Clinicopathological Correlates of Pituitary Adenoma

by

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Institute of Medical Science
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Abstract

Our current understanding of pituitary adenoma (PA) pathobiology is limited. Thus, the purpose of this project is to gain a better understanding of PA biology through the completion of two aims: 1) conducting a retrospective review of PA patients and 2) establishing and characterizing an intracranial xenograft mouse model of PA and using the model to identify novel targeted therapies.

With respect to our first aim, several tumor- and patient specific factors were correlated to PA growth and recurrence. In our second aim, our tumor models were found to express growth hormone (GH) and prolactin as well as proteins in the mTOR pathway. These PA models were responsive to mTOR inhibition using RAD001 and resulted in a reduction in tumor growth rate and the expression of mTOR and GH. A preclinical and clinical study of PA provides a robust understanding of the clincopathologic factors contributing to PA and may ultimately help improve treatment and outcome.
Acknowledgements

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Contributions

Soroush Larjani (Zadeh lab) assisted with data collection in the first aim of this project
Shahrzad Jalali (Zadeh lab) assisted with the in vivo experiments
Amir Alamshebpour (Zadeh lab) performed western blots on tumor lysates
Toru Tateno (Ezzat lab) kindly provided the GH4 cells used for in vivo experiments
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## Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>4EBP1</td>
<td>Eukaryotic translation initiation factor 4E-binding protein 1</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin Biotin Complex</td>
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<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
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<tr>
<td>ADH</td>
<td>Antidiuretic Hormone</td>
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<tr>
<td>CS</td>
<td>Cavernous Sinus</td>
</tr>
<tr>
<td>CSI</td>
<td>Cavernous Sinus Invasion</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’ - Diaminobenzidine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicule-Stimulating Hormone</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Eukaryotic Translation Initiation Factor 4E</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FGFR4</td>
<td>Fibroblast Growth Factor Receptor 4</td>
</tr>
<tr>
<td>GH</td>
<td>Growth Hormone</td>
</tr>
<tr>
<td>HS</td>
<td>Horse Serum</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>ICA</td>
<td>Intracavernous carotid artery</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin Growth Factor-1</td>
</tr>
<tr>
<td>IRS-1 or 2</td>
<td>Insulin Receptor Substrate 1, -2</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ITK-SNAP</td>
<td>Insight Toolkit Snake Automatic Partitioning</td>
</tr>
<tr>
<td>mTOR</td>
<td>mechanistic Target of Rapamycin</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Image</td>
</tr>
<tr>
<td>NFPA</td>
<td>Non-functioning pituitary adenoma’</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>non-obessive severe combined immunodeficiency</td>
</tr>
<tr>
<td>NP40</td>
<td>Noniodet P40</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PA</td>
<td>Pituitary adenoma</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide dependent kinase 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>Pit-1</td>
<td>Pituitary-specific positive transcription factor-1</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>Ptd-FGFR4</td>
<td>pituitary tumor derived – Fibroblast Growth Factor Receptor 4</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphotase and Tensin homolog</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SF-1</td>
<td>Steroidogenic Factor 1</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>TEF</td>
<td>Thyrotroph Embryonic Factor</td>
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<tr>
<td>TMZ</td>
<td>Temozolomide</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Tpit</td>
<td>T-box Transcription Factor</td>
</tr>
<tr>
<td>TSC1,2</td>
<td>Tuberous Sclerosis Complex</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid-Stimulating Hormone</td>
</tr>
<tr>
<td>TVDT</td>
<td>Tumor Volume Doubling Time</td>
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Chapter 1
INTRODUCTION

1.1 Overview of PA and its Clinicopathologic Correlates
The pituitary gland is a small endocrine gland that is located at the base of the brain and consists of the anterior lobe and posterior lobe. Proliferations of hormone secreting epithelial cells of the anterior lobe can sometimes lead to neoplastic transformation giving rise to pituitary adenoma (PA). Although generally a benign neoplasm, PA can become locally invasive and infiltrate sensitive structures surrounding the pituitary fossa such as the optic chiasm and cavernous sinus. Furthermore, particularly aggressive PA can recur following surgery. The following subsections provide a description of pituitary anatomy and an overview of PA and how its growth and recurrence has been associated with various tumor- and patient specific characteristics.

1.1.1 Pituitary Gland

The pituitary is a small endocrine gland that sits at the base of brain in an area of the sphenoid bone referred to as the sella turcica. The pituitary gland is surrounded superiorly by the optic chiasm and hypothalamus, laterally on either side by the cavernous sinus, and inferiorly by the sellar bone. It is encased in fibrous material referred to as the capsule. Outside of the capsule, the pituitary is surrounded by dura which attaches to the inferior aspect of the diaphragma sella. The pituitary is the primary endocrine gland for the body as it regulates many of the hormones secreted from other endocrine glands. In turn, the hormonal activity of the pituitary gland is regulated by hypothalamic hormones, and negative feedback mechanisms from hormones released by the pituitary’s target organs. The pituitary can also regulate its own activity through various autocrine and paracrine functions (Bilezikjian, et al. 2004).

The pituitary is composed of two general areas referred to as the neurohypophysis and adenohypophysis. A third area called the pars intermedia is rudimentary in humans but plays a more prominent role in rodents. The neurohypophysis consists of the infundibulum, pituitary
stalk, and pars nervosa and is essentially composed of axonal projections from the hypothalamus. Consequently, hormones that are stored and secreted from the neurohypophysis, including oxytocin and antidiuretic hormone (ADH), are produced in the hypothalamus. The adenohypophysis is composed of epithelial tissue and consists of the pars distalis, which is the largest component of the adenohypophysis, and the pars tuberalis which extends from the adenohypophysis towards the pituitary stalk. The intercommunication between the hypothalamus and adenohypophysis is achieved via a portal vein network (Asa and Ezzat 2002).

The adenohypophysis, specifically the pars distalis, is composed of acini that contain six hormone-secreting cells within a reticulin rich stroma (Asa and Ezzat 2009). All of these cells are regulated by hypothalamic releasing or inhibiting factors. The specific cells and the hormones that they secrete are as follows: corticotroph cells secrete adenocorticotropic hormone (ACTH); the somatotrophs secrete growth hormone (GH), lactotrophs secrete prolactin (PRL), mammosomatotrophs secrete both GH and PRL, thyrotrophs secrete thyroid stimulating hormone (TSH), and gonadotrophs secrete follicle-stimulating hormone/Luteinizing Hormone (FSH/LH). In addition, the adenohypophysis contains the non-endocrine folliculostellate cells which are sustenacular cells that release cytokines and growth factors important for the maintenance of normal pituitary function (Marin, et al. 1991).

The adenohypophysis is derived from the oral ectoderm to form Rathke’s pouch. A single progenitor cell with the aid of specific transcription factors form the six cells of the adenohypophysis. Essentially, each of the cells is derived from one of three lineages which include pituitary specific positive transcription factor 1(Pit-1), the T-box transcription factor (T-pit), and steroidogenic factor 1(SF-1) (figure 1.1). The somatotrophs, lactotrophs, mammosomatotrophs, and thyrotrophs are derived from precursors expressing Pit-1. Pit-1 is required for GH gene expression and cooperation with ERα allows mammosomatotrophs to develop. A GH silencing mechanism aids in lactotroph differentiation. Coexpression of thyroph embryonic factor (TEF) and GATA-2 is needed to initiate TSH production. Corticotroph development is dependent on T-pit. Stereodogenic factor-1 (SF-1) and GATA-2 are required for gonadotroph differentiation (Asa & Ezzat, 2009) (figure 1.1.1). In addition, there are various transdifferentiation mechanisms that can convert one cell type of Pit1 lineage to
another depending, in part, on certain hormonal requirements by the body at certain times such as pregnancy or puberty, thus a level of fluidity is maintained among Pit1 cells (somatotrophs, lactotrophs, and mammosomatotrophs). Identification of the particular lineage from which a cell is derived is an important pathological criterion for diagnosing a specific tumor of adenohypophyseal origin.

Figure 1.1.1: Pituitary cell lineage as adapted from Asa and Ezzat, 2009

1.1.2 Pituitary Adenomas

Pituitary tumors or adenomas (PA) are benign neuroendocrine proliferations or over growths of adenohypophyseal differentiation. They are a common intracranial neoplasm, evident in up to 20% of the general population(Asa & Ezzat, 2009), though only a smaller percentage require medical treatment. PA are generally derived from one of the six hormone secreting cells of the adenohypophysis and are thought to originate from a single transformed cell and are thus classified as monoclonal lesions((Alexander, et al. 1990). They are characterized based on the expression of pituitary-specific transcription factors, hormones, and overall PA cell structure(Mete & Asa, 2012). Examination of the density of hormone-secreting granules within the PA cells leads to the designation of “densely” and “sparsely” granulated PA. A detailed
pathological examination of PA can lead to the identification of many different histological subtypes.

PAs are further classified based on their biochemical activity. Clinically functioning PA are tumors that oversecrete hormone and cause corresponding hormonal syndromes within the body. PRL-secreting PA are the most common functioning PAs accounting for up to 30% of all PA. Due to PRL overexpression, patients harboring a prolactinoma suffer from amenorrhea, infertility and galactorrhea in females, infertility or impotence in males. GH-secreting PAs are often associated with acromegaly. Clinical presentation of acromegaly is characterized by acral and facial changes, hyperhidrosis (abnormally increased perspiration), headaches, sexual dysfunction, and soft tissue enlargement. ACTH hypersecretion and adrenal overstimulation lead to hypercortisolism, which is responsible for the symptoms of Cushing’s disease. Cushing’s disease gives rise to fat accumulation in the face resulting in moon face, as well as buffalo hump, and truncal obesity. Additionally, the skin gets thinner caused by the loss of the epidermal layer and the tension over the accumulated fat produces purple striae. The development of psychiatric disorders such as depression and psychosis is also associated with Cushing’s disease(Bertagna, et al. 2009). TSH-secreting PAs are rare and present with a mild increase in thyroxin levels with abnormal TSH levels. Symptoms associated with gonadotroph PA that secrete FSH and/or LH may manifest in sexual dysfunction and hypogonadism. Nevertheless, gonadotroph PAs that secrete FSH/LH are very rare and this PA subtype is not usually characterized by hormonal production(Lake, et al. 2013).

On the other hand, clinically non-functioning PA (NFPA) are generally referred to as “silent” and do not cause hormonal abnormalities. Of the non-functioning subtype, gonadotrophs are the most common, comprising approximately 85% of all NFPA((Greenman and Stern 2009)). Other NFPA include the “silent” counterparts to the corresponding functional variant, and null cell adenomas which are of adenohypophysial origin but are negative for any pituitary transcription factor or hormone((Mete and Asa 2012)). Presenting symptoms from the NFPA subtypes are mainly due to mass effect such as headache or visual disturbance. These tumors may also lead to varying degrees of hypopituitarism depending on the amount of adenohypophyseal damage due to the PA((Lindholm, et al. 2006)). The distinction between clinically functioning and
nonfunctioning PA is based on a biochemical assessment rather than a histopathological examination (Mete and Asa 2012).

PAs are also classified radiologically, through examination of computed tomography (CT) or magnetic resonance image (MRI) which provides information on the size of the PA as well as on the degree of local invasion (Bonneville, et al. 2005). MRI provides good resolution of the sellar area while CT is good at depicting tumor that invades into bone. Generally, PA visualized on MRI are categorized based on size with microadenomas referring to PA that are less than 1 cm in diameter while macroadenomas are PA that are greater than 1 cm in diameter (Asa and Ezzat 1998). The designation of ‘giant’ adenoma is sometimes reserved for PA that are ≥3cm (Juraschka, et al. 2014) As noted earlier, the sellar fossa is adjacent to several areas including the optic chiasm, and cavernous sinus. PA that extend to or even invade these areas, typically the larger macroadenomas, make treatment more difficult and are often associated with a poorer outcome ((Rey-Dios, et al. 2014; Woodworth, et al. 2014)).

There are several treatment options available for patients with PA. These include medical therapy, surgery, radiotherapy, and/or gamma knife radiosurgery. There are currently a limited number of medications that are taken to reduce hormone secretion and/or tumor size. These include the dopamine agonists such as bromocriptine used to treat PRL-secreting prolactinomas (Price and Bridges 2014). Dopamine agonists target the D2 dopamine receptor to restore tonic PRL inhibition. Another medication is the somatostatin analogue, such as long acting octreotide, which is used to treat acromegalic patients (Zhang, et al. 2014). However, Octreotide is only effective in particular subtypes of GH PA. The vast majority of PA do not respond to medication. Endonasal microscopic or endoscopic transsphenoidal surgery is the primary treatment for most PA with gross total removal accomplished in most instances (Dallapiazza, et al. 2014). However, the more aggressive or invasive the PA, the lower the probability for complete surgical removal. Radiotherapy and gamma knife radiosurgery are generally reserved for recurrent PA that are not amenable to reoperation. Radiation-induced side effects from these treatment options include visual comprise, cranial neuropathies, or cognitive decline (Brada et al., 1993; Hahn et al., 2009).
Classifying a PA based on its radiological, biochemical, and histopathological features yields the most accurate portrait of a particular PA and the complete profile of the tumor should be incorporated into patient management. Thus, a multidisciplinary team of endocrinologists, neurosurgeons, radiologists, and pathologists should be assembled for the treatment of PA.

1.1.3 Growth Patterns of PA

Although generally a benign brain tumor, PAs have a tendency to compress or invade structures adjacent to the pituitary fossa. The invasive potential of PA has been correlated to tumor size with larger macroadenomas typically engaging in more locally invasive behavior (Scheithauer, Kovacs, Laws, & Randall, 1986). Furthermore, invasion has been thought to be associated with histological subtype. Such aggressive macroadenomas include sparsely granulated somatotrophs, densely granulated lactotroph adenoma, sparsely granulated corticotrophs, thyrotoph adenoma, acidophil stem cell adenoma (PRL- and GH positive PA of Pit-1 lineage), and crooke cell adenoma. (ACTH-positive PA of T-pit lineage)((Mete and Asa 2012)). The factors that are involved in aiding different growth and invasion patterns in PA need to be clarified.

One of the most common extrasellar growth patterns exhibited by PA is superior growth towards the suprasellar cistern and optic chiasm. It is estimated that 80% of macroadenomas present with this pattern (Ramakrishnan et al., 2013; Zada, Lin, & Laws, 2010). It has been reported that it is the NFPA subtype in particular that has a proclivity for suprasellar growth (Zada et al., 2010). Of the NFPA, the silent corticotroph adenomas are more commonly associated with a larger, irregular-shaped, lobulated suprasellar component compared to null cell/gonadotrophs, indicative of more aggressive behavior (Nishioka, Inoshita, Sano, Fukuhara, & Yamada, 2012).

Preferential inferior growth is also evident in different histological subtypes of PA. Lundin et al., analysed 115 PA that included GH-, PRL- and NFPA and reported that prolactinomas are most associated with invasion into the base of skull, and associated with a lower frequency of suprasellar tumor extension (Lundin, Nyman, Burman, Lundberg, & Muhr, 1992). Other groups have noted that GH-secreting adenomas frequently invade the sphenoid sinus and
clivus (Hagiwara et al., 2003; Marro et al., 1997; Zada et al., 2010). Silent GH-PRL, subtype III adenomas and acidophil stem cell adenomas also possess characteristic downward growth with significant bone invasion rather than presenting with the more common suprasellar expansion (Mete & Asa, 2012).

Parasellar growth into the cavernous sinus (CS) represents a surgical challenge due to the sensitive carotid artery, veins and cranial nerves located within this area. Cavernous sinus invasion (CSI) has been reported to occur in up to 20% of cases and is more commonly observed in clinically NFPA rather than functioning PA (Tanaka, et al. 2003)). Within the nonfunctioning group specifically, silent corticotroph adenomas have been reported to invade the CS more frequently than the null cell/gonadotroph subtypes and other hormone inactive subtypes (Jahangiri et al., 2013; Nishioka et al., 2012). An examination of CSI and its extent is discussed below.

1.1.4 Cavernous Sinus Invasion

The CS space is a venous plexus located on either side of the pituitary fossa. Each side is outlined by four dural walls: the roof, medial, lateral and posterior walls. The CS contains the intracavernous segment of the carotid artery (ICA) as well as the oculomotor, trochlear, ophthalmic, and maxillary cranial nerve located within the lateral wall of the CS. The abducens nerve also runs through the middle of the CS alongside the ICA.

The infiltration of the CS space usually occurs unilaterally and is observed more frequently in macroadenomas but can also occur in microadenomas (Cottier et al., 2000; Knosp, Steiner, Kitz, & Matula, 1993; Vieira, Cukiert, & Liberman, 2006). CSI often precludes gross total resection of tumor and increases intra- and postoperative complications (Sepehrnia, et al. 1991)). Therefore, the detection of CSI preoperatively is of critical clinical importance.

One of the ways preoperative diagnosis of CSI is accomplished is by taking into account the boundaries of the CS space. The CS space is surrounded by dura mater and the medial dural wall that separates the CS from the pituitary fossa is an important determinant of CSI. It is
perforation of this medial wall by PA that determines invasion as opposed to compression of the CS. However, localizing the medial wall on MRI is difficult and is usually only defined upon direct surgical observation (Ceylan, Anik, & Koc, 2011). Nevertheless, there are a few studies that were purportedly able to visualize PA induced disruption of the medial wall utilizing either T2-weighted (T2W) images (Sol et al., 2012) or proton-density weighted images (Cao et al., 2013). Aside from examining medial wall defects, the size and symmetry of the CS as well as bulging of its lateral wall due to PA are also sometimes taken into account when determining CSI (Cottier et al., 2000).

Our in-house method commonly utilized by UHN neuroradiologists uses the detection of lateral displacement of the lateral wall of the CS as one of the criteria for CSI (See chapter 3.1.2).

The venous plexus in the CS space is also a helpful feature for determining CSI. The venous content of the CS is divided into several compartments relative to the ICA. There is the medial compartment which is between the ICA and the pituitary fossa, the superior compartment which is above the ICA, the lateral compartment which is lateral to the ICA, and the inferior compartment which is below the ICA. Cottier et al., further divided the inferior compartment into the carotid sulcus compartment (between ICA and carotid sulcus of sphenoid bone) and the inferolateral compartment which is between the carotid sulcus and the lateral compartment (Cottier et al., 2000). The configuration of these compartments depend mainly on the configuration of the carotid siphon and its position within the cavernous sinus space (Knosp, et al. 1993) In the majority of cases, the superior and inferior compartments are the largest (Knosp et al., 1993). Knosp reported that the superior compartment was most often affected when PA entered the CS space (Knosp et al., 1993). However, Cottier et al., reported that PA growth below the ICA was more common in PA that invaded the CS (Cottier et al., 2000). Also, obliteration of the carotid sulcus venous compartment was observed most often when the CS was invaded (Bonneville et al., 1989; Cottier et al., 2000; Vieira et al., 2006). Vieria also noted that when the lateral venous compartment was not depicted the probability of CSI was high (Vieira et al., 2006).

The ICA is very easily observed on MRI due it’s void of high velocity blood flow and as such it is a critical radiological landmark for determining CSI. The diagnosis of CSI is typically
reserved for cases in which the ICA is completely encased by tumor, but this occurs late
((Moreau, et al. 1998)). Establishing criteria that would predict CSI before the PA encases
the ICA is of clinical importance because this may facilitate a greater probability of gross total
removal of PA and improve patient outcome. Knosp et al., assessed the extent of PA
involvement into the CS by dividing the CS into a series of lines drawn between the
supracavernous and intracavernous segments of the carotid artery. There is the medial
intercarotid line, the median intercarotid line ((Knosp et al. 1993)), as well as the lateral
intercarotid line and PA that crosses each successive line from medial to lateral represents a
greater degree of CS involvement and a greater probability of CSI((Knosp et al. 1993)). Most
studies suggest that when PA crosses the medial or lateral intercarotid line the probability of CSI
is high (Cottier et al., 2000; Moreau et al., 1998; Sol et al., 2012; Vieira et al., 2006). A number
of studies have also attempted to establish a threshold value for carotid artery encasement that
would predict CSI. Cottier reports that when 67% of ICA is encased by tumor, CSI is
likely((Cottier, et al. 2000)), Vieria reports a high probability of CSI when at least 45% of the
ICA is encased by PA((Vieira, et al. 2006)). Sol reports a threshold value of 135.5 degrees of
ICA encasement in order to make an accurate diagnosis of CSI((Sol, et al. 2012)).
The UHN neuroradiology department defines CSI when there is greater than 50% encirclement
of the ICA (See chapter 3.1.2).

Determining when the CS is not invaded is also useful clinical parameter. It is suggested that
when there is normal pituitary interposed between the PA, no tumor involvement beyond the
medial intercarotid line, and/or when there is a low percentage of carotid artery encasement, the
CS is not invaded. Several studies have noted that when 25% or less of carotid artery is encased,
there is no CSI(Connor, Wilson, & Hogarth, 2014; Cottier et al., 2000; Vieira et al., 2006).
Depiction of the medial and/or superior compartments is also helpful for excluding the
possibility of CSI although their absence does not necessarily indicate CSI. Conner noted that
when the lateral or inferolateral compartments were visible or for every 25% reduction in ICA
encasement, the probability for complete resection increased greatly.

Assessing PA invasion into the CS and its extent should be conducted by considering the
features of the CS itself, its venous compartments, and the ICA. Determination of CSI on MRI is
clinically relevant and aids in determining appropriate treatment strategies.

1.1.5 PA Residual and Recurrence

Despite gross total resection in a majority of cases, some PA tend to recur following surgery. Tumor recurrence is a clinically relevant scenario observed in up to 46% of cases (Cappabianca, et al. 2000; Losa, et al. 2008). Most PA tend to recur between 1-5 years after surgery (Roelfsema, Biermasz, & Pereira, 2012). In a recent meta-analysis conducted by Chen et al., the tumor-growth free survival (TGFSR) at 5 and 10 years was 71% and 59%, respectively. Furthermore, they noted that the percentage of PA recurrence was higher and TGSFR was lower in cases with residual following surgery compared to cases without residual (Chen et al., 2012).

The postoperative outcome of PA has been shown to relate to histological subtype. It has been reported that prolactinomas have the highest incidence of recurrence while NFPA have the lowest rate of remission (Roelfsema et al., 2012). The prognostic value of invasion in determining PA recurrence has been reported to be highest for PRL and ACTH tumors and lower for FSH/LH and GH tumors (Trouillas et al., 2013). Tumor size was also shown to correlate with long-term disease-free outcome but not with recurrence/progression-free status. According to a recent systematic review by Roelfsema., the postoperative basal (non-stimulated) hormone levels are the only predictor of tumor recurrence in functioning PA while no single factor is of prognostic value for recurrence in NFPA (Roelfsema et al., 2012).

1.1.6 PA Growth Rate and Tumor Volume Doubling Time (TVDT)

As mentioned above, PA have the potential to become locally invasive or recur following surgery. This complicates patient management and necessitates the quantification of PA growth as a means to predict outcome. It has been proposed that monitoring pituitary macroadenoma
growth using 3D volumetric assessment of MRI is more accurate than conventional 2D interpretation of MRI (Ringstad et al., 2012).

An examination of PA volume has been shown to correlate to surgical outcome. Shrestha et al determined that PA with a preoperative volume greater than 8 cm$^3$ were associated with a greater risk of postoperative residual (Shrestha, Qi, Bao, & Wang, 2012). In another study, a greater risk of postoperative residual was associated with a preoperative PA volume of over 5 mL (Jain, Gupta, Pathak, Bhansali, & Bapuraj, 2008).

It has been reported through mathematical modelling that the majority of PA grow exponentially (Honegger et al., 2003; Honegger et al., 2008). Exponential tumor growth is associated with a constant tumor growth rate. If growth rate is constant, PA growth velocity can be described using a tumor volume doubling time (TVDT), which is the time needed for a tumor to double in size.

The TVDT has been employed as a means to estimate tumor growth in a number of other solid tumors including other benign intracranial neoplasms such as meningioma ((Nakaguchi, et al. 1999))

To date, with respect to PA, the TVDT has been reported in only a few studies, primarily for residual NFPA. The reported growth rates of PA exhibit a broad range: the TVDT range in one study was 200-2550 (980 days) ((Ekramullah, Saitoh, Arita, Ohnishi, & Hayakawa, 1996) and 506-537 (mean 1836 days) in another study (Tanaka et al., 2003) Residual NFPA growth rate has been correlated to patient age with elderly patients exhibiting a slower tumor growth rate than younger patients.

Hsu reported the TVDT in a cohort consisting of residual functioning and nonfunctioning PA and reported a TVDT range of 3.7 months to 879.4 months (mean 78.7 months) (Hsu, Guo, Chien, & Ho, 2010).

It is currently unknown whether the preoperative pattern of PA growth in the context of its
growth rate has any predictive value in determining postoperative tumor growth rate. However, currently, a commonly used molecular marker to predict PA growth potential is the examination of the Ki67/MIB-1 labeling profile.

1.1.7 The Ki-67/MIB-1 Labeling Index: A Measure of Cell Proliferation

The Ki-67/MIB-1 index is commonly used to assess the proliferative activity of tumor cells. The nuclear protein Ki-67 is recognized by the MIB-1 monoclonal antibody and is expressed throughout the cell cycle in G1, G2, and M phases but not G0. The percentage of cells immunostained for the Ki-67 protein represents a marker of proliferation in many cancers((Urruticoechea, et al. 2005)). However, despite its widespread use in PA pathological examinations, its usefulness as predictor of PA growth behavior remains unclear(Chacko et al., 2010; Madsen et al., 2011; Prevedello, Jagannathan, Jane, Lopes, & Laws, 2005).

Currently, there are no reliable molecular markers used to predict the growth behavior of PA. However, the examination of the Ki-67/MiB-1 is frequently used in the pathological assessment of PA to gain insight into the growth rate of PA. The Ki-67 has been studied in relation to PA invasion. A study by Chacko et al., defined the biological significance of the MIB LI in 159 surgically excised PA and found that MIB1 correlated with PA invading the CS. Furthermore, bilateral CS invasion had a significantly higher MIB1 than unilateral extension(Chacko et al., 2010). Similarly, Pan et al., 2005 determined that expression of Ki-67 in PA has associations with PA growth into the CS(Pan, Chen, Liu, & Zhao, 2005).

The Ki-67 may also indicate possible PA residual/recurrence. A study by Wildhalm et al., has determined that in NFPA, residual tumors that progressed had a significantly higher MIB-1 relative to the residuals that did not progress(Widhalm et al., 2009). In another study that consisted of GH-secreting PA, Fusco et al determined that Ki-67 was significantly lower in tumors that were cured after surgery versus those that were not (Fusco et al., 2008).
The relationship between Ki-67 and the growth rate of recurrent tumor has also been examined in a few studies. The Ki-67 LI has been shown to be inversely correlated to TVDT, where lower TVDT were associated with higher MIB-1 (Ekramullah et al., 1996).

The impact that the Ki-67 marker has on PA invasiveness and recurrence remains controversial with conflicting reports in the literature. Nevertheless, this marker provides a general estimate of the proliferative potential of PA and is routinely included in PA pathology assessments.

Although PA generally possess a relatively low proliferative index in comparison to other cancers, they still have the capacity for aggressive growth. This has prompted a number of studies to examine molecular signaling pathways that may be involved in cell growth and other proproliferative activities. Recently, a candidate pathway that is frequently upregulated in various endocrine and non-endocrine cancers, including PA, is the PI3K/Akt/mTOR pathway. This particular pathway and its relation to PA is the focus of the next section.

1.2 Overview of PI3K/Akt/mTOR Pathway in Relation to PA

This section was published in Endocine Related Cancer (Monsalves et al., 2014)

The PI3K/Akt/mTOR pathway is a signal transduction cascade involved in cell growth and metabolism. It is ubiquitously upregulated in various endocrine and non-endocrine cancers and has recently been implicated in PA. Several studies have shown that this pathway is involved in human and animal PA cells in vitro and that these PA cells are responsive to mTOR inhibition manifesting in an overall PA cell reduction.

1.2.1 The PI3K/Akt/mTOR Pathway

The PI3k/Akt/mTOR pathway is a signal transduction cascade involved in cell growth and metabolism (Leslie, Biondi, & Alessi, 2001). The pathway is activated by upstream receptor tyrosine kinases (RTK) which feed the signal through the phosphotidylinositide-3-kinase (PI3K) complex. PI3K is a lipid kinase consisting of a regulatory (p85) and catalytic (p110) subunit. It is activated directly via the p85 subunit which interacts with phosphotyrosine residues on the RTK. Alternatively, in the indirect route, PI3K interacts with the RTK via the IRS-1 or 2 adaptor proteins (White, 1998). Both methods of activation lead to the conversion of PIP2 to the second
messenger PIP3. PIP3 recruits the kinases PDK1 and Akt to the plasma membrane. Akt is then phosphorylated by PDK1 and mTORC2, on its threonine and serine residues respectively. These phosphorylation events lead to the activation of Akt (figure 1.2.1).

Akt is the central mediator of the PI3K/Akt/mTOR pathway and phosphorylates several downstream targets which ultimately lead to cell proliferation. These include phosphorylating the apoptosis inducing factor BAD (Datta et al., 1997) and the FKHR/FOXO transcription factors (Medema, Kops, Bos, & Burgering, 2000) which inhibit apoptosis and promote cell survival, or phosphorylating glycogen synthase kinase-3 which removes inhibition of pro-proliferative pathways (van Weeren, de Bruyn, de Vries-Smits, van Lint, & Burgering, 1998).

mTOR is another phosphorylation target of Akt and is the focus of this paper. mTOR is a kinase that plays an important role in cell growth via modulation of cell cycle regulators or maintaining nutrient supplies into the cell (Advani, 2010). It is affected by Akt through the tuberous sclerosis complex (TSC), which is composed of two subunits: TSC1 (hamartin) and TSC2 (tuberin) (Manning, Tee, Logsdon, Blenis, & Cantley, 2002). TSC2 is a negative regulator of mTOR and phosphorylation of TSC2 by Akt relieves TSC2’s inhibitory effect on mTOR (Zhang et al., 2003). Once activated, mTOR phosphorylates its downstream effectors including p70S6K and eIF4E binding protein 1 (4EBP1) which are both involved in protein synthesis (Harrington et al., 2004; Jefferies et al., 1997).

Termination of the PI3K pathway is accomplished via phosphatase-mediated degradation of PIP3 or through the negative feedback induced by the P70S6K-mediated phosphorylation of the upstream IRS1.
1.2.2 PI3K/Akt/mTOR Pathway in Endocrine and Non-Endocrine Neoplasia

Given the key role of the PI3K/Akt/mTOR pathway in cell growth and metabolism, its role in pathological states such as neoplasia has been investigated extensively in the past decades. As described previously, the PI3K/Akt pathway is activated by upstream ligand dependent RTKs. One of the most widely studied RTK is the ERBB2 receptor which is frequently overexpressed
in breast and other cancers. In breast cancer, for example, ERBB2 is positively associated with worse histological grade, aneuploidy, high rate of cell proliferation and poor survival (Revillion, Bonneterre, & Peyrat, 1998). Furthermore, transgenic mice overexpressing ERBB2/HER2 develop mammary tumors and lung metastases(Guy et al., 1992; Muller, Sinn, Pattengale, Wallace, & Leder, 1988).

The PIK3CA gene, which encodes the p110 catalytic subunit of PI3K, appears to be involved in a number of cancers. For example, PIK3CA has been reported to have increased amplification in ovarian carcinoma, increased PIK3CA expression, and subsequent increased PI3K activity (Shayesteh et al., 1999). Somatic mutations in PIK3CA have also been reported in a number of cancers, including tumors of the colon, breast, brain, lung, and parathyroid(Kasaian, et al. 2013) (Samuels & Velculescu, 2004). Furthermore, PIK3CA mutations have been shown to upregulate AKT and promote oncogenic transformation in vitro (Oda et al., 2008) and in vivo (Bader, Kang, & Vogt, 2006).

The tumor suppressor gene PTEN, a negative regulator of PI3K signaling, has also been widely studied in its association to cancer. It has been reported that somatic mutations, allelic inactivation, or gene silencing via promoter hypermethylation are present in glioblastoma, melanoma, endometrial, and colon cancer(Chow et al., 2011; Lahtz, Stranzenbach, Fiedler, Helmbold, & Dammann, 2010; Mhawech-Fauceglia et al., 2014; Nassif et al., 2004). Furthermore, the inactivation tends to be associated with poor clinical outcome. In prostate cancer, for example, Yoshimoto et al., 2007 demonstrated that homozygous PTEN deletion was an indicator of a more advanced disease at surgery and also associated with faster time to biochemical recurrence of disease (Yoshimoto et al., 2007).

AKT activation is a general feature of malignancy, seen in breast, ovarian, and pancreatic cancers (Altomare et al., 2002; Kandel & Hay, 1999; Roy et al., 2002; Tanno et al., 2004). Of particular note, brain tumors such as meningioma and glioblastoma have increased cell proliferation with concurrent activation of the AKT pathway (Johnson, O'Connell, Pilcher, & Reeder, 2010; Rieman Schneider, Betensky, Pasedag, & Louis, 2006) Furthermore AKT activation has been linked with poor prognosis in many human cancers(Nam et al., 2003; Perez-Tenorio & Stal, 2002; Yamamoto et al., 2004). In addition, AKT is associated with resistance to
chemo- and radiotherapy (Brognard, Clark, Ni, & Dennis, 2001; Clark, West, Streicher, & Dennis, 2002; Tanno et al., 2004). It has been shown that a number of cancers have increased mTOR activation. This activation is also associated with clinicopathologic activities. For example, in gastric cancer an increase in phosphorylated cytoplasmic mTOR was associated with depth of tumor invasion, tumor stage, and poorer survival rates (Murayama et al., 2009). Nuclear phosphorylated mTOR expression was also associated with poor survival in endometrial cancer (Yoshida et al., 2010). Based on the wealth of data available in the literature on the role of PI3K/AKT/mTOR pathway, it is clear that this pathway plays a key role in cancer development and correlates with clinical outcome. Therefore, pharmacologic inhibition of this pathway may be an effective treatment strategy for treating cancer.

1.2.3 The Importance of Establishing Novel Therapeutic Targets in PA

PA are typically benign non-metastasizing lesions that may have few characteristics in common with more aggressive cancer types (Asa & Ezzat, 2009). However, a large proportion of PA at diagnosis are macroadenomas that have a tendency to become locally invasive (Mete & Asa, 2012) An examination of the molecular determinants of these invasive properties in PA may reveal certain signalling patterns that are also involved in more malignant tumors.

Due to the aggressive nature of some PA, complete surgical removal may be impossible in the clinical setting. In such cases, the presence of residual tumor may result in tumor recurrence. Furthermore, some PA may recur several times following multiple survival procedures. Recurrent tumors that are not amenable for reoperation undergo radiation therapy, which may lead to adenohypophseal damage leading to hypopituitarism, optic nerve damage, or cognitive deficits ((Brada et al., 1993; Hahn et al., 2009)).

As mentioned previously, most PA at first clinical presentation are large macroadenomas, and these macroadenomas consist primarily of NFPA, which are unresponsive to current medications (Mete & Asa, 2012). Among functional PA, the available medications, including somatostatin
analogs or dopamine agonists, are only effective in certain subtypes of GH PA and PRL PA, respectively (Mete & Asa, 2012). Thus, there remains a need to identify novel targets for therapy for a large set of patients for whom current options are limited.

The pathobiology of PAs is complex and is yet to be elucidated. It has been established that the classical oncogenes and tumor suppressor genes are not commonly altered in PA (Asa & Ezzat, 2009). However, recent interest has focused on exploring whether elements of the PI3K/Akt/mTOR pathway plays a role in the progression of PA, with ultimate hope that targeting this pathway in PAs will provide a novel therapeutic target.

1.2.4 Analysis of the RTK/PI3K/Akt/mTOR Pathway in PA

Several studies have used mouse and rat pituitary cell lines in vitro to study PA tumorigenesis (Table 1.2.1). The reader is referred to reference (Ooi, Tawadros, & Escalona, 2004) for a summary of how current mouse and rat PA cell lines were established.

The PI3K/Akt/mTOR pathway is activated by RTK and RTK activation has been examined in relation to the pituitary. RTKs are important for normal pituitary function and help to modulate hormone production and cell growth (Ezzat, 2001). Aberrant RTK activation possesses the ability to confer proproliferative potential and abnormal hormone production to pituitary cells leading to hyperplastic and/or neoplastic growth. Two important RTKs in normal pituitary development that are also implicated in PA are EGFR and FGFR. Currenty, studies examining RTK activation in PA and subsequent downstream PI3K/Akt/mTOR signalling remain scant.

EGFR has been implicated in PA. Preclinical studies demonstrate that EGF is able to enhance mRNA levels of prolactin in PA cell lines while gefitinib, an EGFR inhibitor, has been shown to block serum-induced cell proliferation and prolactin gene expression (Vlotides et al., 2008). Furthermore, Lapatinib, a dual EGFR and HER2 inhibitor has been reported to be more potent than gefitinib at abrogating PRL expression and PA cell proliferation both in vitro and in vivo (Fukuoka et al., 2011). The EGFR-mediated effects on PA cell hormone regulation and proliferation have been shown to be PI3K-dependent. In the human situation, EGFR is
demonstrable in all types of PA and its expression has been shown to correlate with tumor aggressiveness especially in GH PA and NFPA (Cooper, Vlotides, Fukuoka, Greene, & Melmed, 2011; LeRiche, Asa, & Ezzat, 1996).

The FGF receptor has also been implicated in PA. The FGFR consists of four receptors and mRNA for prototypic isoforms of FGFR1, 2, and 3 are present in the normal pituitary (Abbass, Asa, & Ezzat, 1997). A common alteration in the FGFR that promote PA is the presence of an N-terminally truncated variant of FGFR4 called pituitary tumor derived FGFR4 (ptd-FGFR4) (Ezzat, Zheng, Zhu, Wu, & Asa, 2002). N-terminal truncation of FGFR4 results in a constitutively activated protein that is localized to the cytoplasm where it promotes PA oncogenic transformation in vitro and in vivo. FGFR4 inhibition with a FGFR-selective inhibitor has been shown to restore membranous FGFR4 and inhibit PA proliferation. Ptd-FGFR4 is present in human PA where it has been shown to correlate with tumor aggressiveness (Ezzat, Zheng, & Asa, 2004; Ezzat, Zheng, Winer, & Asa, 2006). Recently, a SNP in the FGFR4 gene, resulting in a glycine to arginine substitution in the transmembrane domain of the FGFR4 protein, has been identified in PA. The presence of the polymorphic variant has been shown to promote PA cell proliferation and hormone deregulation in vitro and in vivo. In humans, the presence of this SNP has been shown to correlate with GH levels and tumor size in GH PA (Tateno et al., 2011). The FGFR alterations with subsequent PI3K/Akt/mTOR signalling have not examined in PA, however, the polymorphic variant of FGFR4 and downstream mTOR signalling has been reported in pancreatic neuroendocrine tumors (Serra et al., 2012).

Consistent with a role for PI3K activation in PA, in study by Lin et al., mutations of the PIK3CA gene were assessed in 353 pituitary tumors. Nine percent of the invasive, but none of the non-invasive tumors harbored PIK3CA gene mutations. Genomic PIK3CA amplifications, defined as more than four copies, were observed in both invasive and non-invasive tumors with a prevalence of 20-40% (Lin et al., 2009). Another study examined PIK3CA mutations and genomic amplifications in 33 PAs including ACTH-, GH-, PRL-producing and NF-PAs and found that PIK3CA mutations were evident in 12.1% of tumors including one non-invasive ACTH tumor. Genomic amplification (defined as copy number ≥4) were found in 21.2% of cases (Murat et al., 2012).
Additional elements of the PI3K/Akt/mTOR pathway have been examined in PA. In human tissue, AKT, PTEN, and p27 (a target of Akt) mRNA and protein expression was analyzed in ACTH-, GH-, PRL-, and NF-PAs, as well as in normal pituitary tissue controls. In PAs, AKT mRNA expression and phosphorylated-AKT protein levels were increased in comparison to normal pituitary tissue. Additionally, the levels of PTEN and p27 were lower in PA (Musat et al., 2005). The immediate downstream effectors of mTOR, pS6 and eIF4E, have also been studied in human PA tumor samples with more frequent activation of S6/eIF4E evident in all recorded PA subtypes relative to normal pituitary controls (33-71% vs. 20%), with GH-PAs exhibiting the highest frequency of overexpression (Sajjad et al., 2013). Mouse models harbouring genetic mutations resulting in the formation of PA have also been reported to exhibit unregulated p70S6k/S6 expression in PA tissue relative to adjacent CNS tissue (Kenerson, Dundon, & Yeung, 2005; Lu, Willingham, Furuya, & Cheng, 2008). In contrast, another study reported that there was no difference in the expression of phosphorylated or total mTOR, TSC2, or p70S6K as compared to controls. However, this group did report an increase in c-myc levels (a target of Akt) in all PA subtypes, as well as a mild activation of cyclin D1 but only in NF-PAs (Dworakowska et al., 2009). Furthermore, elevated levels of cyclin D1 staining in NF-PAs compared to other tumors has been replicated in a number of other studies. In a study including only NF-PAs and GH-PAs, cyclin D1 allelic imbalance, which may indicate gene amplification, was detected in one quarter of the PAs analyzed (Hibberts et al., 1999).

Table 1.2.2 provides a comprehensive list of the different components of the PI3K/Akt/mTOR pathway examined in PA.

Table 1.2.1: Examining elements of the PI3k/Akt/mTOR in models of pituitary adenoma

<table>
<thead>
<tr>
<th>Study</th>
<th>Model</th>
<th>Species</th>
<th>Treatment</th>
<th>Proteins</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vlotides et al., 2008</td>
<td>GH3 cells</td>
<td>Rat</td>
<td>EGF</td>
<td>EGFR</td>
<td>Increase PRL mRNA levels and cell proliferation;</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>expression</td>
<td></td>
</tr>
<tr>
<td>Romano et al., 2006</td>
<td>GH4C1 cells</td>
<td>Rat</td>
<td>IGF-1; P13K/Akt</td>
<td>P13K/Akt</td>
<td>Increased PRL release; P13K/Akt</td>
</tr>
<tr>
<td>Study</td>
<td>Cells/Model</td>
<td>Species</td>
<td>Growth Factors</td>
<td>Inhibitors</td>
<td>Conclusion</td>
</tr>
<tr>
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<tr>
<td>Kowarik et al., 2010</td>
<td>AtT20, MTt/S, TtT/GF</td>
<td>Rat/mouse</td>
<td>PDGF/LY294002</td>
<td>PDGFR, PDK1, Akt</td>
<td>Mixed expression of PDGFR in cells; PDGF-induced cell proliferation and VEGF-A secretion; VEGF-A secretion blocked by LY294002</td>
</tr>
<tr>
<td>Banerjee et al., 2003</td>
<td>GH3 cells</td>
<td>Rat</td>
<td>17-α-estradiol/wortmannin</td>
<td>PI3k/Akt</td>
<td>Increased VEGF-A mRNA expression; decreased by wortmannin</td>
</tr>
<tr>
<td>Fernandez 2003 and 2004</td>
<td>Lactotroph cells</td>
<td>Rat</td>
<td>IGF-1/LY294002</td>
<td>Akt, BAD, bcl-2</td>
<td>Increased cell proliferation and cell survival; reversed by PI3K inhibitor LY294002</td>
</tr>
<tr>
<td>Rose et al, 2004</td>
<td>aT3</td>
<td>Mouse</td>
<td>IGF-1/LHRH</td>
<td>IRS-1, PI3K, Akt</td>
<td>IGF-1 stimulates cell proliferation and survival; cotreatment with LHRH reduces cell survival</td>
</tr>
<tr>
<td>Lu et al, 2008</td>
<td>Genetic mutant</td>
<td>Mouse</td>
<td>Knock in mutation of the thyroid receptor B gene</td>
<td>Akt, mTOR, p70s6k, bcl-2</td>
<td>Decreased apoptosis; LY294002 reduces pituitary growth</td>
</tr>
<tr>
<td>Kenerson</td>
<td>Renal and TSHoma</td>
<td>Rat</td>
<td>mTOR, S6</td>
<td>Akt, mTOR, p70s6k, bcl-2</td>
<td>The mTOR pathway</td>
</tr>
<tr>
<td>Authors, Year</td>
<td>Tumor Type</td>
<td>Cell Line</td>
<td>Genetic Mutations</td>
<td>PI3K/Akt</td>
<td>Description</td>
</tr>
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<tr>
<td>et al 2005</td>
<td>pituitary</td>
<td>mutation of TSC2 gene</td>
<td>activity is critical for tumor progression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Musat et al., 2005</td>
<td>ACTH-, GH-, PRL-NF-Pas</td>
<td>Human</td>
<td>N/A</td>
<td>p-Akt, PTEN, p27</td>
<td>Increased p-Akt expression; lower nuclear p27 and PTEN expression as compared to normal pituitary</td>
</tr>
<tr>
<td>Sajjad et al., 2013</td>
<td>GH-, ACTH-, NF-PAs</td>
<td>Human</td>
<td>N/A</td>
<td>mTOR(pS6/eIF4e)</td>
<td>Increased mTOR activity in all PA subtypes vs controls</td>
</tr>
<tr>
<td>Daworakowska et al., 2009</td>
<td>ACTH-, GH-, PRL-NF-adenomas</td>
<td>Human</td>
<td>N/A</td>
<td>mTOR,TSC2,p70s6k</td>
<td>No difference in mTOR, TSC2, p70s6k expression compared to controls; decreased c-myc and increased cyclin D1 expression only in NF adenomas</td>
</tr>
<tr>
<td>Lin et al., 2009</td>
<td>Pituitary adenomas</td>
<td>Human</td>
<td>Genetic PI3kCA mutations</td>
<td>PI3K/Akt</td>
<td>Some invasive and no non-invasive PA harbored mutation</td>
</tr>
</tbody>
</table>

### 1.2.5 Current mTOR inhibitors

The proposed role of aberrant PI3K/Akt/mTOR signalling in PA makes this tumor group amenable for the use of mTOR inhibitors in the clinic. Results using mTOR inhibitors in preclinical models of PA have provided further support for mTOR pathway involvement in PA.
1.2.6 Effects of Rapamycin (and Rapalog)-induced mTOR Inhibition on PA Cell Proliferation and Viability

Rapamycin (sirolimus) is an immunosuppressant and antiproliferative agent that has previously been shown to be effective at abrogating cancer-related properties in a number of tumors (Douros & Suffness, 1981; Majumder et al., 2004). Specifically, rapamycin binds to the mTORC1 complex affecting downstream signalling events including cell cycle arrest and protein synthesis inhibition. Tumors that harbor upstream mutations from mTOR, such as PTEN deletion or Akt overexpression, are ideal targets for treatment with mTOR inhibitors. Everolimus (RAD001) is an orally available analog to rapamycin (rapalog) and has also been shown to be an effective anticancer agent in a number of in vitro cell lines and animal models (Beuvink et al., 2005).

PAs are typically of low proliferative potential as assessed by the cell cycle antigen, Ki-67 and of low mitotic index. However, the ability of Ki-67 index to predict tumor growth/invasion and recurrence is debatable (Chacko et al., 2010). The presence of large macroadenomas that have a propensity for invasion into extrasellar structures could potentially benefit from antiproliferative agents. The ability of rapamycin and its analogs to inhibit cell proliferation is key to its antitumor efficacy. Several studies have examined the antiproliferative properties of rapamycin and rapalog treatment in pituitary and PA cells (Table 1.2.2). In normal rat pituitary cells, rapamycin was shown to inhibit basal proliferation and insulin-, cAMP-, and estradiol-induced proliferation of cells (Kawashima, Yamakawa, & Arita, 2000). Human prolactin gene is expressed in the GH3 cell line and its transcription was shown to be inhibited by rapamycin (Wera, Belayew, & Martial, 1995; Wera, Zheng, et al., 1995). In an animal model using rats that carry an inactivating germline mutation of the TSC2 gene that results in pituitary tumor formation, rapamycin induced regression in size of the pituitary tumors and a concomitant decrease in levels of phosphorylated-S6 (the target of p70S6K) (Kenerson et al., 2005). A recent study using human PA cells in primary culture has also demonstrated that rapamycin induces mTOR
inhibition in mTOR-active GH-, ACTH-, and NF-PAs, although, in this study, cell viability or proliferation was not assessed (Sajjad et al., 2013). Nevertheless, these studies provide evidence for rapamycin as a possible anti-PA agent through its mTOR-inhibiting effects.

Gorshtein et al., first demonstrated the antiproliferative effects of rapamycin and its orally bioavailable analog RAD001 on GH3 and MtT/S cells as well as on human GH-PA cells in primary culture. They reported that treatment with rapamycin or RAD001 significantly decreased cellular viability and proliferation in a dose- and time-dependent manner, which was reflected by decreased of phosphorylated p70S6K (Gorshtein et al., 2009). Another study used GH3 and the prolactin secreting MMQ cell lines and also noted a time- and concentration-dependent decrease in cellular viability following exposure to rapamycin or RAD001 (Sukumari-Ramesh, Singh, Dhandapani, & Vender, 2011). These findings were attributed to decreased levels of mTOR phosphorylation at the serine-2448 residue, which is a key determinant of mTOR activity. A third study noted a small but statistically significant antiproliferative effect of 1 nM rapamycin on mouse AtT20 cells and no effect at lower doses (Cerovac et al., 2010).

Musat et al. have reported that NFPAs have the highest levels of AKT mRNA expression of all PA subtypes (Musat et al., 2005). Furthermore, it has been shown that high levels of phosphorylated AKT may contribute to early recurrence of NF-PA (Noh et al., 2009). Therefore, it is important to evaluate the antiproliferative efficacy of rapalogs in this histological subtype. Zatelli et al. examined RAD001 sensitivity in 40 NF-PAs and reported that 70% (28/40) displayed a dose-dependent reduction in cellular viability. Furthermore, in these 28 samples, RAD001 blocked IGF-1 induced increases in cell proliferation and VEGF secretion, although RAD001 by itself had no effect on VEGF secretion (Zatelli et al., 2010). Of note, investigation of patient medical records showed that those patients who responded to RAD001 were younger, were more likely to have an invasive macroadenoma, and predominantly female. Lee et al. examined a particular strain of rats with a MENX, a multiple endocrine neoplasia-like syndrome, mutation. MENX is caused by a biallelic frameshift mutation in the cdkn1b gene, encoding p27, which leads to the development of multiple endocrine tumors, including NFPA. Administration of RAD001 to MENX PA cells in primary culture also resulted in a dose-dependent reduction in cellular viability that reached significance at a dose of 100 nM in 45% of their sample (5 out of
11 cultures) (Lee et al., 2011). Finally, Cerovac et al., exposed 28 human NFPAs in primary culture to rapamycin and reported a small (<20%) reduction in cellular proliferation in 29% (8 of 28 tumors) of their samples (Cerovac et al., 2010).

Table 1.2.2: In vitro studies examining effect of mTOR inhibitors on PA cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Species</th>
<th>Hormone</th>
<th>Drug</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactotrophs(1°)</td>
<td>Rat</td>
<td>PRL</td>
<td>Rapamycin</td>
<td>Inhibit basal and growth factor induced proliferation of cells (Kawashima, 2000)</td>
</tr>
<tr>
<td>GH3(CL)</td>
<td>Rat</td>
<td>GH/PRL</td>
<td>Rapamycin</td>
<td>Transcription inhibition of prolactin gene (Wera, 1995); decreased cell viability and proliferation (Gorshtein, 2009, Sukumari, 2011); cotreatment with RT results in radiosentization (sukumari); decrease cell proliferation/increased apoptosis (Dai 2013)</td>
</tr>
<tr>
<td>MtT/S(CL)</td>
<td>Rat</td>
<td>GH</td>
<td>Rapamycin/RAD001</td>
<td>Decreased cell viability and proliferation (gorshtein, sukumari); cotreatment with RT results in radiosentization (sukumari)</td>
</tr>
<tr>
<td>MMQ(CL)</td>
<td>Rat</td>
<td>PRL</td>
<td>Rapamycin</td>
<td>Decreased cell viability and proliferation (Sukumari); decreased cell proliferation/increased apoptosis (Dai 2013)</td>
</tr>
<tr>
<td>Tumor Type</td>
<td>Species</td>
<td>Hormone</td>
<td>Treatment</td>
<td>Effect</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td>---------</td>
<td>-----------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>αT3-1(CL)</td>
<td>Mouse</td>
<td>FSH/LH</td>
<td>Rapamycin</td>
<td>Decreased cell proliferation/increased apoptosis (Dai 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RAD001</td>
<td>Decreased cell viability (Sukumari)</td>
</tr>
<tr>
<td>AtT20(CL)</td>
<td>Mouse</td>
<td>ACTH</td>
<td>rapamycin</td>
<td>Decreased cell proliferation (Cerovac)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Octreotide/Rapamycin</td>
<td>Greater decrease in cell proliferation than rapamycin or octreotide alone (Cerovac)</td>
</tr>
<tr>
<td>GH-adenoma(1°)</td>
<td>Human</td>
<td>GH</td>
<td>Rapamycin/RAD001</td>
<td>Decreased cell viability and proliferation (Gorshtein)</td>
</tr>
<tr>
<td>NFPA(1°)</td>
<td>Human</td>
<td>N/A</td>
<td>Rapamycin</td>
<td>Decreased cell proliferation (Cerovac)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Octreotide/Rapamycin</td>
<td>Greater antiproliferative response than either octreotide or rapamycin exposure</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RAD001</td>
<td>Decreased cell viability; blockage of growth factor induced cell proliferation and VEGF secretion (Zatelli)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SOM230/RAD001</td>
<td>Greater antiproliferative effect seen when drugs were combined versus individually (Zatelli)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NVPBEZ235</td>
<td>Decreased cell viability (Zatelli)</td>
</tr>
<tr>
<td>MENX (1°)</td>
<td>Rat</td>
<td>N/A</td>
<td>RAD001</td>
<td>Decreased cell proliferation; triggers apoptosis (Lee)</td>
</tr>
</tbody>
</table>
One potential pitfall of mTOR inhibition is that mTOR activation normally causes negative feedback on IRS1. O’Reilly et al. reported that inhibition of mTOR by rapamycin does indeed cause a decrease in p70S6K, abolishing its negative feedback to IRS1 and increasing Akt phosphorylation (O'Reilly et al., 2006). This mechanism may explain the partial response to the inhibitor treatments mentioned above. Theoretically, it seems that co-administration of an agent able to decrease Akt phosphorylation upstream could potentiate the antiproliferative action of rapamycin treatment downstream.

Somatostatin receptor type 2 (SSTR2) was shown to deactivate the PI3K pathway by inhibiting p85 tyrosine phosphorylation and thereby decreasing Akt phosphorylation (Theodoropoulou et al., 2006). Somatostatin-analogs such as octreotide are frequently used for the treatment of neuroendocrine tumors (Lamberts, de Herder, & Hofland, 2002). Cerovac et al. demonstrated that the addition of octreotide to rapamycin increased serine-phosphorylated IRS-1 levels but notably decreased rapamycin induced pAkt-Ser levels (Cerovac et al., 2010). In AtT20 cells, the addition of octreotide to rapamycin resulted in a 50% decrease in cell viability, an effect that was significantly greater than the actions of octreotide or rapamycin alone (Cerovac et al., 2010).

Rapamycin combined with somatostatin analogues are effective at inhibition of human PA cell growth in vitro. In one study, among human NFPAs that did not respond to rapamycin treatment alone (20/28), all responded to concurrent rapamycin and octreotide treatment. Moreover, in the
cotreatment group a lower dose of rapamycin was required to maintain a significant antiproliferative effect. This study also noted that cotreatment suppressed proliferation to a greater extent in the cells that did respond to rapamycin alone (8/28) (Cerovac et al., 2010). Similar results were obtained when human NFPAs were exposed to SOM230, a somatostatin receptor multiligand, in combination with RAD001. This study showed higher response rate to RAD001 alone (28/40 cultures responded), but cotreatment with SOM230 significantly potentiated the antiproliferative effect of RAD001 compared to SOM230 or RAD001 alone (Zatelli et al., 2010). Similar antiproliferative results were also obtained when ACTH PA cells in primary culture were exposed to SOM230 and RAD001 in combination (Zatelli, Minoia, Filieri, Tagliati et al., 2010).

The effects of RAD001 on the negative feedback loop which, by inhibiting mTOR, reduces p70S6k phosphorylation and induces IRS-1 expression, was examined in pituitary cell lines. In one study, GH3 and AtT20 cells were exposed to rapamycin, which reduced serine-phosphorylated mTOR and phosphorylated p70S6K levels. In AtT20 cells, rapamycin treatment increased phosphorylated Akt levels, but this effect was reversed by octreotide in a serine-phosphorylated IRS1-dependent mechanism (Cerovac et al., 2010). In contrast, Gorshtein showed that rapamycin or RAD001 treatment of GH3 and MtT/S cells abrogated phosphorylation of p70S6K without an accompanied rise in phosphorylated Akt levels 24 hours after treatment (Gorshtein et al., 2009).

Recently, a new class of compounds have been developed that are designed to inhibit both mTOR and upstream PI3K pathway components. Theoretically, these drugs are designed to inhibit mTOR without stimulating an increasing in upstream Akt activity. One of these agents, NVPBEZ235, is a synthetic small molecule that inhibits both PI3K and mTOR kinase activity by binding to the ATP cleft of these enzymes (Maira et al., 2008). In a study by Lee et al. in 2011, NVPBEZ235 was reported to inhibit the viability of 100% (10 of 10) of MENX rat PAs in primary cell culture. This result was significantly more potent that previous studies of RAD001 effects on this cell line. Furthermore, it was noted that incubation with NVPBEZ235 at concentrations that suppress cellular viability also decreased the phosphorylation of Akt and S6, whereas RAD001 decreased phosphorylated S6, but increased phosphorylated Akt (Lee et al.,
2011). In another study examining NVPBEZ235, NFPA cells in primary cell culture exposed to the dual inhibitor exhibited a 50% decrease in cell viability in 37 of 40 cases (Zatelli et al., 2010).

Dual PI3K/mTOR inhibitors have also been combined with more conventional therapies to yield promising preclinical results in PA cells. One such dual-inhibitor known as XL765 used in combination with temozolomide (TMZ), an orally-available alkylating agent previously shown to be effective at reducing PA cell viability (Ma et al., 2011; Sheehan, Rainey, Nguyen, Grimsdale, & Han, 2011), synergistically inhibited the proliferation of αT3-1, MMQ, and GH3 PA cell lines (Dai et al., 2013).

1.2.8 Effects of mTOR Inhibition on Apoptosis on PA Cells in vitro

Apoptosis, or programmed cell death, is characterized by a rapid sequence of events leading to the elimination of damaged cells. It is defined by morphological changes such cell shrinkage and nuclear demarcation. In neoplastic tissue, apoptosis is typically suppressed in favour of cell survival.

PA are clonal lesions that are thought to derive from a single transformed cell and defects within the apoptotic pathway may preserve the survival of the altered cell and promote PA genesis and growth. However, the clinical utility of using indicators of apoptosis as a prognostic tool in PAs is not consistently reported (Guzzo, Carvalho, & Bronstein, 2014). Nevertheless, induction of PA cell apoptosis via mTOR inhibition has been an important characteristic of their anti-PA properties. Caspases are the main protein family effectors of apoptosis and the study by Zatelli et al study demonstrated that the effects of RAD001 on cellular viability of NFPA may be attributed to increased levels of caspase 3/7 activity (Zatelli et al., 2010). Increases in caspase 3/7 activity were also reported by Lee et al. following NVPBEZ235 treatment of MENX rat PAs in primary culture (Lee et al., 2011) but did not affect caspase activity in AtT20 cells (Cerovac et al., 2010). The use of XL765 and TMZ synergistically increased caspase 3/7 levels and TUNEL-positive αT3-1, MMQ, and GH3 cells compared to either treatment alone (Dai et al., 2013).
1.2.9 Effects of PI3K and mTOR Inhibition on PA Cell Cycle

The cell cycle is an important regulator of cell proliferation. Many cyclins, cyclin dependent kinases, and cyclin kinase inhibitors have been implicated in PA tumorigenesis (Saeger, 2004) and some have been shown to be affected by mTOR inhibition. Previous in vitro studies have reported that induction of cell cycle arrest is an important mechanism by which mTOR inhibitors exert their antiproliferative effects on PA cells (Faivre, Kroemer, & Raymond, 2006). It has been reported that treatment with rapamycin results in the arrest of PA cell lines in the G0/G1 phase.

The early G1 phase is positively regulated by D-type cyclins and their corresponding cyclin-dependent kinases (Sherr, 2000). Rapamycin and its analogs seem to primarily affect cyclin D3 levels while cyclin D1 and the cyclin dependent kinases (CDK4 and CDK6) remain unaffected. The dual PI3K/mTOR inhibitor NVP may be a more potent agent at abrogating PA cell cycle progression by attenuating both cyclin D1 and Cyclin D3.

The KIP1/CIP family of cyclin-dependent kinase inhibitors (CKDI) negatively regulate cyclins and CDKs in the G1 phase of the cell cycle. Gorshtein et al. have demonstrated that p21/CIP levels were reduced by rapamycin in GH3 cells (Gorshtein et al., 2009). A similar down-regulation of p21/CIP was also noted when AtT20 cells were exposed to high concentrations of NVPBEZ235 (Cerovac et al., 2010). On the other hand, p27/KIP1 was reported to have greater transcriptional and protein expression following combined rapamycin/octreotide treatment in AtT20 cells (Cerovac et al., 2010). Increased p27 also seemed to play a role in the antiproliferative action of the dual inhibitor NVPBEZ235. Lee at al. were able to demonstrate that increased levels of p27 positively correlate to the antiproliferative efficacy of NVPBEZ235 in GH3 cells transfected with wild type p27 and in MENX rat PA cells in primary culture (Lee et al., 2011). However, unlike rapamycin/octreotide cotreatment, which increased protein and transcript levels of p27 in AtT20 cells, NVPBEZ235 only increased p27/KIP1 protein levels in GH3 cells, indicating regulation at a post-transcriptional level (Cerovac, Tichomirowa, Stalla et
A major determinant of G1/S progression is retinoblastoma (Rb) phosphorylation. CDK4 when associated with D-type cyclins phosphorylates Rb in the G1 phase. Gorshtein et al. showed that rapamycin inhibits Rb phosphorylation in GH3 and MtT/S cells and reduces subsequent E2F transcriptional activity (Gorshtein et al., 2009). Octreotide and rapamycin cotreatment of AtT20 cells seems to potentiate this Rb inhibition effect (Cerovac et al., 2010).

In response to decreased E2F activity, cell cycle components that contribute to late-G1 phase and S-phase entry, such as the expression of E2F regulated genes cyclin E and CDK2, were also reduced by rapamycin in GH3 cells (Gorshtein et al., 2009). In AtT20 cells, cyclin E expression is reduced even further by rapamycin and octreotide co-treatment (Cerovac et al., 2010).

Thus, it appears that mTOR inhibitors possess the capacity to reduce proliferation of PA cells by inducing apoptosis and restoring some of the inhibitory mechanisms involved in the cell cycle particularly in the G1 phase.

### 1.2.10 mTOR Inhibition and Radiation on PA Cells

Radiotherapy is typically reserved for particularly aggressive PA that are not suitable for surgical resection or are multirecurrent lesions that are resistant to pharmacologic therapies. To date, a number of studies have shown that mTOR inhibition radiosensitizes cancer cells (Altmeyer et al., 2012; Burris, 2013; Mauceri et al., 2012), Existing data suggests that mTOR inhibition may also lead to decreased radioresistance of PA cells in vitro. Ramesh et al. report that inhibition of mTOR radiosensitizes GH3 cells such that 2.5 Gy in combination with 0.5 nM rapamycin or RAD001 reduced cellular viability more effectively than 2.5 Gy or 10 Gy alone (Sukumari-Ramesh et al., 2011). Another study utilized nelfinavir, a protease inhibitor, to increase radiation sensitivity of GH3 cells, as well as MMQ and AtT20 cells. At three days post-radiation, cellular viability was decreased in all cell lines in a radiation-dose-dependent manner (dose range 0-6 Gy) (Zeng et al., 2011). In this study, apoptosis was induced in vitro at higher rates by con- nelfinavir and radiation treatment compared to either intervention alone. The proposed
mechanism of nelfinavir action is decreased phosphorylated S6, a key downstream target of the PI3K/Akt/mTOR pathway. Thus, these data suggest that nelfinavir action may bare similarities to that of rapamycin and its derivatives.

An increase in the radiosensitization of PA cells by mTOR inhibitors is an important therapeutic feature because although radiotherapy is a relatively uncommon form of treatment for PA, it is nevertheless utilized for aiding in the arrest of tumor growth of invasive and aggressive PA. Radiotherapy has been associated with a number of deficits in patients with PA including optic nerve damage, cranial nerve palsies, and adenohypophosphal damage. Thus, when radiotherapy is employed, coupling this form of therapy with mTOR inhibition in PA patients may reduce the required dose of radiation thus minimizing the subsequent radiation-induced adverse effects.

1.2.11 mTOR Inhibition Using Xenograft In Vivo Models of PA.

Xenograft models are an important tool for studying the behaviour of PA. Unlike transgenic models that may take a prolonged period of time to develop a PA, xenograft models can be generated quickly and reliably and are more reflective of the common sporadic PA which do not usually possess underlying genetic mutations (Asa & Ezzat, 1998). Furthermore, some transgenic mouse models first develop pituitary hyperplasia prior to developing a PA which is not characteristic of the human scenario. Two studies to date have used xenograft models to examine the effects of mTOR inhibition on PA. Zeng et al. demonstrated that GH3 cells implanted into the flanks of nude mice treated with a combination of nelfinavir and radiation experienced delayed tumor size quadrupling time. Co-treatment displayed a synergistic effect compared with nelfinavir or radiation treatment alone. Immunohistochemistry of tumor sections from this study confirmed downregulation of phosphorylated S6 following treatment with nelfinavir (Zeng et al., 2011). In another study by Dai et al., 2013, GH3 cells were also used and implanted into the flanks of female nude mice. These mice were treated with a combination of XL765 and TMZ which was shown to inhibit tumor growth and induce apoptosis, inhibit GH and PRL secretion and downregulate Akt, mTOR, and S6 phosphorylation (Dai et al., 2013).
Unfortunately, there are currently no intracranial mouse models of PA in which to study mTOR inhibition. Intracranial models may be more reflective of the human situation by mimicking the intracranial microenvironment of PA. An intracranial xenograft model may provide a more accurate portrait of the nature of sporadic PA growth behaviour following mTOR inhibition. One factor hampering the establishment of an intracranial mouse model is the difficulty in accessing the mouse pituitary fossa. Nevertheless, more robust xenograft models in general and intracranial models specifically are needed to better characterize PI3K/Akt/mTOR pathway signalling and how it impacts PA growth dynamics. Utilization of improved preclinical models of PA may provide stronger evidence for the role of this oncogenic pathway in PA and pave the way for clinical trials involving mTOR inhibition in PA patients.
Chapter 2
Outline and Overview of Aims

2.1 Purpose

The overall goal of my research is to gain a better understanding of pituitary adenoma (PA) biology. I have two main aims for this project. The first aim is to correlate PA biology with relevant clinical characteristics in order to provide a comprehensive understanding of the mechanisms contributing to tumor growth behaviour. The second aim will be to establish an intracranial model of PA and identify signal transduction pathways involved in PA in order to identify appropriate targeted treatment strategies.

2.2 Hypothesis 1

To establish whether the growth and extension pattern of PA can predict postoperative growth rate and recurrence in addition to whether the PA growth rate correlates with proliferation and growth factor expression.

2.2.1 Aim#1: Examine growth patterns of pituitary adenomas and histopathological correlates

To retrospectively review clinical, radiological and histopathological features of PA and correlate them with growth patterns and rate of PA

2.2.2 Rationale for Aim#1

PA are a frequently occurring intracranial neoplasm, evident in up to 20% of the general population (Asa and Ezzat 2009). PA comprise several histopathological subtypes each
displaying characteristic presenting symptoms based on specific hormonal imbalances in the body and/or symptoms of tumor mass, such as visual disturbance. First-line therapy for the majority of PA is typically endonasal surgery although medications such as dopamine agonists or somatostatin analogs, are useful in certain PA subtypes.

Despite its often benign and indolent nature, the majority of PA are large macroadenomas at diagnosis. Macroadenomas usually grow out of the sellar compartment and compress or invade adjacent structures. Parasellar PA growth into the CS is particularly problematic as it often precludes complete tumor removal and complicates patient management (Sepehrnia et al. 1991). It has been suggested that different histopathological subtypes have a proclivity for different patterns of extrasellar growth (Mete and Asa 2012). Furthermore, the rate of tumor growth has been suggested to be different for different subtypes. Specific molecular markers that are involved in the cell cycle, such as Ki-67, are often employed as a means to assess PA growth potential (Chacko, et al. 2010). However, it is still a matter of debate as to how accurate this marker is for estimating PA growth behavior.

Postoperatively, there is a probability of tumor residual especially in cases in which the CS is involved. The extent of CS involvement often correlates to a greater probability of PA residual. Several studies have attempted to establish preoperative criteria for assessing PA involvement into the CS as a means to predict surgical outcome (Cottier et al. 2000; Knosp et al. 1993). Even when the PA is completely removed, some PA have a tendency to recur. The probability of tumor recurrence varies based on its histopathological subtype and biochemical status (Roelfsema, et al. 2012). Particularly aggressive PA are often multirecurrent and require reoperation and/or adjuvant therapy.

The clinical and molecular factors that are involved in PA growth and residual/recurrence are not well-understood. Thus, the objective of the first aim was to correlate patient demographic, MRI, histopathological parameters to the growth behavior of PA both pre- and postoperatively with the goal of identifying predictive factors of PA growth behavior.
2.3 Hypothesis 2

To investigate whether the mTOR pathway is expressed in an intracranial xenograft mouse model of PA harboring wild type or mutated FGFR4 and to determine a possible differential response to mTOR inhibition between these tumors.

2.3.1 Aim#2: Examine preclinical intracranial mouse model of PA for interrogating therapeutic strategies: role of mTOR inhibitors

To establish an intracranial model of PA and to use this pre-clinical model for the investigation of targeted therapeutics against the mTOR pathway.

2.3.2 Rationale for Aim#2

The pathogenesis of PA is poorly understood. The molecular markers that contribute to PA formation and progression remain elusive. Furthermore, many of the classical oncogenes and tumor suppressor genes involved with other forms of neoplasia are rarely altered in PA (Asa and Ezzat 2002).

Recently the fibroblast growth factors and their receptors have been implicated in PA pathology. A specific subtype of the FGF receptor, FGFR4, has garnered particular interest in the pathobiology of PA after the identification of an N-terminally truncated variant that is only present in PA, not in the normal pituitary, and is referred to as pituitary tumor derived FGFR4 (ptd-FGFR4)(Ezzat, et al. 2002). Ptd-FGFR4 has been shown to confer oncogenic characteristics to pituitary cells both in vitro and in vivo, an effect not seen in wild-type full length FGFR4(Ezzat, et al. 2006).

Shortly after the discovery of ptd-FGFR4 in PA, a polymorphism in the gene encoding the FGFR4 protein was identified(Bange, et al. 2002). This polymorphism occurs at codon 388 and results in the substitution of an uncharged glycine residue for a charged argine residue in the
transmembrane domain of the FGFR4 protein (FGFR4-G388R). At least one allele of this SNP is present in approximately 50% of the population. It is associated with poor prognosis in several cancers including breast and prostate (Thussbas, et al. 2006; Wang, et al. 2004). There is a paucity of data implicating this SNP in PA. However, one study has identified distinct signaling and hormone regulatory properties that distinguish FGFR4-R388 from the prototypic FGFR4-G388 form in GH-PA (Tateno, et al. 2011) and another in ACTH-PA (Nakano-Tateno, et al. 2014).

The activation of the PI3K/Akt/mTOR pathway signaling may serve as a viable downstream target that may also distinguish the FGFR4-R388 SNP variant from wild type FGFR4-G388. The PI3K/Akt/mTOR pathway is activated via upstream receptor tyrosine kinases (RTKs), which includes FGFR4. Also, elements of this pathway have been shown to upregulated in PA in vitro and in vivo models, including samples of human PA of various histotypes (Musat, et al. 2005; Sajjad, et al. 2013). Moreover, PA cells respond to mTOR inhibition with a downregulation of mTOR pathway expression resulting in a reduction of proliferation and induction of apoptosis (Gorshtein, et al. 2009; Zatelli, et al. 2010).

A major factor impeding the study of the mechanisms contributing to the formation and progression of PA is the lack of studies utilizing a robust intracranial model of PA. Current in vivo models involve transgenic mutants that may or may not develop a PA even after a prolonged period of time. Alternatively, xenograft of PA cell lines are used but these are implanted subcutaneously and may not be characteristic of the human scenario. Thus, the first part of aim 2 (aim 2a) is to establish an intracranial model of PA that can be conducted quickly and reliably. The second part of aim 2 is to use this PA model to identify the mTOR pathway as a possible therapeutic target that distinguishes wild type FGFR4-G388 from FGFR4-R388 and helps to establish mTOR inhibitors as a viable treatment option for PA patients.
Chapter 3
Growth Patterns of PA and Histopathological Correlates

This chapter was published in the Journal of Clinical Endocrinology and Metabolism (JCEM, 2014) IF=6.430 (Monsalves, et al. 2014)

3.1 Introduction

Pituitary adenomas (PA) are common in neurosurgical practice, accounting for up to 25% of all intracranial neoplasms (Asa and Ezzat 2009). The majority of pituitary macroadenomas are clinically nonfunctioning or hormonally inactive and exhibit gonadotroph differentiation, (Mete and Asa 2012). Pituitary adenomas exhibit a wide range of behaviour (Mete, et al. 2012) and some have a tendency to compress or invade structures adjacent to the sella, such as the optic chiasm or cavernous sinus (CS), causing significant morbidity and impeding complete surgical removal. Furthermore, PA sometimes recurs despite gross total resection (Alahmadi, et al. 2012; Benveniste, et al. 2005).

The factors that govern and can aid in predicting the behaviour of PA, such as clinical growth of residual tumour following subtotal resection are not well understood. Currently, it is thought that the most reliable tool for predicting the growth behaviour of PA is to determine the histopathological subtype (Lundin, et al. 1992; Scheithauer, et al. 1986; Zada, et al. 2010). In addition, the MIB-1 labeling index (MIB-1 LI), a measure of cell proliferation, has been used as the main predictor of tumor recurrence and to some extent tumor invasiveness in PA (Chacko et al. 2010; Noh, et al. 2009; Prevedello, et al. 2005). However, our current understanding of all of the factors involved in tumour regrowth, invasiveness and recurrence is lacking.

There have been some recent attempts to identify other factors that may aid in the prediction of growth patterns and recurrence rates for PAs. For instance, preoperative PA volume has been shown to correlate to the presence of residual tumor (Jain, et al. 2008; Ringstad, et al. 2012; Shrestha, et al. 2012). A few studies have shown a positive correlation between residual PA growth and the MIB-1 LI (Ekramullah, et al. 1996; Honegger, et al. 2003; Hsu, et al. 2010; Kawamoto, et al. 1995; Saeger, et al. 2008; Tanaka et al. 2003). However, overall there is limited
understanding of whether preoperative tumor volume and growth rate across serial radiological examinations is predictive of postoperative growth rate of a residual PA.

In our study, we examined the association between pre- and postoperative PA growth characteristics in association to patient demographics, MRI parameters and histopathological factors that may predict growth rate of PA both pre- and postoperatively.

3.2 Materials and Methods

3.2.1 Patient Population

Following institutional research ethics approval, we retrospectively reviewed all patients who had surgery for resection of a histologically proven PA between 1999-2011. All surgeries done prior to 2004 were microscopic and thereafter purely endoscopic for the patient cohort included in this study. All patients in this study have been reviewed and managed by our institutional multidisciplinary clinic that includes neurosurgeons, neuro-endocrinologists and neuropathologists. All patients routinely have a complete hormonal biochemical analysis prior to surgery by our endocrinologist (SE), including dynamic studies when indicated by baseline biochemical assessments.

3.2.2 Magnetic Resonance Imaging (MRI)

Patients were required to have a minimum of two pre- and two postoperative MRI examinations to allow for measurement of tumor volume doubling time (TVDT). Cases requiring urgent surgery due to acute presentation with visual compromise were not included in this study. The first preoperative MRI was the first recorded image and the second preoperative MRI was the one taken immediately prior to surgery. A minimum of three months between the first and second scan was required. The first postoperative image had to be at least three months after surgery to allow for a reduction in postoperative changes related to scarring and inflammation, and the last postoperative MRI was the last recorded image which was at least 12 months from the first postoperative scan. Additionally, none of the patients had received medical therapy for
their PA, except for some prolactinomas that were medically refractory.

All patients included in this study underwent a sella/pituitary specific imaging protocol. Coronal and sagittal sections of T1-weighted MR images, with and without contrast enhancement, were used for the two preoperative scans. For postoperative assessment, coronal and sagittal sections of T2-weighted MR images were used, as T2 scans were more sensitive than T1 at detecting subtle postoperative changes. Slice thickness including MRI magnet strength were standardized.

For volumetric assessment, MR images were uploaded into ITK-SNAP (Insight ToolKit Snake Automatic Partitioning) (University of Pennsylvania, www.itksnap.org) (Yushkevich et al., 2006). ITK-SNAP is free software available on-line that allows for segmentation and measurement of target volumes on MRI and has been used by our group previously in several publications (Hayhurst, et al. 2012; Lwu, et al. 2013) Ebinu et al., 2013). Once the MRIs are uploaded, the tumors are manually contoured slice by slice and at the end a volume is generated along with a 3D reconstruction of the tumor (Figure 3.2.1). The reader is referred to (Yushkevich et al., 2006)(www.itksnap.org) for a more thorough description of ITK-SNAP.
3.2.3 Growth Pattern and MRI Characteristics

The pattern and direction of tumor growth was recorded as superior, inferior, anterior, posterior and lateral relative to the sellar fossa. Many tumors exhibited a combination of these growth patterns. Superior growth was noted if the tumor extended into the suprasellar cistern and/or compressed the optic chiasm as observed in the coronal plane. Additionally, a line was drawn in the sagittal plane extending from the posterior clinoid to the tuberculum and if tumor grew above this line, it was noted as superior growth. Inferior growth was recorded as tumor that appeared to be eroding through the sellar floor and entering into the sphenoid sinus. Anterior growth was tumor that extended over the planum sphenoidale and in some cases extended beneath the inferior surface of the frontal lobes. Posterior growth was observed when the tumor extended into the interpeduncular cistern/prepontine area and/or compressed the brainstem. Lateral growth and extension towards the CS was defined according to Knosp criteria for CS
Knosp criteria are divided into five grades (0-4) based on a series of lines drawn through the supra- and intracavernous segments of the carotid arteries as observed in mid-sella coronal sections. In our study, PA extending to at least the lateral boundary of the intracavernous carotid segments (Knosp grade ≥2) was considered true PA extension into the CS as previously correlated to surgical findings.

**Figure 3.2.2**: midsagital coronal diagram of pituitary fossa depicting different PA extension patterns into the Cavernous Sinus. Knosp grading is as follows: Grade 0 = tumor does not extend past medial wall of intracavernous carotid artery. Grade 1 = tumor extends midway between supra- and intracavernous segments of carotid artery (intercarotid line); Grade 2 = tumor extends to lateral wall of ICA Grade 3 = tumor extends beyond lateral wall of ICA. Grade 4 – complete encasement of ICA by PA. Knosp Grade 2+ definition of CSI in my cohort.
Figure 3.2.3: Recorded PA growth patterns in (A) the coronal plane and (B) the sagittal plane as depicted in Monsalves et al., 2014. Inferior growth into sphenoid sinus (erosion of sphenoid bone by PA), superior growth towards optic chiasm, lateral growth towards and into CS (Knosp grading), anterior growth above the planum, and posterior growth towards brainstem were noted.
As an alternative, we used an in-house UHN method for determining CS invasion as proposed by Dr W Kucharczyk(Figure X). In this model, the CS is invaded if either of the following criteria are met: a) at least 50% of the carotid artery is encircled by tumor, b) the lateral wall of the CS is displaced, and/or c) there is increased amount of tissue between the lateral wall of the CS and the carotid artery. This proposed model corresponds to approximately Knosp grade 3 or 4.
The presence of a heterogeneous mass on T1 or a hyperintense signal on T2, suggesting either cystic or hemorrhagic changes was also recorded. Growth patterns and tumor morphology were only determined preoperatively. Analysis of postoperative growth patterns was limited to alteration of CS extension, as other growth patterns could not definitively be ascertained given the postoperative scarring and changes seen as a consequence of nasal-septal flap reconstruction.

An increase in size of image characteristics from the very first postoperative scan to a scan one year apart was taken as a sign of tumor and not postoperative changes/scar. Furthermore, we ensured that the MRIs were reviewed by three independent observers: our senior neuroradiologist (WK), first author (EM) and neurosurgeon (BG) and an interobserver consistency.
score was recorded to ensure no bias in review of imaging.

### 3.2.4 Tumor Volumetry and Growth Rate

The TVDT was calculated using the previously established formula for tumor growth rate measurements: \( \text{TVDT} = \frac{t \cdot \log_2 \frac{V_f}{V_0}}{\log_2(V_f/V_0)} \), where \( t \) is the time interval between the first and second MRI examinations, \( V_0 \) is the initial tumor volume, and \( V_f \) is the final tumor volume (Nakaguchi et al., 1999). TVDT was calculated both for the pre- and postoperative MRIs. To minimize measurement error, postoperative residuals were grouped into one of 3 categories: 1. Growth (≥25% increase in volume), 2. Reduction (≥25% reduction in volume), or 3. Stability (±24.9% change in volume).

For the subgroup of patients who had more than two pre and two post-operative images, the TVDT was calculated for all available imaging to ensure the rate was not significantly different. We had compared the TVDT at different intervals and since there was no significant variability we only reported the TVDT for two time points to standardize the results.

### 3.2.5 Correlating Tumor Growth Rate to MIB-1, FGFR4, P27 Age, Sex, Functional Status, and Histopathology of PA

Each patient in our cohort was reviewed in a systematic manner by two expert endocrine pathologists (Drs. Ozgur Mete and Sylvia L. Asa) at the time of initial diagnostic assessment. The status of the MIB-1 labelling index, p27 and N-terminally truncated FGFR4 were extracted from standardized diagnostic synoptic pathology reports. The MIB-1 LI was assessed in the hot spot regions by counting preferably 1000 tumor cells; however, given the nature of some specimens less than 1000 tumor cells were assessed for this evaluation. Cytoplasmic positivity for FGFR4 was considered to be positive. The extent or the intensity of staining patterns were not scored. The loss of p27 or positivity for p27 is recorded. The extent of positive nuclei was not scored.

Correlations were made between preoperative TVDT, patient age, gender, functional status, histopathology, and PA growth patterns. The preoperative TVDT was also correlated to the postoperative TVDT. PA were analysed in multiple different ways: based on the entire group,
stratified based on functional status (clinically functioning vs. non-functioning) and stratified based on histopathological subtype.

3.2.6 Statistical Analysis

A Cronbach’s alpha was generated for PA volumes in order to determine interobserver reliability. Correlations were made using Pearson’s correlation coefficient and Spearman rank coefficient. Mean comparisons were made using t-tests or ANOVAs. Categorical variables were examined using Chi-square, Fisher’s Exact test, and Odds Ratios (OR). All statistical calculations were performed using SPSS v.20.0 (Chicago, IL). Significance values that were p<0.05 were considered statistically significant.

3.3 Results

3.3.1 Clinical Demographics and Radiological Characteristics

We reviewed all patients treated between 1999 and 2011 at our institution and identified 500 consecutive patients who had complete follow-up data to be analyzed for this study. Of the 500 we identified 153 patients that satisfied our inclusion criteria. Of the 153 patients, 82(53.6%) were female and 71(46.4%) were male. The mean±SD age was 53±15 years (range=25-87). The preoperative imaging interval was variable and to eliminate some variability, cases with at least three month imaging intervals were selected. The mean preoperative interscan imaging interval was 17.3 months (range 3-113.9 months). Postoperative imaging intervals were more uniform as patients were followed closely by our multidisciplinary PA clinic with regular scans at 3, 6, 12, 24 etc months. The mean postoperative PA interscan interval was 29.2 months (range=12-84 months). All recorded PA were considered macroadenoma(diameter >1cm). PA histological and growth characteristics are listed in tables 3.3.1 and 3.3.2.
Table 3.3.1: Pituitary adenoma histological subtypes. All major subtypes of PA represented in my cohort. Nonfunctioning gonadotrophs were the most common as expected. Plurihormonal PA were defined as those secreting two or more hormones.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Number(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonadotroph</td>
<td>63(41.2)</td>
</tr>
<tr>
<td>Null Cell</td>
<td>24(15.7)</td>
</tr>
<tr>
<td>Prolactinoma*</td>
<td>10(6.5)</td>
</tr>
<tr>
<td>Somatotroph</td>
<td>16(10.5)</td>
</tr>
<tr>
<td>Corticotroph</td>
<td></td>
</tr>
<tr>
<td>Silent</td>
<td>26(17.0)</td>
</tr>
<tr>
<td></td>
<td>5(19.2)</td>
</tr>
<tr>
<td>Plurihormonal</td>
<td>13(8.5)</td>
</tr>
<tr>
<td>Thyrotroph</td>
<td>1(0.7)</td>
</tr>
<tr>
<td>Total</td>
<td>153(100)</td>
</tr>
</tbody>
</table>

*prolactinomas were medically refractory

Table 3.3.2: Characteristic growth patterns in pituitary adenoma. Most PA presented with superior extension towards the optic chiasm and this was the most frequent indication for surgical referral. A significant proportion also extended into the CS. All included PA were macroadenomas (≥1 cm in diameter)

<table>
<thead>
<tr>
<th>Growth Pattern</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>22(14.4%)</td>
</tr>
<tr>
<td>Posterior</td>
<td>46(30.1%)</td>
</tr>
<tr>
<td>Suprasellar</td>
<td>123(80.4%)</td>
</tr>
<tr>
<td>Sphenoid Sinus</td>
<td>37(24.2%)</td>
</tr>
<tr>
<td>Cavernous Sinus (Knosp≥2)</td>
<td>78(51%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>153(100%)</strong></td>
</tr>
</tbody>
</table>

3.3.2 Preoperative PA Volume and Tumor Growth Rate (TVDT)

Interobserver consistency for measuring tumor volume on ITK-SNAP was measured and the respective alpha levels for the first preoperative, second preoperative, first postoperative, and second postoperative scans were: α=0.996, 0.997, 0.914, 0.909. An alpha level above 0.7 is generally considered acceptable as a measure of good internal consistency(A. Christmann, 2006).
A subset of patients had multiple preoperative and postoperative scans.

The mean±SD preoperative TVDT for the study population was calculated to be 1147±870 days (range=60-3478 days), with functioning tumors demonstrating a significantly shorter TVDT at 747±564 days compared with clinically non-functioning PA at 1334±926 days (p=0.0001). The mean±SD TVDT based on histopathological subtypes were null cell 1579±1235, gonadotrophs 1097±668, prolactinomas 1222±1223, somatotrophs 895±612, corticotrophs 2215±2098, and plurihormonal tumors were, 941±502 days (Figure 3.3.1). The presence of a cyst/haemorrhage was evident in 22% of the cohort. However, the cystic/haemorrhagic components remained small (5-20% of tumor volume) and did not grow over time.

Figure 3.3.1: Growth rates (TVDT) of different PA histological subtypes included in cohort

The preoperative TVDT was positively correlated to age (r=0.361; p<0.0001). In addition, a shorter mean TVDT was significantly associated with suprasellar extension in somatotrophs (330 vs 1146 days, p=0.003) and the presence of a cyst/hemorrhage in both somatotrophs (32% cystic/hemorrhagic) (307 vs 1002 days, p=.004) and null cell tumors (33%...
cystic/hemorrhagic) (831 vs 1927 days, p=0.01). The preoperative TVDT was also inversely correlated to the MIB-1 LI (r=-0.256, p=0.005). Gonadotrophs were the only histological subtype to display a significantly shorter mean TVDT with both FGFR4 positivity (983 days vs 1379 days, p=0.047) and p27 negativity (559 vs 1386 days, p=0.007) (Table 3.3.3). No other factors were significantly related to preoperative TVDT.

Table 3.3.3: Factors associated with a shorter preoperative TVDT identified by whole group analysis, stratification by histological subtype, and stratification by hormonal status.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Entire cohort</th>
<th>Tumor Histotype</th>
<th>Hormonal Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>P&lt;0.001</td>
<td>Gonadotrophs**</td>
<td>Functioning***, Nonfunctioning***</td>
</tr>
<tr>
<td>S Growth</td>
<td>NS</td>
<td>Somatotrophs**</td>
<td>NS</td>
</tr>
<tr>
<td>C/H Component</td>
<td>NS</td>
<td>Somatotrophs**, Null Cell*</td>
<td>NS</td>
</tr>
<tr>
<td>MIB-1 LI</td>
<td>P&lt;0.01</td>
<td>NS</td>
<td>Nonfunctioning*</td>
</tr>
<tr>
<td>Negative p27 status</td>
<td>NS</td>
<td>Gonadotrophs**</td>
<td>Nonfunctioning*</td>
</tr>
<tr>
<td>Positive FGFR4 status</td>
<td>NS</td>
<td>Gonadotrophs*</td>
<td>NS</td>
</tr>
</tbody>
</table>

*p<0.05  NS=Nonsignificant; S = Suprasellar; C/H = Cystic/Hemorrhagic  
**p<0.01  
***p<0.001

3.3.3 Association of Residual Volume with Growth Patterns and Patient Characteristics

Fifty three cases (34.6%) exhibited postoperative residual volumes while the remaining 100 did not. Of the 53 residuals, the majority (80.8%) were clinically nonfunctioning. The presence of a residual volume was associated with older patient age (57 vs 51, p=0.038). The presence of residual was also associated with several preoperative growth patterns: 28.3% of residual had anterior (p=0.001)(OR=5.24), 54.7% had posterior (p<0.0001)(OR=5.90), 94.2% had suprasellar (p=.002)(OR=5.73), and 71.7% had CS extension (p<0.0001)(OR=3.80), 42.4% of which continued to have definitive extension into the CS (p<0.0001)(OR=14.6) post-operatively (Table 3.3.4).
3.3.4) The presence of a residual or its subsequent growth behavior (growth, stability, regression) was not associated with any other factor.

Table 3.3.4: Factors associated with residual volume following surgery

<table>
<thead>
<tr>
<th>Factors</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>P=0.038</td>
</tr>
<tr>
<td>Cavernous Sinus Invasion</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Suprasellar Extension</td>
<td>P=0.002</td>
</tr>
<tr>
<td>Posterior Growth</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Anterior Growth</td>
<td>P=0.001</td>
</tr>
</tbody>
</table>

3.3.4 Postoperative Tumor Volume and Tumor Growth Rate (TVDT)

There were a total of 23(43.3%) tumors with postoperative residuals that demonstrated growth, with 17(73.9%) nonfunctioning and 6(26%) functioning PA. The mean±SD age was 51±12; 13(56.5%) females and 10(43.5%) males. The overall mean±SD postoperative TVDT for both the functioning and nonfunctioning PA was 1164±615 days with a mean radiological follow-up period of 29.2 months(range=12-84 months). The mean preoperative TVDT of PA that demonstrated postoperative growth was 1165±178 days. This was not significantly different from postoperative TVDT (p=0.106). The TVDT for nonfunctioning PA was 1272±637 and 857±461 days for functioning PA. The difference between functioning and non-functioning TVDT was not significant (p=0.089).

The postoperative TVDT was positively correlated to preoperative TVDT (r= 0.497, p=0.026) and to patient age in non-functioning(r=0.596, p=0.015) but not in functioning PA. A shorter mean TVDT was associated with female gender in functioning PA only(689 vs 1695 days, p=0.017) but not in any particular subtype. No other factors were associated with postoperative TVDT. However, due to the fact that there are only 6 functioning tumor postoperatively, these results should be interpreted with caution.
3.3.5 Correlation of Growth Rate with Expression of MIB-1, FGFR4, and p27

92.6% of cases had sufficient material for detailed examination of MIB-1 LI. The overall mean MIB-1 LI was 3.19%(range=7.5). Clinically functioning PA had a mean MIB-1 LI of 3.88% and non-functioning tumors had a mean LI of 2.86% and this difference was statistically significant(p=0.007). In terms of subtype, the mean MIB-1 LI for gonadotroph, null cell, prolactinoma, somatotroph, corticotroph, and plurihormonaltumors were 2.53%, 3.07%, 3.79%, 2.72%, 3.99%, 5.1%, respectively. The single thyrotroph adenoma in the series had a MIB-1 LI of 5.0%. Furthermore, the mean MIB-1 LI was marginally but nonsignificantly greater for those residual masses that grew compared to those that remained stable or regressed (3.15%, 2.71%, 2.66%, respectively).

The MIB-1 was positively correlated with age (r=0.215, p=0.011) and was significantly higher in the female gender, specifically in those with the corticotroph subtype(5.07% vs 1.97%, p<0.0001). The MIB-1 was also higher in gonadotrophs that exhibited anterior extension (3.29% vs 2.41%, p=0.025) and in null cell tumors with CS extension(3.90% vs 2.23%, p=0.016). In functioning PA, a higher MIB-1 was associated with suprasellar (4.55% vs 3.07%, p=0.019) and posterior extension (5.9% vs 3.9%, p=.019) as well as with the presence of hemorrhage or cystic change (5.04% vs 3.49%, p=0.026).

FGFR4 positive PA (53.9% of cases) were associated with patients of older age (60 vs 48 years old, p=.016) and 1.83 times more likely to be non-functioning than functioning PA(p<0.0001). FGFR4 positive PA were also 2.16 times more likely to have a cystic change or a hemorrhagic component(p=0.05).

In contrast to FGFR4, p27 negative PA (5.2% of cases) were associated with patients of younger age (44 vs 57 years old, p=.003) and 4.13 more likely to be functioning than nonfunctioning tumors(p=0.042). The p27 expression profile was inversely related to the MIB-1(r=-0.187, p=.032) but not related to FGFR4.
Figure 3.3.2: Schematic summary of Aim 1

**Demographic Parameters**
- Older age
- Gender*

**Predictors of residual and/or recurrent PA**
- Increased likelihood of residual and/or recurrent PA
- Increase patient surveillance

**Growth (radiological) Parameters**
- Reduced preop tumor volume doubling time (TVDT)
- Suprasellar growth**
- Anterior and posterior growth
- Cavernous sinus invasion
- Cyst/hemorrhage***

**Histopathological Factors**
- Hormone expression
- Histosubtype
- MIB-1
- Positive FGFR4****
- Negative p27 ****

*Female with corticotroph PA
**Somatotroph PA
***Somatotroph and Null cell PA
****Gonadotroph PA
3.4 Discussion

To gain insight into PA growth behavior, we opted to examine the preoperative growth direction and rate of PA and correlate it to postoperative growth rate. To date, attempting to correlate PA growth rate pre- and postoperatively has never been reported in the literature. The postoperative TVDT has, however, been examined in relation to PA in a limited number of studies, primarily in residual NFPA (Ekramullah et al. 1996; Tanaka et al. 2003). Our cohort which included both functioning PA and NFPA revealed that the preoperative and postoperative growth rate as assessed by TVDT were correlated. Previous studies suggested that preoperative PA volumes were able to predict postoperative outcome but growth rates were not assessed in these studies (Jain et al. 2008; Shrestha et al. 2012). Our data suggests that an understanding of preoperative PA growth dynamics will provide insight into how these tumors will behave postoperatively in terms of radiological growth which will help enable the selection of appropriate strategies in a more timely fashion and may even be able to preempt future PA recurrence.

Additionally, our data suggest that the growth rate of PAs are influenced by various patient and tumor-specific characteristics including the age and sex of the patient, the specific histologic subtype of PA, it’s hormonal activity, it’s preponderance for different growth directions relative to the pituitary fossa. Some of these factors have been reported in connection to PA growth behavior previously. For example, it has been shown that PA that invade into the CS or sphenoid sinus are associated with a more aggressive phenotype with increased likelihood of postoperative residual and recurrence (Trouillas, et al. 2013). The current study aimed to provide a more complete growth profile of PA and use this information to create a model that may help predict PA residual, growth, and recurrence.

The immunohistological profile has also been correlated to growth rate in our study. This includes the MIB-1 LI, a marker of cell proliferation, which has been previously correlated to postoperative PA TVDT in some studies where a higher MIB-1 is associated with a faster (lower) TVDT (Hsu et al. 2010; Tanaka et al. 2003). In our study, the MIB-1 was inversely correlated with TVDT consistent with what other have reported but our correlation was only seen with respect to preoperative TVDT and not to postoperative TVDT. The status of biomarkers,
including ptd-FGFR4 and the early cell cycle inhibitor p27 were also included in our study as gleaned from institutional synoptic PA pathology reports. ptd-FGFR4 is an N-terminally truncated variant of normal FGFR4; it is constitutively active and is localized to the cytoplasm. It has been previously reported to be prevalent in 40-50% of all PA, with a specific tendency for expression in null cell and gonadotroph PA (Ezzat, et al. 2004; Ezzat et al. 2002). ptd-FGFR4 has been correlated previously to PA invasiveness (Morita, et al. 2008). Although positivity for ptd-FGFR4 was seen in approximately 50% of our cohort primarily in null cell and gonadotroph PA, it was not associated with growth or invasiveness. The status of p27 has been shown previously to be positive in most PA suggesting that the early cell cycle is not adversely affected in the majority of cases. Additionally, consistent with other studies (Hewedi, et al. 2011; Zhao 1999), p27 negativity was not shown to correlate with PA growth or recurrence in our cohort in general. However, gonadotrophs that were both p27 negative and FGFR4 positive were associated with a faster rate of growth implicating the prognostic value of these biomarkers in this subtype specifically. The concurrent examination of p27 and FGFR4 has not reported previously in the PA literature.
CHAPTER 4
Preclinical Model of PA for Interrogating Therapeutic Strategies: Role of mTOR inhibitors

4.1 Introduction

Identification of molecular mechanisms that are involved in PA initiation and progression are not clearly defined. Some molecular initiators of pituitary tumorigenesis have been identified. For example, somatic mutations resulting in G-protein abnormalities and subsequent increases in cAMP levels have been identified in a subset of GH-PA (Vallar, et al. 1987). RAS mutations also occur but these are limited to the rare metastatic pituitary carcinoma (Pei, et al. 1994). Other more common molecular events that modulate PA growth in general have not been well characterized.

Modulation of growth factors and their receptors are necessary for normal pituitary growth and function. Dysregulation of these factors may lead to abnormal pituitary growth and tumor development. For example, FGF-2 mRNA has been shown to be overexpressed in PA ((Ezzat, et al. 1995)). Recent interest has focused on FGFR4, which is a 110 kDa transmembrane kinase that is involved in mitogenesis and angiogenesis (Abbass, et al. 1997). A 60kDa N-terminally truncated pituitary-tumor derived variant of FGFR4 (ptd-FGFR4) has been identified in 40-50% of PA but not in normal pituitary (Ezzat et al. 2002). Preclinical evidence suggests that ptd-FGFR4 but not full-length FGFR4 confers oncogenic properties to pituitary tumor cells. In human PA tissue, positivity for ptd-FGFR4 has been associated with increased PA aggressiveness (Ezzat et al. 2004).

Germline allelic alterations in FGFR4 have also been identified in PA. An adenine to guanine SNP at codon 388 of the FGFR4 gene results in a glycine to arginine substitution in the transmembrane domain of the FGFR4 protein (FGFR4-G388R) (Bange et al. 2002). This SNP results in increased growth and growth hormone production in GH4 cell lines and human GH PA tissue (Tateno et al. 2011). In ACTH PA, the FGFR4-R388 variant is associated with the
presence of silent ACTH macroadenomas while wild-type FGFR4 (G388) is associated with ACTH production (Nakano-Tateno et al. 2014).

Downstream molecular mechanisms from FGFR4 have not been examined in detail. Ezzat et al., 2006 have reported that ptd-FGFR4 promotes oncogenic transformation by disturbing the NCAM/b-catenin complex thereby disrupting normal cell to cell interactions. In their study, an FGFR4 inhibitor was used to restore the NCAM complex and reduce cell and tumor growth (Ezzat et al. 2006). In the FGFR4-G388R SNP src and STAT3 serine phosphorylation was shown to promote growth while STAT3 tyrosine phosphorylation modulated hormone secretion (Tateno et al. 2011).

Other signaling mechanisms in PA need to be identified. Recent interest has focused on the PI3K/mTOR pathway which is a signaling cascade important for maintaining growth and homeostasis within the cell (see chapter 3). It is ubiquitously overexpressed in cancer and has been shown to be overexpressed in PA, particularly in NFPA and GH-PA (Musat et al. 2005; Sajjad et al. 2013). It has been demonstrated that the mTOR inhibitors are effective in PA cells by reducing cell viability and proliferation and also arresting cells early in the cell cycle (Gorshtein et al. 2009; Zatelli et al. 2010).

The PI3K/mTOR pathway is frequently initiated by activation of RTKs which may include FGFR4. The aim of this study is to determine a differential expression pattern of the mTOR pathway between FGFR4-G388 and R388 tumors and how this expression may influence sensitivity to mTOR inhibition using RAD001 and affect tumor growth rate and hormone levels.
4.2 Material and Methods

4.2.1 Cell lines and cultures

As there are no human-derived hormone-producing pituitary cell lines, we used rat pituitary GH4 mammosomatotroph cells. These cells were obtained from Dr Ezzat's lab from his senior research fellow, Dr Toru Tateno, and propagated in Ham F10 medium 12.5% horse serum (HS)(Sigma, Oakville, ON) and 2.5% fetal bovine serum (FBS; Sigma, Oakville, ON) 2mM glutamine, 100 IU/ml penicillin, 100 ug/ml streptomycin (37°C, 95% humidity, 5% CO2 atmosphere incubation). The cell lines were maintained as described previously by Tateno et al., 2011 (Tateno et al. 2011)

4.2.2 Plasmids and Transfections

Plasmids encoding human prototypic FGFR4 (G388) or the polymorphic form FGFR4- R388 were generated and stably transfected into GH4 as previously described (Tateno et al. 2011). Plasmids were obtained in collaboration with Dr T Tateno (PDF) in my co-supervisors lab (Dr S Ezzat). Construct fidelity was confirmed by DNA sequencing after introduction into pcDNA 3.1 as described in Tateno et al., 2011. Cells were transfected using Lipofectamine 2000 (Life Technologies, Rockville, MD) according to the manufacturer’s instructions. Stable clones were selected using neomycin (G418) at a concentration of 0.7 ug/ml and maintained in this conditioned media throughout the experiments

4.2.3 Animals

All animal experiments and protocols were approved by the University Health Network (UHN) Animal Care and Use Committee. Immunocompromised, non-obese severe combined immunodeficiency (NOD-SCID) male mice were purchased from Jackson Laboratory (Sacramento, CA, USA) and used for intracranial studies. Cell lines used were GH4 cells, used as
parental control, G388, and R388 representing the SNP variant FGRF4 expressing cells. GH4 empty vector (PC) transfected cells were also used as controls.

4.2.4 In vivo Tumor Models

Six week old NOD-SCID mice were anesthetized with intraperitoneal injections of Avertin [1.25% solution, 0.2 mL/10 g body weight approximately 0.45 mL for 6 week old mice]. Mice were used at age 6-8 weeks. Intracranial xenografts were generated as described previously (Burrell et al., 2014). Briefly, the head was cleaned with 70% ethanol, the scalp was opened with a 1 cm incision midline and the usual stereotactic landmarks were used for injections. A burr hole was created in the skull with a dental drill and cells were injected using a Hamilton syringe. 5*10^5 were injected using a hamilton syringe via stereotactic coordinates 3mm deep into the frontal cortex on the right side of the brain. All animals were injected on the same day, with assistance of senior research associate Dr Shahrzad Jalali. In order to establish the model that allowed for tumor growth and sufficient time for growth to permit MRI examination and drug studies we did a serial dilution of 10^5 and 10^6 cells injected intracranially to optimize the best model for PA xenografts as previous such studies do not exist.

Injection of 10^6 cells resulted in rapid tumor development precluding imaging and detailed analysis of growth rate studies or response to therapy for alteration in tumor growth.

Injection of 10^5 cells resulted in tumors with very long growth latency periods. We decided to inject 5*10^5 cells for our next set of experiments. Each mouse received one injection containing 5*10^5 cells suspended in 10 ul PBS. Mice were monitored closely by serial magnetic resonance imaging (MRI) starting at week 2 and sacrificed at the end of drug treatment period or sooner if signs of illness developed, typically characterized by hunched or abnormal posture, decreased movement, lethargy, paralysis or weight loss.

4.2.5 Magnetic Resonance Imaging (MRI)

MRI was performed with a 7 Tesla Biospec 70/30 (Bruker Corporation), using the B- GA12 gRTient coil insert and 7.2 cm inner diameter linearly polarized volume resonator coil for
radiofrequency (RF) transmission as detailed previously (Chung et al., 2012).

The protocol provided a stack of transverse 2D slices with shared geometric prescription (16 x 16 mm field-of-view or FOV) testing multiple contrast mechanisms, as follows: i) T2-weighted-RARE anatomical imaging (echo time or TE=72 ms; repetition time or TR=5000 ms; rapid acquisition relaxation enhancement or RARE factor=16; readout bandwidth or RBW=50 kHz; 25×125×500 μm voxels) for; 1 min and 20 sec; ii) Contrast enhanced T1-weighted RARE (TE=8 ms; TR=1200 ms, RARE factor=4; RBW=81.5 kHz; 125×125×500 μm voxels; start imaging at 5 min post-contrast) for 1 min and 20 sec.

4.2.6 Treatment Schedule.

After intracranial injection of cells, the mice were monitored by weekly MRI. After tumor development was noted, as determined by T2 MRI, mice were randomly stratified into control or treatment arms, ensuring approximate equal tumor volume distribution into each arm. Treatment consisted of daily intraperitoneal I.P. injections of 5mg/kg RAD001 (Cedarlane, Burlington, ON) or DMSO dissolved in ddH2O (control). Treatment lasted for three weeks at which point the mice were sacrificed and their brains extracted and placed into formalin for histological analysis. For 5 mice in each of the three cell groups, tumor was isolated using a surgical microscope and preserved in liquid nitrogen for western blot analysis (see below for details below). MRI was performed weekly on all animals while on treatment to allow tumor volume analysis.

4.2.7 Tumor Size Measurement

Serial MR images were used to establish the tumor volumes and growth rates. ITK-SNAP was used to analyze the MRI images. Tumor region of interest (ROI) on T2-weighted (T2w) images was manually defined and used to measure the tumor volume. Growth rate studies using both T1 and T2 images were correlated and found to show good correlation. Interobserver correlations were generated statistically using Cronbach's alpha and an excellent reliability score was achieved (α =0.89) Furthermore, T1 and T2 images were compared to differentiate tumor from post-surgical changes.
These studies were done by two independent examiners (EM) and (SJ). Since, T2 MR images paralleled the T1 images closely, were primarily used for calculating tumor volume and growth rate in this study for longitudinal studies using T2 images.

4.2.8 Immunohistochemistry

Immunostaining was conducted on formalin-fixed paraffin embedded on lesional tissue in brain sections of control and treated animals. The sections were deparaffinized in xylene and rehydrated in graded ethanol and rinsed in dH20. Heat-induced antigen retrieval was used by pressure cooking the sections for 20 minutes in Antigen Retrieval Solution (low pH). Slides were incubated with primary antibodies with appropriate conditions. Detection was performed using vectastain ABC reagent and DAB chromagen (Vector Labs, Burlingame, CA). Slides were counterstained in Meyer’s Haematoxylin for 5 minutes, dehydrated with ethanol (70%, 95%, 100%) and coverslipped. Primary antibodies were MIB-5/Ki-67 (Dako), p-S6 (Cell Signalling), p-4EBP1 (Cell Signalling), growth hormone and prolactin antibodies were kindly provided by the Dr. Ezzat’s lab. All slides were scanned using Zeiss Mirax Scanner and analysed using Mirax Viewer software.

4.2.9 Western Blot Analysis

To quantify protein expression in tumor tissue, brains were extracted, tumors immediately isolated and placed in liquid nitrogen for snap-freezing. Lysis buffer (0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40 and 1X PBS) containing proteinase and phosphatase inhibitors was added to tumors which were then homogenized using a mechanical tissue homogenizer. Total cell lysates were incubated on ice for 30 mins followed by micro-centrifugation at 10 000g for 10 min at 4°C, and the supernatent was collected. Protein concentration was determined by BCA (bicinchonic) assay as per manufacturers specifications (Pierce Chemicals Co., Rockford, IL). A total of 40ug was separated on a 10% SDS polyacrylimide gel electrophoresis (SDS PAGE) and blotted onto nitrocellulose membranes (Bio RAD, Hercules CA) using a semi-dry turbo transfer apparatus (Bio-Rad, Hercules, CA). After blocking the membrane with PBS (or TBS) containing 5% nonfat dry milk and 0.05% Tween 20
(Sigma-Aldrich) for 1 hour the membranes were incubated with primary antibodies overnight at 4 degrees. The following antibodies were used: 1:1000 p-4EBP1 (Cell Signaling); 1:2000 p-S6 (Cell Signaling); 1:1000 Growth Hormone (Dr. Ezzat lab); 1:1000 Prolactin (Dr Ezzat lab); 1:500 ki-67 (Dako). Membranes were washed and later incubated with IRDye secondary antibodies against the species the primary antibody was derived from (Bio-RAD, Hercules, CA). Protein bands were detected using and quantified using LI COR Software.

4.2.10 ELISA

Mouse whole blood was extracted from the right ventricle of the heart and placed in Microvette CB 300z serum extraction tubes. Whole blood was centrifuged for 5 minutes at 5000 RPM at 4C. Serum was extracted and stored at -80C. ELISA was used to measure serum IGF-1 (Quantikine) levels according to manufacturer’s protocol.

4.2.11 Statistical Analysis

Nonparametric tests (Kruskall-Wallis, Mann Whitney U) were used to examine median differences in tumor growth rates, IGF-1 levels. H-Scoring was used to semi-quantify IHC data. Intensity of Western Blot protein bands were quantified using LICOR software.

4.3 Results

4.3.1 In vivo tumor formation assay

To examine the biologic impact of the FGFR4 SNP, we introduced FGFR4-G388 or FGFR4-R388 into pituitary adenoma GH4 cells. Initially, we injected 10^6 GH4 cells but we noted rapid tumor growth that precluded our ability to track tumor growth rate so 5*10^5 cells were used in later experiments. Targeting the frontal intraparenchymal region was chosen as the model to investigate for the purposes of my masters thesis. Evidence of a proliferative GH4 tumor co-expressing GH and PRL was noted (Figure 4.3.1) in pc, G388, and R388 mice. The R388 tumors
expressed more GH relative to the G388 and pc tumors which is in line with what has been reported previously using these cells (Tateno et al. 2011)

Figure 4.3.1: The FGFR4-R388 polymorphism deregulates pituitary hormone production intracranial xenograft mouse tumor. Growth hormone (GH), prolactin (PRL), and Ki-67 protein expression were examined in pituitary GH4 mammosomatotroph intracranial tumors stably expressing empty vector (pc) (control), the prototypic FGFR4 receptor (G388), or the human polymorphic FGFR4 variant (R388). G388 produce a proliferating tumor with enhanced PRL production and diminished GH production whereas injection of R388 cells produce a proliferating tumor with diminished PRL expression and enhanced GH expression. (n=5)(magnification 40X; scale: 50um)

4.3.2 MRI analysis

We first established the growth rate of the PA grown as frontal ic xenografts. MRI examination of tumor growth was performed using T1W MR images with gadolinium enhancement (Figure 4.3.2). However, given that T1W images with contrast on each animal every week is associated with high number of animal loss, we opted for T2W MR images without contrast for subsequent measurements of tumor volume and growth rate. Correlative studies were conducted between
T2W and T1W images and were found to have a good correlation. Tumor development was noted on MRI after 4 weeks in all 3 tumor groups (Figure 4.3.2)

Figure 4.3.2: Injection of GH4 cells into the frontal intraparenchymal area in NOD-SCID mice results in radiologically determined tumor growth. T1W MR images with gadolinium contrast enhancement in the axial plane demonstrating tumor development three weeks post intracranial injection of $5 \times 10^5$ GH4 cell stably transfected with either pc, G388, and R388 mice.

4.3.3 Tumor volume and growth rate.

Weekly MR images allowed for an examination of tumor growth rate. Tumor volumes were found to increase exponentially over time with pc tumors exhibiting a significantly longer latency for tumor development relative to the G388 or R388 tumors ($p=0.034$)(figure 4.3.3A) and was also associated with a slower growth rate (increased TVDT) (Figure 4.3.3B)
Figure 4.3.3: FGFR4-G388R cells result in intracranial tumors that exhibit short latency periods followed by rapid tumor expansion. A. Analysis of serial MRI in axial plane using ITK-SNAP for volumetric assessment revealed that intracranial xenograft mouse models of GH4 mammosomatotroph tumors transfected with either G388 or R388 resulted in significantly larger tumors than those transfected with PC at week 4. B. Volumetric data from the intracranial GH4 tumors transfected with either pc, G388, or R388 were incorporated into the formula for estimating tumor volume doubling time (TVDT) and a growth rate was generated (in days). pc had a significantly longer TVDT (slower growth rate) than G388 or R388 tumors. *p<0.05. N=8 in each group (pc, G388, R388)

4.3.4 mTOR pathway expression

We next wanted to determine mTOR pathway expression in our GH4 tumors using the immediate downstream effectors, S6 and 4EBP1 as a readout for mTOR expression. There was abundant staining of both p-S6 and p-4EBP1 particularly in the G388 and R388 tumors (Figure 4.3.4) suggesting that our model is ideal for mTOR inhibition therapy.
Figure 4.3.4: Intracranial GH4 tumors transfected with either prototypic FGFR4 (G388) or polymorphic FGFR4 (R388) exhibit abundant mTOR signaling. Immunostaining of p-S6 and p-4EBP1 in intracranial xenograft mouse GH4 tumors transfected with either G388 or R388 reveal widespread expression. p-S6 and p-4EBP1 are the immediate downstream effectors of mTOR and are therefore the most typically used readouts for mTOR (n=5) (magnification: 40X; scale: 50um)

4.3.5 mTOR Inhibition Treatment Using RAD001

Validation of our initial experiments was conducted. A total of 20 mice were randomly stratified into the RAD001 (mTOR inhibitor) treatment arm (n=10) or the DMSO control arm (n=10) once tumor development was noted. This procedure was repeated sequentially for the mice GH4 xenografts transfected with empty vector (PC), FGFR4-G388, and FGFR4-R388.

4.3.6 The Growth Rate of GH4 Tumors

A significant reduction in tumor volume and growth rate was noted in mice that were treated with RAD001. This significant reduction was seen in PC and the G388 tumors and R388 tumors( Figure 4.3.5 a, b, c). The differences in growth rates for untreated tumors in the three groups did not differ significantly. However, the magnitude of the growth rate reduction was of similar magnitude in all three cell groups: 2.1X, 2.2X, 2.4X in pc, G388, and R388 tumors, and
these differences were not statistically significant.
Figure 4.3.5: The mTOR inhibitor RAD001 decreases intracranial GH4 tumor volume regardless of FGFR4 status a) Intracranial GH4 tumor in control (DMSO) group exhibiting representative large, hemorrhagic tumors (top) as indicated on both hematoxylin and eosin (H&E) stained coronal formalin fixed paraffin embedded (FFPE) brain tissue sections and axial T2W MRI. Intracranial GH4 tumors treated with 5mg/kg RAD001 were typically smaller than in control as evident in representative bottom coronal H&E FFPE section and T2W MRI. B) The growth rates for intracranial GH4 tumors transfected with either pc, G388 or R388 as determined by serial MRI and reported as TVDT are displayed as boxplot. The median growth rates are reported. The median growth rates in all three control groups were not significantly different from each other. However, the growth rates in all 3 groups decreased following RAD001 treatment although the magnitude of the decrease was similar in all groups.

4.3.7 mTOR Expression Patterns Following RAD001 Treatment

Following a examination of growth rate after treatment with RAD001, we determined mTOR signalling patterns and found that there was a substantial decrease in p-S6 expression particularly in the G388 and R388 tumors(Figure 4.3.6a,b,c). There appeared to be no treatment effects with p-4EBP1 as the expression pattern of this protein did not change following treatment (Figure4.3.7a,b,c).

Figure 4.3.6 (below): RAD001 decreases p-S6 expression in intracranial xenograft GH4 mouse tumor transfected with either prototypic FGFR4 (G388) or polymorphic FGFR4 (R388) A) Immunohistochemical analysis of p-S6 expression in GH4 tumors transfected with either pc, G388 or R388 shows a decrease following treatment with RAD001 (n=5)(magnification =40X; scale=50um) B. Equal amounts of tumor lysates were resolved by SDS-PAGE and analyzed by immunoblotting with p-S6 (32 kDa) and Tubulin (50kDA). Quantification of protein was conducted using Li-Cor software and the intensity of the p-S6 band was compared to intensity of the loading control(tubulin). Subsequent results are reported in 'relative fluorescence units' and displayed in boxplot format. Median p-S6 band intensity was significantly attenuated in both G388 and R388 tumors following mTOR inhibition with RAD001(n=5).
Figure 4.3.7: RAD001 has no effect on p-4EBP1 expression in intracranial GH4 tumors transfected with FGFR4-G388R. 

A) Immunohistochemical analysis of p-4EBP1 expression in GH4 tumors transfected with either pc, G388 or R388 shows no change in expression following treatment with RAD001 (n=5)(magnification =20X; scale=50um). 

B. Equal amounts of tumor lysates were resolved by SDS-PAGE and analyzed by immunoblotting with p-4EBP1 (20 kDa) and Tubulin (50kDa). Quantification of protein was conducted using Li-Cor software and the intensity of the p-4EBP1 band was compared to intensity of the loading control (tubulin). Subsequent results are reported in 'relative fluorescence units' and displayed in boxplot format. Median p-4EBP1 band intensity was not significantly altered in either G388 or R388 tumors following mTOR inhibition with RAD001 (n=5).

4.3.8 Hormone Expression Patterns Following RAD001 Treatment

The hormone expression profile of our GH4 tumors following RAD001 treatment was examined next. Growth hormone is expressed in tumors with greatest intensity of expression in R388 tumors (Figure 4.3.8 a,b,c). Expression significantly decrease in the R388 group following inhibition with RAD001 (Figure 4.3.8c).
Figure 4.3.8: RAD001 decreases GH expression in intracranial GH4 xenograft tumors harboring the FGFR4 polymorphism (R388) A) Immunohistochemical analysis of GH expression in GH4 tumors transfected with either pc, G388 or R388. R388 tumors exhibit enhanced GH expression with decreased expression levels following treatment with RAD001 (n=5)(magnification =40X; scale=50um) B. Equal amounts of tumor lysates were resolved by SDS-PAGE and analyzed by immunoblotting with GH (25kDa) and Tubulin (50kDa). Quantification of protein was conducted using Li-Cor software and the intensity of the GH band was compared to intensity of the loading control(tubulin) band. Subsequent results were reported in 'relative fluorescence units' and displayed in boxplot format. Median GH protein band intensity was enhanced in R388 groups and demonstrated a significant reduction in GH protein levels following mTOR inhibition with RAD001(n=5).

4.3.9 Mouse Serum IGF-1 Levels Following RAD001 Treatment

Serum measurements of IGF-1 (systemic hormone stimulated by GH) were analysed following mTOR inhibition. Untreated control IGF-1 levels were significantly higher in
R388 relative to pc and G388 tumors. Following RAD001 treatment, all groups experienced a decrease in IGF-1 levels but the decrease was only significant in the R388 group (Figure 4.3.9).

Figure 4.3.9: RAD001 decreases serum IGF-1 levels in intracranial xenograft GH4 tumors harboring the FGFR4 polymorphism (R388) Circulating IGF-1 levels as a measure of GH4 tumor output are noted in pc, G388 and R388 mice. Median IGF-1 levels are enhanced in the R388 group and display a significant decrease in IGF-1 levels following mTOR inhibition with RAD001 in the R388 group only (p=0.005).
4.4 Discussion

Our preclinical in vivo mouse model of PA has enabled us to delineate some of the pathophysiological mechanisms contributing to the PA disease process. Our target pathway was the PI3K/Akt/mTOR pathway as this pathway has been shown to be upregulated in PA both in human and animal PA cells at the in vitro level. Some in vivo experiments examining mTOR pathway expression in subcutaneous PA models have also been conducted. In our study, we opted to grow tumor cells in the intracranial environment which, in our opinion, reasonably recapitulated the actual PA milieu.

Differential mTOR pathway expression was examined with respect to cells and tumors transfected with either wild-type FGFR4 or the SNP variant of FGFR4. FGFRs are important for normal pituitary growth and hormone modulation. Prototypic FGFR4(G388) is a 110 KDa membrane-bound protein consisting of three extracellular Ig-like domain, a transmembrane domain, and an intracellular tyrosine kinase domain(Bange et al. 2002). In our mouse models, FGFR4 harbored a polymorphism at codon 388 resulting in the transmembrane domain possessing an arginine residue instead of wild-type glycine (R388). The presence of this SNP has been shown to correlate with increased aggressive behavior in various cancers and in GH and ACTH secreting PA cell lines has shown to phosphorylate src and the serine residue of STAT3 to increase cell proliferation(Nakano-Tateno et al. 2014; Tateno et al. 2011). In the in vivo mouse harboring the FGFR4 SNP we found increased expression of GH relative to wild-type
FGFR4 and empty vector controls (pc). R388 tumors were also associated with increased serum IGF-1 levels compared to pc, and G388 tumors. G388 cells and tumors were associated with higher PRL levels compared to pc, and R388 cells. The GH and PRL expression patterns found in our tumor models have been reported previously in these cells (Tateno et al. 2011). Downstream mTOR pathway expression as determined by p-S6 and p4EBP1 was also noted. Finally, positive Ki-67 staining indicated a proliferating GH4 tumor model.

Once we established and characterized our intracranial model, we were able to target the mTOR pathway using an mTOR inhibiting agent called RAD001. This agent has been used previously in human and animal PA cells to reduce cell proliferation and viability while arresting cells in the early stages of the cell cycle and downregulating mTOR (Gorshtein et al. 2009; Zatelli et al. 2010). We did see a reduction in tumor growth rate following mTOR inhibition. However, the magnitude of tumor inhibition was similar in all groups (pc, G388, and R388). The decrease in tumor growth was associated with lower expression in p-S6. One unexpected finding was the concommitant reduction in GH levels in R388 tumors following treatment with RAD001. The reduction was also noted in serum IGF-1 levels. These findings suggest that patients with a GH-secreting tumor would benefit from RAD001 treatment. RAD001 has been shown by the current study to be efficacious in reducing tumor growth rate regardless of the FGFR4 genotype.
Chapter 5
Discussion

There were two aims to my project, one was a clinical retrospective review of PA patients with the goal of identifying factors that would aid in predicting radiologically determined PA growth. The second aim was preclinical and sought to establish an intracranial mouse xenograft model of PA and then to use that model to identify novel targeted therapies, namely inhibitors targeting the mTOR pathway. Although these two aims were under the umbrella of PA pathology, they were conducted independently from one another with no interconnecting components. Therefore, the two aims will be discussed below sequentially.

In our clinical retrospective study we have attempted to establish a correlation between preoperative and postoperative growth rate for PA and investigate factors that may predict growth rates of PA and residual postoperative growth rates of PA.

To date, only two studies report on postoperative TVDT for non-functioning adenomas with 930±180 days in one study and 1836±3445 days in the other study (Ekramullah et al., 1996; Tanaka et al., 2003). Another study included both functioning and non-functioning tumors together and reported a postoperative TVDT of 2361±4449 days (Hsu et al., 2010). Our recorded postoperative TVDT, which included both functioning and nonfunctioning PA, was 976±382 days. Our study is the only one to date that attempts to make a correlation between pre- and postoperative TVDT.

The discrepancy in TVDT values across studies may be due to differences in methods for measuring tumor volume. For example, in previous studies, PA volume was calculated using a formula based on tumor diameter. In our study, in contrast, a program was used to manually contour the tumor borders which, in our opinion, is more accurate in capturing differences in tumor volume due to the heterogeneity of tumor shape. The discrepancies in TVDT may also be due, in part, to true biological differences. For example, the
composition of histological subtypes in our study was different from the study by Hsu et al. Although our study included a majority of gonadotrophs and null cells as reported previously, other subtypes were more represented in our sample, for example, corticotroph and somatotroph composition were 16.9 and 10.4% in our study compared to 3% and 0% in the study by Hsu et al. Furthermore, corticotrophs comprised the third largest proportion of our cohort.

In our study, the TVDT correlated to specific patient demographics. Preoperative TVDT was related to age, specifically in non-functioning adenomas. This significant trend was also evident for postoperative TVDT. A similar finding has been reported by Tanaka et al, who report patients under 61 harbouring a gonadotroph PA displayed a faster TVDT of their residual than patients over 61 years of age. Gender was also related to the postoperative TVDT only, where females with functioning tumors had a faster TVDT than did males(Tanaka et al., 2003).

The MIB-1 LI is currently used as a predictor of aggressive behaviour in PA with the general understanding that higher MIB-1 LI indicates a faster recurrence (Chacko et al., 2010; Noh et al., 2009; Prevedello et al., 2005). However, to date no studies have examined the correlation of pre-operative growth rate to MIB-1 LI in PA. Several studies have found significant inverse correlations between TVDT and MIB-1 LI for residual PA indicating that a higher cell proliferation index is associated with a faster PA growth rate. In our study, the preoperative TVDT was inversely related to the MIB-1 LI in non-functioning PA but this correlation was not seen between postoperative TVDT and MIB-1 LI. Also, MIB-1 LI was higher in clinically functioning tumors than in non-functioning PAs in keeping with what has been reported previously (Thapar et al., 1996). The MIB-1 LI was also associated with preoperative presence of CS growth. Other growth patterns that exhibited a significantly higher MIB-1 LI include PA with preoperative suprasellar, anterior, and/or posterior extension. There was an inverse correlation between MIB-1 LI and age, and other authors have found similar inverse correlations in PA. The MIB-LI is also significantly higher in females with functioning adenomas relative to males. Wolfsberger et al. found similar results in females with PA exhibiting a higher MIB-1 LI
than males (Wolfsberger et al., 2004).

It should be noted that in our study there was no direct relation between pre- and postoperative CS extension: only 42% of preoperative cases had continued postoperative CS extension. Furthermore, a small percentage of cases (10.4%) developed postoperative CS extension that was not evident preoperatively. These discrepancies can in part be as a consequence of how accurately CS invasion is defined on MR imaging and how well Knosp criteria correlates to intraoperative presence of true invasion.

Besides MIB-1 LI, other biological markers may predict PA growth characteristics. One such candidate protein is FGFR4. The FGFs and their receptors comprise several different isoforms that help regulate mitogenesis and angiogenesis. FGFR4 expression has been previously studied in relation to PA clinical characteristics in a few studies (Morita et al., 2008; Qian et al., 2004; Zhao, Tomono, & Nose, 1999). Qian et al. examined 137 PA patients and reported that the majority of their cohort expressed positive cytoplasmic ptd-FGFR4 staining (59.1%), which is similar to our findings (53.9% FGFR4 positivity) and congruent with preclinical studies on ptd-FGFR4 positivity in PA (Qian et al., 2004). Ptd-FGFR4 mRNA was previously reported to correlate with CS invasiveness (but not tumor size) in somatotrophs (Morita et al., 2008), and its protein expression was also shown to correlate with PA invasiveness, primarily in gonadotroph and null cell tumors (Ezzat et al., 2006). Our results confirm that the majority of gonadotrophs and null cell tumors express cytoplasmic ptd-FGFR4, however, in this series FGFR4 positivity was not related to invasiveness. In the study by Morita et al., age was not significant correlated to FGFR4 (Morita et al., 2008), whereas in our cohort we found a significant association of older age and FGFR4 positivity in null cell tumors, a subtype which was not included in their study. It has been reported that FGFR4 is correlated to the MIB-1 LI. In the study by Qian et al., which included 137 PA patients, it was demonstrated based on immunohistochemical analysis that there was a significant positive correlation between FGFR4 and MIB-1 LI expression (Qian et al., 2004). This finding was not replicated by our study. The difference in our results may be explained by variation in PA subtype distribution between the two studies (66% nonfunctioning PA in our study vs 39% nonfunctioning PA in their study) or differences in staining and quantification techniques,
since their mean MIB-1 LI was much lower (1.50% vs 3.99%), even though the proportions of FGFR4 positivity were similar (53.9% vs 59.1%). On the other hand, we report an inverse correlation between p27 and MIB-1 LI which has been demonstrated previously (Korbonits et al., 2002), and provides some confidence in the consistency of our data more generally.

The expression of p27, which blocks early cell cycle progression, is another candidate for use as prognostic tool in PA. The p27 expression is reduced in a number of cancers and is associated with poor clinical outcome (Chu, Hengst, & Slingerland, 2008). It has been shown that nuclear p27 expression is attenuated in human PA relative to normal pituitary tissue (Musat et al., 2005). However, the p27 status of most PA in our study were positive suggesting that the early stages of cell cycle regulation (G1 to S phase) are not adversely affected in most PA, at least in this patient population. With respect to the expression profiles of p27 among functioning and nonfunctioning PA, corticotrophs have been reported to express the lowest amount of p27 (Komatsubara et al., 2001). In our study, the majority of corticotrophs were positive for p27. When growth characteristics of PA were examined, we found no correlations to p27 expression. To date, several studies have also failed to show correlation between p27 expression and PA invasiveness or recurrence (Hewedi, Osman, & El Mahdy, 2011; Zhao et al., 1999) and currently no studies have attempted to correlate PA growth rate with p27 expression.

To date, no study has examined how p27 and FGFR4 expression may correlate to one another in PA. We did not find a significant association between FGFR4 and p27 expression. However, gonadotrophs that were both FGFR4 positive and p27 negative were significantly associated with a faster rate of growth. Thus, in these subtypes specifically, both the FGFR4 and p27 biomarkers may have prognostic value.

Histopathological subtyping of PA is another means of predicting PA growth behaviour. In our cohort, somatotroph and null cell subtypes with a cystic or hemorrhagic component had a faster TVDT. Tanaka et al. recorded the presence intratumoral cyst which occurred with a similar prevalence as in our cohort (27.5% vs 22% in our study) but found no correlations to TVDT. Also in our study, only somatotroph subtypes that exhibited suprasellar extension with optic chiasm compression had significant
associations with a faster preoperative TVDT. Our findings suggest that growth towards
the optic chiasm may be an indication for monitoring somatotrophs more closely than for
other subtypes with similar growth patterns. The tendency for somatotrophs to invade the
sphenoid sinus as reported by Zada et al. was not present in our study.

Another factor relevant to predicting PA growth is the presence of postoperative residual
tumor. A residual mass after surgery can herald potential tumor re-growth and so it is
important to determine predictive factors that may be associated with its occurrence,
which may help to establish guidelines for postoperative tumor surveillance. We report
residual volumes in 34.6% of our cohort, with 41.5% of these cases showing evidence of
actual tumor growth. This amounts to tumor recurrence in 14.5% of total cases in our
study. Due to the heterogeneity of PA subtypes and surgical techniques, rates of
recurrence reported in the literature are variable and range from approximately 7-
46%(Alahmadi, Dehdashti, & Gentili, 2012; Cappabianca et al., 2000; Chen et al., 2012;
Losa et al., 2008; Rudnik et al., 2006). In our study, a number of preoperative growth
patterns including suprasellar, posterior, and anterior growth as well as pre- and
postoperative cavernous sinus extension patterns were predictive of the presence of a
residual after surgery. However, neither the recorded growth patterns nor the preoperative
growth rate were predictive of the growth rates of the residual PA, whether it istumor
growth, regression, or stability. We did not find the MIB-1 LI to significantly correlate
with growth characteristics of the residual in our study though MIB-1 LI has previously
been correlated to postoperative residual tumor progression (Widhalm et al., 2009), the
difference in our results and literature reports can be explained by how one defines
growth of residual PA post-operatively, which varies widely between studies.
Due to the heterogeneity of PA, no single predictor of PA growth behaviour can be taken
in isolation as a means to predict its outcome. These predictors must be combined in
order to formulate the most accurate estimation of PA growth which in turn will inform
sound clinical management. A recent study by Trouillas has taken multiple
clinicopathological variables such as tumor proliferation rate, size, and degree invasion
and used this information to predict postoperative outcome (Trouillas et al., 2013). They
found that invasive (into the sphenoid sinus and/or cavernous sinus), and proliferative
tumors (Ki-67 >1%, p53 positivity and/or the presence of mitotic nuclei) had a poorer prognosis, with increased probability of tumor persistence or recurrence compared to non-invasive tumors. Similarly, our article which has examined various clinicopathological factors for PA based on their histology and functional status can also serve as a guide to make a more informed prognosis for PA.

Dysregulated signaling cascades that subserve the clinicopathological observations outlined above have yet to be identified. Consistent molecular aberrations that underlie the formation and progression of sporadic PA are not clearly defined. In order to identify and characterize the biological impact of a proposed pathophysiologic mechanism involved in PA, a robust in vivo model of PA must be established. To date, a number of models of PA have been generated but these have primarily harbored genetic mutations. For example, transgenic mice possessing a dopamine D2 receptor knockout or p27 deletion do develop PA but these are uncharacteristic of the human situation in that, in humans, these genes are not generally altered (Friedman et al., 1994; Ikeda, Yoshimoto, & Shida, 1997). Furthermore, these PA models sometimes progress through a hyperplastic stage before becoming neoplastic (Asa, Kelly, Grandy, & Low, 1999; Friedman et al., 1994) which is not a common phenomenon that is seen clinically. In our study we have reliably generated an intracranial xenograft model of PA which may more closely approximate the sporadic nature of the vast majority of PA. Subcutaneous xenograft models of PA have been established previously but these may not mimic the intracranial microenvironment in which PA resides.

The tumor growth rate, as assessed by TVDT, was examined in our cell lines as described previously. Our preliminary experiments showed that the GH4 cells transfected with empty vector (pc) grew the slowest over time relative to the wild-type FGFR4-G388 or the FGFR4-R388 tumors. Validation of our initial experiments revealed that the pc tumors tended to grow the slowest (longest TVDT) but the growth rates between the untreated tumors of the pc, G388 and R388 tumors were not significantly different from each other. Our in vivo data does not parallel in vitro findings where the R388 cells have been shown to grow significantly faster than the pc and G388 cells (Tateno et al. 2011).
The discrepancy between our in vivo growth observations and in vitro data reported by others may stem from biological factors that are introduced once the cells are injected into the intracranial location. For example, the tumor microenvironment exposes the cells to various growth factors and cytokines that may have changed the morphology and growth dynamics of the cells. Another factor that may have contributed to the discrepant growth rates may have been the presence of tumor hemorrhage. A number of the GH4 tumors had an extensive hemorrhagic component especially in the pc control group and thus tumor growth may have been somewhat overestimated.

In our model, we examined the expression of the mTOR pathway. The mTOR pathway, which is frequently upregulated in various cancers, has been shown to be a viable molecular marker for PA (Musat et al., 2005). Several papers have shown that various upstream and downstream components from mTOR, including mTOR itself, are upregulated in animal and human PA tissue (Kenerson et al., 2005; Lu et al., 2008; Musat et al., 2005). This has indeed been confirmed in our GH4 tumors, particularly with respect to the abundant expression patterns of mTOR’s two target proteins, S6 and 4EBP1, which are the typical readouts for mTOR expression and are both critical for the positive regulation of protein synthesis.

The mTOR inhibiting agents rapamycin and its analogs including RAD001 are potent immunosuppressants which are also used for their antiproliferative properties. Rapamycin/RAD001 have been shown to be effective in various endocrine and non-endocrine cancers and have also demonstrated efficacious preclinical treatment effects in PA. Our data demonstrates mTOR pathway expression in our GH4 xenograft mouse models and these models, which harbour different FGFR4 genotypes, are equally susceptible to mTOR inhibition. There is currently one other study implicating mTOR signaling with respect to the FGFR4-G388R SNP in pancreatic neuroendocrine cells. In their study, it was revealed that the wild-type FGFR4-G388 tumors responded to RAD001 while the tumors harboring the SNP were resistant to mTOR inhibition (Serra et al., 2012). In our model, RAD001 treatment resulted in reduced tumor volume and growth rate (as assessed by a TVDT) but these reductions were of similar magnitude in
all cell groups (~2x reduction in growth for pc, G388, R388 tumors). Reduced growth rate was associated with a downregulation of p-S6 expression. It has been demonstrated previously by other groups that, following mTOR inhibition, rapamycin resistance is promoted by the reduction of S6 leading to an elimination of the aforementioned negative feedback loop to IRS-1 resulting in Akt activation. It has been reported that this phenomenon is not seen in the GH cell lines (Gorshtein et al. 2009). This was also not seen in our GH4 tumors as we initially used octreotide, which abrogates Akt expression, in addition to RAD001 and the combination did not reveal any synergistic antiproliferative effects. Furthermore Akt expression in our tumor model was weak suggesting that mTOR activation may have occurred by other signaling mechanisms.

The parallel downstream effector of mTOR, 4EBP1, was upregulated but the expression pattern was not affected by RAD001 in our animals. There is evidence supporting this phenomenon where long term treatment promotes a rebound effect of p-4EBP1 and reinitiation of protein translation (Choo, et al. 2008). It has been demonstrated that p-4EBP1 may become rapamycin (or rapalog) insensitive over time. The treatment period we implemented lasted for a period of three weeks which may have been long enough to promote rapamycin insensitivity. The rebound effect of p-4EBP1 re-stimulates mRNA translation and may provide a possible explanation for our GH4 tumors exhibiting a slower growth rate but not a complete cessation of growth or regression. Other studies have shown a reduced expression pattern of mTOR pathway following mTOR inhibition but correlating this attenuation with a quantified lower growth rate has not been performed previously.

The cells that we used to generate our model were transfected with FGFR4. Prototypic FGFR4 is a 110 KDa membrane protein consisting of three extracellular Ig like domains, a transmembrane domain, a split tyrosine kinase cytoplasmic domain, and a COOH-terminal tail. It is important to note that the FGFR4 examined in our clinical cohort was not the same as FGFR4 examined in our mouse GH4 tumors. In our clinical study, ptd-FGFR4 was examined which was truncated on the extracellular side and localized to the
cytoplasm. The FGFR4 used in our GH4 models was membrane-bound and harbored a SNP in its transmembrane domain (discussed below). Our GH4 tumors transfected with wild-type FGFR4 were quite aggressive but did respond to mTOR inhibition with reduced mTOR signaling and a lower growth rate. Previous work with human data demonstrates that wild-type FGFR4 was not associated with tumor size in patients harbouring a somatotroph adenoma. However, in ACTH-PA, the FGFR4 allele was associated with small hormonally active ACTH-secreting microadenomas. Another study showed that a correlation exists between homozygous wild-type FGFR4 genotype and postoperative recurrence in patients with Cushing’s disease((Brito, et al. 2010)) Thus, the wild-type FGFR4 may play a more integral role in the pathology of human ACTH tumors relative to GH PA(Nakano-Tateno et al. 2014).

In addition to prototypic FGFR4, we also used an FGFR4 variant that harboured a germline SNP at codon 388 resulting in the presence of a glycine to arginine substitution in the transmembrane domain of the FGFR4 protein. At least one allele for this SNP is present in approximately half of the general population indicating that this is a clinically relevant polymorphism. The literature regarding the role of this FGFR4-G388R SNP in cancer has largely been correlative and suggests that is associated with poorer clinical outcome. Data regarding downstream mechanisms from the FGFR4 SNP remain unclear. There are two studies to date implicating this SNP in ACTH- and GH PA and suggests that src and serine STAT3 phosphorylation is responsible for cell proliferation while tyrosine STAT3 phosphorylation modulates hormone secretion(Nakano-Tateno et al., 2014; Tateno et al., 2011). Clinically, the SNP allele is associated with increased tumor size and GH levels as well as the presence of large hormonally-inactive corticotroph macroadenomas in humans.

In our GH4 tumors, GH expression and IGF-1 secretion was noted but PRL expression and secretion was absent. This is contrary to PRL expression and secretion noted in other mouse models of PA tumors. The reason for this discrepancy may be that in other models transgenic mice were used where pituitary tumors develop in the correct location and can thus utilize the pituitary-hypothalmic portal system to sustain hormone
expression and secretion. Since our PA were intracranial but ectopic, the cells may have lost this capability due to the lack of a portal system. Interestingly, following mTOR inhibition with RAD001, GH expression in GH4 tumors experienced a reduction with a significant attenuation in IGF-1 levels seen only in the R388 mice. It maybe that RAD001 disrupts a connection where GH is unable to stimulate IGF-1 secretion, specifically in tumors with aberrant FGFR4. Recently, it has been demonstrated that tyrosine phosphorylation of STAT3 helps to regulate GH levels (Tateno et al. 2011) and it may be through this pathway that RAD001 exerts it’s hormone attenuating effects. There is one study to date that has reported, at the in vitro level, mTOR inhibition abrogates IGF-1 mediated effects in human PA cells(Zatelli et al. 2010). In sum, our finding is meaningful and warrants further investigation as it has important therapeutic implications in patients with GH tumors who harbour the FGFR4-R388 allele.

5.1 Clinical applications for mTOR inhibitors in PA

Despite a promising role for mTOR inhibitors in preclinical models of PA, data regarding mTOR inhibitors as efficacious therapeutic agents remains limited in clinical management of PA. To date, there is one case report published on the combined use of everolimus and octreotide in a patient with an ACTH pituitary carcinoma resistant to temozolomide treatment(Jouanneau, et al. 2012). Pituitary carcinomas are extremely rare malignant pituitary tumors which are differentiated from PA by their potential for metastatic spread. The combined everolimus/octreotide treatment was unable to control tumor growth in this patient who eventually died shortly after treatment was initiated. It should be noted that the studies attesting to the efficacy of mTOR inhibition in the PA preclinical setting were conducted using human NFPA or GH-secreting PA(Gorshtein et al. 2009; Zatelli et al. 2010). At this stage, though preclinical results are promising, their enthusiasm for their efficacy in clinical practice has to be moderated by the fact that objective clinical data is lacking to confirm the therapeutic value of mTOR inhibitors in PA patients.
5.2 Conclusions

Current understanding of PA pathology is limited. Our data has identified several preclinical and clinical factors that may be involved in PA growth behavior. Clinically, our data suggest that the growth rate of PAs are influenced by various patient and tumor-specific characteristics including the age and sex of the patient, the specific subtype of PA, its hormonal activity, its immunohistochemical profile including the MIB-1 LI status, and its preponderance for different growth directions relative to the pituitary fossa. Furthermore, the pre- and postoperative PA growth rates were correlated suggesting that postoperative PA growth rates can be predicted, in part, by preoperative growth rates thus better informing postoperative outcome.

Our preclinical in vivo model of PA has enabled us to identify the mTOR pathway as a possible treatment target, specifically in GH4 tumors with altered FGFR4 genotype. We have shown that the mTOR pathway is differentially expressed in the R388 tumors compared to the G388 tumors. Both G388 and R388 tumors display a reduction in tumor volume, growth rate, and mTOR expression following treatment with RAD001 with a similar reduction in all groups. The R388 group had the highest levels of GH and serum IGF-1 levels and is the only group that experienced a significant reduction in these levels following RAD001 treatment. These data suggest that PA, specifically GH PA can benefit from mTOR inhibition therapy with a overall reduction in growth rate and a reduction in hormone levels in those GH PA harboring an FGFR4 polymorphism.

5.3 Limitations

In our first aim, we conducted a retrospective review. There are a number of limitations inherent in such a study design. For instance, when retrieving archival data, missing values or data that is not reported is an issue. It would have been ideal to correlate our radiological finding of PA growth to surgical outcome, as this would have provided definitive evidence for a particular PA growth pattern. However, often times intraoperative findings of PA growth were not reported and thus our study, based solely
on radiological data, may have been an over- or underestimation of the true incidence of PA growth. Our inclusion of pathological markers MIB-1, p27, and FGFR4 were obtained from historical pathology reports and these markers were not always reported for each case. Furthermore, the p27 and the FGFR4 status were only examined qualitatively. Ideally, the tissue should have been collected for each case and reassessed for the purposes of this study. However, for the majority of tissue samples there was a limited amount of archival material which precluded reinvestigation of samples. Another issue may have been the introduction of selection bias into the study. Patients were excluded if they did not have adequate preoperative and postoperative radiological data for comparison, even if their tumors were large. Our definition of tumor recurrence was based on radiological outcome and we did not consider biochemical recurrence in our definition, which also excluded a few patients.

In our in vivo study, the main question remains, as with all other preclinical models of brain tumor the validity and relevance of this model to human disease. For example is injection of frontal intraperhancyma an accurate model for PA, and most likely injectionof the pit fossa is a more reliable model. However given technical limitations we chose to proceed the initial step of this project with frontal lobe injection. This raises the issue of how well our model recapitulates the PA microenvironment. We believe that our ectopic intracranial model of PA does mimic the microenvironment as the brain consists of necessary cytokines and growth factors required to facilitate PA development. There was also an issue concerning the heterogeneity of tumor growth rates. Although cells were injected in equal numbers, tumors of the same cell type grew at different rates thus complicating treatment initiation and endpoints. The variable tumor growth rates had to do with the exponential growth patterns of these tumor models, which consisted of a growth latency period shortly after intracranial injection followed by rapid tumor expansion. In some mice, the tumor was much more aggressive than in other mice and this lack of uniformity sometimes hampered our ability to extract meaningful treatment data.
5.4 Future Directions

With respect to our retrospective review of PA patients, increasing the sample size by including additional patients treated after 2011 may be an option to expand the project. Also, including a multi-centric or even nationwide database of PA parameters may increase the validity of the study findings. Comparing biochemical relapse to our finding of radiological tumor recurrence within the functioning PAs may be of clinical value and should also be considered.

The xenograft mouse project would benefit from the incorporation of human in vitro data to complement the in vivo findings. Human PA surgical samples could be dispersed in primary culture and mTOR expression could be examined following mTOR inhibition therapy. There are currently no human PA cell lines in which to study RAD001 effects on human PA cells so developing such a model would be a task in itself. In fact, the original aim of this study was to generate a stable human cell line. After several attempts this proved to be very difficult and we were ultimately unsuccessful (See Appendix 1 for protocol). The cells would remain viable for one passage and die shortly thereafter. Perhaps to mimic the necessary microenvironment in which PA grow, several growth factors, hormones, and cytokines should have been added to the culture media. Nevertheless, if human PA cell lines are established, exposure to RAD001 and subsequent mTOR expression could be examined. Colony formation assays could be performed to assess cell proliferation and viability following drug treatment. RAD001 is a known cytostatic agent so FAC sorting should be performed for cell cycle analysis using either human PA cells in primary culture or PA cell lines. All of these experiments should be performed in the context of the FGFR4 polymorphism.
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Appendix 1: Human Primary Culture Protocol

Human Pituitary Adenoma Primary Culture Protocol

Day 1
1. Obtain autoclaved scissors and forceps; one 60 mm plate; one 30 mm plate; syringe
2. Take tumor falcon tube (FT) and record information (tissue bank # and MRN)
3. Aspirate most of the old media from the tumor FT and pour onto 60 mm plate
4. Cut tumor with scissors holding with forceps
5. Obtain 10 mL FT and transfer minced tissue from plate to FT using a pipette
6. Centrifuge FT for 5 min at 1000rpm
7. While centrifuging, obtain collagenase type v and poly-L-lysine (sigma) as well as 3 chamber slides
8. After centrifugation, aspirate old media; flick tube to break pellet
9. Put 1 mL collagenase into FT and use pipette to transfer onto 30 mm plate
10. Incubate for 30 min at 37°C
11. While incubating coat each well of the chamber slides with lysine (keep for at least 10 min and then remove)
12. Get out of incubator and put a little more of media onto plate (refer to Lei’s protocol for making media)
13. Suck up all media and tissue and put into cell strainer with a 30 mm plate underneath and mash the tissue
14. Transfer the strained liquid into a FT and centrifuge the tube for 5 min
15. Aspirate and then add 1.4 mL of media
16. Add 200 microL of media to each well and 50 microL of cells to each well
17. Incubate ON

Day 2 – Drug Treatment
RAD001 cat # E4040 LOT BDE103 (LC Laboratories)
TMZ – (Sigma Aldrich)
1. Obtain 8 falcon tubes 1(0), 2(1), 3(10), 4(100) – TMZ; 1(0), 2(1), 3(10), 4(100) – RAD001
2. For TMZ- Add 1.5 mL of DMEM to each tube and 1.5 microL of drug to each tube
3. For RAD001 add 0.9 mL media: 0.9 microL drug to each tube for 1, 10 microM; for 100 microM add 2.1 mL media: 2.1 microL drug
4. Aspirate old media from chamber; add pbs; aspirate PBS
5. Add 300 microL of respective drug to each chamber; just add straight media to insulin and IGF1 chambers
6. Incubate at 37C ON
7. Top vs side: TMZ vs P73; 1/50; 1/200 vs pmTOR; TMZ and RAD001 vs MIB1

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Day 3- Fixing the cells
1. Make 1% formalin (in PBS)
2. Obtain 2 FT (one for Ins another for IGF1)
3. Obtain IGF1 and Ins from 4C and spin them down before use
4. Put 10 mL of DMEM into each tube
5. Add 4 microL IGF1; 1 microL of Ins
6. Take out chamber slide from incubator
7. Aspirate old media with drug in it
8. Add 300 microL C; IGF1; Ins to respective well
9. Incubate for 10 min at 37C
10. Aspirate after incubation
11. Add cold PBS to each well; aspirate; add formalin to each well
12. Put in 4C for 3 hr
13. Aspirate then add PBS; put in 4C ON