyPhen-2 score ranges from 0.0 (tolerated) to 1.0 (deleterious). Variants with scores of 0.0 are predicted to be benign. Values closer to 1.0 are more confidently predicted to be deleterious. The mutation was predicted to be probably damaging with a score of 0.983 out of 1.0.

Prediction was verified using PROVEAN. PROVEAN scores equal to or below a predefined threshold (e.g. -2.5) are predicted to have a "deleterious"

effect, while scores above the threshold predict a "neutral" effect. The PROVEAN score for the identified mutation was -3.626, suggesting it as deleterious.

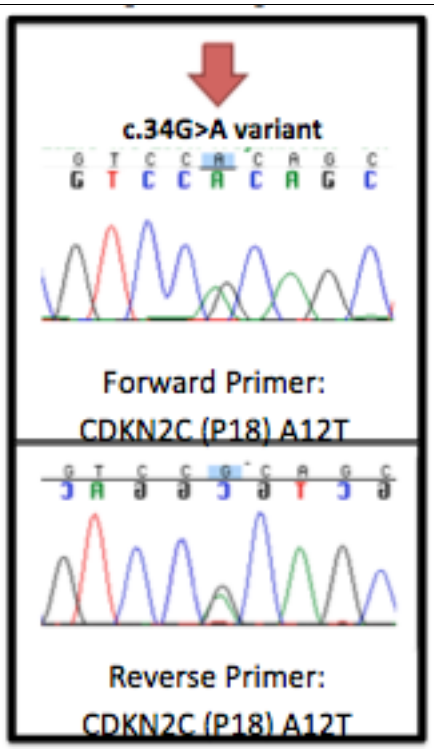


Figure 14. CDKN2C gene Germline variant c.34G>A
These chromatograms show a novel c.34G>A variant for the germline sequence. A mutation in the *CDKNC* gene was found at position 34, encoding a codon at amino acid position 12. This alters the codon from an alanine (GCA) to threonine (ACA) residue also, displaying heterozygosity [guanine/adenine].

Discussion

In this study several cyclin dependent kinase inhibitors were analyzed in a patient with phenotypic MEN1 but normal *MEN1* gene status. A novel variant c.34G>A, in CDKN2C, resulting in a p.A12T change in p18 protein, was found. The protein variant is predicted to have a "deleterious" effect by the software

program PROVEAN and polyphen-2. As expected for a germline mutation, this variant was heterozygous. This specific variant was not found in National Center of Biotechnology Information (NCBI), Catalogue of Somatic Mutations in Cancer (COSMIC), or in the single nucleotide polymorphism database (dbSNP).

The evidence, pending confirmation, of the variant c.34G>A in *CDKN2C* contributing to the clinical phenotype of MEN1 syndrome would be strengthened by directly determining an abnormal impact of the mutation on *CDKN2C activity.* The significance of this finding can be verified through experiments that demonstrate a relationship between the genotype with the variant and the clinical phenotype by incorporating a cellular or animal model (Cassanova et al., 2014).

Further studies, in which other affected and unaffected family members are examined is another way to strengthen the evidence of the *CDKN2C* mutation's relevance. Concordance of the candidate genotype and clinical phenotype in family members of the patient would help demonstrate the relevance of the *CDKN2C* variant. However, the inability to identify this variant in family members with the same clinical manifestations would negate its significance (Cassanova et al., 2014).

The germline DNA of a single patient was sequenced in order to identify any mutations in the CDKIs that could contribute to the molecular pathogenesis of this patient's adenomas, linked to MEN1 syndrome. The Identification of a missense mutation in the *CDKN2C* gene could potentially suggest that it plays a role in the phenotypic presentation of the MEN1 syndrome. Further knowledge on this inherited mutation might be useful in the management of disease by

providing guidance for best course of action for treatment for others in the same family. The patients could benefit from a positive or negative status report. For instance, a positive status could direct them to the best monitoring and treatment options. On the other hand, individuals with a negative status will be spared from unnecessary screenings and checkups. In general, an increased patient sample pool together, with powerful tools like Next Generation Sequencing technology, will assist in the discovery of more variants associated with parathyroid related diseases.

List of Abbreviations

CASR	Calcium Sensing Receptor
CCND1	Human Cyclin D1 proto-oncogene
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CDKN1A	Human cyclin dependent kinase inhibitor 1A gene, p21
CDKN2A	Human cyclin dependent kinase inhibitor 1A gene, p16
CDKN1B	Human cyclin dependent kinase inhibitor 1B gene, p27
CDKN2B	Human cyclin dependent kinase inhibitor 2B gene, p15
CDKN1C	Human cyclin dependent kinase inhibitor 1C gene, p57
CDKN2C	Human cyclin dependent kinase inhibitor 2C gene, p18
CDKN2D	Human cyclin dependent kinase inhibitor 2D gene, p19
CTNNB1	Human catenin (cadherin-associated protein), beta 1 gene
DNA	Deoxyribonucleic acid
FHH	Familial hypocalciuric hypercalcemia
HPT-JT	Hyperparathyroidism-jaw tumor syndrome
HRPT2	Human Hyperparathyroidism 2 gene, encoding parafibromin
(CDC73)	
MEN1	Multiple endocrine neoplasia type 1
MEN1	Human multiple endocrine neoplasia type 1 gene
MEN2	Multiple endocrine neoplasia type 2
MEN2A	Multiple endocrine neoplasia type 2A
MEN2B	Multiple endocrine neoplasia type 2B

MTC	Medullary thyroid cancer
PCR	Polymerase chain reaction
PTH	Parathyroid hormone
RET	Human RET (rearranged during transfection) proto-oncogene
SNP	Single nucleotide polymorphism

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