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Molecular and Clinical Characteristics of Pituitary Adenoma Predisposition (PAP)

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Academic dissertation

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II **Heliövaara E***, Raitila A*, Launonen V, Paetau A, Arola J, Lehtonen H, Sane T, Weil R, Vierimaa O, Salmela P, Tuppurainen K, Mäkinen M, Aaltonen LA, Karhu A. The expression of *AIP*-related molecules in elucidation of cellular pathways in pituitary adenomas. Am J Pathol 2009, 175(6):2501-2507

III Raitila A, Lehtonen HJ, Arola J, **Heliövaara E**, Ahlsten M, Georgitsi M, Jalanko A, Paetau A, Aaltonen LA, Karhu A. Mice with Inactivation of *Aryl Hydrocarbon Receptor Interacting Protein (Aip)* Display Complete Penetrance of Pituitary Adenomas with aberrant ARNT Expression. Am J Pathol 2010, 177(4):1969-1976

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V **Heliövaara E**, Tuupanen S, Ahlsten M, Hodgson S, de Menis E, Kuismin O, Izatt L, McKinlay Gardner RJ, Gündogdu S, Lucassen A, Arola J, Tuomisto A, Mäkinen M, Karhu A, Aaltonen LA. No evidence of *RET* germline mutations in familial pituitary adenoma. J Mol Endocrinol 2011, 46(1):1-8

*Equal contribution

Abbreviations

aa	amino acid
ACTH	adrenocorticotropin
AIP	aryl hydrocarbon receptor interacting protein (or ARA9, XAP2)
AHR	aryl hydrocarbon receptor
ARA9	aryl hydrocarbon receptor-associated protein-9 (or AIP, XAP2)
ARNT	aryl hydrocarbon receptor nuclear translocator (or HIF1-β)
ARNT2	aryl hydrocarbon receptor nuclear translocator 2
aSU	alpha subunit
bp	base pair
BRCA1	breast and ovarian cancer 1
BRCA2	breast and ovarian cancer 2
bSU	beta subunit
cAMP	cyclic adenosine monophosphate
CD34	cluster of differentiation 34
CDKN1B	cycline-dependent kinase inhibitor 1B
cDNA	complementary deoxyribonucleic acid
CEPH	Centre d'Étude du Polymorphisme Humain
CNC	Carney complex
CYP	cytochrome P450
DAB	3,3'-diaminodenzidine
DNA	deoxyribonucleic acid
DRE	dioxin response element
E	(mouse) embryonic day
EBNA-3	Epstein-Barr virus encoded nuclear antigen-3
EEL	enhancer element locator
ERα	estrogen receptor α
ERβ	estrogen receptor β
ES cells	embryonic stem cells
ETV	ETS variant gene 6
FGFR	fibroblast growth factor receptor
FIPA	familial isolated pituitary adenoma
FKBP	FK605 binding protein
FSH	follicle-stimulating hormone
G_{13}	G protein subtype 13
GDNF	glial cell line-derived neurotrophic factor
GH	growth hormone
GHRH	growth hormone-releasing hormone
GLUT1	glucose transporter 1
GNRH	gonadotropin-releasing hormone
GNAS	guanine nucleotide-binding protein alpha stimulating activity polypeptide
GR	glucocorticoid receptor
HBV	hepatitis B virus
HE	haematoxylin-eosin
HEK293	human embryonic kidney 293 cells
HET	heterozygote
HIF	hypoxia inducible factor
HIF1-α	hypoxia inducible factor 1, alpha subunit
HIF1-β	hypoxia inducible factor 1, beta subunit (or ARNT)
HLRCC	hereditary leiomyomatosis and renal cell cancer
HRE	hypoxia response element
Hsc70	heat-shock cognate protein 70
HSP90	heat-shock protein 90
iASPP	inhibitor of apoptosis stimulating protein of p53
IFS	isolated familial somatotropinoma
IGF-I	insulin-like growth factor 1
IHC	immunohistochemistry
IVS	intronic variable sequence
iXRE	inhibitory xenobiotic response element
kb	kilobase
kDa	kilodalton
LH	luteinizing hormone
LOH	loss of heterozygosity
MAS	McCune-Albright syndrome
MCS	multi-species conserved sequence
MEF	mouse embryonic fibroblast
MEN1	multiple endocrine neoplasia type 1
MEN2A	multiple endocrine neoplasia type 2A
MEN2B	multiple endocrine neoplasia type 2B
MEN4	multiple endocrine neoplasia type 4 (or MEN1-like syndrome)
MENX	MEN-like syndrome in the rat
MLPA	multiplex ligation-dependent probe amplification
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NFPA	non-functioning pituitary adenoma
OGTT	oral glucose tolerance test
PAP	pituitary adenoma predisposition
PCR	polymerase chain reaction
PDE2A	phosphodiesterase 2A
PDE4A5	phosphodiesterase 4A5
PI	proliferation index
Pit-1	pituitary transcription factor-1
PKA	protein kinase A
PPAR- α	peroxisome proliferation-activated receptor alpha
PRKAR1A	protein kinase A regulatory subunit 1 alpha
PRL	prolactin
PTTG	pituitary tumor transforming gene
qPCR	quantitative polymerase chain reaction
RET	rearranged during transfection
RSUME	RWD-containing sumoylation enhancer
S	serum
siRNA	small interfering ribonucleic acid
SNP	single nucleotide polymorphism
SRF	serum response factor
SSA	somatostatin analog
T_3	triiodothyronine
T_4	thyroxine
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TOMM20	translocase of the outer membrane of mitochondria 20
TPR	tetratricopeptide repeat
TRβ1	thyroid hormone receptor beta 1
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
UFC	urinary free cortisol
UTR	untranslated region
VEGF	vascular endothelial growth factor
WT	wildtype
XAP2	hepatitis B virus x-associated protein 2 (or AIP, ARA9)

In addition, standard one-letter codes are used to denote amino acids and bases.

Abstract

Pituitary adenomas are common intracranial neoplasms that generally arise sporadically. However, a small minority occurs in a familial setting, often as aggressive and difficult-to-treat adenomas in patients who are relatively young. Familial syndromes with phenotypes including pituitary adenomas include multiple endocrine neoplasia type 1 (MEN1), Carney complex and MEN4. Recently, a fourth gene underlying pituitary adenomas was discovered in Northern Finland in a cluster of familial acromegaly. Heterozygous mutations in the *aryl hydrocarbon receptor interacting protein (AIP)* gene caused this condition, designated as pituitary adenoma predisposition (PAP). PAP confers incomplete penetrance of pituitary adenomas, and patients often lack a strong familial background of adenomas. *AIP* mutation positive (*AIP*mut+) patients are often young at disease onset and have mostly growth hormone (GH) secreting adenomas. Loss of heterozygosity of *AIP* in tumors and functional evidence suggest that *AIP* is a tumor suppressor gene.

Elucidation of the molecular mechanisms of PAP is a requirement for better understanding of the detailed genesis of these pituitary adenomas. Moreover, clarification of the clinical characteristics of PAP may be beneficial in establishing genetic testing protocols to recognize individuals at risk for developing tumors and to improve patients' clinical outcome. Development of novel treatment regimes relies on detailed knowledge of tumorigenesis. This thesis work aims to clarify the molecular and clinical characteristics of PAP.

Applying the multiplex ligation-dependent probe amplification (MLPA) assay, we searched for large genomic *AIP* deletions in apparently *AIP* mutation negative (*AIP*mut-) familial pituitary adenoma patients. For the first time, genomic *AIP* deletions were found in two families, suggesting that this mutation type accounts for a subset of PAP. Therefore, MLPA could be considered in cases with a phenotype indicative of PAP but when no *AIP* mutations are found with conventional sequencing.

To clarify molecular mechanisms of AIP-mediated tumorigenesis, we elucidated the expression of AIP-related molecules in *AIP*mut+ and *AIP*mut- pituitary tumors. The expression of aryl hydrocarbon receptor nuclear translocator (ARNT) protein was reduced in *AIP*mut+ pituitary adenomas, whereas the nuclear expression of aryl hydrocarbon receptor (AHR) was somewhat increased. This result was endorsed by underexpression of ARNT in an *Aip* knockdown rat mammosomatotroph cell line. These results suggest that ARNT, AHR or both may play a role in AIP-related tumorigenesis, possibly via pathways involving phosphodiesterases and cyclic adenosine monophosphate.

We generated an *Aip* mouse model to examine pituitary tumorigenesis *in vivo*. Heterozygous *Aip* mutations conferred complete penetrance of pituitary adenomas in these mice, and the vast majority of adenomas were GH-secreting. Thus, the tumor phenotype of the *Aip* mouse is similar to that in human PAP patients. As in the study on human tumors, aberrant ARNT, but also ARNT2 expression, was evident in mouse pituitary adenomas that were *Aip*-deficient. Our results suggest that *AIP* may function as a candidate gatekeeper gene in somatotrophs. Furthermore, this disease model is an

excellent tool in further elucidation of the molecular mechanisms of pituitary tumorigenesis, and it also has potential in developing therapeutic approaches.

We studied the clinical characteristics and the response to therapy of *AIP*mut+ pituitary adenoma patients, with sporadic acromegaly patients as a control population. *AIP*mut+ adenomas conferred an aggressive disease phenotype with young age at disease onset. *AIP*mut+ adenomas were most often large, expansive and invasive at diagnosis. Patients were predominantly male, and GH-secreting adenomas appeared in nearly 80%. *AIP*mut+ adenomas also seemed to have many difficult-to-treat clinical characteristics. The aggressive nature of *AIP*mut+ adenomas is further supported by increased expression of the Ki-67 proliferation marker in mouse pituitary adenomas that are *Aip*-deficient. We conclude that the improvement in treatment outcomes for PAP patients would require efficient identification of *AIP*mut+ patients, as well as earlier diagnosis of the pituitary adenomas.

The possible role of the *rearranged during transfection* (*RET)* proto-oncogene in tumorigenesis of familial *AIP*mut- pituitary adenomas was evaluated. Five novel germline heterozygous *RET* variants were found in the patients; however, none of these could be considered causative of pituitary tumorigenesis. Surprisingly, RET immunohistochemistry suggested possible underexpression of RET in *AIP*mut+ pituitary adenomas – an observation that merits further investigation.

Review of the Literature

1. The genome and tumorigenesis

The human body is composed of approximately 3 x 10^{13} cells, nearly all of which contain the same genetic material. This material resides in the nucleus as chromosomes that contain the deoxyribonucleic acid (DNA) sequence, which encodes the ~22 000 genes of the genome. In addition, a small fraction of DNA is contained within the mitochondria. Genes encoded by the DNA sequence constitute the templates for amino acid (aa) sequences of proteins that carry out the genes' purposes in the cells; the genotype creates the phenotype of an individual through proteins. Gene expression is stringently regulated in time and space. An important example of this is the regulation of the expression of genes involved in cell division of somatic cells. Proper tissue architecture is maintained by appropriate proportions of constituent cell types, replacement of missing cells and discarding of unneeded cells. This process of normal growth involves a delicate balance between growth factors and growth-inhibitory factors in the surroundings of cells (Weinberg 2007 p.9, 19, 43, 121).

Deviations from normal growth can involve hyperplasia, designating an excessive numbers of cells; metaplasia, when certain cells are displaced by cells of another type that are normally not encountered in that site; dysplasia, when cells have reached a cytologically abnormal stage; and ultimately neoplastic and metastatic lesions. The genesis of tumors results from the abnormal proliferation of normal cells, which is accompanied by the accumulation of genetic defects in these cells (Weinberg 2007 p.36-39, 43). A succession of genetic changes confers a growth advantage, leading to the progressive conversion of normal cells into cancer cells (Hanahan & Weinberg 2000). Since the karyotypes of cancer cells are usually abnormal, cancer can be seen as a genetic disease of somatic cells (Knudson 2002). Recent evidence suggests that cancers of distinct subtypes within an organ may be derived from different 'cells of origin', and that these are the cells that acquire the genetic changes that culminate in the initiation of cancer (Levy 2008, Vankelecom 2011, Visvader 2011).

Tumors are initiated by the first genetic alteration that renders a fitness advantage to a cell, and tumor progression is the multi-step evolution of a normal cell into a tumor cell (Weinberg 2007 p.G20, Bozic *et al.* 2010). The mutations that are essential in the initiation of tumorigenesis are known as "drivers" and they confer a growth advantage, causing the positive selection of the cell in which they occur. However, the majority of somatic mutations are "passengers", which are expected to be biologically neutral since they do not confer a growth advantage to the cell in which they occur and they are not causative of oncogenesis (Greenman *et al.* 2007). It has been reported that, for example, in typical breast and colorectal cancers there are ~80 aa-altering mutations in the tumor DNA and that ~15 of these mutations are likely to be responsible for driving initiation, progression or maintenance of the tumor (Wood *et al.* 2007).

It has been postulated that cancer cells comprise six essential alterations in cell physiology that define their malignant growth. These are self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), a limitless replicative potential of cells, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg 2000). Also genomic instability, for example resulting from mutations in DNA repair genes, has recently been

suggested to be a hallmark of cancer (Negrini *et al.* 2010, Hanahan & Weinberg 2011). Emerging views also suggest that the genetic changes in cancer cells are not sufficient and that tumor progression is dependent on ancillary processes provided by the tumor environment but not necessarily cancerous themselves, such as inflammation and a shift in cellular metabolism (Rakoff-Nahoum 2006, Tennant *et al.* 2009, Hanahan & Weinberg 2011).

In contrast to malignant cancer, benign tumors are by definition confined to a specific site of a tissue and give no evidence of invading adjacent tissues (Weinberg 2007 p.G2). They are composed of well-differentiated cells that closely resemble their normal counterparts, and their rate of growth is usually slow (Kumar *et al.* 2003 p.168-173). However, benign tumors can cause significant morbidity and even mortality, for example pituitary adenomas can do so by compressing critical brain structures (Elston *et al.* 2009).

1.1 Oncogenes

Tumor cells can exhibit abnormal activation of certain normal genes to promote tumorigenesis. These genes are called oncogenes and one mutated allele of such a gene is sufficient to confer a selective growth advantage to the cell. Oncogene activation can be caused by chromosomal translocations, gene amplifications or intragenic mutations affecting crucial residues that regulate the activity of the gene product (Vogelstein & Kinzler 2004). Translocations and intragenic mutations can occur either as initiating effects of tumorigenesis or during tumor progression, whereas gene amplification usually occurs during tumor progression (Croce 2008). Oncogenes encode proteins that can control cell proliferation, apoptosis or both. The products of these oncogenes can be classified into six groups of molecules: transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transducers and apoptosis regulators. Known oncogenes of the six classes include *v-myc* (transcription factor), *ALL1* (chromatin remodeler), *KS3* (growth factor), *rearranged during transfection* (*RET*) proto-oncogene (growth factor receptor), *K-RAS* (signal transducer) and *BCL2* (apoptosis regulator) (Croce 2008, Table 1).

Table 1. Oncogenes and tumor suppressor genes, data from Bronner *et al.* 1994, Kinzler & Vogelstein 1998, Soussi 2000, Weber *et al.* 2006 and Croce 2008.

Gene	Mechanism in tumorigenesis
Oncogene	Activating mechanism
v-myc	deregulated activity of transcription factor
ALL1	chromatin remodelling
KS3	constitutive production of growth factor
RET	constitutive action of growth factor receptor
K-RAS	signal transduction
BCL2	inhibition of apoptosis
Tumor suppressor gene	Inactivating mechanism
p53	evading apoptosis (gatekeeper)
RB1	uncontrolled proliferation (gatekeeper)
MLH1	genome instability (caretaker)
BRCA1	genome instability (caretaker)
POLD1	generating unstable stroma (landscaper)

1.2 Tumor suppressor genes

Tumor suppressor genes are antigrowth genes whose involvement in tumor formation occurs when these genes are inactivated or lost (Weinberg 2007 p.209-210). A model for tumor suppressor gene associated cancer development was proposed in 1971, when Alfred Knudson published his "two-hit" hypothesis based on epidemiological studies on retinoblastoma patients. He suggested that a mutational event must occur in both alleles of a tumor suppressor gene for the affected cell to acquire a growth advantage (Knudson 1971).

Mutations in tumor suppressor genes have the opposite effect to oncogene mutations, since they reduce the activity of the gene product. Such inactivation can arise from amino acid changes at residues that are essential for the activity of the gene product, from mutations that result in a truncated protein, from gene deletions or insertions, or from epigenetic silencing (Vogelstein & Kinzler 2004). Interestingly, some tumor suppressor genes exert a selective advantage on the cell even when only one allele is inactivated and the other remains functional, a situation that is called haploinsufficiency (Santarosa & Ashworth 2004). However, inactivation of both alleles of a tumor suppressor gene is generally required to confer a selective advantage to the cell. This situation often arises through an intragenic mutation in one allele, coupled with a deletion of the other allele via a gross chromosomal event (Knudson 2002). Generally, this phenomenon where the second allele is lost is called loss of heterozygosity (LOH) (Weinberg 2007 p.219-224).

Tumor suppressor genes can have functions over the control of cellular proliferation directly acting as "gatekeepers", or they can function in maintaining the integrity of the genome as "caretakers". Inactivation of a caretaker gene does not promote tumor initiation directly, but does so indirectly by leading to genetic instability which results in increased mutation rates of all genes including gatekeepers (Kinzler & Vogelstein 1997). Classical tumor suppressor genes from these groups include *p53* (gatekeeper), *MLH1* (caretaker) and *breast and ovarian cancer 1* and *2* (*BRCA1* and *BRCA2*) (caretakers) (Bronner *et al.* 1994, Kinzler & Vogelstein 1998, Soussi 2000, Table 1). Interestingly, some mutant forms of *p53* can also act as oncogenes (Harris & Hollstein 1993). A third group of tumor suppressor genes are the "landscapers". It is postulated that alterations in landscaper genes can cause proliferation of stromal cells. This genetically unstable stroma results in an abnormal microenvironment that may promote neoplastic transformation of associated epithelial cells (Kinzler & Vogelstein 1997, Kinzler & Vogelstein 1998, Weber *et al.* 2006, Table 1).

1.3 Genetic predisposition to tumorigenesis

Inherited predisposition is known for virtually every type of human cancer (Knudson 2002). The predisposed individual carries a defect in the germline, such as a mutated allele of a gene, which predisposes him or her to the formation of tumors. This predisposition can also be transferred to the offspring of the individual. Usually a certain amount of loss of germline mutations occurs in every generation, for example if the condition produces mortality before the end of the age of reproduction. However, mutational equilibrium can be attained by a low rate of occurrence of new germline mutations in the population (Knudson 2002, Weinberg 2007 p.43, 224-226).

There are currently about 100 genes known to cause Mendelian-inherited cancer syndromes (Cazier & Tomlinson 2010). Clinical characteristics of inherited cancer syndromes include, among others, a positive family history of cancer, a typical inheritance pattern of tumors, multiple primary tumors and early age of onset (D'Orazio 2010). It has been argued that these syndromes affect about 1 % of cancer patients, and in children it is estimated that 5 to 10% of cancer can be explained by a certain genetic mutation (Fearon 1997, D'Orazio 2010). Inherited predisposing mutations in oncogenes have been identified in several well-established syndromes that cause dominant heredity of cancer, such as *RET* mutations causing thyroid, parathyroid and adrenal tumors in multiple endocrine neoplasia type 2A (MEN2A) (Mulligan *et al.* 1993, Salmela and Ebeling in Välimäki *et al.* 2009 p.474-480, Table 1). Inherited mutations in tumor suppressor genes can also confer a dominant pattern of heredity. These include for example *RB1* mutations causing retinoblastomas and *FH* mutations causing hereditary leiomyomatosis and renal cell cancer (HLRCC) (Knudson 1971, Tomlinson *et al.* 2002, Table 1). Hereditary cancer predisposition can also be caused by mutations in stability genes (also called caretakers), such as *BRCA1* and *BRCA2*, resulting in dominant inheritance of breast and ovarian cancer, and *FANCA* mutations, causing recessive inheritance of leukemia (Butturini *et al.* 1994, Futreal *et al.* 1994, Miki *et al.* 1994, Wooster *et al.* 1995, Vogelstein & Kinzler 2004). The known Mendelian-inherited cancer syndromes explain only a minor part of the familial clustering of cancers. Thus, in most cases, the increased familial relative risk of cancer must involve several risk alleles with low or moderate penetrance (Cazier & Tomlinson 2010). It has indeed been estimated in twin studies that hereditary factors could significantly contribute to prostate cancer (42% of risk may be explained by heritable factors), colorectal cancer (35%) and breast cancer (27%) (Lichtenstein *et al.* 2000).

It is known that a certain germline mutation does not suffice for carcinogenesis, but subsequent somatic mutations are required. These can be caused by various environmental factors, such as ionizing radiation, dietary factors and consumption of tobacco. These factors can also affect the penetrance of cancer, i.e. the proportion of genetically predisposed individuals that ultimately develop tumors (Knudson 2002, Weinberg 2007 p.47, Cazier & Tomlinson 2010).

2. The pituitary gland

The pituitary gland is a crucial part of the endocrine system. It co-ordinates the body's internal physiology, regulates its development throughout life and helps it to adapt to change in the external environmental by secreting hormones that act on their target tissues (Brook & Marshall 2001 p.34). The pituitary is composed of three lobes: the anterior lobe (adenohypophysis; contains the pars distalis and pars tuberalis) is mainly glandular tissue; the posterior lobe (neurohypophysis; contains the pars nervosa and infundibulum) is neural tissue that stores oxytocin and antidiuretic hormone; and between them the intermediate lobe (pars intermedia), which is atrophic in humans (Sane in Välimäki *et al.* 2009 p.76-77). The pituitary lies in a bony cavity, the sella turcica of the sphenoid bone. It is connected to the overlying hypothalamus by a stalk that carries a system of portal veins from the hypothalamus to the pituitary, as well as axons to the neurohypophysis (Brook & Marshall 2001 p.35-37).

During embryogenesis, the pituitary emerges from two distinct ectodermal components. One of these is the Ratkhe's pouch, a dorsal outgrowth of the buccal cavity, which forms the anterior pituitary. The second is a downgrowth of neuroectoderm from the floor of the third ventricle, which develops into the pituitary stalk and the posterior pituitary (Brook & Marshall 2001 p.35). The distinct cell types of the anterior pituitary arise from a pool of self-renewing and proliferating progenitor cells present in the epithelium of Ratkhe's pouch (Vankelecom 2010). Mitotic activity of the adult pituitary is seen in 1-2% of cells that are active oligopotent stem cells. They undergo mitoses at a steady rate that gradually decreases with age (Levy 2008). Interestingly, the adult pituitary seems to retain plasticity and is able to flexibly remodel its hormone-producing cell compartment in response to changing endocrine demands, for example during pregnancy and puberty. Recently, plausible candidates for stem cells of the adult pituitary have been proposed, that express stem cell-associated markers and signaling factors, and display multipotency and a niche-like organization (Vankelecom 2010).

The anterior pituitary contains five populations of secretory cells that secrete by exocytosis the six pituitary hormones into the bloodstream. Growth hormone (GH) is secreted by somatotrophs, prolactin (PRL) by lactotrophs, thyroid-stimulating hormone (TSH) by thyrotrophs, adrenocorticotropic hormone (ACTH) by corticotrophs, and both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are secreted by gonadotrophs. FSH and LH are dimeric proteins that share a common alpha subunit (aSU), but their beta subunits (bSU) are unique and derived from different genes encoding distinct proteins (Brook & Marshall 2001 p.38, Ooi *et al.* 2004, Bernard *et al.* 2010, Figure 1).

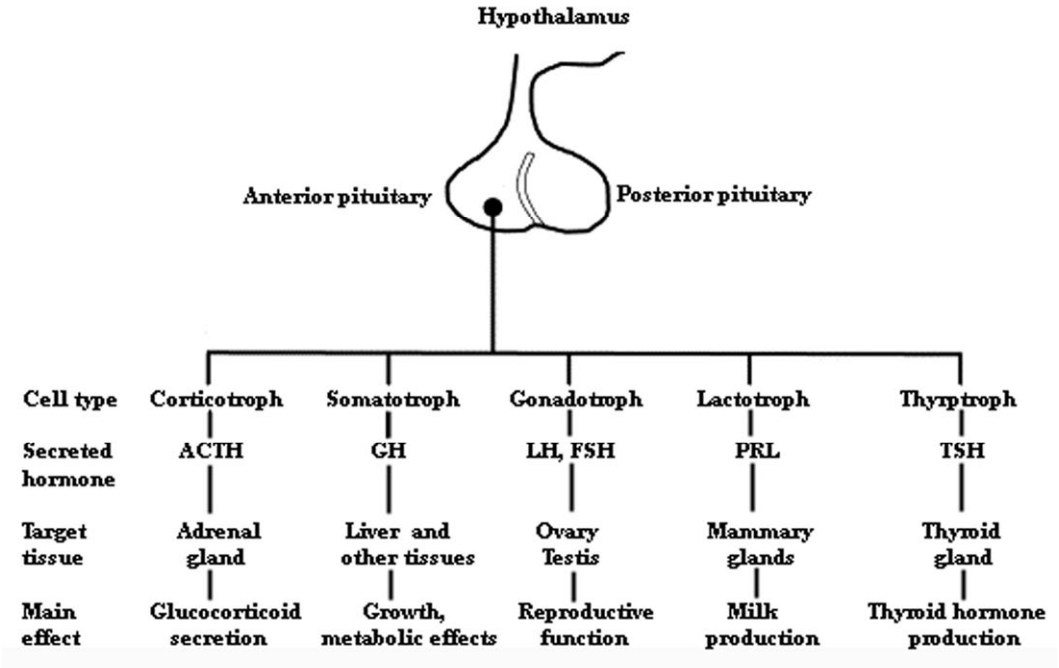

Figure 1. Pituitary gland. A schematic presentation of the cells in the anterior pituitary, the hormones they secrete, and the target tissues and main effects of these hormones. Modified from Ooi *et al.* 2004 with the permission from Elsevier.

The anterior pituitary lies under the stringent control of the hypothalamus, which controls the release of pituitary hormones by releasing hypothalamic hormones such as growth hormone-releasing hormone (GHRH) and gonadotropin-releasing hormone (GNRH) to the hypophysial portal vasculature. In addition, some neuromessengers can directly control the release of pituitary hormones; for example dopamine is the main inhibitor of PRL secretion. Also feedback effects of systemic circulating hormones and cytokines acting in a paracrine or autocrine fashion regulate the secretion of pituitary hormones. (Crowley 1999, Brook & Marshall 2001 p. 38-40, Haedo *et al.* 2009, Sane in Välimäki *et al.* 2009 p.69, 75-76).

3. Pituitary adenomas

3.1 Benign adenomas of the anterior lobe

Pituitary adenomas account for about 15% of intracranial neoplasms. They can arise from any cell type(s) of the anterior pituitary and accordingly present a variety of clinical manifestations based on their size, location and function. Frequently encountered clinical manifestations relate to excessive hormone secretion of the tumor, hormone deficits of the pituitary hormones (i.e. hypopituitarism) and expansion of the tumor mass. However, pituitary adenomas can also be asymptomatic. Although some adenomas are invasive, the vast majority of them are considered as histologically benign lesions, and they metastasize exceedingly rarely. Recent advances in molecular biology, immunocytochemistry and imaging, and the introduction of new treatment options have improved the knowledge of these adenomas and their management (Arafah & Nasrallah 2001, Melmed 2003, Karhu & Aaltonen 2007, Sane in Välimäki *et al.* 2009 p.98-126).

3.2 Incidence and prevalence

According to data obtained from autopsy and radiological imaging series, pituitary adenomas occur very commonly in the general population. Most of these tumors are found incidentally and present no obvious clinical impact (Daly *et al.* 2009). In an early study on pituitary adenoma prevalence, adenomas were found in nearly one in every four autopsy cases (Costello 1936). In a recent systematic review, the overall prevalence of pituitary adenomas was found to be 16.7% (14.4% based on autopsy studies and 22.5% based on radiological studies) (Ezzat *et al.* 2004). Incidence rates of pituitary adenomas generally increase with age and are higher in women in early life and higher in men in later life. The difference in incidence rates between sexes could be due to their different symptomatology, such as earlier and more noticeable symptoms of hyperprolactinemia in women (e.g. amenorrea and galactorrhea). Men are on average diagnosed with larger tumors than women since their diagnosis may be delayed, giving the tumor a chance to grow larger before clinical detection (Ciccarelli *et al.* 2005, McDowell *et al.* 2010). Also race may affect the incidence of pituitary adenomas, for example incidence rates for women of African descent are about three times as high as for Caucasian women (Heshmat *et al.* 1976). The overall incidence rate of pituitary adenomas in the United States has been noted to be 2.7 cases per 100 000 patient years in 2004-2007 (McDowell *et al.* 2010). In contrast, incidences noted in England have been lower, only 0.75 cases per 100 000 patient years in 1999-2003. In England, there has been a pattern of initial increase (0.91 per 100 000 in 1989-1993) followed by stabilization, and this change in incidence has mainly been seen in the elderly age group (Arora *et al.* 2010). In Northern Finland, overall incidence of pituitary adenomas in 1992-2007

was 4.0 cases per 100 000 patient years, with a gender-specific incidence of 2.2 per 100 000 in males and 5.9 per 100 000 in females (Raappana *et al.* 2010).

3.3 Tumor classification

Pituitary adenomas are traditionally classified as microadenomas if they are <10mm in diameter and located totally within the sella turcica, in contrast to macroadenomas that are >10mm in diameter and can be totally intrasellar, but are often associated with extrasellar extension. In addition, giant adenomas have been defined as extending >40mm from the midpoint of the jugum sphenoidale or extension to within 6mm of the foramen of Monro (Majós *et al.* 1998, Arafah & Nasrallah 2001). However, the general classification of pituitary adenomas is based on characteristics of hormone staining, electron microscopic changes, clinical signs and symptoms. Hereby, adenomas are classified as prolactinomas, somatotropinomas, adrenocorticotropinomas, gonadotropinomas, thyrotropinomas, null-cell adenomas and oncocytomas (Arafah & Nasrallah 2001, Table 2). The World Health Organization's complete clinicopathological five-tier scheme for pituitary adenoma classification includes assessment of endocrine activity, imaging, operative findings, histology, immunocytochemistry and ultrastructure of the pituitary adenoma (Kovacs *et al.* 1996).

Table 2. Classification and characteristics of pituitary adenoma types. Modified from Arafah & Nasrallah with the permission from the Society for Endocrinology, data also from Sane in Välimäki *et al.* 2009 p.100 and Melmed 2003.

Tumor type	Secretion	Prevalence	Symptoms of hormone secretion	Laboratory diagnosis
Prolactinoma	PRL	27-45%	hypogonadism, galactorrhea	S-PRL↑
Somatotropinoma	GH	15-20%	acromegaly, gigantism	OGTT, S-IGF-I↑, S-GH↑
Adrenocorticotropinoma	ACTH	9-12%	Cushing's disease	Dexamethasone test, 24hUFC↑
Gonadotropinoma	LH, FSH, a/bSU	9-15%	none, hypergonadism or hypogonadism	S-FSH↑, S-LH↑, S-a/bSU↑
Thyrotropinoma	TSH	1-2%	hyperthyroidism	TRH test, T_3↑, T_4↑, TSH↑
NFPA[1]	none	5-25%	none	none

PRL, prolactin; GH, growth hormone; ACTH, adrenocorticotropic hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; a/bSU, alpha/beta subunit; TSH, thyroid-stimulating hormone; NFPA, non-functioning pituitary adenoma; OGTT, oral glucose tolerance test; S-IGF-I, serum insulin-like growth factor 1; TRH, thyrotropin-releasing hormone; T_3, triiodothyronine T_4, thyroxine; UFC, urinary free cortisol
[1] Including null-cell adenomas and oncocytomas

3.4 Clinical features and diagnosis

The majority of pituitary adenomas are asymptomatic and have no clinical impact (Daly *et al.* 2009). However, based on population studies the prevalence of clinically relevant adenomas is higher than previously thought at about 94 in 100 000 patients in the general population (Clayton 1999, Daly *et al.* 2006b). In addition to hormone hypersecretion, there are two main mechanisms by which pituitary adenomas can cause clinical symptoms. These are compressive pituitary failure, causing e.g. hypogonadism, thyroid failure or adrenal failure, and central mass effects, causing e.g. visual field disturbances, headaches and cranial nerve palsies (Melmed 2003).

Prolactinomas are the most common type of pituitary adenomas, accounting for 27-45% of all pituitary adenomas (Arafah & Nasrallah 2001, Sane in Välimäki *et al.* 2009 p.100, Table 2). However, even higher prevalence of prolactinomas (66.2% of pituitary adenomas) has been suggested (Daly *et al.* 2009). Prolactinomas occur more frequently in women than men until after the fifth decade of life when their frequency equalizes between the sexes (Mindermann & Wilson 1994). The most common clinical features of prolactinomas are hypogonadism and/or galactorrhea in both males and females, and they cause amenorrhea, oligomenorrhea or infertility in females and decreased libido or diminished sexual potency in males. Due to this different symptomatology in sexes, women usually present earlier than men and often exhibit microprolactinomas at diagnosis; men present later and with a higher frequency of macroprolactinomas and attendant mass effects (Ciccarelli *et al.* 2005). The diagnosis is based on symptoms, an elevated serum PRL (S-PRL) level and magnetic resonance imaging (MRI). Other causes of hyperprolactinemia should be ruled out in prolactinoma diagnostics, such as pregnancy and consumption of certain medications (Colao 2009).

GH-secreting somatotropinomas account for approximately 15-20% of pituitary adenomas and cause acromegaly in adults and gigantism if they occur in children before epiphyseal plate fusion (Arafah & Nasrallah 2001, Keil & Stratakis 2008, Sane in Välimäki *et al.* 2009 p.100, Table 2). The incidence of somatotropinomas is about 3-4 cases in a million patient years (Lissett *et al.* 1998, Kauppinen-Mäkelin *et al.* 2005). About 25% of somatotropinomas also co-secrete PRL. These mixed adenomas can be dimorphous adenomas composed of GH and PLR cells. They can also be monomorphous mammosomatotroph adenomas derived from mammosomatotrophs that can secrete both GH and PRL. A third possibility is that they are derived from a more primitive acidophil stem cell, which is the progenitor of somatotrophs and lactotrophs. The clinical manifestations of acromegaly are derived from the major organ systems of the body: the musculosceletal, integumentary, gastrointestinal, cardiovascular, pulmonary, endocrine and metabolic systems. Symptoms can be subtle signs of acral overgrowth (arthralgias, jaw prognathism and frontal bone bossing), soft-tissue swelling, fasting hyperglycemia and hyperhidrosis. At the other end of the spectrum are symptoms of florid osteoarthritis, diabetes mellitus, hypertension and respiratory and cardiac failure. Before epiphyseal plate closure, excessive GH leads to linear growth acceleration and gigantism (Melmed 2006, Sane in Välimäki *et al.* 2009 p.113-116). The mortality rate of acromegalics is double that of the general population. However, it has been reported that mortality reverts to expected levels when there is reduction of GH to less than 1µg/l or normalization of insulin-like growth factor 1 (IGF-I), which is the prime mediator of the effects of GH on target tissues (Holdaway *et al.* 2004). In a survey of mortality in acromegaly in Finland, posttreatment GH less than 2.5µg/l was associated with a normal life-span (Kauppinen-Mäkelin *et al.* 2005). Apart from typical symptoms and imaging, the diagnosis of acromegaly can be based on the inability of the patient to suppress GH levels during an oral glucose-tolerance test (OGTT), the excessive peripheral biologic effects of GH reflected by elevations in S-IGF-I levels, as well as elevated S-GH levels (Melmed 2006, Sane in Välimäki *et al.* 2009 p.116-119).

ACTH-secreting adenomas, known as adenocorticotropinomas, account for 9-12% of pituitary adenomas and are seen predominantly in females. They cause Cushing's disease, a state of hypercortisolism caused by excess pituitary secretion of ACTH, which stimulates the secretion of cortisol by the adrenal glands. Symptoms of hypercortisolism include central obesity, easy bruising,

proximal myopathy, striae, hypertension, hirsutism, menstrual irregularity, mood changes, poor wound healing, osteoporosis and hyperglycemia. Initial screening of suspected Cushing's disease patients is achieved by an overnight 1-1.5mg dexamethasone suppression test where dexamethasone fails to suppress S-cortisol levels in Cushing's patients, or a 24h urinary free cortisol (UFC) measurement, followed by pituitary MRI (Arafah & Nasrallah 2001, Sane in Välimäki *et al.* 2009 p.100, Table 2).

Gonadotropinomas account for 9-15% of pituitary adenomas. They can secrete LH, FSH or both, or their respective subunits aSU and bSU. However, their hormone secretion is often minimal or inefficient and the clinical behavior is thus often that of an inactive tumor (Samuels & Ridgway 1995). If present, the most common clinical presentations of gonadotropinomas are related to mechanical effects of the expanding macroadenoma, and it is common that the adenomas are large and extend beyond the sella turcica at diagnosis. In rare cases, patients may have symptoms of excessive hormone secretion, for example increased libido in men and ovarian hyperstimulation syndrome in women (Arafah & Nasrallah 2001, Table 2). Diagnosis of gonadotropinomas is based on measurements of serum hormone concentrations of intact FSH, intact LH and a/bSU, and tumor imaging with MRI (Daneshdoost *et al.* 1991, Young *et al.* 1996, Arafah & Nasrallah 2001).

Thyrotroph adenomas that secrete TSH are rare, accounting for 1-2% of pituitary adenomas. Patients often have goiter and evidence of mild hyperthyroidism, such as hyperhidrosis and increased appetite. By the time of diagnosis, tumors are often large and have extrasellar extension, causing signs of hyperthyroidism and symptoms of mechanical compression. However, 30% of tumors also show increased secretion of GH or PRL, which can complicate the symptoms in these patients. Diagnosis of thyrotropinomas is based on increases in S-TSH and the serum thyroid hormone levels thyroxine (T_4) and triiodothyronine (T_3). Also a thyroid-releasing hormone (TRH) stimulation test without a rise in S-TSH indicates the possibility of a thyrotropinoma, and diagnosis should be followed with MRI scanning (Arafah & Nasrallah 2001, Roelfsema *et al.* 2009, Sane in Välimäki *et al.* 2009 p.123-124, Table 2).

Approximately 30% of pituitary adenomas are endocrinologically silent i.e. they cause no clinical symptoms related to excessive hormone secretion. These are often true non-functioning pituitary adenomas (NFPA) that include null-cell adenomas or oncocytomas. They usually present with mechanical effects of the adenoma and variable degrees of hypopituitarism, and their diagnosis is based on MRI scanning (Arafah & Nasrallah 2001, Heaney & Melmed 2004; Sane in Välimäki *et al.* 2009 p.125, Table 2).

3.5 Treatment and management

Goals in the treatment of pituitary tumors include controlling clinical and biochemical signs of excessive hormone secretion, preserving normal pituitary function whenever possible, reversing or treating impaired pituitary function and controlling the growth of the tumor and its mechanical effects on surrounding structures (Arafah & Nasrallah 2001).

In most pituitary adenomas, the primary treatment approach is transsphenoidal surgical adenomectomy. Generally, it is a very effective operation with low morbidity and mortality. However,

a subsequent transsphenoidal approach may be needed to resect the residual suprasellar part of the tumor that descended after the first operation. A craniotomy may be needed in patients with a residual, suprasellar tumor that did not descend during transsphenoidal approaches. Repeated operations may also be needed if there is recurrence of the tumor. Radiation therapy is rarely recommended as the primary form of treatment, but it can be used as an adjunctive therapy in selected patients (Landolt 1999, Arafah & Nasrallah 2001, Sane in Välimäki *et al.* 2009 p.103). If hypopituitarism persists after surgery, it is managed by replacement of the deficient hormone(s) (Arafah & Nasrallah 2001, Melmed 2003).

There is also medical therapy available for the treatment of many types of pituitary adenomas. For example, the dopamine agonists bromocriptine and cabergoline reduce the size and hormonal hypersecretion of prolactinomas, and they are most often the primary treatment of prolactinomas. In acromegaly patients, the first-line pharmacological treatment is somatostatin analog (SSA) therapy. SSAs are beneficial especially in patients with post-operative residual tumor activity, or in patients who are poor surgical candidates. Patients are most often treated with octreotide or lanreotide, which reduce plasma GH and IGF-I concentrations and in some cases also cause a moderate decrease in tumor size. In addition, a novel SSA, pasireotide, is currently in clinical trials. Also chimeric compounds with both somatostatin receptor and dopamine receptor affinity are being developed. Novel drugs also include pegvisomant, a GH receptor antagonist that can be used for treatment of persistently high IGF-I levels and that can also be used in combination therapy with SSAs (Barkan *et al.* 1988, Molitch *et al.* 1997, Arafah & Nasrallah 2001, Manjila *et al.* 2010).

Recurrence of pituitary adenomas after apparently complete surgical resection is reported in 10-25% of patients, usually within the first four years of operation. Therefore, periodic hormonal testing and repeated imaging studies are recommended to pituitary adenoma patients. It has further been recommended that follow-up of patients is maintained indefinitely (Arafah & Nasrallah 2001, Sane in Välimäki *et al.* 2009 p.126, 128).

Apart from clinically relevant adenomas that require effective treatment, some pituitary adenomas are found incidentally by radiological imaging of asymptomatic patients. These tumors are called incidentalomas. Their management is suggested to be based on periodic hormonal, clinical and radiological follow-up, particularly in cases having neither hormonal abnormalities nor clinical signs of the incidentaloma (Daly *et al.* 2007a).

4. Genetics of pituitary adenomas

Pituitary adenomas arise from the monoclonal expansion of a pituicyte that evades apoptosis and acquires unlimited replicative potential. Etiologic factors that have been implicated in pituitary tumorigenesis include genetic events, hormonal stimulation and growth factors. It is likely that all of these interact to initiate transformation and promote tumor-cell proliferation (Melmed 2003, Asa & Ezzat 2009, Tanase *et al.* 2009). In the following sections, a number of genetic aberrations are described that are encountered either as somatic events in sporadic pituitary adenomas or as inherited defects in the context of familial susceptibility to pituitary tumors.

4.1 Sporadic pituitary adenomas

The vast majority of pituitary adenomas are sporadic. Their tumorigenesis has been studied with for example candidate gene approaches, genome-wide allelotyping and comparative genomic hybridization. The studies have identified LOH at putative tumor suppressor gene loci, putative markers of tumor progression and early alterations in tumors. Also hotspots that may indicate an unstable chromatin structure that is susceptible to deletions or epigenetic gene-silencing events and chromosomal aberrations have been discovered (Simpson *et al.* 2003, Pack *et al.* 2005). However, in many cases it is still unclear which of these alterations are involved in initiation and progression of sporadic pituitary oncogenesis.

4.1.1 *GNAS/gsp* oncogene

The *guanine nucleotide-binding protein alpha stimulating activity polypeptide (GNAS)* gene (20q13) encodes the guanosine nucleotide-binding protein $G_s\alpha$. It is required for the activation of adenylyl cyclase and subsequent generation of cyclic adenosine monophosphate (cAMP) in the cell. Acting as a cellular second messenger, cAMP binds to the cAMP-dependent protein kinase A (PKA) receptor and regulates a vast number of cellular processes, such as cell proliferation, differentiation and apoptosis. Activating mutations in *GNAS* cause increased activity of $G_s\alpha$, leading to increased cAMP levels (Vallar *et al.* 1987, Akintoye *et al.* 2002, Chin *et al.* 2002, Boikos & Stratakis 2007b). The term *gsp* oncogene has been assigned to these activating *GNAS* mutations due to their association with certain neoplasms. The *gsp* oncogene is found in 30-40% of GH-secreting adenomas, in a low percentage of NFPA and ACTH-secreting adenomas and in differentiated thyroid carcinomas. In addition, it is reported that $G_s\alpha$ messenger ribonucleic acid (mRNA) levels can be high in some somatotropinomas without the *gsp* oncogene itself. The increased production of cAMP conferred by these mutations leads to overactivation of specific pathways involved in cell proliferation and specific programs of cell differentiation (Landis *et al.* 1989, Spada *et al.* 1998, Picard *et al.* 2007).

McCune-Albright syndrome (MAS) is a rare sporadic condition characterized by a triad of café-au-lait skin pigmentation, polyostotic fibrous dysplasia of the bone and hyperfunctioning endocrinopathies. These include excess GH, hyperthyroidism and Cushing's syndrome. The molecular etiology of this genetic but not inherited disease is an early embryonic postzygotic activating mutation of *GNAS* that results in constitutive $G_s\alpha$ activation and elevated cAMP levels in the affected individual (Vallar *et al.* 1987, Akintoye *et al.* 2002, Boikos & Stratakis 2007b).

4.1.2 Other features of sporadic adenomas

Apart from the well-defined *GNAS/gsp* oncogene activation in sporadic pituitary adenomas, research of other pathways and factors is vigorously on-going. Putative mechanisms in sporadic pituitary tumorigenesis involve classic oncogenic signals, dysregulated growth factors and their receptors, epigenetically silenced tumor suppressor genes and chromatin remodeling (Asa & Ezzat 2009). Examples of these mechanisms include activation of the proto-oncogene *pituitary tumor transforming gene* (*PTTG*), downregulation of the *fibroblast growth factor receptor* (*FGFR*) *2* or inactivation of *RB1* (Woloschak *et al.* 1996, Abbass *et al.* 1997, Hunter *et al.* 2003). There are also other proteins

emerging in recent studies, that may be involved in pituitary tumorigenesis, such as inhibitor of apoptosis stimulating protein of p53 (iASPP) and RWD-containing sumoylation enhancer (RSUME) (Fuertes *et al.* 2010, Pinto *et al.* 2010).

4.2 Familial pituitary adenomas

A minority (~5%) of pituitary adenomas occurs in a familial setting. The identification of genetic and molecular mechanisms underlying these conditions has greatly improved the understanding of them. This process classically involves initial linkage analysis studies, the mapping and identification of relevant gene(s) and deciphering how abnormal protein expression leads to neoplastic changes at the molecular level (Daly *et al.* 2005, Tichomirowa *et al.* 2009). In the following sections, four familial conditions will be outlined where a known genetic defect leads to pituitary tumorigenesis. The two last sections will focus on familial pituitary adenomas with a yet unknown genetic background.

4.2.1 Multiple endocrine neoplasia type 1 (MEN1)

Multiple endocrine neoplasia type 1 (MEN1) (OMIM 131100) is an autosomal dominant disorder characterized by different combinations of tumors in the parathyroids, pancreas and the anterior pituitary. In addition, some patients may develop adrenal cortical tumors, gastrointestinal or thoracic neuroendocrine tumors, facial angiofibromas, collagenomas and lipomas. MEN1 arises from germline mutations in the *MEN1* gene (11q13) (Chandrasekharappa *et al.* 1997, Elston *et al.* 2009, Thakker 2010, Table 3). Pituitary adenomas occur in about 30% of patients. Most often these are prolactinomas, although somatotropinomas, corticotropinomas or NFPAs are occasionally diagnosed as well (Trump *et al.* 1996, Tichomirowa *et al.* 2009, Thakker 2010). The prevalence of MEN1 has been estimated to be 0.02 – 0.2 in 1000 (Cazabat *et al.* 2009). MEN1 is characterized as familial if an affected individual has at least two of the three above-mentioned main MEN1 tumors and at least one first-degree relative has one of the three tumors (Marx *et al.* 1999, Brandi *et al.* 2001). Although most MEN1 patients have inherited the disorder, molecular genetic studies have confirmed *de novo* mutations of the *MEN1* gene in approximately 10% of patients with MEN1 (Lemos & Thakker 2008). Approximately 10% of clinically suspected MEN1 patients do not have *MEN1* mutations, suggesting that other predisposition genes may play a role in this phenotype (Hai *et al.* 2000; Daly *et al.* 2005).

The *MEN1* gene consists of 10 exons encoding a 610 aa protein referred to as menin. It is a predominantly nuclear protein that has a role in transcriptional regulation, genome stability, cell division and proliferation. MEN1-associated tumors frequently exhibit LOH at the *MEN1* locus, which is consistent with the tumor suppressor role of *MEN1*. Furthermore, although occasional somatic abnormalities of *MEN1* have been reported in endocrine tumors, *MEN1* is very rarely mutated in sporadic pituitary adenomas (Chandrasekharappa *et al.* 1997, Zhuang *et al.* 1997, Thakker 2010). To date, over 1300 *MEN1* mutations have been identified, of which the majority are predicted to lead to the truncation of menin. Interestingly, the phenotype of MEN1 is variable and shows an absence of phenotype-genotype correlations. Therefore, clinical features vary between patients of MEN1 families, even between identical twins (Bahn *et al.* 1986, Lemos & Thakker 2008, Thakker 2010).

Table 3. Familial conditions with pituitary adenomas, data from Vierimaa *et al.* 2006, Agarwal *et al.* 2009, Tichomirowa *et al.* 2009, Gadelha & Frohman 2010, Kirschner 2010 and Thakker 2010.

Condition	Predisposing gene (locus)	Pituitary adenomas
MEN1	*MEN1* (11q13)	30% of patients; mostly prolactinomas
MEN4	*CDKN1B* (12p13)	few patients discovered; some present
	p15 (9p21), *p18* (1p32), *p21* (6p21)?	with pituitary adenomas (GH, ACTH)
CNC	*PRKAR1A* (17q22-24)	10% GH-secreting adenomas ; 75%
	unidentified (2p16)	show GH/PRL overactivity
PAP	*AIP* (11q13)	low penetrance of pituitary adenomas; mostly GH-secreting adenomas
IFS	*AIP* (11q13) in ~40%; *unidentified*	acromegaly; gigantism
FIPA	*AIP* (11q13) in ~15%; *unidentified*	all pituitary tumor types; GH- and PRL-secreting adenomas the most common

4.2.2 MEN4 (MEN1-like syndrome)

A recessively inherited MEN-like syndrome (MENX), causing multiple endocrine cancers including pituitary tumors, was first identified when occurring spontaneously in the rat. The MENX gene was later shown to be *cyclin-dependent kinase n1b (cdkn1b)* (Fritz *et al.* 2002, Piotrowska *et al.* 2004, Pellegata *et al.* 2006). In humans, the corresponding *CDKN1B* encodes the protein cyclin-dependent kinase inhibitor $p27^{Kip1}$ on chromosome 12p13. The first heterozygous germline *CDKN1B* mutation (W76X) was found in a German family with acromegaly, primary hyperparathyroidism, renal angiomyolipoma and testicular cancer. Pedigree analysis revealed mutation segregation with the phenotype. Although the wildtype allele was retained in the tumor tissue, immunohistochemical staining of $p27^{Kip1}$ showed no protein expression in the tumors. This supported an association between germline *CDKN1B* mutations and a heritable human MEN1-like condition called MEN4 (OMIM 610755) (Pellegata *et al.* 2006, Table 3). So far, only a handful of *CDKN1B* mutations have been found in suspected MEN1 cases with no *MEN1* mutations (Georgitsi *et al.* 2007b, Agarwal *et al.* 2009). Interestingly, one of these (K25fs) was in a patient with an ACTH-secreting adenoma, but three others (P95S, -7G>C and X>Q) were in patients with no pituitary manifestation (Georgitsi *et al.* 2007b, Agarwal *et al.* 2009).

The *CDKN1B* gene consists of three exons encoding 198 aa. It is a well-established cyclin-dependent kinase inhibitor that negatively regulates cell cycle progression by inhibiting cyclin and cyclin-dependent kinase complexes in the nucleus (Pellegata *et al.* 2006, Lee & Kim 2009). It is reported that $p27^{Kip1}$ is underexpressed or even absent in most pituitary adenomas (Lidhar *et al.* 1999). Intriguingly, the regulation of $p27^{Kip1}$ expression involves both menin, and aryl hydrocarbon receptor interacting protein (AIP) through aryl hydrocarbon receptor (AHR) (Kolluri *et al.* 1999, Karnik *et al.* 2005, Milne *et al.* 2005, see section 5.2.1). The precise role and pathways of $p27^{Kip1}$ in pituitary tumorigenesis are, however, yet to be elucidated. Interestingly, Besson *et al.* reported that independently of its role as a CDK inhibitor and tumor suppressor, $p27^{Kip1}$ can act as an oncogene *in vivo*, promoting stem cell expansion and tumorigenesis in multiple tissues (Besson *et al.* 2007). In addition, there are reports of variations in other CDK inhibitor genes (*p15*, *p18* and *p21*) that have been suggested to lead to a MEN1-like phenotype (Agarwal *et al.* 2009).

4.2.3 Carney complex (CNC)

Carney complex (CNC) (OMIM 160980) is a complex of myxomas, schwannomas, spotty skin pigmentation and endocrine overactivity (Carney *et al.* 1985, Tichomirowa *et al.* 2009, Table 3). It is a rare autosomal dominant disease that has been described in about 500 patients (Boikos & Stratakis 2007a). The median age at diagnosis is about 20 years, and the most common clinical manifestation at the time of presentation is spotty skin pigmentation. CNC patients have a decreased life-span, mostly due to heart-related causes such as cardiac myxomas (Stratakis *et al.* 2001). The main endocrine abnormalities seen in CNC are primary pigmented nodular adrenocortical disease, thyroid tumors and nodules, testicular tumors and acromegaly (Stergiopoulos & Stratakis 2003). Acromegaly occurs in roughly 10% of cases, but even 75% of patients have elevated GH, IGF-I or PRL levels or abnormal responses to dynamic pituitary testing (Pack *et al.* 2000, Stratakis *et al.* 2001). CNC-related acromegaly is distinguished by multifocal hyperplasia of mammosomatotropic cells that includes nonadenomatous pituitary tissue within the tumors (Kurtkaya-Yapicier *et al.* 2002).

Two candidate gene loci have been identified, one on chromosome 17q22-24 and the other on chromosome 2p16 (Stratakis *et al.* 1996, Casey *et al.* 1998). While no predisposing gene(s) have been found in the 2p16 locus, the 17q22-24 locus contains the gene encoding PKA regulatory subunit 1 alpha (*PRKAR1A*), which comprises 11 exons and encodes a protein of 381 aa. Mutations in *PRKAR1A* have been identified in up to 65% of CNC patients (Stratakis *et al.* 1996, Veugelers *et al.* 2004). *PRKAR1A* is a tumor suppressor gene, and most *PRKAR1A* mutations lead to mRNA instability, decreased or absent protein expression, and PRKAR1A haploinsufficiency in CNC tumors (Kirschner *et al.* 2000). LOH at 17q22-24 and allelic loss have been shown in CNC tumors. PRKAR1A is the main component of PKA, which regulates most of the kinase activity catalyzed by the PKA holoenzyme in response to cAMP. This pathway is involved in the regulation of metabolism, cell proliferation, differentiation and apoptosis. The loss of PRKAR1A function enhances signaling through the PKA pathway. In the pituitary, the GHRH receptor uses the cAMP/PKA pathway to stimulate synthesis and the release of GH, suggesting that this could be one mechanism involved in the oncogenesis of somatotropinomas (Mayo *et al.* 1995, Kirschner *et al.* 2000, Groussin *et al.* 2002, Bossis & Stratakis 2004, Kirschner 2010).

4.2.4 Pituitary adenoma predisposition (PAP)

A fourth condition with familial pituitary adenomas, designated as pituitary adenoma predisposition (PAP) (OMIM 102200), was discovered by Vierimaa *et al.* in 2006 in three clusters of familial pituitary adenomas from Northern Finland. Two of the clusters could be linked by genealogy data. The patients displayed low-penetrance susceptibility to somatotropinomas, prolactinomas and mixed adenomas. To identify the predisposing gene, whole-genome single nucleotide polymorphism (SNP) genotyping was performed. This was followed by linkage analysis, which provided evidence for linkage in 11q12-11q13, a region also previously implicated in isolated familial somatotropinoma (IFS) (Gadelha *et al.* 1999, Gadelha *et al.* 2000, Soares *et al.* 2005, Vierimaa *et al.* 2006).

The candidate locus was fine-mapped and the two pedigrees shared the linked haplotype, which segregated perfectly with somatotropinomas. Out of the 295 genes in the linked region, expression

profiles showed the lowest values for probes representing *aryl hydrocarbon receptor interacting protein* (*AIP*), also named *aryl hydrocarbon receptor-associated protein-9* (*ARA9*) or *hepatitis B virus x-associated protein 2* (*XAP2*). *AIP* was chosen as the prime candidate for mutation analysis. A protein-truncating *AIP* mutation (Q14X) was found to perfectly segregate with the GH-secreting adenoma phenotype in both of the families (Vierimaa *et al.* 2006, Table 3). *AIP* screening was also performed on 45 acromegaly patients in a population-based cohort in Northern Finland that included four cases from the two families (Kauppinen-Makelin *et al.* 2005, Vierimaa *et al.* 2006). Six Q14X mutations and one intronic variable sequence (IVS) 3-1G>A mutation affecting the splice acceptor site of exon 4 were identified. Thus, *AIP* mutations accounted for 16% of the acromegaly patients in the population-based cases, and 40% of those that were diagnosed under the age of 35 years. In addition, two Q14X mutations were found in ten unselected Finnish sporadic acromegaly patients and a R304X mutation was found in Italian siblings with somatotropinomas (Vierimaa *et al.* 2006).

AIP LOH in tumors was detected in all the mutation carriers that were studied in the study by Vierimaa *et al*., indicating a tumor suppressor role for *AIP*. The authors concluded that the penetrance of PAP appeared to be low and patients did not necessarily have a strong familial background of pituitary adenomas. The phenotype was characterized by young age at onset and occurrence of at least somatotropinomas, prolactinomas and mixed adenomas (Vierimaa *et al.* 2006). Since gene identification, studies have shown that *AIP* mutations are predominantly associated with somatotropinomas (Daly *et al.* 2007b, Georgitsi *et al.* 2008, Leontiou *et al.* 2008, Cazabat *et al.* 2009). *AIP* mutation screening has been performed in a variety of sporadic non-pituitary tumors, but no relevant *AIP* mutations have been found (Georgitsi *et al.* 2007a, Raitila *et al.* 2007). Interestingly, *AIP* LOH was recently reported in an adrenocortical carcinoma of an acromegaly patient with a R81X *AIP* germline mutation, suggesting putative *AIP* implication also in non-pituitary tumorigenesis (Toledo *et al.* 2010). However, the 11q13 LOH could also be accompanied by a germline defect in another, yet unidentified tumor suppressor gene at 11q13. Furthermore, the existence of such a gene related to adrenocortical tumorigenesis has strongly been suggested by previous genetic studies (Kjellman *et al.* 1999, Toledo *et al.* 2010). Interestingly, one recent report has also suggested *MEN1* and *AIP* deletions to be involved in the pathogenesis of brown fat tumors hibernomas (Nord *et al.* 2010).

The *AIP* gene (11q13) comprises six exons that encode a protein of 330 aa in length. AIP has a FK605 binding protein (FKBP) homology domain in the amino-terminus, and the carboxy-terminal half contains three tetratricopeptide repeat (TPR) domains that mediate protein-protein interactions (Carver & Bradfield 1997, Petrulis & Perdew 2002). Since gene discovery, about fifty different *AIP* mutations have been identified, including deletions, insertions, frameshift, nonsense, missense, splice site and promoter mutations, as well as deletions of the whole *AIP* gene (Barlier *et al.* 2007, Cazabat *et al.* 2007, Daly *et al.* 2007b, Georgitsi *et al.* 2007a, Iwata *et al.* 2007, Naves *et al.* 2007, Raitila *et al.* 2007, Toledo *et al.* 2007, Georgitsi *et al.* 2008, Leontiou *et al.* 2008, Montanana 2008, Yaneva 2008, Jennings *et al.* 2009, Khoo *et al.* 2009, Montanana 2009, Igreja *et al.* 2010, Naves *et al.* 2010, Stratakis *et al.* 2010, Toledo *et al.* 2010, Figure 2). However, some of these may be rare polymorphisms and not disease-causing mutations. The vast majority of the mutations result in the deletion of the C-terminal end of the AIP protein (stop codons or frameshifts resulting in stop

codons), although the missense variants and an in-frame segmental duplication mostly affect the TPR domains of the C-terminal α-helix. These findings are supported by earlier data suggesting that the third TPR domain and the last five carboxy-terminal aa are necessary for the biological activity of AIP (Petrulis & Perdew 2002, Daly *et al.* 2007b, Leontiou *et al.* 2008). Based on the *AIP* mutation screening from apparently sporadic pituitary adenomas in several studies, the estimated prevalence of *AIP* mutations is 2% for all these patients and 2.7% for acromegaly patients (Vierimaa *et al.* 2006, Barlier *et al.* 2007, Cazabat *et al.* 2007, Georgitsi *et al.* 2007a, Iwata *et al.* 2007, Raitila *et al.* 2007, Toledo *et al.* 2007, Buchbinder *et al.* 2008, Georgitsi *et al.* 2008, Leontiou *et al.* 2008, Montanana 2008, Yaneva 2008, Chahal *et al.* 2010, Stratakis *et al.* 2010). Interestingly, no somatic *AIP* mutations have been found in pituitary adenomas to date (Barlier *et al.* 2007, Leontiou *et al.* 2008, Vargiolu *et al.* 2009, Chahal *et al.* 2010). However, the first apparently *de novo AIP* mutation was recently identified in a young prolactinoma patient (Stratakis *et al.* 2010).

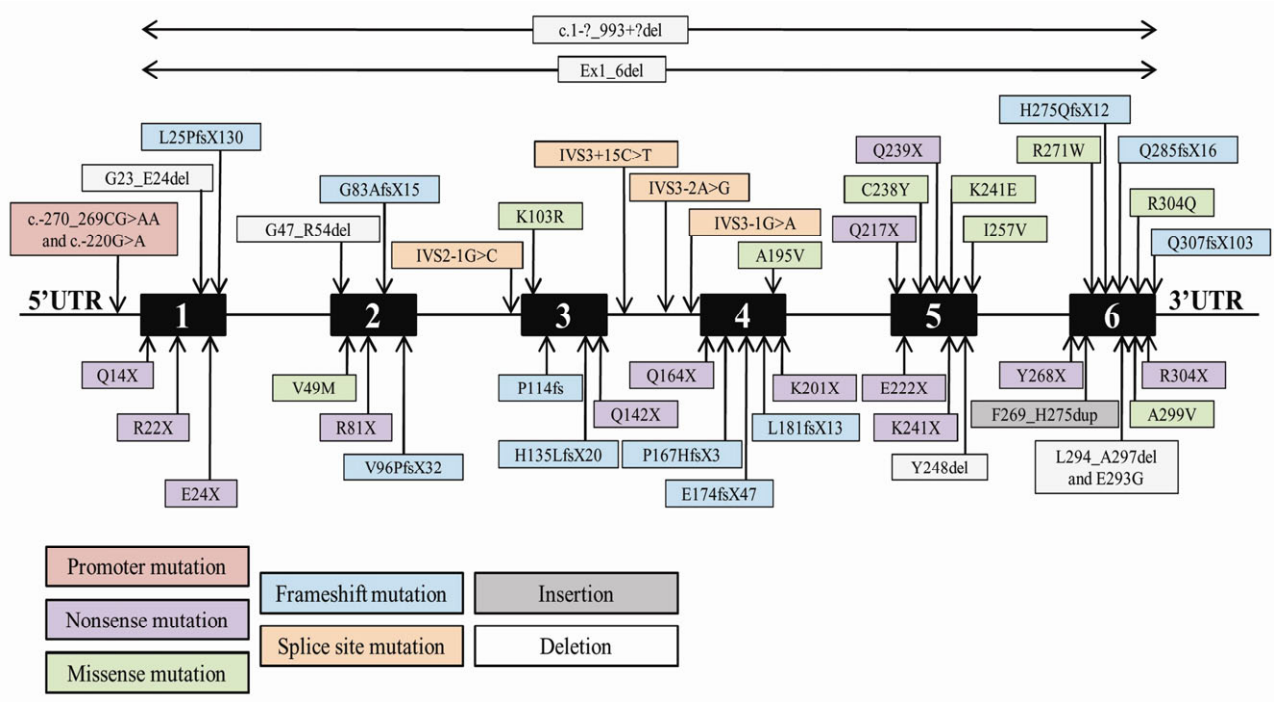

Figure 2. The *AIP* gene and reported mutations. The *AIP* variants are shown according to their location in the *AIP* gene. Exons are shown as numbered black boxes and different mutation types are color coded (see above). See references in Cain *et al*. 2010.

4.2.5 Isolated familial somatotropinoma (IFS)

Isolated familial somatotropinoma (IFS) (OMIM 102200) is defined by at least two individuals with acromegaly or gigantism in a family without diagnosis of MEN1 or CNC (Gadelha *et al.* 1999, Table 3). The patients are typically young and are most often diagnosed with macroadenomas, of which about half co-secrete prolactin (Soares & Frohman 2004). In efforts to identify a predisposition locus,

LOH at 11q13 without *MEN1* mutations was shown, and it was concluded that loss-of-function of a tumor suppressor gene distinct from MEN1 would be responsible for IFS (Gadelha *et al.* 1999, Gadelha *et al.* 2000, Soares *et al.* 2005). Subsequently, this area was reported to contain truncating mutations in *AIP* (Vierimaa *et al.* 2006). Since gene discovery, *AIP* mutations have been found in several IFS families (e.g. Daly *et al.* 2007b, Iwata *et al.* 2007, Leontiou *et al.* 2008). Approximately 40% of the families with IFS harbor an *AIP* mutation. These tumors are diagnosed at a young age and are larger than tumors in IFS families without *AIP* mutations, indicating a more aggressive disease (Gadelha & Frohman 2010).

4.2.6 Familial isolated pituitary adenoma (FIPA)

Pituitary tumors that occur in a familial setting without diagnosis of MEN1 or CNC constitute a condition termed familial isolated pituitary adenoma (FIPA) (OMIM 102200) (Verloes *et al.* 1999, Daly *et al.* 2006a). Comprising all pituitary adenoma types, FIPA is a much broader entity than IFS. In FIPA, pituitary tumors of one type can present in the affected members of a family (homogeneous presentation) or affected members can have different types of tumors (heterogeneous presentation) (Daly *et al.* 2006a). The reported frequencies of different pituitary tumor types in FIPA families are: prolactinomas 41%, somatotropinomas 30%, NFPAs 13%, mammosomatotropinomas 7%, gonadotropinomas 4%, Cushing's disease 4% and thyrotropinomas 1%. There is a first-degree relationship between affected family members in about 75% of FIPA families. The pituitary adenomas of FIPA patients generally occur about four years earlier than in their sporadic counterparts. Macroadenomas are seen in 63% of FIPA kindreds (Tichomirowa *et al.* 2009, Table 3).

A study on FIPA patients revealed that 15% of families had germline *AIP* mutations. Patients with an *AIP* mutation were significantly younger at diagnosis than FIPA patients without an *AIP* mutation. Tumors were also larger in the *AIP* mutation positive families when compared with the remainder of the cohort. The existence of kindreds with a strong family history of pituitary adenomas and with no *MEN1*, *PRKAR1A*, *CDKN1B* or *AIP* mutations indicates that other, yet unidentified genes may underlie FIPA (Daly *et al.* 2007b, Tichomirowa *et al.* 2009).

5. Molecular function of the AIP protein

5.1 Features and function of AIP

The *AIP* gene (11q13) comprises six exons and encodes a co-chaperone protein of 330 aa with a molecular mass of 38 kilodaltons (kDa). An FKBP homology domain is located in the amino-terminus of AIP and the carboxy-terminal half contains three TPR domains that mediate the various protein-protein interactions of AIP (Carver & Bradfield 1997, Petrulis & Perdew 2002). AIP has a ubiquitous tissue distribution (Kuzhandaivelu *et al.* 1996). In the normal pituitary, AIP has been identified only in GH- and PRL-secreting cells, where it associates with cytoplasmic secretory vesicles. In sporadic pituitary tumors, however, AIP is expressed in somatotropinomas, prolactinomas, corticotropinomas and NFPAs. In these lesions, AIP resides in the cytoplasm, except for in somatotropinomas where it is expressed in secretory vesicles, similar to normal somatotrophs (Leontiou *et al.* 2008). Interestingly,

low AIP expression has recently been shown to be a better marker of invasiveness in sporadic somatotropinomas than the proliferation marker Ki-67 and p53 (Kasuki Jomori de Pinho *et al.* 2010).

To date, the function of the AIP protein has been poorly characterized, although it has been evaluated regarding its putative tumor suppressor role and also in *Aip* knockout models. The tumor suppressor role of *AIP* is supported by the occurrence of LOH in pituitary adenomas of *AIP* mutation carriers and by the ability of transient overexpression of wildtype AIP to reduce cell proliferation in cell culture (Vierimaa *et al.* 2006, Leontiou *et al.* 2008). Mice with a homozygous *Aip* deletion have been reported to die of congenital cardiovascular abnormalities such as a double-outlet right ventricle, ventricular septal defects and pericardial edema during embryonal development at the embryonic age of E10.5-E14.5. Thus, AIP seems to have a crucial function during embryogenesis (Lin *et al.* 2007). In addition, a hypomorph mouse model expressing 10% of normal AIP did not show the severe congenital cardiovascular abnormalities seen in the *Aip*-null mice; however it had a patent ductus venosus, suggesting that AIP may have a role in AHR-mediated hepatovascular development. Heterozygous *Aip* mice did not manifest any congenital cardiovascular abnormalities (Lin *et al.* 2008). The tumor phenotype or possible pituitary adenoma formation of *Aip* mice was not assessed in these two studies (Lin *et al.* 2007, Lin *et al.* 2008).

5.2 AIP-related cellular pathways

A number of studies have identified several cellular interaction partners of AIP (Table 4, Figure 3). However, the role of these proteins in AIP-mediated tumorigenesis is not yet clear and this should be evaluated in further studies. The following sections will present the interactions found thus far.

5.2.1 Role of AIP in the xenobiotic response

AIP is best characterized as having a role in AHR signaling in the xenobiotic response. AIP resides in the cytoplasm where it forms a complex with AHR and two 90kDa heat-shock proteins (HSP90) that are complexed with p23 (Carver & Bradfield 1997, Kazlauskas *et al.* 2000, Figure 3). AHR is a transcription factor that mediates the effects of environmental toxins that ultimately cause e.g. teratogenesis and tumor promotion (Bunger *et al.* 2003). However, AHR has also been suggested to have a physiologic role in cell proliferation and differentiation as well as in immune system and liver function (Barouki *et al.* 2007). AIP is involved in the cytoplasmic retention of AHR and decreases its proteosomal degradation by protecting it against ubiquitination (Meyer & Perdew 1999, Kazlauskas *et al.* 2000, Petrulis & Perdew 2002, Pollenz & Dougherty 2005). In the presence of ligands such as dioxins or dioxin-like chemicals, for example 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), AHR is shuttled to the nucleus where it forms a heterodimer with AHR nuclear translocator (ARNT), also called hypoxia inducible factor 1β (HIF1-β). The binding of the AHR-ARNT complex to sequences called dioxin response elements (DREs) regulates the transcription of a multitude of genes encoding e.g. drug metabolizing proteins such as cytochrome P450 (CYP) enzymes (Reyes *et al.* 1992, Hankinson 1995, Ramadoss & Perdew 2005). Under hypoxia, ARNT heterodimerizes with hypoxia inducible factor 1 alpha (HIF1-α) to form the HIF1 transcription factor that binds hypoxia response elements (HREs) to regulate target gene expression (Gu *et al.* 2000, Rankin & Giaccia 2008).

Table 4. Cellular interaction partners of AIP, modified from Chahal *et al.* 2010 with the permission from Elsevier.

Interacting protein	Putative effect of interaction	Reference
AHR-HSP90-p23	Stabilization of AIP-HSP90-AHR complex in the cytoplasm, protection of AHR from ubiquitination	Carver & Bradfield 1997
PDE4A5	Inhibition of PDE4A5 enzyme activity	Bolger *et al.* 2003
PDE2A	Lowered cAMP levels and AHR retention in cytoplasm	de Oliveira *et al.* 2007
Survivin	Elevation of anti-apoptotic threshold, regulation of survivin stablility	Kang & Altieri 2006
RET	Inability of AIP to bind and protect survivin from degradation	Vargiolu *et al.* 2009
HBV X antigen	Involvement of AIP pathway in virus-induced cell transformation	Kuzhandaivelu *et al.* 1996
EBNA-3	Involvement of AIP pathway in virus-induced cell transformation	Kashuba *et al.* 2000
PPARγ	Reduction of PPARγ activity	Sumanasekera *et al.* 2003
TRβ1	TRβ1-mediated transcription of TRH	Froidevaux *et al.* 2006
GR	Delay of nuclear entry and inhibition of the transcriptional activity of GR	Laenger *et al.* 2009
TOMM20	Maintenance of import competency of the mitochondrial translocator complex	Yano *et al.* 2003
G_{13}	Inhibition of AIP-AHR binding leading to reduced AHR signaling	Nakata *et al*. 2009
Hsc70	Preferential binding of Hsc70 than HSP90 in the absence of AHR	Yano *et al.* 2003

There is currently no definitive correlation between dioxin exposure and pituitary tumorigenesis. A population-based study on pituitary adenoma incidence was conducted on subjects exposed to dioxin following an industrial accident in 1976 in Italy. However, no statistically significant increase in pituitary tumor incidence was noted in the contaminated area, although a tendency towards a higher risk was seen in subjects exposed to high to intermediate dioxin concentrations in comparison with the unexposed population (Pesatori *et al.* 2008).

Interestingly, AHR has been shown to inhibit cell cycle progression in dioxin-induced rat hepatoma cells by directly inducing the cell cycle inhibitor $p27^{Kip1}$ at the mRNA level, while germline mutations in *CDKN1B* encoding $p27^{Kip1}$ cause MEN4 syndrome with pituitary adenomas (Kolluri *et al.* 1999, Pellegata *et al.* 2006, see section 4.2.2). Another mechanism by which AHR has anti-proliferative potential is through the direct interaction with RB1, a negative regulator of the cell cycle G1/S transition. It has been shown that in the presence of mitogenic signals, AHR binds RB1 and co-operates in repressing the transcription of target genes involved in G1/S transition (Puga *et al.* 2000). Loss of the *Rb1* chromosomal region appears to be related to aggressive pituitary tumor behavior (Donangelo *et al.* 2005). Finally, a recent study reported that *Ahr* functions *in vivo* as a tumor suppressor gene in murine liver carcinogenesis, and that its silencing may be involved in cancer progression (Fan *et al.* 2010).

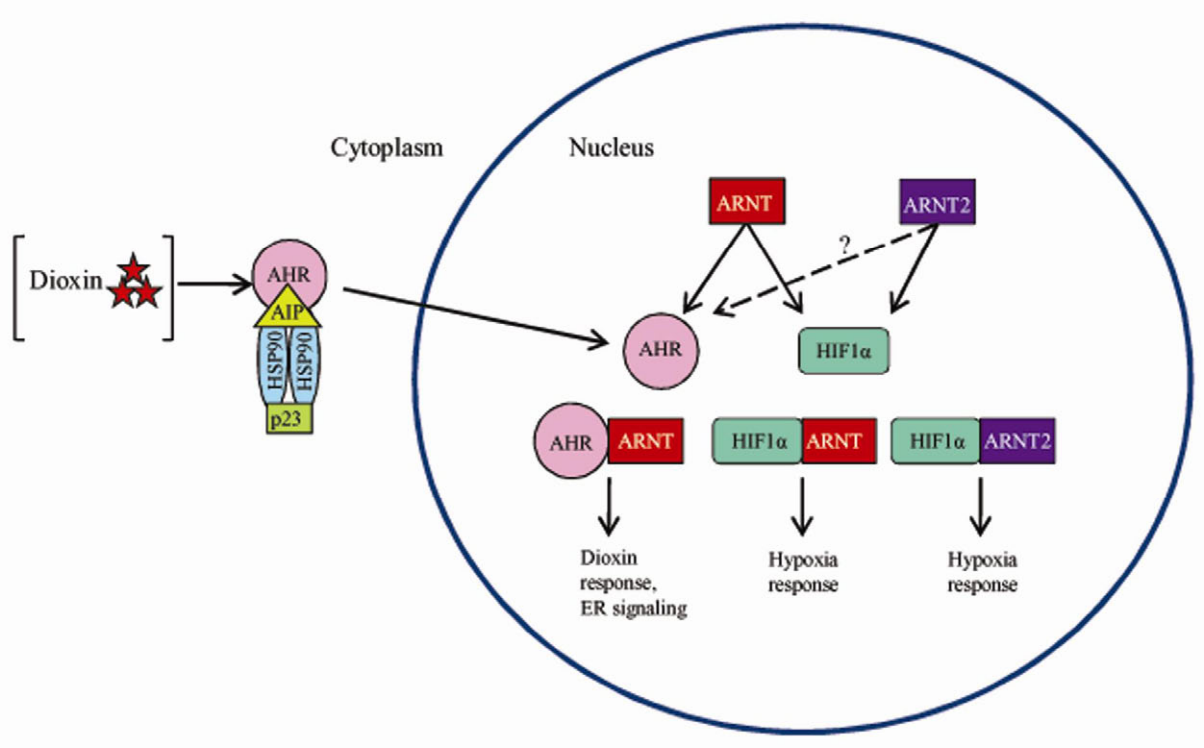

Figure 3. AIP-related pathways. AIP resides in the cytoplasm complexed with AHR, a dimer of HSP90 and p23. Ligands (e.g. dioxin) can induce translocation of AHR to the nucleus where it dimerizes with ARNT and regulates the transcription of genes involved in the dioxin response and in modulation of estrogen receptor (ER) pathways. ARNT2 can also affect ER signaling, although it is not known whether this involves heterodimerization with AHR. Hypoxia leads to heterodimerization of ARNT and HIF1α and the transcription of genes involved in the hypoxia response. Alternatively, ARNT2 can bind HIF1α and induce the hypoxia response (see Discussion 3.1 and 3.2).

5.2.2 AIP in the regulation of cAMP

An altered cAMP-PKA pathway is known to be involved in somatotroph tumorigenesis via the *gsp* oncogene (somatic *GNAS* mutations in somatotroph adenomas; MAS) or *PRKAR1A* mutations (CNC) and it is also upregulated in sporadic somatotropinomas (Spada *et al.* 1998, Boikos & Stratakis 2007b, Kirschner 2010, see sections 4.1.1 and 4.2.2). Phosphodiesterases (PDEs) degrade and deactivate cAMP and other cyclic nucleotides. Thus, the interaction between AIP and PDE4A5 and PDE2A is intriguing since it could provide a logical molecular link between somatotroph tumorigenesis and AIP (Bolger *et al.* 2003, Oesch-Bartlomowicz *et al.* 2005, de Oliveira *et al.* 2007). It has also been reported that cAMP activates AHR and regulates its cytoplasmic-nuclear translocation (Oesch-Bartlomowicz *et al.* 2005). AIP binding to the cAMP-specific PDE4A5 inhibits its enzyme activity and attenuates the ability of cAMP-dependent protein kinase to phosphorylate PDE4A5. In addition, it was recently shown that mutant AIP loses the ability to bind PDE4A5 (Bolger *et al.* 2003, Leontiou *et al.* 2008). PDE2A binding to AIP has been shown to inhibit cAMP-induced nuclear translocation of AHR (de Oliveira *et al.* 2007). It was suggested that AIP-PDE2A binding may result in retention of

the AHR complex in the cytoplasm as a result of lower cAMP levels (Oesch-Bartlomowicz *et al.* 2005, de Oliveira *et al.* 2007). It is currently not known what role PDE4A5, PDE2A, possibly other PDEs and cAMP play in AIP-mediated pituitary tumorigenesis.

5.2.3 Interaction of AIP with RET and survivin

Survivin belongs to the family of inhibitors of apoptosis, but it also has implications in cell division, chromosomal segregation, mitotic spindle formation and cellular stress responses. AIP has been found to be an interaction partner of survivin and it has been shown that knockdown of AIP by small interfering ribonucleic acid (siRNA) or competition of the survivin-AIP complex by peptidyl mimicry destabilizes survivin levels in cells. This enhances apoptosis but causes no changes in cell cycle progression. Thus, AIP regulates survivin stability and elevates the anti-apoptotic threshold of cells (Kang & Altieri 2006).

There are implications that the *RET* proto-oncogene is also involved in survivin-AIP interactions. RET is a receptor tyrosine kinase for glial cell line-derived neurotrophic factor (GDNF). Gain-of-function germline mutations of *RET* lead to multiple endocrine neoplasia type 2A or 2B (MEN2A or MEN2B) or medullary thyroid carcinoma, whereas loss-of-function mutations of *RET* are associated with Hirschsprung's disease. RET promotes cell survival in the presence of GDNF, but induces apoptosis in the absence of its ligand. RET also stimulates the expression of pituitary transcription factor-1 (Pit-1) and p53, and induces apoptosis in somatotrophs and potentially restrains somatotroph proliferation (Canibano *et al.* 2007).

AIP was recently identified as a novel interaction partner of RET (Vargiolu *et al.* 2009). The pro-apoptotic domain of RET interacts with AIP and this interaction prevents the AIP-survivin complex formation. Therefore, in the presence of RET, AIP is unable to bind and protect survivin from degradation and from a consequent increase in apoptosis. In this study, none of the pathogenic *AIP* or *RET* mutations analyzed disrupted the AIP-RET interaction, however, the relevance of these interactions in pituitary tumorigenesis remains to be elucidated (Vargiolu *et al.* 2009).

5.2.4 Other cellular interactions of AIP

AIP was first identified as the partner of viral protein hepatitis B virus (HBV) X antigen. It has been suggested that the X viral gene product can contribute to HBV-induced tumorigenesis. The X protein is also capable of inducing transformation of NIH3T3 cells and mouse hepatocytes (Kuzhandaivelu *et al.* 1996, Meyer *et al.* 1998). Another viral protein that binds AIP is Epstein-Barr virus encoded nuclear antigen-3 (EBNA-3), which plays a role in the effect of the Epstein-Barr virus on cell transformation (Kashuba *et al.* 2000, Krauer *et al.* 2004). The binding of AIP to the transforming proteins of the two viruses might indicate the involvement of AIP pathways in virus-induced cell transformation.

In addition to AHR, other nuclear receptors capable of binding AIP are peroxisome proliferation-activated receptor α (PPARα) and thyroid hormone receptor beta 1 (TRβ1). Also the glucocorticoid receptor (GR) can bind AIP indirectly through HSP90 (Sumanasekera *et al.* 2003, Froidevaux *et al.*

2006, Laenger *et al.* 2009). PPARα has roles in lipid metabolism and homeostasis. *In vitro* binding assays have revealed that cells co-expressing PPARα and AIP have a reduced peroxisome proliferator response, suggesting a repressor effect of the PPARα-HSP90-AIP complex on PPARα activity (Sumanasekera *et al.* 2003, Yang *et al.* 2008). TRβ1 and TRβ2 are central feedback regulators of the hypothalamic-hypophyseal-thyroid axis and of thyroid hormone homeostasis, with TRβ1 having a more important activating role in TRH transcription. The TPR domain of AIP has been reported to interact with TRβ1. In a siRNA assay, knockdown of AIP resulted in the abrogation of TRβ1-mediated activation of hypothalamic transcription of TRH (Froidevaux *et al.* 2006). However, patients with *AIP* mutations have not been reported to have specific thyroid axis abnormalities. In addition, a recent study revealed that AIP can bind the HSP90-GR complex, delay its nuclear entry and inhibit the transcriptional activity of GR (Laenger *et al.* 2009).

AIP has also been reported to interact with the translocase of the outer membrane of mitochondria 20 (TOMM20), which is part of a translocator complex that imports mitochondrial preproteins into mitochondria. *In vitro* import assays showed that AIP maintains the import competency of the translocator complex (Yano *et al.* 2003). Another interaction of AIP has been shown to occur with G protein subtype 13 (G_{13}), and the AIP-G_{13} interaction was able to inhibit the binding of AIP to AHR, leading to reduced AHR signaling (Nakata *et al.* 2009). In addition to binding HSP90, AIP can also bind another heat-shock protein, the heat-shock cognate protein 70 (Hsc70), which belongs to the HSP70 family. It seems that AIP preferentially forms a complex with Hsc70 rather than HSP90 in the absence of AHR (Yano *et al.* 2003, Chahal *et al.* 2010).

Aims of the Study

The primary goal of this work was to clarify the molecular and clinical characteristics of PAP. The specific aims of studies I-V were as follows:

I	To identify large genomic *AIP* deletions in apparently *AIP* mutation negative familial pituitary adenoma patients
II	To elucidate the expression of AIP-related molecules in *AIP* mutation positive and negative tumors to clarify molecular mechanisms of AIP-mediated tumorigenesis
III	To create a mouse model of the disease phenotype of PAP patients and to examine AIP-mediated tumorigenesis *in vivo* in the *Aip* mouse
IV	To study the clinical characteristics and response to therapy of PAP patients compared with sporadic pituitary adenoma patients
V	To evaluate the possible role of *RET* in familial pituitary tumorigenesis of *AIP* mutation negative pituitary adenoma patients

Subjects and Methods

1. Subjects (I, II, IV, V)

1.1 Familial pituitary adenoma patients

In study I, the probands from 21 families with pituitary adenomas from the United Kingdom (n=8), Italy (n=7), Finland (n=4), Germany (n=1) and Turkey (n=1) were analyzed with the multiplex ligation-dependent probe amplification (MLPA) assay. All patients had previously tested negative for germline *AIP* and *MEN1* mutations by conventional sequencing (Georgitsi *et al.* 2007a, Georgitsi *et al.* 2008, unpublished data). The tumors of index cases secreted GH (n=10) or PRL (n=4). Seven tumors were NFPAs. In addition, 32 sporadic Finnish GH-secreting adenoma cases aged 40 years or less at diagnosis and 35 sporadic Italian pediatric pituitary adenoma patients were analyzed (Table 5).

Study IV involved 96 *AIP*mut+ patients without MEN1, MEN4 or CNC from 36 medical centers in Belgium, Finland, France, Italy, Spain, Germany, Bulgaria, the Netherlands, Brazil, Argentina, the United States, Australia, New Zealand and Lebanon. The patients were originally diagnosed with pituitary adenomas in 1970-2009, and *AIP* mutations were found in 2006-2009. Previous studies have reported that *AIP* mutations are predominantly associated with somatotropinomas (Vierimaa *et al.* 2006, Daly *et al.* 2007b, Georgitsi *et al.* 2008, Leontiou *et al.* 2008, Cazabat *et al.* 2009). A suitable control population was obtained from databases of collaborating study centers and comprised 232 *AIP*mut-, non-MEN1, non-CNC acromegaly patients. The control group was randomly extracted to match the *AIP*mut+ group in terms of decade of diagnosis and geographic region to give ≥3 control cases for each *AIP*mut+ case (Table 5).

In study V, 16 patients from *AIP*mut- families with pituitary adenomas were included in the screening of *RET* mutations. Twelve of these families were the same as in study I, and all the patients had previously tested negative for *AIP* mutations (Vierimaa *et al.* 2006, Georgitsi *et al.* 2007a, unpublished data). The patients were from the United Kingdom (n=6), Italy (n=5), Finland (n=3), Turkey (n=1) and New Zealand (n=1). The tumors secreted GH (n=8), PRL (n=3) or ACTH (n=1). Four adenomas were NFPAs. DNA samples from family members, if available, were analyzed when segregation of *RET* variants was evaluated (Table 5).

1.2 Assessing patient characteristics of PAP

In study IV, anonymized patient information on demographics, diagnosis, genetics, hormonal profiles at diagnosis and radiological criteria was collected. Therapeutic responses for each patient following neurosurgery, SSA therapy, radiotherapy, dopamine agonists and pegvisomant were collected. Long-term responses to therapy (≥12 months post-treatment) included information on hormonal, clinical and radiological disease status, treatment modalities used and the presence of hypopituitarism. Tumor size was measured as the maximum diameter on CT or MRI imaging and tumors were classified accordingly as microadenomas (<10mm), macroadenomas (≥10mm) or giant adenomas (≥40mm). Information, if available, on extrasellar extension and invasion into surrounding structures was also collected from radiological reports or from surgical notes. Long-term disease control criteria (≥12 months of follow-up post-therapy) were defined according to tumor type. In all cases tumor size had

to be stable without growth or expansion. For patients with somatotropinoma, disease control at last follow-up was defined as the absence of clinical activity, an age/sex appropriate IGF-I and a valid random GH level <1ng/ml. In prolactinoma, serum PRL had to be age/sex appropriate. For NFPAs, disease control was defined as long-term tumor size stability. In thyrotropinoma, patients had to be symptom-free and have normal serum TSH, T_4 and T_3 levels.

Pre-defined comparison between the *AIP*mut+ and the control group was performed on the following disease and treatment characteristics: gender ratio, age at diagnosis and at first symptoms, tumor size and classification, proportion of patients with extrasellar extension and invasion, GH and IGF-I levels at baseline, PRL co-secretion at baseline, treatment characteristics (number/type of surgery, use of radiotherapy, hormonal and radiological responses to medical therapies), proportion of patients with controlled and active disease, disease control as a function of cumulative therapies, and frequency of hypopituitarism among patients with controlled and active disease.

Table 5. *AIP*mut+ and *AIP*mut- patients and tumors.

Study	Patients/tumors analyzed	Study	Patients/tumors analyzed
Study I	88 patients	**Study IV**	96 *AIP*mut+ patients
	48 GH-secreting tumors		75 GH-secreting tumors
	23 prolactinomas		13 prolactinomas
	14 NFPA		7 NFPA
	3 ACTH-secreting tumors		1 TSH-secreting tumor
Study II	14 *AIP*mut+ tumors		232 *AIP*mut- GH-secreting adenoma patients
	11 GH-secreting tumors	**Study V**	16 *AIP*mut- patients
	3 prolactinomas		8 GH-secreting tumors
	53 *AIP*mut- tumors		3 prolactinomas
	35 GH-secreting tumors		4 NFPA
	7 prolactinomas		1 ACTH-secreting tumor
	10 NFPA		7 *AIP*mut+ GH-secreting tumors
	1 ACTH-secreting tumor		10 *AIP*mut- GH-secreting tumors

GH-secreting tumors include mixed adenomas with GH secretion

1.3 Human pituitary adenoma samples

In study II, 67 formalin-fixed, paraffin-embedded blocks from pituitary adenoma tissue were analyzed for immunohistochemical (IHC) expression of AIP-related proteins. Fourteen of the samples were from *AIP*mut+ patients harboring four different mutations (Q14X, 824insA, IVS3-1G>A and IVS2-1G>C). These adenomas secreted GH (n=5), PRL (n=3), and GH+PRL (n=6). The 53 *AIP*mut- sporadic adenomas secreted GH (n=19), PRL (n=7), GH+PRL (n=14), ACTH (n=1), GH+PRL+ACTH (n=1) and GH+PRL+LH+TSH (n=1). Ten adenomas were hormonally silent. Patients were from Finland (n=65) and the United States (n=2) (Table 5).

In study V, RET IHC was performed on paraffin-embedded blocks from pituitary adenoma tissue from seven *AIP*mut+ somatotropinoma patients and ten *AIP*mut- somatotropinoma patients. The tissue sections were in part from the same patients as in study II (Table 5).

1.4 Healthy controls

In study I, DNA from seven healthy, anonymous blood donors was used as a negative control for MLPA experiments. In addition, healthy, unrelated individuals from the United Kingdom (n=74), Germany (n=18) and the Centre d'Étude du Polymorphisme Humain (CEPH) (n=4) were used as controls for the mutation validation experiments.

In study V, control samples included 279 UK Caucasians (Human Random Control DNA panels, Sigma-Aldrich, Porton Down, Salisbury, Wiltshire, United Kingdom) and 41 samples from Italy.

2. MLPA assay and validation of results (I)

Gene dosage analysis was carried out using the SALSA MLPA kit P244 designed to detect deletions or amplifications in *AIP* and *MEN1* genes (MRC-Holland, Amsterdam, the Netherlands). The polymerase chain reaction (PCR) products were run on an ABI3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Initially, electropherograms were visualized with GeneMarker software v.1.4 (Softgenetics LLC, State College, PA, USA). The data were exported using Peak Scanner software v.1.0 (Applied Biosystems). Final gene dosage analysis was performed with Coffalyser v.6.0 (MRC-Holland). Probes with a dosage quotient DQ of less than 0.65-0.7 (for deletions) or higher than 1.3- 1.35 (for amplifications) were examined for consistency by repeated testing. Negative controls (no DNA) were included throughout the MLPA experiments.

Deletions detected by MLPA were confirmed by long range PCR. Fragments were amplified from genomic DNA by Phusion DNA Polymerase (Finnzymes, Espoo, Finland) or from complementary deoxyribonucleic acid (cDNA) by AmpliTaq Gold (Applied Biosystems). PCR products corresponding to aberrant alleles were extracted either from 1% low-melt agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) or 1% SeaKem LE Agarose gel (Lonza, Rockland, ME, USA) using the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany), and sequenced using BigDye 3.1 termination chemistry on an ABI3730 DNA sequencer (Applied Biosystems).

The whole genomic region of *AIP* and 2 kilobases (kb) upstream of the 5' untranslated region (UTR) (NCBI36:11:67,005,097:67,015,750 and Ensembl release 48) was scanned for possible *Alu* repeats using GEMS Launcher – ModelInspector software (release 5.4.3, May 2007) (Genomatix Software GmbH, Munich, Germany) and the Repeat Masker program (v.3.1.9). Sequence identities of *Alu* repeats were evaluated by NCBI BLAST 2 Sequences (BLASTN, v.2.2.17).

3. Immunohistochemistry of pituitary adenomas (II, III, V)

In studies II, III and V, IHC was performed according to standard procedures on 4-5 µm sections of paraffin-embedded pituitary adenoma specimens, followed by anonymous evaluation by a pathologist.

The Power Vision rabbit or rabbit/mouse Poly-HRP IHC Kit (ImmunoVision Technologies, Norwell, MA, USA), Dako ENVISION Kit (Dako, Glostrup, Denmark), or PowerVision Poly-HRP IHC Detection System kit (PV6104; Leica Biosystems Newcastle Ltd, Newcastle, United Kingdom) was used for antibody detection. 3,3'-diaminodenzidine (DAB) was used as a chromogen and haematoxylin as a counterstain.

In study II, the stained proteins were ARNT (SC-5580; 1:200; Santa Cruz, CA, USA), AHR (ab2770; 1:2000; Abcam, Cambridge, United Kingdom) HIF1-α (610958; 1:100; BD Biosciences, San Jose, CA, USA), $p27^{Kip1}$ (610244; 1:500; BD Biosciences) and cluster of differentiation 34 (CD34) (M7165; 1:50; Dako). AHR staining was scored as negative (0) or positive (1). The staining intensity of ARNT, HIF1-α, and $p27^{Kip1}$ was scaled as negative (0), weak (1), intermediate (2), or high (3). In addition, the fraction of the positively staining cells was evaluated in the case of HIF1-α. Specimens with less than 1% of staining cells were scored as 0, 1-10% of positive cells as 1, 10-50% of positive cells as 2, and more than 50% of positive cells as 3. The density of CD34-vessels per square millimeter was recorded. To avoid imprecision, the mean of two separate vessel counts was calculated in most samples.

In study III, the hormonal status of the mouse pituitary adenomas was assessed by GH (A0570; 1:400; Dako), PRL (A0569; 1:4000; Dako) and ACTH (PA1-36035; 1:2000; AH diagnostics, Århus, Denmark) IHC. In addition, expression of AIP (ab48833; 1:100; Abcam), ARNT (ab14829; 1:50; Abcam), ARNT2 (sc5581/clone M-165; 1:100; Santa Cruz), HIF1-α (NB100-479; 1:200; Novus Biologicals, Littleton, CO, USA), estrogen receptor α (ERα) (ab80922; 1:100; Abcam) and the Ki-67 proliferation marker (ab15580; 1:250; Abcam) was investigated. The AIP protein and the hormone IHCs were scored either as negative or positive. The staining intensity of ARNT, ARNT2 and HIF1-α was scaled as negative (0), weak (1), intermediate (2), or high (3). In the case of macroadenomas, the Ki-67 proliferation index (PI=the number of Ki-67 positive cells among the total number of resting cells) was evaluated from 100-500 tumor cells in the area of strongest expression. If the pituitary tumor contained less than 100 cells, all the cells were counted. Only distinctly stained nuclei were considered as immunopositive. ERα intensity was scaled as negative (0), weak (1), intermediate (2), or high (3). The percentage of ERα positive cells was evaluated on a scale of 0 to 4 (0%=0, 1-25%=1, 26- 50%=2, 51-75%=3, and >75%=4). Finally, the Q-score method (intensity score + % cells stained, range 0-7) was used to quantify ERα expression (Lee *et al.* 2002).

In study V, RET (ab51122; 1:75; Abcam) expression was scored as negative (-) or positive (+). In cases where the tumor stained partly negative and partly positive, the tumor was scored as (+/-).

4. Cell culture studies (II)

4.1 *Aip* silencing and cell proliferation assay

Human embryonic kidney (HEK293) cells, HeLa cells, rat mammosomatotroph GH3 cells and *Aip*-null, heterozygote (HET) and wildtype (WT) mouse embryonic fibroblasts (MEFs) from embryonic day (E) 12.5 mouse embryos were cultured in 95% air, 5% CO_2 at 37°C. HEK293 and HeLa cells were transfected with 30nM duplex siRNA strands of *AIP* siRNA or non-targeting control siRNA

containing a pool of four different oligos (Dharmacon, Lafayette, CO, USA) using Dharma FECT™1 transfection reagents (Dharmacon). GH3 cells were electroporated using AmaxaTM nucleofector (Amaxa Biosystems, Gaithersburg, MD, USA) with 100nM siRNA oligos (Dharmacon). In all studied cell lines, RNA was extracted with the RNeasy Mini Kit (Qiagen), cDNA was produced by standard methods and the relative expression levels of *AIP/Aip* were determined using TaqMan chemistry and the 7500 Fast Real-Time PCR system (Applied Biosystems). *AIP/Aip* TaqMan probes for human, mouse, and rat transcripts were Hs00610222_m1, Mm00479316_m1, and Rn00597273_m1 (Applied Biosystems), respectively. The relative mRNA copy numbers were normalized against the β-actin housekeeping gene (4326315E for human, 4352341E for mouse, and 4352340E for rat transcripts; Applied Biosystems). The proliferative status of *AIP/Aip* siRNA transfected and non-treated HEK293, HeLa and GH3 cells was determined by the MTS assay (G3580; Promega, Madison, WI, USA). Conversion of MTS into formazan was detected at the absorbance of 490nm according to the manufacturer's instructions (Promega). Measurement time points for HEK293 and HeLa cells were from 20h until 72h, and for GH3 the timescale was from 6h until 72h.

4.2 Western blot analyses

Proteins from MEFs, HEK293, HeLa and GH3 cells were extracted with M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) or RIPA buffer (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with proteinase inhibitor (Roche, Mannheim, Germany). Twenty-five μg of protein was loaded into a 10% Tris-HCL gel (Bio-Rad Laboratories). Primary antibodies against AIP (NB100-127; 1:1000 in MEFs and 1:500 in HEK293, HeLa, and GH3 cells; Novus Biologicals) and ARNT (ab14829; 1:200; Abcam) were used. α-Tubulin (T5168; 1:5000; Sigma-Aldrich) was used as a loading control protein. Proteins were visualized using the Amersham™ ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, United Kingdom). Western blot band intensities were calculated with FluorChem™ 8800 using the Spot Denso analysis tool (Alpha Innotech Corporation, San Leandro, CA, USA). The program calculates integrated density values for the bands based on band areas and intensities. ARNT band values were normalized against band values from α-Tubulin of the same sample. Band intensity was reported as a percentage relative to the control siRNA band.

5. Statistical analysis (II, III, IV, V)

In studies II, III and V, Fisher's exact test or the Fisher Freeman-Halton extension test was used to test statistical significances.

In study III, the chi-square ($\chi 2$) -statistic was used to investigate the deviation of genotypes of live *Aip* embryos from the expected Mendelian 1:2:1 ratio. Comparisons between the groups were drawn through the parametric Student's t-test, and in cases of non-normally distributed variables the non-parametric Wilcoxon-Mann-Whitney U test was applied. Correlations were assessed by Spearman rank correlation.

In study IV, continuous data were represented as medians and ranges. For non-normally distributed data, comparisons were made using a non-parametric test, Wilcoxon's signed-rank test. For count data, values were placed in a contingency table and compared with a chi-square test. Where

continuous data were plotted as density graphs, a kernel density approximation was computed using a Gaussian kernel and bandwidth calculated using Silverman's "rule of thumb". The kernel density was finally plotted as a continuous curve.

6. The *Aip* mouse model (III)

6.1 Generation of *Aip* mutant mice

Embryonic stem cells (ES cells) containing the gene trap vector construct in an intronic region of genomic DNA between *Aip* exons 2 and 3 (ENSMUST00000117831) (BayGenomics, University of California, Davis, CA) were injected into blastocysts, and chimeras were then identified (Stanford *et al.* 2001). The inbred mouse strain C57BL/6Rcc was used for generation of congenic mice. Mice were genotyped as *Aip* HET or WT by a multiplex PCR reaction from cDNA or genomic DNA, amplifying the WT and mutant alleles (Figure 4). Total RNA or DNA was extracted from the ear using the RNeasy Mini Kit (Qiagen) or the DNeasy Blood & Tissue Kit (Qiagen) and cDNA was produced according to standard protocols.

6.2 Collection and staining of tissues

HET and WT mice were followed up in a cohort study in which necropsies were performed at three-month intervals, from 3 to 21 months of age. Mice used in the study had 89-100% C57BL/6Rcc genetic background. Analysis in each age group included HET mice and age-matched WT controls from the same litters with both genders represented close to 1:1. Following CO_2 anesthesia and neck dislocation, the pituitary, thyroid/parathyroid, adrenal glands, pancreas, brain, kidneys and liver were dissected. Other tissues were also collected if macroscopic abnormalities were detected. The total weight of the mice and the relative weights of their liver, spleen, and kidneys (organ weight/total weight of mouse x 100) were measured. Small tissues were fixed up to two hours and larger ones overnight in cold 4% paraformaldehyde. The pituitary was retained on top of the skull and fixed for 1.5 hours followed by decalsification of two hours. Fixed tissues were embedded in paraffin and sectioned at 5μm. Haematoxylin-Eosin (HE) staining of the pituitary was performed approximately in every 50μm to thoroughly analyze the histopathological features. From other tissues, all macroscopic abnormalities were further examined at the microscopic level.

6.3 LOH analysis

To assess the presence of LOH, fresh pituitary tumor DNA from two heterozygote mice was sequenced. Multiplex PCR was performed with one forward primer targeted in the WT sequence, another forward primer in the insertion and a reverse primer in the WT sequence. Allelic imbalance was scored by comparing the ratios of the allele peak heights between normal and tumor samples as described previously. The cutoffs for LOH were <0.60 and >1.67 (Canzian *et al.* 1996, Pastinen *et al.* 2004).

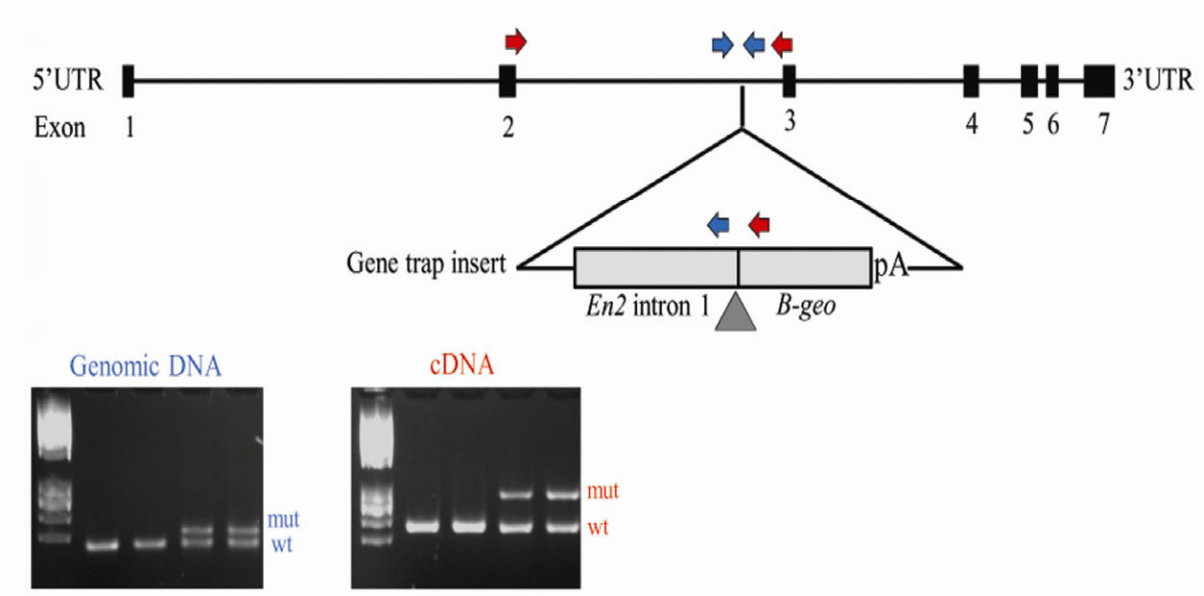

Figure 4. *Aip* mouse construct and genotyping. Genomic *Aip* sequence and gene trap vector construct inserted in IVS2. The gray triangle indicates the splice acceptor site after mouse *En2* intron 1 sequence. The *B-geo* fusion gene contains *β-galactosidase* and *neomycin*, and pA is a polyadenylation signal. Blue arrows designate genomic primers and red arrows designate cDNA primers used in genotyping PCR. Gel pictures show results of genomic and cDNA genotyping with wildtype (wt) and mutant (mut) bands.

6.4 *Igf-1* expression analysis

RNA was extracted from the liver of 18-month-old HET and WT mice with the RNeasy Mini Kit (Qiagen) and cDNA was produced by standard methods. The relative expression of *Igf-1* was determined using TaqMan chemistry and the 7500 Fast Real-Time PCR system (Applied Biosystems). The *Igf-1* probe was Mm00439560 (Applied Biosystems) and the relative mRNA copy numbers were normalized against the *β-actin* housekeeping gene (4352341E; Applied Biosystems).

7. *RET* sequencing and Enhancer Element Locator (EEL) analysis (V)

RET mutation screening was performed on genomic DNA. PCR products were purified using the ExoSAP-IT PCR purification kit (USB Corporation, Cleveland, OH, USA). DNA sequencing was performed using BigDye 3.1 termination chemistry on an ABI3730 DNA sequencer (Applied Biosystems). The whole coding region of *RET* (Ensembl version 55 gene ENSG00000165731, transcript ENST00000340058) and flanking intronic sequences of exons were sequenced, as well as 5'UTRs and 3'UTRs, promoter regions and a non-coding *RET* variant (rs2435357). Evaluation of sequences was done with Mutation Surveyor software v.3.24 (SoftGenetics).

EEL is a computational tool that predicts enhancer elements based on e.g. analysis of transcription factor binding affinity (Hallikas *et al.* 2006). Genomic human and mouse sequences 50 kb up- and

downstream from *RET* and *Ret* (GRCh37:10:43522517:43675799:1 and NCBIM37:6:118051766:118197762:1, respectively) were aligned and the EEL computer algorithms were applied as previously described to detect possible enhancer elements in and around *RET* (Palin *et al.* 2006); 149 previously described transcription factor binding site matrices were used (Hallikas *et al.* 2006, Badis *et al.* 2009, Jaspar2).

8. Ethical issues (I, II, III, IV, V)

Studies I, II and V were approved by the Ministry of Social Affairs and Health and the Ethics Review Committees of the hospital district of Helsinki and Uusimaa.

Permission to use the patient samples (studies I, II and V) was obtained either by appropriate informed consent of patients or by the permission of the National Authority for Medicolegal Affairs.

All the animal experiments were authorized by the appropriate review committee (National Animal Experiment Board) and regulations concerning the use of animals in research were adhered to in study III.

In study IV, all patients provided informed written consent for genetic testing at their medical center, and the study was approved by the Ethics Committee of the Centre Hospitalier Universitaire de Liège.

Results

1. Identification of large genomic deletions in *AIP* (I)

Two out of 21 (9.5%) *AIP* and *MEN1* mutation negative families were found to harbor one distinct deletion each. Copy number changes were not detected either among 32 sporadic Finnish GH-secreting adenoma cases or in 35 Italian pediatric pituitary adenoma patients.

1.1 Exon 2 deletion

A heterozygous *AIP* exon 2 deletion (Ex2del) was found in a British family where the index case had NFPA and his brother had a GH-secreting adenoma. Also, a maternal second cousin had been diagnosed with NFPA, but no clinical data or tumor sample was available. The Ex2del results in the in-frame ablation of 60 amino acids (A34_K93del), which corresponds to three-quarters of the FKBP12-like domain (Meyer *et al.* 1998). Sequencing of the aberrant allele revealed Ex2del as part of a larger deleted fragment of 1562 basepairs (bp). The deletion was absent in 74 healthy population-matched controls. To examine whether Ex2del occurs due to an *Alu*-mediated recombination mechanism, *in silico* search of the chromosome 11 region 67,005,097- 67,015,750 was performed. Interestingly, it revealed the presence of eight *Alu* repetitive elements: four were located upstream of the 5'UTR, and four spanned exon 2. Both breakpoints of the deleted fragment occurred within *Alu* repeats, resulting in the retention of the IVS1 *Alu* repeat in the mutant allele, whereas the most part of the IVS2 *Alu* was lost (Figure 5, see Discussion 1).

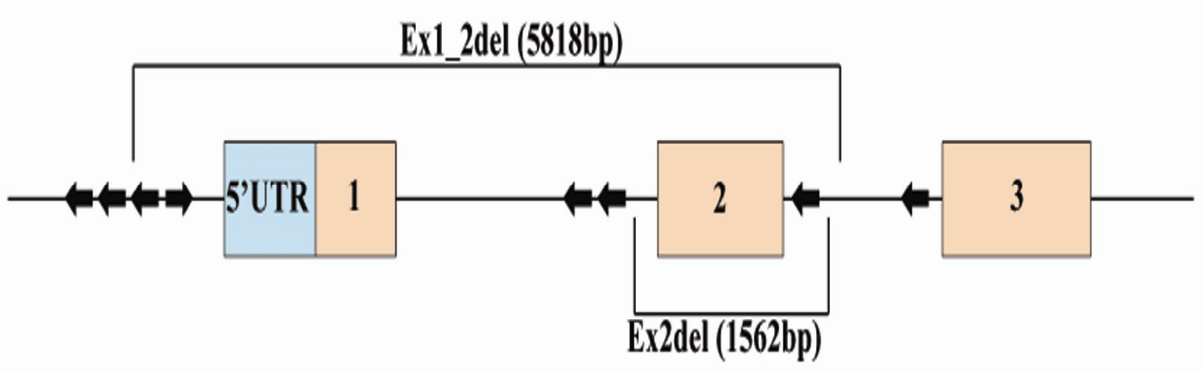

Figure 5. *AIP* and *Alu* repeats. This is a schematic illustration of the 5' *AIP* sequence with black arrows indicating *Alu* repeats flanking exons 1, 2 and 3. Ex1_2del and Ex2del are also depicted.

1.2 Exon 1 and 2 deletion

The second *AIP* deletion was detected in the proband of a previously reported German family with two acromegaly patients (Ackermann *et al.* 1999, Vierimaa *et al.* 2006). The heterozygous deletion encompasses *AIP* exons 1 and 2, including the 5'UTR (Ex1_2del). Sequencing of the aberrant allele revealed a deletion of 5818 bp, encompassing 1104 bp upstream of the 5'UTR and 578 bp of IVS2. The deletion was absent in 22 healthy Caucasian controls. The deletion breakpoints did not occur within *Alu* repeats, but in close proximity, suggesting the involvement of an *Alu*-mediated deletion (Figure 5).

2. Elucidating the expression of AIP-related proteins in pituitary adenomas (II)

To study the impact of *AIP* mutations on the expression of AIP-related proteins, IHC was performed on a set of *AIP*mut+ and *AIP*mut- pituitary adenomas. Since ARNT was found to be underexpressed in *AIP*mut+ adenomas, the ARNT expression was then analyzed in *AIP/Aip* silenced and *Aip* knockout cell lines. Last, the proliferation rates of *AIP/Aip* silenced cell lines were determined.

2.1 Immunohistochemistry results

When negative (score 0) and weak (1) intensities were considered as negative staining and intermediate (2) and high (3) intensities as positive staining, ARNT was found to be more frequently expressed in *AIP*mut- tumors compared with *AIP*mut+ tumors (p=0.001, Table 6). This association remained significant when also weak (1) intensity was considered positive staining (p=0.0016). No correlation between the expression of ARNT and the hormone secretion status of the adenoma was found. By contrast, the expression of ARNT in adjacent normal tissue was consistent in *AIP*mut+ and *AIP*mut- samples. Nuclear AHR was more frequently expressed in *AIP*mut+ samples compared with *AIP*mut- samples, although the result was not statistically significant (p=0.067). The cytoplasmic expression of AHR and the staining intensity and fraction of HIF1-α positive tumor cells were even between tumor types (p=0.51, p=0.09 and p=0.25, respectively). Adjacent normal pituitary tissue did not display HIF1-α staining. Expression of $p27^{Kip1}$ was even between tumor types (p=0.32), and all *AIP*mut+ tumors showed a prominent $p27^{Kip1}$ expression. No statistical difference between the density of CD34-vessels in *AIP*mut+ samples (mean 319.5/mm²) compared with *AIP*mut- samples was detected (mean 253.1/mm²) (p=0.11).

Table 6. ARNT and ARNT2 immunohistochemistry in human and mouse pituitary adenomas.

Protein expression	*AIP*/*Aip* mut+	*AIP*/*Aip* mut-	p-value
Human ARNT	6/13	40/44	0.001[1]
Mouse ARNT	40/54	14/14	<0.00001[1,2]
Mouse ARNT2	14/54	14/14	

[1] Fisher's exact test

[2] loss of either ARNT or ARNT2 in *Aip*-deficient GH-secreting adenomas

2.2 ARNT expression in cell lines

The expression of AIP and ARNT was studied in HEK293, HeLa, GH3 and MEF cells. Quantitative PCR (qPCR) showed lack of *Aip* transcription in *Aip*-null MEFs when compared to HET and WT MEFs. Western blot analysis of an *Aip*-null MEF line confirmed the lack of AIP protein**.** However, *Aip*-null and WT MEF cells showed no differences in the amounts of ARNT in western blot analysis. *AIP* siRNAs reduced the expression of *AIP/Aip* in HEK293, HeLa and GH3 cell lines by approximately 85%, 90%, and 25-87%, respectively. Western blot analysis showed a notable reduction of AIP protein in *AIP* siRNA treated HEK293 and HeLa cells at 48h, but equal presence of ARNT when compared with control siRNA-treated cells. Instead, pituitary adenoma cell line GH3 showed notable reduction of ARNT after *Aip* siRNA transfection by electroporation (with 60% transfection efficiency). At a 6h time point, the ARNT band intensity of *Aip* siRNA-treated cells was 51.6% in contrast to the ARNT band intensity of control siRNA transfected cells. At a 48h time point with 59% transfection efficiency, no reduction of ARNT was observed.

2.3 Cell proliferation

To evaluate the possible effect of *AIP/Aip* silencing on cell proliferation, *AIP/Aip* siRNA-treated HEK293, HeLa and GH3 cells were analyzed with the MTS assay. *AIP* siRNA-treated HeLa cells showed no difference in proliferation compared with control siRNA-treated cells. HEK293 cells showed slightly increased proliferation 72 hours after transfection. In contrast, *Aip* siRNA transfection of GH3 cells resulted in a clearly increased rate of proliferation already at 48h (Figure 6).

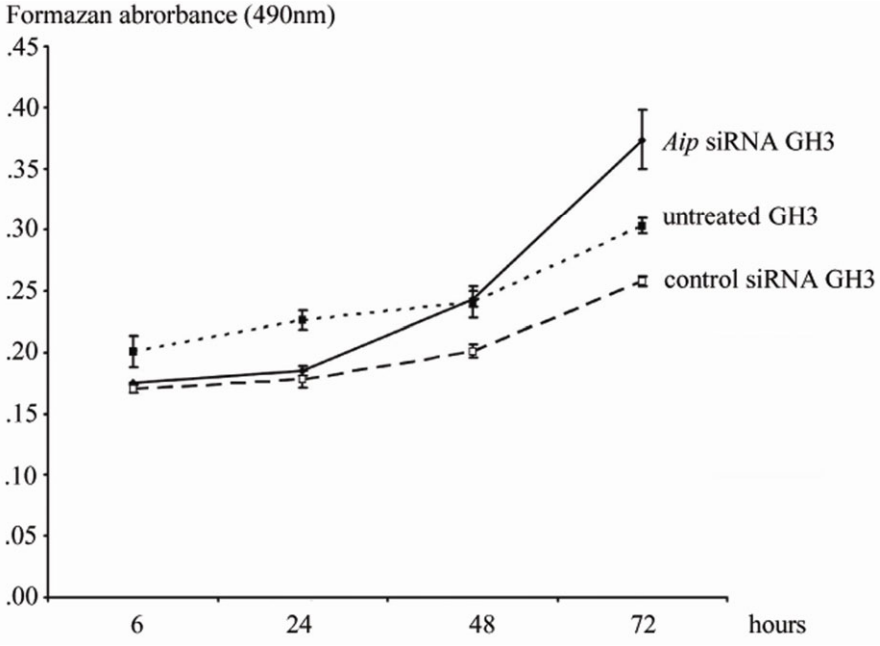

Figure 6. MTS assay shows increased cell proliferation in GH3 cells after *Aip* knockdown with siRNA, compared with control siRNA and untreated cells.

3. Creating *Aip* mutant mice prone to pituitary adenomas (III)

The *Aip* mutation was generated by inserting a gene trap vector construct into an intronic region of genomic DNA between *Aip* exons 2 and 3 (ENSMUST00000117831) (BayGenomics, University of California, Davis, CA) (Stanford *et al.* 2001, Figure 4). The inserted vector construct created an artificial splicing site after 34 codons (34/331). No leakage of the construct was observed when tested by qPCR and western blotting (see study II). The crossings of heterozygous mice yielded one live *Aip*-null, 37 HET, and 22 WT embryos when analyzed at E12.5 stage. This deviates significantly from the expected Mendelian 1:2:1 ratio ($\chi 2=17.97$, $p<0.001$). No living *Aip*-null pups were born.

3.1 Phenotype and tumor spectrum of *Aip* mice

Viability or total weight did not differ between WT and HET mice. The relative weights of the liver, kidneys, and spleen were the same between HET mice and their WT littermates up to 12 months. However, a trend towards increased relative organ weights of ≥15-month-old HET mice was seen compared to WT mice. Observation of 21 months did not reveal excess of any other tumor type than pituitary adenomas in HET mice compared with WT littermates. AIP IHC for tumors of the lung (n=1), liver (n=5) and kidney (n=2) from HET mice was performed, but all showed AIP expression, suggesting that AIP was not associated with the formation of these tumors. A slight excess of macroscopically visible hyperplasia of adrenal glands was detected in HET mice compared with WT mice (p=0.16). Histopathological examination, however, did not reveal any neoplastic growth.

Altogether 88 HET and 58 WT mice were examined. Of HET mice, 69 out of 88 (78.4%) developed one or more pituitary tumors, while only 12 out of 58 WT mice (20.7%) displayed this tumor type ($p<10^{-6}$). HET mice showed the first pituitary lesions at six months of age. Several macroscopically visible macroadenomas were detected among HET mice in older age groups. The pituitary tumor phenotype reached full penetrance in HET mice at the age of 15 months and it was not affected by variable purity of genetic background (89-100% C57BL/6Rcc). No differences in pituitary adenoma formation between sexes was detected (p=0.21). HET mice were prone to developing multiple primary pituitary tumors already at the age of six months, whereas multifocal pituitary tumors were detected among the WT mice at much older age groups (15-21 months). The majority of HET mice developed GH-secreting adenomas (61/69, 88%) (Figure 7). The remaining HET tumors were mostly prolactinomas, but also two mixed GH+PRL adenomas and one ACTH-secreting tumor were seen. The majority of the adenomas in WT mice were prolactinomas (25/27, 92.6%). Also two GH+PRL tumors were detected (7.4%). WT mice did not develop purely GH-secreting adenomas.

3.2 Loss of *Aip* in pituitary tumors

LOH was assessed from fresh tumor tissue of two *Aip* HET mice. The cutoffs for LOH were <0.60 and >1.67 for mutant and WT alleles, respectively (Canzian *et al.* 1996). The allele peak ratios in the normal/tumor pairs were 2.19 and 2.08, indicating reduction of the WT allele in these tumors. Complete loss of the WT allele was not seen due to normal tissue contamination. AIP IHC of all studied GH-secreting adenomas from HET mice revealed negative AIP immunostaining. Also the majority of the HET prolactinomas (22/26, 84.6%) as well as one ACTH-secreting tumor showed lack of AIP. Four HET prolactinomas showed positive AIP staining, suggesting that these lesions are not related to the *Aip* mutation. All WT pituitary adenomas showed positive AIP expression.

3.3 Proliferation index of tumors

Ki-67 IHC analysis was performed to evaluate the proliferation rate of HET and WT tumors. The Ki-67 protein is expressed in all phases of the active cell cycle (G1, S, G2 and M phase), but is absent in resting (G0) cells. HET tumors had a significantly higher proliferation rate compared with WT adenomas (p=0.014). No correlation between age and proliferation rate was detected (Spearman rank correlation; rho=-0.12, p=0.59). There was no clear correlation between the hormonal status of tumors of HET mice and the proliferation index (PI) values (p=0.05, Student's t-test), although *Aip*-deficient prolactinomas showed a higher average PI when compared to *Aip*-deficient GH-secreting tumors, 10.1±3.6 (s.d.) vs. 6.1±4.7 (s.d.), respectively.

3.4 *Igf-1* expression in mice with *Aip*-deficient somatotropinomas

GH functionality of HET somatotropinomas was assessed by measuring the expression of liver *Igf-1* by qPCR. Seven HET mice and 11 WT mice were studied. Three of the WT mice had GH+PRL-secreting adenomas. The mean *Igf-1* expression for seven HET mice with somatotropinomas was 1.9±0.26 (s.d.), and for the eight WT mice without GH-secreting adenomas 1.4±0.22 (s.d.). The relative *Igf-1* expression value for the WT mice with GH-secreting adenomas was 1.8±0.11 (s.d.), thus in line with the expressions measured from the HET mice. Altogether, the HET mice had significantly

elevated *Igf-1* expression levels compared with the WT mice that did not have GH-secreting adenomas (p=0.002, Student's t-test).

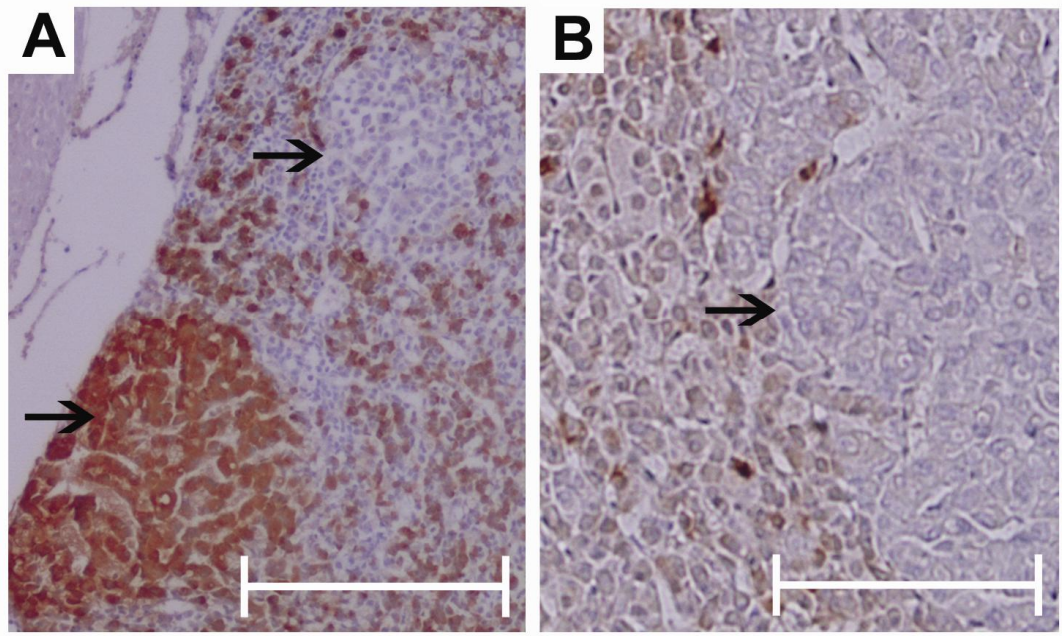

Figure 7. Immunohistochemistry of GH and AIP. (A) One GH positive and one GH negative pituitary adenoma in an *Aip* HET mouse. The GH negative tumor stained positive for PRL in subsequent PRL immunohistochemistry. (B) Negative AIP staining in the pituitary adenoma of an *Aip* HET mouse. Tumors are indicated by black arrows. Scale bars 200µm (A) and 100µm (B).

3.5 Estrogen receptor α expressions in *Aip*-deficient and -proficient tumors

IHC was used to assess the expression of ERα. The study comprised 30 *Aip*-deficient adenomas (14 PRL- and 16 GH-secreting tumors) and eight *Aip*-proficient prolactinomas. All *Aip*-proficient tumors and 29/30 of *Aip*-deficient tumors showed a distinct nuclear expression of ERα. The *Aip*-proficient tumors showed significantly higher ERα expression compared with the *Aip*-deficient adenomas (GH and PRL), mean 4.6 ±0.70 (s.d) vs. 3.8±1.37 (s.d.), respectively (p=0.02, Student's t-test). However, no statistically significant difference between *Aip*-deficient and -proficient tumors was found when only prolactinomas were compared (p=0.11). ERα expression did not correlate with the proliferation rate or gender (rho=-0.31, p=0.65, Spearman rank correlation; p=0.34, Student t-test, respectively). No statistical significance between ARNT or ARNT2 deficiency and ERα expression was detected (p=0.37, Student's t-test). ERα was present in all normal pituitaries and the expression was similar between HET and WT mice, 5.7±0.8 (s.d.) and 5.6±0.7 (s.d.), respectively.

3.6 ARNT/ARNT2 imbalance

ARNT and ARNT2 IHC showed a total lack of ARNT in 14 and of ARNT2 in 40 *Aip*-deficient tumors. GH-secreting *Aip*-deficient tumors more often showed lack of ARNT2 than ARNT ($\chi 2=7.28$, $p<0.007$, Table 6). Remarkably, almost all *Aip*-deficient tumors expressed only ARNT or ARNT2 (49/53, 92.5%, $p<10^{-5}$, Fisher's exact test, Table 6). In contrast, both proteins were present in all *Aip*-proficient tumors (10/10 in WT animals and 4/4 in HET mice). No difference in proliferation rates between *Arnt*-deficient (n=4) and *Arnt2*-deficient (n=9) tumors was detected; median 4% (range 2-8%) vs. 4% (2-10%), respectively (p=0.73, Mann-Whitney U test). To examine the hypoxia response in *Aip*-deficient and -proficient tumors, HIF1-α IHC was performed. A total of 50 *Aip*-deficient and 13 *Aip*-proficient tumors were studied. HIF1-α was present in 46/50 of *Aip*-deficient tumors and in all *Aip*-proficient samples, and staining intensity averages were 1.8±0.9 (s.d.) and 2.0±0.4 (s.d.), respectively (p=0.26, Student's t-test). *Aip*-deficient and -proficient prolactinomas showed significantly higher HIF1-α expression when compared with *Aip*-deficient somatotropinomas (p=0.002). Expression of HIF1-α was found to be even between ARNT and ARNT2 negative tumors. (p=0.92, the Freeman-Halton extension of Fisher's exact probability). Similarly, there was no correlation between HIF1-α intensity and the proliferation rate (p=0.51, Mann-Whitney U test). Adjacent normal pituitary tissue had weak cytoplasmic or absent HIF1-α staining.

4. Assessing clinical characteristics of PAP (IV)

The study population comprised 96 *AIP*mut+ pituitary adenoma patients with 43 separate *AIP* mutations. Most patients presented in FIPA kindreds (59.4%) and the rest had no known familial background of pituitary adenomas. There were no statistical differences in clinical or therapeutic characteristics among patients with different types of *AIP* mutations.

4.1 PAP patient demographics

The *AIP*mut+ population was predominantly male (63.5%) and the median age at first symptoms was 18.0 years, indicating that half of the patients were children or adolescents at clinical onset. Tumors were overwhelmingly macroadenomas (93.3%) including 12 giant adenomas, and 56.3% had invaded local structures at diagnosis. No statistically significant differences existed between characteristics in *AIP*mut+ male and female patients.

4.2 Characteristics of *AIP* mutation positive pituitary adenomas

Somatotropinomas were diagnosed in 78.1% of *AIP*mut+ patients. This *AIP*mut+ group comprised mainly of males (61.3%), and there was a significantly higher male-to-female ratio than in *AIP*mut- controls (p=0.027). The median age at first symptoms was 20.5 years earlier in *AIP*mut+ patients vs. controls ($p<0.000001$); first symptoms occurred as children or adolescents in 52.2% of the *AIP*mut+ cohort. Similarly, the *AIP*mut+ cohort was diagnosed nearly two decades before *AIP*mut- controls ($p<0.000001$). Gigantism was more frequent in the *AIP*mut+ cohort ($p<0.000001$). All 24 patients with gigantism in the *AIP*mut+ group were males as compared with 10/15 (66.7%) male patients with gigantism in the control group. The median maximum tumor diameter was larger (p=0.00026) and the

proportion of patients with macroadenomas was higher in the *AIP*mut+ group (p=0.026). Giant adenomas were seen in 9.3% of the *AIP*mut+ group as compared with 1.3% among controls. There was a higher frequency of extrasellar extension (p=0.018) and a trend towards more frequent invasion of local structures in the *AIP*mut+ cohort versus controls. Larger tumor size in the *AIP*mut+ group was associated with significantly higher median levels of GH at diagnosis than in controls (p=0.00068); median IGF-I levels did not differ (p=0.48). Co-secretion of GH and PRL was nearly twice as frequent in the *AIP*mut+ group as it was in controls (p=0.00023).

Thirteen *AIP*mut+ patients had prolactinomas. Most of these patients were male (76.9%). Patients had young median ages at first symptoms (18.0 years) and diagnosis (22.0 years) and median prolactin levels at diagnosis were 2520.0ng/ml; range 74.0-60,000.0ng/ml. Median maximum tumor diameter was large (31.0mm; range: 6.0-85.0mm), 12/13 tumors were macroadenomas and 11 of these had extrasellar extension and nine were invasive at the time of diagnosis.

There were also seven patients with NFPAs in the *AIP*mut+ cohort. The median age at diagnosis was younger than commonly described for this disease (31.0; range: 12.0-74.0 years) (Ferrante *et al.* 2006). All tumors were macroadenomas; six had suprasellar extension and four were invasive at diagnosis. Two patients presented with pituitary apoplexy. At diagnosis, all patients had mildly elevated prolactin levels. In addition, one patient had hypogonadism and one had hypofunction of the adrenal, thyroid and gonadal axes at the time of diagnosis.

In addition, one *AIP*mut+ patient presented with elevated T_3 and T_4 levels and a normal TSH level, and had a non-invasive pituitary macroadenoma detected by MRI. No other hormonal abnormalities were noted at diagnosis.

4.3 Response to therapy

In somatotropinomas, combinations of different treatment modalities or duration of follow-up post-diagnosis did not differ between *AIP*mut+ and *AIP*mut- groups. Among 71 *AIP*mut+ somatotropinoma patients with >12 months of follow-up, disease control was achieved in 50 cases (70.4%) and acromegaly remained active in 21 cases (29.6%). The long-term disease control rate was higher in control patients (182/226; 80.5%) (p=0.06). Among patients with a higher cumulative treatment burden (≥3 distinct modalities), long-term disease control rates were poorer in the *AIP*mut+ group versus controls (p=0.01). A similar proportion of patients had pituitary neurosurgery in the *AIP*mut+ and control groups; re-operation was significantly more frequent in the *AIP*mut+ group than in controls (p=0.00069). There was a trend toward more frequent use of radiotherapy in the *AIP*mut+ group than in controls (p=0.15). Percentage reduction in GH and IGF-I was similar for primary, pre- and post-operative SSA use in both groups. In the *AIP*mut+ group the median SSA-induced reduction in GH and IGF-I was lower than that seen in the control patients treated with SSA (p=0.0004 and p=0.028, respectively). The median magnitude of tumor shrinkage achieved with SSA was significantly higher in the control group vs. *AIP*mut+ patients (p<0.000001). Concomitant radiotherapy use was similar among patients that were controlled versus not controlled by SSA in the two groups. Unlike in the *AIP*mut+ group where three of four patients were uncontrolled by pegvisomant therapy, all 19 control acromegaly patients that received pegvisomant therapy had

controlled IGF-I levels at follow-up. The frequency of hypopituitarism was similar in the *AIP*mut+ and control groups, but the *AIP*mut+ group had a significantly higher number of deficient axes than control patients ($p<0.000001$).

In *AIP*mut+ prolactinomas, all but one patient received primary dopamine agonist therapy, which was associated with reduction of 50-99% from baseline PRL. Initial normalization of PRL secretion occurred in five cases; one patient developed secondary dopamine agonist resistance and tumor growth despite high-dose cabergoline and needed two transsphenoidal surgeries plus radiotherapy to achieve disease control. Six patients were initially uncontrolled with dopamine agonists and underwent surgery, one of which underwent three transsphenoidal and one transcranial intervention plus radiotherapy, while another two patients had two surgical interventions each. Radiotherapy was eventually undertaken by three operated patients. Long-term control of PRL secretion was achieved in 8/13 (61.5%) patients and two patients developed hypopituitarism.

In *AIP*mut+ NFPAs, six patients underwent surgery and one patient who underwent a transcranial approach received radiotherapy due to a large remnant. Long-term control of tumor size was achieved in all cases. All patients had mildly elevated prolactin levels at diagnosis, and in three patients that received dopamine agonists, two achieved normal prolactin levels (no tumor shrinkage). At diagnosis, one patient had hypogonadism and one had hypofunction of the adrenal, thyroid and gonadal axes, which did not resolve post-therapy.

The patient with a TSH-secreting adenoma underwent transsphenoidal surgery twice, followed by octreotide treatment, which resulted in hormonal normalization but no change in residual tumor size.

5. Evaluating the role of *RET* in familial pituitary adenomas (V)

Patients from 16 *AIP*mut- pituitary adenoma families were screened for *RET* germline mutations to assess whether *RET* could play a role in pituitary adenoma predisposition, similar to *AIP*. The *RET* region was also analyzed with EEL to identify *RET* regulatory elements and to see if the found *RET* changes resided in these. Finally, expression of RET was examined in *AIP*mut- and *AIP*mut+ somatotropinomas by IHC.

5.1 *RET* heterozygous changes

Five novel heterozygous *RET* variants were found when sequencing the *RET* region. A heterozygous c.785T>C (V262A) change was found in *RET* exon 4 in an Italian prolactinoma patient. However, this variant was absent in the patient's acromegalic aunt, and the change was not found in 41 Italian controls. A heterozygous 3'UTR change c.1560*G>A was detected in a British prolactinoma patient and her sister with prolactinoma. The change was not found in an unaffected sister or in 279 British Caucasian controls. In the same patient we also found an unreported heterozygous IVS17+105delG. However, the affected sister did not share the deletion, and it was also found in the unaffected sister and 10 of 91 analyzed British Caucasian controls. A heterozygous -1285G>A change upstream of *RET* was found in a British NFPA patient of Vietnamese origin. It was also present in the patient's

mother with NFPA. No Asian controls were available. The mother also had a heterozygous -1491C>T change that was not present in her child.

5.2 EEL analysis

The EEL tool was used to predict whether the two segregating *RET* variants, c.1560*G>A and -1285G>A, could reside in putative enhancer elements of the *RET* region. The hundred highest scored alignments were mapped in the *RET* region. No overlap between the variants and the predicted enhancer elements was found. The highest scored element (score 258.42) was a 295 bp long fragment beginning 5624 bp upstream of *RET* and comprising nine putative transcription factor binding sites (Figure 8). We also screened a previously described non-coding *RET* variant (rs2435357 C>T) associated with the risk of Hirschsprung's disease and situated within a conserved enhancer-like sequence in *RET* intron 1. The disease-associated allele is T and the wild-type allele is C (Emison *et al.* 2005). The T/T genotype was detected in three samples, T/C alleles were present in three samples and C/C in ten samples. Near this variant, EEL predicted a putative enhancer element (score 184.23) of 152 bp and comprising five transcription factor binding sites. The element begins IVS1+9343, which is 66 bp downstream of rs2435357 (IVS1+9277) (Figure 8).

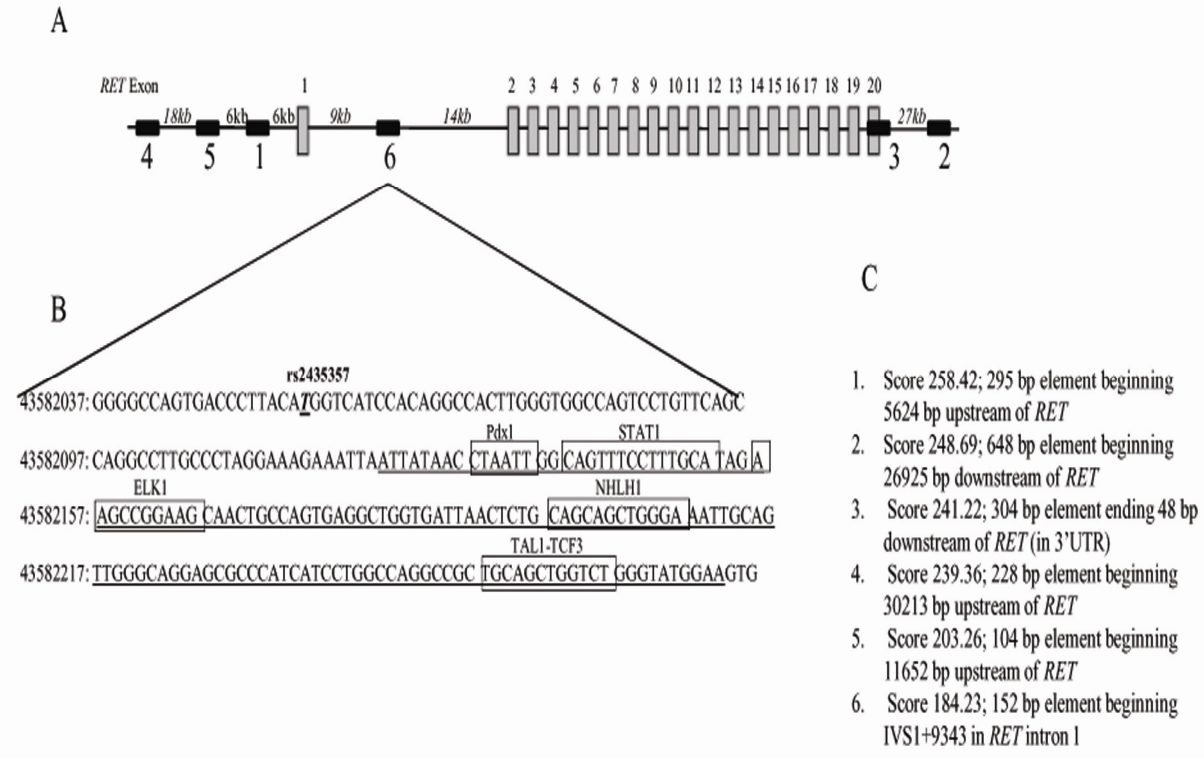

Figure 8. Results of EEL analysis. (A) Schematic illustration of *RET* gene with exons as gray boxes and putative enhancer elements as black boxes numbered according to best scores. (B) *RET* IVS1 sequence with rs2435357 (bold, underlined) and the sixth element (underlined) with putative transcription factor binding sites (boxes).(C) Details of the six highest scoring elements predicted by EEL.

5.3 RET immunohistochemistry

RET expression was positive (+) in 9/10 (90.0%) *AIP*mut- somatotropinomas. One *AIP*mut- somatotropinoma stained partly negative and partly positive (+/-). *AIP*mut+ somatotropinoma results were as follows: negative (-) in 3/7 (42.9%) samples, (+/-) in 2/7 (28.6%) samples and (+) in 2/7 (28.6%) samples. When considering (-) staining vs. (+) and (+/-) staining with Fisher's exact test, the two-sided p-value was 0.05.

Discussion

1. Large genomic *AIP* deletions account for a subset of *AIP* mutations (I)

The development of the MLPA technique has emerged as a methodological advance for identification of large genomic rearrangements (Schouten *et al.* 2002, Taylor *et al.* 2003). Recent studies have identified large genomic deletions in genes underlying familial pituitary adenoma, such as *MEN1* and *PRKAR1A* (Kikuchi *et al.* 2004, Fukuuchi *et al.* 2006, Horvath *et al.* 2008). However, the possibility of such deletions in *AIP* has remained unsolved. Previously, an MLPA assay with custom-made probes for *AIP* and *MEN1* was applied on pituitary tumor samples (Barlier *et al.* 2007). In one out of 41 tumors, a germline R22X mutation was found in one *AIP* allele, and the other allele showed *AIP* LOH. A tumor sample from another patient had LOH of both *AIP* and *MEN1*, but no *AIP* or *MEN1* mutations were found in the other allele (Barlier *et al.* 2007). Thus, no germline *AIP* deletions have been found so far. In this study, for the first time, two out of 21 families were found to harbor large germline *AIP* deletions (Table 5, Figure 5).

The *AIP* Ex2del (1562 bp) was identified in a family with NFPAs and GH-secreting adenomas from the UK, resulting in an in-frame ablation of 60 amino acids (A34_K93del) of *AIP*. It is likely that this large deletion (60/330 aa) leads to aberrant AIP protein function. The second *AIP* deletion, Ex1_2del (5818 bp), encompasses the 5' end of *AIP*. It was found in previously reported German family with two acromegaly patients (Ackermann *et al.* 1999, Vierimaa *et al.* 2006). As Ex1_2del comprises a deletion of the whole 5' end of the gene, it is predicted to be functionally equivalent to a whole gene deletion since the translation initiation codon and most likely part of the promoter region are lost.

Alu elements are retrotransposons of the genome that are capable of generating genomic rearrangements such as deletions through e.g. the ectopic recombination between non-allelic homologous *Alu* elements (Cordaux & Batzer 2009). Partial and whole-gene deletions have previously been explained by the occurrence of *Alu*-mediated recombination events, for instance in *LKB1*, *BRCA1*, *MLH1*, *MSH2*, *BRCA1* and *MEN1* tumor suppressor genes (Nystrom-Lahti *et al.* 1995, Mauillon *et al.* 1996, Petrij-Bosch *et al.* 1997, Puget *et al.* 1997, Kikuchi *et al.* 2004, Fukuuchi *et al.* 2006, Volikos *et al.* 2006). We examined whether the identified *AIP* deletions could be explained by a similar mechanism. An *in silico* search revealed the presence of eight *Alu* repetitive elements; four were located upstream of the 5'UTR and four spanned exon 2 (Figure 5). Indeed, an *Alu*-mediated recombination event may be likely for Ex2del since the deletion breakpoints are flanked by two reversely oriented *Alu* elements that have 89% sequence identity. This event would result in the retention of the IVS1 *Alu* repeat in the mutant allele, whereas most of the IVS2 *Alu* would be lost (Figure 5). The deletion breakpoints of Ex1_2del did not occur within *Alu* repeats, but in close proximity, so we hypothesize that this deletion might also occur due to *Alu*-mediated recombination between the *Alu* repeats upstream of the 5'UTR and the repeats in IVS2 (Figure 5).

No copy number changes were detected among 35 sporadic Italian pediatric pituitary adenoma patients or 32 sporadic Finnish GH-secreting adenoma cases, although the patient series had been enriched for young cases, diagnosed at 40 years of age or less. According to previous reports, small intragenic germline *AIP* mutations are rare in sporadic pituitary adenoma patients (Barlier *et al.* 2007,

Cazabat *et al.* 2007, Georgitsi *et al.* 2007a). The present study suggests that the same could to be true for large genomic germline *AIP* alterations.

The MLPA technique has certain disadvantages. For example, it merely allows the determination of relative copy number changes without identifying, for example, deletion breakpoints (Taylor *et al.* 2003). Moreover, deletions may even remain undetected if their breakpoints occur outside a probe's hybridization sequence (Knappskog *et al.* 2006). False negative results may also be due to contaminations, to which the MLPA assay is sensitive. It has also been reported that sequence variations at the binding sites of probes may prevent probe hybridization, leading to false positive results for deletions (MRC-Holland). In our set of samples, false positive results are highly unlikely since direct *AIP* sequencing covering the hybridization sites had been performed previously and the exact deletion breakpoints were successfully characterized by long range PCR. In conclusion, positive MLPA findings should optimally be further validated by sequencing on genomic DNA or cDNA level.

This study shows, for the first time, that large genomic deletions in *AIP* underlie a subset of PAP. Special techniques such as MLPA, although not simple to perform, give the possibility to thoroughly screen for large alterations in *AIP*. We conclude that MLPA could be applied in PAP-suspected young patients with GH-secreting adenomas undergoing *AIP* genetic testing if conventional sequencing detects no *AIP* mutation.

2. ARNT is underexpressed in *AIP* mutation positive pituitary adenomas (II)

Although it is generally acknowledged that germline *AIP* mutations cause PAP, little is known about the exact molecular mechanisms leading to tumorigenesis (Vierimaa *et al.* 2006). AIP has multiple cellular interaction partners and, thus, *AIP* mutations have the potential to interfere with a large array of cellular and environmental signals (Table 4). In this study, IHC of *AIP*-related molecules was applied to compare their expression in *AIP*mut+ and *AIP*mut- pituitary adenomas (Table 5). The xenobiotic response pathway involving AIP, AHR, ARNT and a DRE target gene *CDKN1B1* ($p27^{Kip1}$) was studied (Hankinson 1995, Carver & Bradfield 1997, Kolluri *et al.* 1999, Figure 3, see Review of the Literature 5.2.1). Also the hypoxia pathway, including the ARNT-HIF1-α complex function, was evaluated with HIF1-α and CD34 IHC (Gu *et al.* 2000, Figure 3, see Review of the Literature 5.2.1). Our main finding was the reduced expression of ARNT in *AIP*mut+ pituitary adenomas compared to *AIP*mut- samples (p=0.001, Table 6). Also a trend for increased nuclear expression of AHR in *AIP*mut+ adenomas was perceived, although the finding was not statistically significant (p=0.06). In a study by Jaffrain-Rea *et al*., no nuclear immunostaining of AHR was detected in *AIP*mut+ adenomas (Jaffrain-Rea *et al.* 2009). Such a discrepancy might arise from differences in the IHC protocol or between the types of mutations studied. In contrast, another study by Nakata *et al*. observed weak nuclear accumulation of AHR after *Aip* siRNA treatment in an *Arnt*-deficient mouse hepatoma cell line, supporting our observation (Nakata *et al.* 2009).

Although AHR and ARNT expression was altered between *AIP*mut+ and *AIP*mut- adenomas, the expression of the target gene $p27^{Kip1}$ was uniform between the two groups of tumors. In fact, all seven successfully stained *AIP*mut+ tumors showed prominent $p27^{Kip1}$staining (intensity score 3). In the

*AIP*mut- tumors, there was more variation in intensities (scores 0 to 3). Similarly, in previous reports, $p27^{Kip1}$ has been underexpressed or even lost in human pituitary tumors (Lidhar *et al.* 1999). However, additional *AIP*mut+ adenomas would be needed to ascertain the role of $p27^{Kip1}$ in AIP-mediated tumorigenesis.

Hypoxia affects the expression of oxygen-responsive genes, leading to upregulation of angiogenic factors and downregulation of inhibitors of angiogenesis (Ameln *et al.* 2005, Yoshida & Teramoto 2007). Hypoxia in tumors leads, for example, to hypoxia-induced hematopoiesis and subsequent increases in the oxygen supply of the neoplasm (Hao *et al.* 2004). This process involves e.g. the stabilization of HIF1-α, which correlates with the expression of the endothelial marker CD34 (Wei *et al.* 2006). Previously, HIF1-α has been reported to be present in all types of pituitary adenomas (Vidal *et al.* 2003). Indeed, it is suggested that the exposure of pituitary adenoma cells to hypoxia may be a major driving force favoring tumor progression (Yoshida & Teramoto 2007). In this study, the expression of HIF1-α was found to be similar between *AIP*mut+ and *AIP*mut- adenomas. Moreover, we applied IHC of CD34 to detect possible changes in vasculature resulting from HIF1-α-ARNT complex imbalances and for cues of possible differences in the hypoxia responses of *AIP*mut+ and *AIP*mut- pituitary adenomas. The expression and density of CD34-positive vessels was found to be high and uniform in both tumor types (p=0.11). However, the standard deviation of CD34-vessels was high (319.5/mm^2 ± 140.0 in *AIP*mut+ samples and 253.1/ mm^2 ± 120.8 in *AIP*mut- samples). This was due to the small size of the adenoma pieces, which made the analysis technically challenging. Nevertheless, the high vessel density detected in *AIP*mut+ tumors with underexpression of ARNT suggests that the hypoxia response is functional despite the lack of ARNT. Obtaining of tumor material is the limiting factor in studies of *AIP*mut+ adenomas. Future research with a larger set of tumors would be desirable to assess possible differences in the hypoxia responses of *AIP*mut+ and *AIP*mut- adenomas.

2.1 Implications of ARNT in other cellular pathways

It has been reported that only 15% of the ARNT pool is sequestered by HIF1-α when the pathway is saturated and that induction of expression of certain HIF1-α target genes requires only small amounts of HIF1-α-ARNT (Pollenz *et al.* 1999, Tomita *et al.* 2000). Thus, it is possible that when ARNT expression is not totally abolished, a required amount of ARNT exists to induce the expression of hypoxia-regulated genes. On the other hand, the ARNT homolog ARNT2 has been shown to be able to compensate for the lack of ARNT through binding with HIF1-α in response to hypoxia (Keith *et al.* 2001, Sekine *et al.* 2006, Figure 3, see section 3.1).

ARNT is a constitutively stable protein and it is ubiquitously present in nearly all cell types (Aitola & Pelto-Huikko 2003). Interestingly, ARNT has been implicated in diseases such as diabetes, breast cancer and lung cancer (Gunton *et al.* 2005, Kang *et al.* 2006, Weir *et al.* 2007). Also an *ETS variant gene 6* (*ETV*)-*ARNT* fusion gene has been reported in T-lymphoblastic and acute myeloblastic leukemias (Salomon-Nguyen *et al.* 2000, Otsubo *et al.* 2010). These remarks support our assumption that ARNT may also have a role in pituitary pathogenesis.

The downregulation of ARNT in *AIP*mut+ tumors could be connected to an imbalance in the AHR/ARNT complex formation arising from aberrant cAMP signaling, which is often detected in pituitary tumors (Boikos & Stratakis 2007b). It was recently introduced that PDE2A is targeted by AIP to the cytoplasmic AHR complex (de Oliveira *et al.* 2007). Consequently, PDE2A inhibits the nuclear translocation of AHR by lowering the local cAMP levels (de Oliveira *et al.* 2007). Hence, it is conceivable that the lack of functional AIP in *AIP*mut+ tumors could result in the nuclear abundance of AHR in the presence of elevated cAMP levels. It has also been shown that cAMP-mediated AHR is able to adopt a unique structure and prevent the formation of the AHR-ARNT complex in the nucleus (Oesch-Bartlomowicz *et al.* 2005). Disturbances in AHR-ARNT and possibly HIF1-α-ARNT complexes may unbalance the transcription of specific target genes leading to pituitary tumorigenesis in *AIP*mut+ individuals.

2.2 ARNT underexpression requires a pituitary tumor environment

Our *Aip* siRNA assay showed partial reduction of ARNT in the GH3 mammosomatotroph cell line, but not in the non-pituitary cell lines (*Aip*-null and WT MEFs or *AIP* siRNA-treated HEK293 or HeLa cells) studied. Reduction of ARNT was detected already six hours after the electroporation-based *Aip* siRNA silencing. However, ARNT protein levels were restored 48 hours after the knockdown of *Aip*. These results suggest that ARNT might have a tissue-specific role in AIP-related tumorigenesis of the pituitary. GH3 was also the only cell line where we were able to show that knockdown of *Aip* leads to a clear increase in the cell proliferation rate, supporting the tumor suppressor role of *AIP* (Figure 6). This finding is in line with previous work by Leontiou *et al.*, who noticed that overexpression of wildtype AIP in HEK293, GH3 and human diploid embryonic lung fibroblast TIG3 cells led to a reduction in cell proliferation (Leontiou *et al.* 2008).

3. *Aip* heterozygous mice are extremely prone to pituitary adenomas (III)

Recently, Lin *et al.* published the first *Aip* mouse model, in which homozygous germline mutations in *Aip* were embryonic lethal. However, the possible tumor formation in these mice was not reported (Lin *et al.* 2007, Lin *et al.* 2008). Thus, we decided to generate an *Aip* mouse to study for the first time the tumor spectrum of *Aip* HET mice and to model the PAP phenotype. We were successfully able to show that *Aip* HET mice had a substantially increased incidence of pituitary adenomas. First tumors in *Aip* HET mice were detected as early as six months of age (Figure 7). No tumors were detected at three months. This could be explained by the true rarity of pituitary adenomas in this age group or possibly by the lesions being too small to be detected with routine stainings. No excess of any other tumor types than pituitary adenomas were detected when comparing *Aip* WT and HET mice. GH-secreting adenomas dominated in the *Aip* HET group, although prolactinomas, two mixed GH+PRL, and one ACTH-secreting adenoma were also seen. Compared to human *AIP* mutation carriers, mixed GH/PRL adenomas were proportionally a less frequent tumor type in the *Aip* mouse. It has been estimated that the percentage of mammosomatotrophs is relatively high in the human pituitary (~25-50%) (Lloyd *et al.* 1988). In mice, however, the proportion of these cells has been reported to be much smaller (less than 20%) (Seuntjens *et al.* 2002). This difference between species may explain the relatively low frequency of mixed adenomas in *Aip* HET mice. All in all, the *Aip* mouse is extremely prone to pituitary adenomas also when comparing it with other mouse models

available (Williams *et al.* 1994, Nakayama *et al.* 1996, Franklin *et al.* 1998, Asa 2001, Crabtree *et al.* 2001, Yin *et al.* 2008, Table 7).

Overall, the *Aip* mouse model greatly resembles human PAP with a close to identical tumor phenotype with predominance of somatotropinomas. This suggests that the factors underlying *Aip/AIP*-deficient tumorigenesis are similar in both species (Vierimaa *et al.* 2006, Daly *et al.* 2007b, Georgitsi *et al.* 2007a, Leontiou *et al.* 2008). Full penetrance of pituitary tumors in *Aip* HET mice was reached at 15 months, emphasizing the fundamental importance of AIP for tumorigenesis in this organ. However, the penetrance of pituitary adenomas in PAP patients is distinctly lower than in *Aip* HET mice (15-45% vs. full penetrance, see section 4). This could be due to species-specific differences in the susceptibility to pituitary adenomas, although the overall prevalence of pituitary adenomas has been estimated to be quite similar between mice and humans. Indeed, 21% of *Aip* WT mice developed pituitary adenomas, while the overall prevalence of these lesions in humans is estimated to be 17% (14% in autopsy studies and 23% in radiographic studies) (Ezzat *et al.* 2004). Another explanation in the difference of penetrances could be the difficulty in comparing the lifespan of mice and humans.

As noted above, mouse models have been widely used to study pituitary development, function and disease, and recently Lin *et al.* published an *Aip* mouse model revealing that homozygous germline mutations in *Aip* are embryonic lethal and that these embryos die *in utero* due to cardiovascular malformations. Moreover, most mice with reduced *Aip* expression showed a patent ductus venosus resulting in reduced liver size. However, the possible tumor formation of these mice was not reported (Lin *et al.* 2007, Lin *et al.* 2008). In our study, *Aip* HET mice had the same or even slightly increased relative liver weights compared with their WT littermates. This discrepancy between the studies could be explained by differences in the strategy of generation or location of the germline *Aip* mutation, or possibly by the different C57BL substrains used for inbreeding (Strachan & Read 2004 p.605, Lin *et al.* 2007, Lin *et al.* 2008). In our study, crossings of *Aip* HET mice yielded one live *Aip*-null, 37 *Aip* HET and 22 *Aip* WT embryos when analyzed at E12.5 stage. This deviates significantly from the expected Mendelian ratio ($p<0.001$). No living *Aip*-null mice were born. These results are in accordance with the previous study, where half of the *Aip*-null embryos died before E10.5 and none of the remaining fetuses survived past E14.5 (Lin *et al.* 2007).

Acromegaly, which results from excessive GH secretion, is known to cause overgrowth of bones, joints and soft tissues in humans (Heaney & Melmed 2004). GH regulates the expression of *Igf-1* in the liver and causes elevated systemic IGF-I levels that mediate the effects of the secreted GH (Ohlsson *et al.* 2009, Sane in Välimäki *et al.* 2009 p.82-84). It was shown that the expression of *Igf-1* in the liver of somatotropinoma-bearing *Aip* HET mice was higher than in control animals, clearly indicating that the GH secreted by *Aip*-deficient somatotropinomas is functional. We also detected signs of increased relative organ weights in *Aip* HET mice (≥15 months), although these weight differences were not statistically significant. However, the lack of significance may be due to the relatively small number of mice in each group.

Table 7. Mouse models that are prone to pituitary adenomas.

Defective gene	Pituitary adenomas and other phenotypic effects in mice	Reference
Men1	26% PRL-secreting adenomas (heterozygous mice), tumors of the pancreas, parathyroid, thyroid and adrenal cortex	Crabtree *et al.* 2001
Prkar1a	48% different pituitary tumor types (tissue-specific knockout mice), no other phenotypic effects	Yin *et al.* 2008
p27(Kip1)	~50% intermediate lobe pituitary tumors (knockout mice), e.g. enlarged size, female sterility, retinal dysplasia	Nakayama *et al.* 1996
Rb	Full penetrance of intermediate lobe pituitary tumors (knockout chimeras), e.g. cataracts, hyperplasia of the adrenal medulla, enlarged cells in liver	Williams *et al.* 1994
p18(Ink4)	Nearly full penetrance of intermediate lobe pituitary tumors (knockout mice), e.g. increased size, hyperplasia of the spleen	Franklin *et al.* 1998

3.1 Aberrant ARNT/ARNT2 expression in *Aip*-deficient pituitary adenomas

IHC of ARNT and ARNT2 revealed the total lack of either protein in *Aip*-deficient mouse pituitary tumors compared with *Aip*-proficient tumors. This finding parallels our earlier work where ARNT protein was significantly reduced in human *AIP*mut+ pituitary tumors (see study II). Strikingly, we found out that nearly always there was loss of either ARNT or ARNT2, in a mutually exclusive manner, in *Aip*-deficient tumors ($p<0.00001$, Table 6). The presence of HIF1-α indicated the activation of the hypoxia response both in *Aip*-deficient and -proficient adenomas. The level of HIF1-α was higher in *Aip*-proficient prolactinomas than in *Aip*-deficient somatotropinomas, and it has accordingly been reported in humans that prolactinomas have a tendency to show a higher HIF1-α protein expression level than GH-secreting adenomas (Yoshida *et al.* 2005). In addition, all five stained *Aip*-deficient prolactinomas showed strong HIF1-α staining.

Tumors often constitute a hypoxic environment, reflecting their increased demand for oxygen. Although hypoxia is toxic to cells, cancer cells may undergo genetic and adaptive changes allowing their survival and proliferation. Under hypoxia, ARNT can heterodimerize with HIF1-α to generate an active complex HIF1 that regulates the expression of target genes. Thus, ARNT has an essential part in the response to hypoxia. HIF1-α has been shown to contribute to proliferation, angiogenesis, metastasis and resistance to radiation therapy of tumors (Graeber *et al.* 1996, Gu *et al.* 2000, Harris 2002, Rankin & Giaccia 2008, Figure 3). ARNT2 is a homolog of ARNT that was initially discovered to be expressed in neural tissue and the kidney (Hirose *et al.* 1996). In this study, we showed that ARNT2 is also expressed in the pituitary. While much is known about the function of ARNT, the dimerization partners and roles of ARNT2 are less clear. Interestingly, ARNT2 has been shown to be able to compensate for the lack of ARNT through binding with HIF1-α, but seemingly has a limited

ability to influence AHR-mediated signaling (Hirose *et al.* 1996, Maltepe *et al.* 2000, Sekine *et al.* 2006, Dougherty & Pollenz 2008, Figure 3). However, other studies have suggested that AHR and ARNT2 are dimerization partners (Kretzschmar *et al.* 2010, Lee *et al.* 2011, Figure 3). ARNT proteins and AIP are not known to be direct interaction partners, and the connection between both ARNT proteins and AIP is through AHR (Figure 3). However, the effect of this connection is currently unclear both in physiology and tumorigenesis, and the roles of these proteins have not been thoroughly defined in previous studies. Overall, while the mechanism behind the lack of ARNT or ARNT2 observed in *Aip*-deficient pituitary adenomas remains to be elucidated, it can be suggested that signaling through these proteins is a key factor in pituitary tumorigenesis after loss of AIP function.

Interestingly, previous knockout mice models of *Aip*, *Arnt*, *Arnt2*, *Hif1-α* and *Ahr* show signs of e.g. vascular and pituitary abnormalities (Fernandez-Salguero *et al.* 1995, Maltepe *et al.* 1997, Kotch *et al.* 1999, Hosoya *et al.* 2001, Lin *et al.* 2007, Table 8). However, not much is currently known about the expression of hypoxia target genes in pituitary adenomas. It has been suggested that the expression of, for example, the hypoxia target gene vascular endothelial growth factor (VEGF) would not be an important vasculogenic pathway in pituitary adenomas under hypoxia (Kim *et al.* 2005). It has also been reported that glucose transporter 1 (GLUT1), another hypoxia responsive gene, is under the regulation of GH (Tai *et al.* 1990). Therefore, the expression of these genes would not necessarily correlate exclusively with the hypoxia in pituitary adenomas, and that is why the expression of hypoxia responsive genes was not studied in this work.

Table 8. Phenotypes of homozygous knockout mouse models of genes encoding AIP-related proteins.

Defective gene	Phenotype	Reference
Aip	Embryonic lethality: decreased blood flow to head and limbs, heart deformations	Lin *et al.* 2007
Arnt	Embryonic lethality: defective angiogenesis of the yolk sac and compromised capillary development in solid tissues	Maltepe *et al.* 1997
Arnt2	Death shortly after birth: defective development of hypothalamic secretory neurons, hypoplastic posterior pituitary	Hosoya *et al.* 2001
Hif1-α	Embryonic lethality: cardiac and vascular abnormalities	Kotch *et al.* 1998
Ahr	Half die shortly after birth: immune system impairment, liver size reduction	Fernandez-Salguero *et al.* 1995

3.2 Other characteristics of *Aip*-deficient and -proficient pituitary adenomas

The ER signaling pathway acts in the biosynthesis and secretion of hormones of the anterior pituitary and stimulates the proliferation of lactotrophs and gonadotrophs (Pereira-Lima *et al.* 2004). ARNT and ARNT2 both have a potent role in the regulation of ER signaling (Brunnberg *et al.* 2003, Matthews & Gustafsson 2006, Swedenborg & Pongratz 2010, Figure 3). The AHR-ARNT complex can, for example, directly bind inhibitory xenobiotic response elements (iXREs) regulating ER target gene expression, cause increased proteasomal degradation of ER, or alter estrogen synthesis or metabolism through increases in aromatase, CYP1A1 and CYP1B1 expression (Brunnberg *et al.*

2003, Matthews & Gustafsson 2006). We detected uniform expression of ERα between *Aip*-deficient and -proficient prolactinomas. In contrast, *Aip*-deficient GH-secreting adenomas had lower ERα expression compared to *Aip*-deficient and -proficient prolactinomas. This is in accord with earlier studies that report that prolactinomas have a tendency to show higher ERα levels than GH-secreting adenomas (Nakao *et al.* 1989, Zafar *et al.* 1995). Although we were not able to compare ERα expression between *Aip*-deficient and -proficient GH-secreting adenomas due to their rarity in *Aip* WT mice, our results rather suggest that ERα would not be a key factor in AIP-related pituitary tumorigenesis. Estrogen receptor β (ERβ) expression was not evaluated in this work because of the lack of functional ERβ antibodies.

IHC analysis of the proliferation marker Ki-67 showed that *Aip* HET pituitary tumors had higher proliferation rates than *Aip* WT adenomas (p=0.014). The average proliferation index of 3.6% detected in tumors of *Aip* WT mice is comparable with values detected in human sporadic pituitary tumors (1-4%) (Knosp *et al.* 1989, Mastronardi *et al.* 1999, Jaffrain-Rea *et al.* 2002). In humans, *AIP*mut+ tumors have been reported, for example, to be larger and to have a poorer response to SSA therapy (Daly *et al.* 2007b, Leontiou *et al.* 2008, see study IV). Also our result suggests that *AIP*mut+ adenomas may have more aggressive characteristics than their sporadic counterparts.

This study reveals for the first time that *Aip* HET mice display a disease phenotype that is strikingly similar to PAP patients. Importantly, the phenotype conferred by human and mouse *AIP/Aip* germline mutations appears to contain only pituitary adenomas, with GH-secreting adenomas accounting for 78% and 88%, respectively (see study IV). This dramatically increased risk of somatotropinomas makes *AIP* an attractive candidate gatekeeper gene of somatotrophs, an issue that requires further studies for verification. All in all, the generation of this mouse model provides an ideal tool to further elucidate the molecular basis of pituitary tumorigenesis, for example the hypoxia and estrogen responses and cAMP signaling in *AIP/Aip*-deficient adenomas. It may also have potential in efforts to develop therapeutic strategies in the management of difficult-to-treat pituitary adenomas.

4. *AIP* mutation positive patients display an aggressive disease phenotype (IV)

This study reports the clinical features and therapeutic responses of 96 *AIP*mut+ patients, compared with a control population of 232 *AIP*mut- acromegalics (Vierimaa *et al.* 2006, Barlier *et al.* 2007, Daly *et al.* 2007b, Georgitsi *et al.* 2007a, Naves *et al.* 2007, Toledo *et al.* 2007, Georgitsi *et al.* 2008, Jaffrain-Rea *et al.* 2009, Jennings *et al.* 2009, Table 5). Nearly 80% of *AIP*mut+ patients presented with somatotropinomas, and more than half co-secreted GH and PRL. Every third *AIP*mut+ somatotropinoma patient had gigantism. Prolactinomas were present in 13.5% of patients, and also NFPA was a clear feature of the tumor spectrum of PAP. This study also included the first reported *AIP*mut+ TSH-secreting adenoma. As TSH-secreting tumors are rare, it remains to be seen if *AIP* mutations could be frequent in this setting (Beck-Peccoz *et al.* 1996, Arafah & Nasrallah 2001). Cushing's disease seems to be very rare in *AIP*mut+ patients with only two cases described in the literature and none in the current series (Georgitsi *et al.* 2007a, Stratakis *et al.* 2010). This was the first international and multicenter retrospective case collection/database analysis of the characteristics of *AIP*mut+ patients.

The reason for predominance of somatotropinomas among *AIP*mut+ patients is still unclear. However, these patients had specific features compared to an *AIP*mut- control somatotropinoma group. *AIP*mut+ somatotropinoma patients were diagnosed with pituitary adenomas two decades earlier than control patients. Their adenomas were larger, with more frequent extrasellar extension and PRL hypersecretion. In addition, the *AIP*mut+ somatotropinomas were associated with higher levels of GH secretion at baseline versus controls. These features appeared to impact therapeutic responses, with poorer disease outcomes seen in the *AIP*mut+ group. Large, invasive and extensive macroadenomas and high GH secretion were associated with a lower rate of disease control with primary neurosurgery, leading to higher rates of re-operation in the *AIP*mut+ cohort (Buchfelder & Schlaffer 2009). A trend towards more frequent radiotherapy and failure of pegvisomant to control IGF-I was seen in *AIP*mut+ individuals compared with control patients. In addition, SSA therapy led to a smaller decrease in GH and IGF-I baseline and less tumor shrinkage in the *AIP*mut+ group than in controls. The reason for poorer responses to SSAs is not known and this would be an interesting issue for further study, particularly since somatostatin receptor expression and the activity of vital determinants of SSA function such as ZAC1 in *AIP*mut+ somatotropinoma cells remain unknown (Theodoropoulou *et al.* 2009). Large tumor size and a poor SSA response in such cases might require tumor debulking to favor eventual control with SSA (Petrossians *et al.* 2005, Karavitaki *et al.* 2008).

Gigantism is a rare manifestation of GH oversecretion, with a little more than 100 cases reported so far in the literature (Eugster & Pescovitz 1999, Schoof *et al.* 2004, Rix *et al.* 2005, Müssig *et al.* 2007, Goldenberg *et al.* 2008). In exceptional cases, gigantism may occur in MEN1, CNC, or MAS (Eugster & Pescovitz 1999). In contrast, 32% of *AIP*mut+ somatotropinoma patients displayed gigantism compared to 6.5% of controls, suggesting that gigantism may be a frequent finding among *AIP*mut+ patients and perhaps not as rare as previously thought in *AIP*mut- patients either. Gigantism occurred in a familial setting in 63% of *AIP*mut+ cases; additionally there were nine giants with no family history of pituitary adenomas. In contrast, Leontiou *et al.* found no *AIP* mutations among seven sporadic giants, although gigantism appeared to occur frequently among their FIPA kindreds (Leontiou *et al.* 2008). The frequent gigantism in *AIP*mut+ patients most likely results from large somatotropinomas secreting high levels of GH that become symptomatic before epiphyse closure in young patients.

In the *AIP*mut+ group, there was an unequal representation of males and females, with about two-thirds of patients being male. In contrast, there is generally a female preponderance of pituitary adenomas, with a female:male ratio ranging from 1.23 to 2.05 (Thapar *et al.* 2001 p.61). In study III, we did not detect differences between sexes in the pituitary adenoma formation of *Aip* HET mice. The sex imbalance of PAP patients was especially remarkable in the *AIP*mut+ prolactinoma group, which was 76.9% male. These patients had large tumors that were often uncontrolled by dopamine agonists, multiple surgery and radiotherapy. The male sex is generally known to be associated with a higher rate of aggressive or treatment-resistant prolactinomas, and possible underlying *AIP* mutations could explain part of those cases (Ciccarelli *et al.* 2005, Colao 2009). Overall, the male preponderance seen in this study differs markedly from the pituitary disease characteristics in MEN1, where 69% of patients are female. This difference may be due to the fact that prolactinomas comprise 62% of pituitary tumors in MEN1 and are more frequent in women (Verges *et al.* 2002). Due to the

differences in symptomatology, women usually present earlier than men (Ciccarelli *et al.* 2005). Interestingly, prolactinomas in MEN1 patients are relatively difficult to treat, similar to *AIP*mut+ cases. CNC is also a disease with a strong female preponderance (63%). While acromegaly is a recognized phenotypic component of CNC, it is relatively uncommon, making valid comparisons with *AIP*mut+ patients difficult (Bertherat *et al.* 2009).

The penetrance of PAP remains an unsolved question. Based on the current figures (over 100 asymptomatic *AIP*mut+ carriers related to patients in this study), the penetrance of pituitary adenomas among FIPA kindreds with *AIP* mutations is 15-45%. Although most (nearly 90%) *AIP* mutation-related adenomas present before the age of 40, many younger *AIP* mutation carriers will need extended follow-up in order to definitively determine the penetrance. Current penetrance determination suffers from various sources of bias, such as small families, limited possibilities for clinical and genetic assessment, inadequate follow-up time of families, as well as seemingly sporadic patients where *AIP* genetic testing in the family is not possible. In contrast, *Aip* HET mice displayed pituitary adenomas starting from six months (6/18 mice; 33%) reaching complete penetrance at 15 months. At six months, no pituitary adenomas were seen in *Aip* WT mice (0/10 mice; 0%). However, we would have required larger groups of young HET mice to determine whether the penetrance of these young mice could be similar to that of young PAP patients.

As demonstrated by large families, for example in Finland and Italy, founder mutations of *AIP* seem to exist (Vierimaa *et al.* 2006, Naves *et al.* 2007, Jennings *et al.* 2009, Occhi *et al.* 2010). This suggests that the *AIP* mutation status does not greatly impair biological fitness, unlike in some aggressive genetic tumor syndromes (Wang *et al.* 1999). It remains to be determined whether some specific *AIP* mutations could confer a lower disease penetrance than others. The fact that more than half of *AIP*mut+ patients present with extensive pituitary macroadenomas as children or adolescents suggest that the *AIP* germline mutation confers a predisposition to rapid tumor growth, evidenced by the short time (2.0 yr) from first symptoms to diagnosis.

The tumorigenesis caused by the known human *AIP* mutations has not been thoroughly studied yet. It can, however, be perceived that *AIP* nonsense mutations are spread along the gene. Instead, *AIP* missense mutations mostly tend to cluster in the carboxy-terminus of AIP. This region harbors the TPR domains that mediate protein-protein interactions of AIP, suggesting that missense mutations in this region could impair cellular interactions of AIP (Carver & Bradfield 1997, Petrulis & Perdew 2002, Figure 2).

The clinical characteristics of PAP have not yet been fully elucidated, which may impede the treatment of patients and the recognition of putative PAP families. We found *AIP*-related pituitary adenomas to be most often large, expansive and invasive at diagnosis. Patients are predominantly males. Half of cases present during childhood or adolescence, manifesting remarkably frequent gigantism. *AIP*-related pituitary adenomas appear also to have many aggressive and difficult-to-treat clinical characteristics. The aggressive nature of *AIP*mut+ adenomas is endorsed by the increased expression of the Ki-67 proliferation marker in *Aip*-deficient mouse pituitary adenomas (see study III). Interestingly, it was recently shown that low AIP expression is a better marker of invasiveness in sporadic somatotropinomas than Ki-67 and p53 (Kasuki Jomori de Pinho *et al.* 2010). These results

suggest that to improve outcomes of *AIP*-associated pituitary tumors, earlier diagnosis is required. Thus, it is important to explore the most appropriate ways to identify new PAP patients, allocating genetic screening especially for FIPA kindreds and young patients with large tumors (Beckers & Daly 2007).

5. No evidence of *RET* mutations in familial pituitary adenoma patients (V)

The *RET* proto-oncogene (10q11) is a tyrosine kinase transmembrane receptor (Trupp *et al.* 1996, Trupp *et al.* 1998). Gain-of-function mutations of *RET* cause MEN2A, MEN2B and familial medullary thyroid carcinoma, while loss-of-function mutations of *RET* produce the neurodevelopmental disorder Hirschsprung's disease (Mulligan *et al.* 1993, Romeo *et al.* 1994, Arighi *et al.* 2005). AIP was recently shown to interact *in vivo* in the pituitary with the RET proto-oncogene (Table 4). AIP interacts with the pro-apoptotic domain of RET, and clinically pathogenic *RET* or *AIP* mutations that were introduced to cell constructs did not impair the interaction. In the same study, no somatic *RET* mutations were found in the 28 screened somatotropinomas (Vargiolu *et al.* 2009). *RET* mutations have also previously been searched for in human pituitary adenomas but no relevant mutations have been found (Komminoth *et al.* 1996, Yoshimoto *et al.* 1999, Vieira Neto *et al.* 2007). However, previous studies have not assessed *RET* germline mutations in familial pituitary adenomas, and it is not known whether *RET* mutations could cause a rare familial pituitary adenoma phenotype, similar to *AIP* mutations. In *AIP*mut- familial pituitary adenoma patients, altogether five previously unreported *RET* variants were found, of which two segregated with the phenotype. In a British prolactinoma patient we found a heterozygous c.1560*G>A change in the 3'UTR, which was also present in the affected sister but absent in 279 control samples and an unaffected sister. Also, a heterozygous -1285G>A change was found in a Vietnamese patient and her affected child. However, this variant is quite far from the coding region of *RET* and is not located on any reported promoter or regulatory *RET* region (Guo *et al.* 2007).

5.1 EEL results provide data on regulation of RET transcription

The EEL tool has recently been applied in detection of enhancer elements of genes causing colorectal cancer predisposition (Tuupanen *et al.* 2009). In this study, EEL was applied to predict possible enhancer elements overlapping the two segregating *RET* changes, but no such elements were found. Thus, it seems unlikely that these variants would be related to pituitary tumorigenesis. We also analyzed the noncoding *RET* variant rs2435357 C>T within a conserved enhancer-like sequence, which is a low-penetrance risk allele for Hirschsprung's disease. The disease-associated T allele reduces *in vitro RET* enhancer activity and decreases transcription (Emison *et al.* 2005). The risk allele was present in three samples in the homozygous form. Another three specimens were heterozygous, and the remaining ten were homozygous for the wildtype C allele. Patients harboring the T allele (n=6) had heterogenous demographics and tumor types. All in all, the allele frequencies we found are similar to previously described population frequencies of C and T alleles (Emison *et al.* 2005). Thus, rs2435357 seems unlikely to have an impact on pituitary tumorigenesis. Previously, rs2435357 has been found to reside within a predicted serum response factor (SRF) binding site (Grice *et al.* 2005). Also, the enhancer-like sequence containing rs2435357 was demonstrated to function as a tissue-specific enhancer *in vivo* (Grice *et al.* 2005). EEL predicted an enhancer element

beginning 66 bp downstream of rs2435357, with the first putative transcription factor binding site beginning 75 bp downstream of it (Figure 8). Notably, four out of the six best scored elements that were predicted were located within previously reported multi-species conserved sequences (MSC) of human and zebrafish (Grice *et al.* 2005, Fisher *et al.* 2006). Finding overlapping sequence elements with different techniques increases their reliability. However, these four MCSs are much longer than the predicted EEL elements that they contain. It is plausible that increasing the amount of transcription factor binding matrices in EEL could enlarge the predicted element and increase the number of putative transcription factor binding sites (Emison *et al.* 2005, Grice *et al.* 2005, Fisher *et al.* 2006). Our predicted RET enhancer with the highest score was a 295 bp long element beginning 5624 bp upstream of *RET*, comprising nine transcription factor binding sites (Figure 8). Grice *et al.* showed that MCS-5.2, a multi-species conserved sequence comprising our predicted enhancer, enhanced luciferase expression in neuronal cells (Grice *et al.* 2005). Located near the *RET* promoter area, this predicted element could have a role in the control of *RET* expression, although further functional and *in vivo* studies are needed to verify this.

5.2 RET underexpression in *AIP* mutation positive somatotropinomas

In the normal pituitary gland, RET is expressed in somatotrophs where it is associated with apoptosis and differentiation (Urbano *et al.* 2000, Japon *et al.* 2002, Canibano *et al.* 2007). In pituitary adenomas, RET has been shown to be present in somatotropinomas and in a subset of corticotropinomas (Japon *et al.* 2002). Our RET IHC results on *AIP*mut+ and *AIP*mut- somatotropinomas indicate possible RET underexpression in *AIP*mut+ somatotropinomas ($p=0.05$). This finding could suggest a putative role for RET in AIP-related pituitary tumorigenesis. It is possible that the loss of AIP protein in *AIP*mut+ adenomas would prevent the interaction between AIP and RET. By an unknown mechanism, this could lead to loss of RET in the tumor, causing inhibition of apoptosis and subsequent pituitary tumorigenesis (Canibano *et al.* 2007). Since RET is expressed exclusively in somatotrophs, it could be logically linked to the tumorigenesis of somatotropinomas, which are the most common phenotype of PAP patients. However, *AIP* mutations also predispose to other tumor types such as prolactinomas, and the correlation between RET and these tumors remains an open question. All in all, reduced RET expression may play a role in AIP-related genesis of somatotropinomas; it would be important to study the expression of RET in a larger set of somatotropinomas in the future, since this study only included a small number of samples.

Conclusions and Future Prospects

This work aimed to clarify the molecular mechanisms and clinical characteristics of familial pituitary adenoma. The main conclusions drawn from this study are summarized as follows:

I) Large genomic deletions were discovered as a new mutation type in *AIP*, underlying a subset of PAP cases. Therefore, *AIP* MLPA could be applied in suspected PAP patients undergoing *AIP* genetic testing if conventional genomic sequencing analysis of *AIP* remains mutation negative.

II) Expression of ARNT was reduced in *AIP*mut+ pituitary adenomas compared with *AIP*mut- adenomas. This suggests that ARNT may play a role in AIP-mediated pituitary tumorigenesis, possibly via pathways that involve PDEs and cAMP.

III) *Aip* HET mice and PAP patients displayed a strikingly similar disease phenotype, with GH-secreting adenomas accounting for 78% and 88% of pituitary tumors, respectively. The dramatically increased risk for somatotropinomas suggests that *AIP* is a candidate gatekeeper gene of somatotrophs. Supporting the finding of ARNT underexpression in *AIP*mut+ adenomas in study II, mutually exclusive ARNT or ARNT2 underexpression was evident in mouse *Aip*-deficient pituitary adenomas. This may indicate an ARNT/ARNT2 imbalance and an aberrant ARNT/ARNT2 function in AIP-related tumorigenesis. All in all, the generation of this mouse model provides an ideal tool to further elucidate the molecular basis of pituitary tumorigenesis. It may also have potential in efforts to develop therapeutic strategies in the management of difficult-to-treat pituitary adenomas.

IV) *AIP*mut+ adenomas conferred an aggressive disease phenotype, with young age at disease onset and difficult-to-treat clinical characteristics. These results are supported by the aggressive nature of *Aip*-deficient mouse pituitary adenomas in study III. Improving the treatment outcomes of PAP patients would require their efficient identification, as well as earlier diagnosis of pituitary tumors.

V) We found novel heterozygous *RET* variants in *AIP*mut- familial pituitary adenoma patients with an as yet unidentified mechanism underlying adenoma formation. However, none of these *RET* variants were considered causative of pituitary tumorigenesis. Interestingly, underexpression of RET protein in *AIP*mut+ pituitary adenomas was observed, although additional studies are necessary to verify this.

Important questions remain to be answered regarding AIP-related pituitary tumorigenesis. It is postulated that tumors may arise from different ‘cells of origin’. The adult pituitary has been shown to contain stem cells, although whether pituitary pathogenesis could be related to trophic activity of these cells, and what role germline *AIP* mutations could have in this setting, is still unclear.

In future studies, the molecular mechanisms and cellular pathways of AIP-related pituitary tumorigenesis should be elucidated. Whole-genome RNA-based expression profiles from *Aip*-null and WT MEF cell lines would be most desirable, to provide insight into the pathways in which *AIP* plays a crucial role.

In addition, it would be interesting to compare the RNA expression profiles of *AIP*-deficient and *AIP*-proficient pituitary adenomas. In humans, collecting such adenomas is time-consuming, since fresh tumor material from pituitary adenenomectomies of *AIP*mut+ patients is exceedingly rarely available. However, the *Aip* mouse has the potential to provide large numbers of *Aip*-deficient and -proficient tumors. This presents the possibility to study *AIP*-mediated tumorigenesis in the relevant tissue and in a microenvironment with the appropriate molecular background.

AIP mutations confer an aggressive disease phenotype that causes both severe morbidity in PAP patients and concern for unaffected mutation carriers. To improve the long-term outcome for *AIP*mut+ patients, the PAP diagnosis should lead to possible tailored treatment, as well as to optimal follow-up of *AIP*mut+ patients and healthy mutation carriers. These are issues that should be addressed in future studies on PAP penetrance and the response of PAP patients to various treatment modalities.

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The Human Protein Atlas: http://www.proteinatlas.org/

Repeat Masker Program: http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker

Statistical analysis: http://www.R-project.org and http://www.quantitativeskills.com/sisa

MRC-Holland (MLPA): http://www.mrc-holland.com/

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