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GENETIC BASIS OF PITUITARY ADENOMA PREDISPOSITION

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ACADEMIC DISSERTATION

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"A Rare Disorder, Yes; an Unimportant One, Never"

Angelo M. DiGeorge, 1975

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LIST OF ORIGINAL PUBLICATIONS

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II M. Georgitsi*, A. Raitila*, A. Karhu, K. Tuppurainen, M.J. Mäkinen, O. Vierimaa, R. Paschke, W. Saeger, R.B. van der Luijt, T. Sane, M. Robledo, E. De Menis, R.J. Weil, A. Wasik, G. Zielinski, O. Lucewicz, J. Lubinski, V. Launonen, P. Vahteristo, L.A. Aaltonen (2007) Molecular diagnosis of pituitary adenoma predisposition, caused by aryl hydrocarbon receptor interacting protein gene mutations. *Proceedings of the National Academy of Sciences of the United States of America* 104(10):4101-4105.

III M. Georgitsi, A. Karhu, R. Winqvist, T. Visakorpi, K. Waltering, P. Vahteristo, V. Launonen, L.A. Aaltonen (2007) Mutation analysis of *aryl hydrocarbon receptor interacting protein* (*AIP*) gene in colorectal, breast, and prostate cancers. *British Journal of Cancer* 96(2):352-356.

IV M. Georgitsi*, E. De Menis*, S. Cannavò, M.J. Mäkinen, K. Tuppurainen, P. Pauletto, L. Curtò, R.J. Weil, R. Paschke, A. Wasik, G. Zielinski, J. Lubinski, P. Vahteristo, A. Karhu, L.A. Aaltonen (2008) *Aryl hydrocarbon receptor interacting protein* (*AIP*) gene mutation analysis in children and adolescents with sporadic pituitary adenomas. *Clinical Endocrinology* 69(4):621-627.

* Equal contribution

Publication I was included in the thesis of Dr. Outi Vierimaa, MD, PhD ("Multiple Endocrine Neoplasia Type 1 (MEN1) and Pituitary Adenoma Predisposition (PAP) in Northern Finland"-D973, Oulu 2008) from University of Oulu.

ABBREVIATIONS

A	adenine
aa	amino acid
ACTH	adrenocorticotrophin
AIP	aryl hydrocarbon receptor interacting protein gene
AHR	aryl hydrocarbon receptor (or dioxin receptor)
ALK	anaplastic lymphoma kinase gene
ARA9	aryl hydrocarbon receptor interacting protein (or AIP, XAP2)
ARNT	aryl hydrocarbon receptor nuclear translocator
α/β-SU	alpha/beta-subunit
bp	base pair
BRCA1	breast and ovarian cancer 1 gene
BRCA2	breast and ovarian cancer 2 gene
C	cytosine
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CDKN1B	cyclin-dependent kinase inhibitor 1B gene
cDNA	complementary deoxyribonucleic acid
CEPH	Centre d'Étude du Polymorphisme Humain
CGH	comparative genomic hybridization
CNC	Carney complex
CRC	colorectal cancer
CRH	corticotrophin-releasing hormone
C-terminal	carboxyterminal
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DRE	dioxin response element
E	(mouse) embryonic day
FIPA	familial isolated pituitary adenomas
FISH	fluorescence *in situ* hybridization
FKBP	FK506 binding protein
FMM	familial malignant melanoma
FSH	follicle-stimulating hormone
G	guanine
GH	growth hormone
GHRH	growth hormone-releasing hormone
GI	gastrointestinal
GNAS	guanine nucleotide-binding protein, alpha stimulating activity polypeptide
HLRCC	hereditary leiomyomatosis and renal cell cancer
HNPCC	hereditary nonpolyposis colorectal cancer
HSP90	heat-shock protein 90
IFS	isolated familial somatotropinoma
IGF-I	insulin-like growth factor I
IHC	immunohistochemistry
IVS	intronic variable sequence
kb	kilobase
kDa	kiloDalton
LGALS12	galectin-12 gene
LD	linkage disequilibrium
LH	luteinizing hormone
LOD	logarithm of the odds
LOH	loss of heterozygosity
MAS	McCune-Albright syndrome
Mb	mega base pairs
MEN1	multiple endocrine neoplasia type 1
MEN2	multiple endocrine neoplasia type 2
MENX	multiple endocrine neoplasia type X
MIM	Mendelian Inheritance in Man
MLH1	MutL E. coli homologue 1
MLPA	multiplex ligation-dependent probe amplification
mRNA	messenger ribonucleic acid
MSI	microsatellite instability
MSS	microsatellite stability
NCBI	National Center for Biotechnology Information
NFPA	non-functioning pituitary adenoma
NLS	nuclear localization signal
NMD	nonsense-mediated mRNA decay
PAP	pituitary adenoma predisposition
PCR	polymerase chain reaction
PDE2A	phosphodiesterase 2 A
PDE4A5	phosphodiesterase 4 A5
PDE8B	phosphodiesterase 8 B
PDE11A	phosphodiesterase 11 A
PKA	protein kinase A
PPARα	peroxisome proliferator-activated receptor alpha
PRKAR1A	cAMP-dependent protein kinase A type 1-alpha regulatory subunit gene
PRL	prolactin
q	long arm of a chromosome
pRb	retinoblastoma protein
RET	rearranged during transfection protooncogene
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
T	thymine
T3/T4	thyroid hormone
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TPR	tetratricopeptide repeat
TRH	thyrotrophin-releasing hormone
TSG	tumor suppressor gene
TSH	thyroid-stimulating hormone
UTR	untranslated region
XAP2	hepatitis B virus X-associated protein 2 (or AIP, ARA9)

In addition, standard one-letter codes are used to denote aminoacids.

ABSTRACT

Much of the global cancer research is focused on the most prevalent tumors; yet, less common tumor types warrant investigation, since *"A rare disorder is not necessarily an unimportant one"*. The present work discusses a rare tumor type, the benign adenomas of the pituitary gland, and presents the advances which, during the course of this thesis work, contributed to the elucidation of a fraction of their genetic background.

Pituitary adenomas are benign neoplasms of the anterior pituitary lobe, accounting for approximately 15% of all intracranial tumors. Pituitary adenoma cells hypersecrete the hormones normally produced by the anterior pituitary tissue, such as growth hormone (GH) and prolactin (PRL). Despite their non-metastasizing nature, these adenomas can cause significant morbidity and have to be adequately treated; otherwise, they can compromise the patient's quality of life, due to conditions provoked by hormonal hypersecretion, such as acromegaly in the case of GH-secreting adenomas, or due to compressive effects to surrounding tissues.

The vast majority of pituitary adenomas arise sporadically, whereas a small subset occur as component of familial endocrine-related tumor syndromes, such as Multiple Endocrine Neoplasia type 1 (MEN1) and Carney complex (CNC). MEN1 is caused by germline mutations in the *MEN1* tumor suppressor gene (11q13), whereas the majority of CNC cases carry germline mutations in the *PRKAR1A* gene (17q24). Pituitary adenomas are also encountered in familial settings outside the context of MEN1 and CNC, but unlike in the latter syndromes, their genetic background until recently remained elusive. Evidence in previous literature supported the notion that a tumor suppressor gene on 11q13, residing very close to but still distinct from *MEN1*, causes genetic susceptibility to pituitary tumors.

The aim of the study was to identify the genetic cause of a low penetrance form of Pituitary Adenoma Predisposition (PAP) in families from Northern Finland. The present work describes the methodological approach that led to the identification of *aryl hydrocarbon receptor interacting protein* (*AIP*) as the gene causing PAP. Combining chip-based technologies (SNP and gene expression arrays) with traditional gene mapping methods and genealogy data, we showed that germline *AIP* mutations cause PAP in familial and sporadic settings. PAP patients were diagnosed with mostly adenomas of the GH/PRL-secreting cell lineage. In Finland, two *AIP* mutations accounted for 16% of all patients diagnosed with GH-secreting adenomas, and for 40% of patients being younger than 35 years of age at diagnosis. *AIP* is suggested to act as a tumor suppressor gene, a notion supported by the nature of the identified mutations (most are truncating) and the biallelic inactivation of *AIP* in the tumors studied. AIP has been best characterized as a cytoplasmic interaction partner of aryl hydrocarbon receptor (AHR), also known as dioxin receptor, but it has other partners as well. The mechanisms that underlie AIP-mediated pituitary tumorigenesis are to date largely unknown and warrant further investigation.

Because *AIP* was identified in the genetically homogeneous Finnish population, it was relevant to examine its contribution to PAP in other, more heterogeneous, populations. Analysis of pituitary adenoma patient series of various ethnic origins and differing clinical

settings revealed germline *AIP* mutations in all cohorts studied, albeit with low frequencies (range 0.8-7.4%). Overall, PAP patients were typically diagnosed at a young age (range 8-41 years), mainly with GH-secreting adenomas, without strong family history of endocrine disease. Because many PAP patients did not display family history of pituitary adenomas, detection of the condition appeared challenging. AIP immunohistochemistry was tested as a molecular pre-screening tool on mutation-positive versus mutation-negative tumors, and proved to be a potentially useful predictor of PAP.

Mutation screening of a large cohort of colorectal, breast, and prostate tumors did not reveal somatic *AIP* mutations. These tumors, apart from being the most prevalent among men and women worldwide, have been associated with acromegaly, particularly colorectal neoplasia. In this material, *AIP* did not appear to contribute to the pathogenesis of these common tumor types and other genes seem likely to play a role in such tumorigenesis.

Finally, the contribution of *AIP* in pediatric onset pituitary adenomas was examined in a unique population-based cohort of sporadic pituitary adenoma patients from Italy. Germline *AIP* mutations may account for a subset of pediatric onset GH-secreting adenomas (in this study one of seven GH-secreting adenoma cases or 14.3%), and appear to be enriched among young (≤25 years old) patients.

In summary, this work reveals a novel tumor susceptibility gene, namely *AIP*, which causes genetic predisposition to pituitary adenomas, in particular GH-secreting adenomas. Moreover, it provides molecular tools for identification of individuals predisposed for PAP. Further elaborate studies addressing the functional role of AIP in normal and tumor cells will hopefully expand our knowledge on endocrine neoplasia and reveal novel cellular mechanisms of pituitary tumorigenesis, including potential drug targets.

REVIEW OF THE LITERATURE

1. The human genome and tumorigenesis

Tumors are lesions caused by the abnormal growth of cells in various tissues. They are broadly divided into two categories: The benign tumors that remain localized, without invading adjacent tissues, such as the adenomas, and the malignant tumors (i.e. cancer) that acquire invasive potential towards adjacent tissues and can spawn metastases elsewhere in the body. The development of tumors is a complex phenomenon attributed to many causes; these are regarded as external – including tobacco use, exposure to chemicals and ionizing/ultraviolet radiation, exposure to infectious microorganisms, dietary habits, alcohol consumption, or obesity – or internal, including inherited DNA mutations causing genetic predisposition to tumor development, acquired (i.e. somatic) genomic alterations, or prolonged exposure to hormones and growth factors.

The vast majority of tumors occur due to mutations that human cells accumulate during one's lifetime. The spontaneous mutation rate in mammalian cells from normal tissues is exceedingly low ($<10^{-8}$) (Bielas *et al.*, 2006) and accumulation of a considerable number of mutations is required for transformation from normal cells to neoplastic ones. Some tumor types (i.e. colorectal and breast cancers) have been found to harbor around 15 mutations likely to be involved in driving initiation, progression, or maintenance of the tumor (Wood *et al*., 2007). These mutations, known as "drivers", confer a growth advantage on the cell in which they occur, and are, thus, positively selected. On the other hand, "passenger" mutations are expected to be biologically neutral, since they do not confer growth advantage and are not causative of tumorigenesis (Greenman *et al*., 2007). Apart from the well recognized genomic alterations that occur in specific tumor types, the genomes of tumor cells display genomic instability in the form of greatly elevated frequencies of random mutations. Thus, it was proposed that tumor cells exhibit a mutator phenotype (Loeb, 1991; Bielas *et al*., 2006). These altered genotypes constitute a permissive environment for a tumor cell to acquire novel physiological capabilities, such as: a) self-sufficiency in growth signals, b) insensitivity to growth-inhibitory signals, c) evasion of apoptosis, d) limitless replicative potential, e) sustained angiogenesis, f) tissue invasion and metastasis (Hanahan & Weinberg, 2000) and possibly g) evasion of immune system-mediated elimination (Weinberg, 2007).

Approximately 380 genes, representing about 1% of all human genes, have been implicated via mutation in tumorigenesis (Futreal *et al*., 2004; The Wellcome Trust Sanger Institute, August 2008 at www.sanger.ac.uk/genetics/CGP/Census). In their vast majority (90%), these genes are somatically mutated, and only a small subset harbor mutations in the germline (20%), or both germline and somatic level (10%) (Futreal *et al*., 2004). The distinct gene categories implicated in tumorigenesis are the oncogenes, the tumor suppressor genes (TSGs), and the "caretaker" genes, which are the genes that maintain the genomic stability.

1.1 Oncogene activation

Oncogenes are the mutated versions of normal genes – called proto-oncogenes – that code for proteins involved in a variety of key cellular processes, including cell proliferation, differentiation, and apoptosis. The oncogenic protein products can be distinguished in the following categories, based on their subcellular localization and functional roles: Growth factors (e.g. PDGF-β), growth factor receptors (e.g. EGFR), signal transducers (e.g. RAS), transcription factors (e.g. ETS family), chromatin remodelers (e.g. MLL), and apoptotic regulators (e.g. BCL2) (Croce *et al*., 2008).

The activation of proto-oncogenes to oncogenes may be the result of a number of genomic alterations, such as activating point mutations, gene fusions due to chromosomal rearrangements, juxtaposition of proto-oncogenes to enhancer elements, or gene copy number amplifications. Point mutations and translocations usually occur as initiating events, or during tumor progression, whereas amplification usually occurs during tumor progression (Croce *et al*., 2008). These alterations result in either increased expression of the normal protein, due to constitutive gene activation, or expression of an aberrant protein, such as chimeric proteins that result from chromosomal translocations (Table 1). The latter are the most common class of somatic mutations occurring mainly in hematopoietic malignancies (i.e. leukemias), but also in epithelial neoplasms, such as sarcomas (Delattre *et al*., 1994), thyroid carcinoma (Kroll *et al*., 2000), and prostate cancer (Tomlins *et al*., 2005).

Because oncogenic activation is manifested even if only one gene copy is altered (in terms of alterations on the genomic sequence level or the expression level), oncogenes are thought to act in a dominant manner at the cellular level.

1.2 Loss of tumor suppression

Alfred Knudson proposed a seminal model for tumor development, which, since its elucidation, expanded to include many tumor types associated with mutational events that occur in TSGs. Knudson's so-called "two-hit" hypothesis, based on his epidemiological studies on pediatric retinoblastoma (Knudson, 1971), suggests that two "hits" (i.e. two mutational events) must occur in a TSG, one on each allele, in order for an affected cell to acquire a growth advantage, as a prerequisite for its clonal expansion. Thus, on the somatic level, TSG inactivation occurs in a recessive manner.

In hereditary tumors, the first mutational event is an inherited germline mutation, which is present in all cells of an affected individual. The "second hit" occurs as a somatic (i.e. acquired) mutation in a single cell, resulting in the inactivation of the remaining wild type gene copy, thus leading to gene inactivation. Because the probability of a single somatic mutation in one allele is much higher than the probability of acquisition of two hits in both alleles, people already carrying an inherited mutation of a TSG face higher risk of developing a tumor than the general population (Kinzler & Vogelstein, 1997).

The "second hit" is most often the loss of the remaining gene copy by a mechanism known as loss of heterozygosity (LOH). This allelic loss can result from erroneous mitotic recombination or gene conversion events, but it may also be observed on tumor DNA level

as a large deletion, encompassing anything between few megabases (Mb) up to whole chromosomal arms or whole chromosome losses (Weinberg, 2007). It appears that large genomic deletions are not easily tolerated on genomic DNA level, because they may not be compatible with life; instead, somatic deletions are far more common in individual cells, and may occur spontaneously (Tomlinson *et al*., 2001). Chromosomal losses on the somatic level may result in the ablation of nearby genes, which may have further implications in tumor growth and progression. Epigenetic changes, such as promoter hypermethylation, are a common mechanism of gene silencing, estimated to occur in approximately 50% of TSGs in sporadic tumors (Jones & Baylin, 2002; Weinberg, 2007). Point mutations and intragenic deletions are less common; these types of "second hits" may compromise the gene expression or protein function, depending on the type and location of the mutation (i.e. promoter region, splice-sites, or coding sequence) (Table 1).

The evolution of tumors requires much more than just two hits, though; no tumors have been found to occur exclusively due to two hits on a single genomic locus. In reality, tumor growth and progression is a multistep process, characterized by the stepwise accumulation of several mutational events, including loss-of-function of TSGs, as well as gain-of-function of one or more oncogenes (Loeb, 1991; Knudson, 2001). In colorectal cancer, for instance, 25-50% of tumors have been shown to harbor more that nine (Fearon & Vogelstein, 1990) or even up to 15 mutations (Wood *et al*., 2007).

Yet, a number of TSGs involved in tumorigenesis do not follow the "two-hit" model. Haploinsufficiency, described for a number of TSGs mainly in murine models, has been proposed to lead to accelerated tumorigenesis, since only one defective gene copy is sufficient to compromise the corresponding protein's role in the cell (reviewed in Payne & Kemp, 2005) (Table 1). An example of such an atypical, haploinsufficient TSG is *CDKN1B*, which codes for the cyclin-dependent kinase inhibitor $p27^{Kip1}$, a cell cycle regulatory protein that inhibits the G1/S phase transition (Sherr & Roberts, 1999). *CDKN1B* genomic sequence and mRNA expression levels have been found unaltered in most human or murine tumors analysed (Slingerland & Pagano, 2000); yet $p27^{Kip1}$ protein expression is markedly reduced in common human epithelial cancers, a fact associated also with poor prognosis (reviewed in Chu *et al*., 2008). These data indicate that other mechanisms, possibly on the post-translational level, compromise the cellular functionality of $p27^{Kip1}$ (Hengst & Reed, 1996; reviewed in Chu *et al*., 2008).

1.3 Loss of genomic stability

The stability genes, also known as "caretakers", code for proteins that monitor and repair the genomic alterations that normally occur in the cells. Caretaker genes belong to the mismatch repair system (MMR), the nucleotide-excision repair system (NER), the base-excision repair system (BER), and the double-strand break repair system (DSBR) (Hoeijmakers, 2001; Weinberg, 2007). If mutations occur in these genes and render them inactive or defective, the genome inevitably accumulates genomic alterations that remain unrepaired. Thus, stability genes act in a different way than oncogenes or TSGs: Caretakers do not promote tumorigenesis themselves, but rather through the accumulation of aberrations in other crucial genes, such as TSGs; contrary, oncogenes and TSGs directly affect cellular mechanisms leading to neoplasia (Kinzler & Vogelstein, 1997).

Stability genes have been found mutated in a number of hereditary cancer syndromes, such as hereditary nonpolyposis colorectal cancer (HNPCC) (MMR genes *MLH1*, *MSH2*, *MSH6*, *PMS2*), xeroderma pigmentosum (XP) (NER genes *XPA-G*), and breast and ovarian cancer (DSBR genes *BRCA1* and *BRCA2*) (Table 2) (Hoeijmakers, 2001; Weinberg, 2007).

Table 1. Mechanisms of gain- or loss-of-function events in tumor predisposing genes.

Mechanism of activation/inactivation	Functional result on transcript/protein level
Oncogenes (Gain-of-function)	
Point mutation (missense)	Constitutively active protein
Chromosomal translocations or inversions resulting in fusion genes	Fusion transcripts leading to fusion proteins with aberrant activity
Chromosomal translocations or inversions resulting in juxtaposition to an enhancer element	Overexpression of the normal protein
Gene amplification	Overexpression of the normal protein
Promoter hypomethylation	Activation of transcription of a silenced proto-oncogene
TSGs and stability genes (Loss-of-function)	
Point mutation (nonsense, missense)	
Early nonsense mutation	Loss of protein
Late nonsense or missense mutation	Hypomorphic or dominant-negative protein
Insertions or deletions resulting in frameshift	
Premature stop codon	Loss of protein
Late stop codon	Hypomorphic or dominant-negative protein
Elongated transcript	Loss of protein or dominant-negative protein
Small in-frame deletions	Loss of functional or interaction domains Hypomorphic or dominant-negative protein
Large genomic deletions	
Whole gene deletion	Loss of protein
Partial (exon(s)) deletion	Loss of protein or dominant-negative protein
LOH	Loss of protein
Epigenetic silencing by promoter hypermethylation	Transcription silencing
Haploinsufficiency	Reduced expression of the normal protein

1.4 Genetic predisposition to tumor development

Inherited tumor susceptibility accounts for a small subset of all neoplasias (5-10%) (Nagy *et al*., 2004); yet, the elucidation of a variety of rare familial tumor syndromes has had a great impact on our understanding of tumor genetics and has confirmed that cancer, in its essence, is a disease of the genome (Vogelstein & Kinzler, 2004). In addition, it has provided ground for the study of sporadic tumors as well, and has greatly improved our knowledge on novel molecular pathways implicated in tumorigenesis (Fearon, 1997).

The majority of inherited tumor susceptibility syndromes are transmitted as autosomal dominant traits, with varying penetrance (i.e. disease expressivity) observed either between affected members of the same family or between affected members from different families with the same predisposing mutations (Marsh & Zori, 2002). Most of these syndromes are caused by germline mutations in more than 30 TSGs and oncogenes, a fraction of which is presented in Table 2. The latter category includes only a handful of examples, such as *RET*, *MET*, *KIT*, *CDK4*, and *ALK* as the predisposing genes in Multiple Endocrine Neoplasia type 2 (MEN2) (Mulligan *et al*., 1993), hereditary papillary renal cell carcinoma (HPRCC)

(Schmidt *et al*., 1997), familial gastrointestinal stromal tumors (GIST) (Nishida *et al*., 1998), familial malignant melanoma (FMM) (Zuo *et al*., 1996), and familial neuroblastoma (Mosse *et al*., 2008), respectively. A fairly small number of hereditary tumor predisposition syndromes are transmitted as autosomal recessive traits (Table 2); these cases are rare and are not expected to be enriched in the general population, due to the often life-limiting character of the disease and the hampered potential of the affected individuals to produce offspring.

In highly penetrant conditions, affected individuals manifest the disease phenotype at a considerably younger age than their sporadic counterparts; this is due to the shorter time elapse before a "second hit" occurs in a predisposed tissue that already harbors a germline genetic defect. Other features that typically characterize highly penetrant familial tumor syndromes include: a) several affected cases in the family with the same rare tumor phenotype; b) several generations affected; c) bilateral disease, such as bilateral breast tumors in breast and ovarian cancer (BRCA), or multiple disease sites in one organ, such as the colonic polyps in polyposis syndromes of the gastrointestinal (GI) tract; d) multiple primary cancers in a single individual, such as the colorectal and endometrial tumors in HNPCC patients; e) disease phenotype in the less affected sex, such as the male breast cancer seen in BRCA2 families; and f) tumors associated with other conditions, such as the skin pigmentation seen in Carney complex (CNC) patients (Marsh & Zori, 2002; Nagy *et al*., 2004). The highly penetrant tumor predisposing alleles are rarely encountered in the general population, in other words predisposing mutations are rare in sporadic counterparts.

On the contrary, low-penetrance alleles may be more common in the general population, since the presence of a predisposing allele does not necessarily cause a disease-associated phenotype, or it may be associated with age-related penetrance and gender-specific risks (Fearon, 1997; Nagy *et al*., 2004). Interactions between, yet unidentified, low-penetrance genes or between genes and environmental factors may account for another 15-20% of all human cancers (Nagy *et al*., 2004). For this reason, low-penetrance susceptibility conditions are more difficult to identify, presumably owing to the lower frequency of clustering of affected cases in pedigrees. Moreover, low-penetrance tumor syndromes are more challenging in terms of genetic counseling and management of the mutation carriers.

Table 2. Highly penetrant hereditary tumor syndromes and the predisposing genes (adapted from Fearon, 1997; Marsh & Zori, 2002; Nagy *et al*., 2004; Garber & Offit, 2005).

Clinical syndrome	Predisposing gene	Gene function	Tumor spectrum
Autsomal dominant pattern of inheritance			
Carney complex (CNC)	*PRKAR1A*	TSG	Cardiac and other myxomas, anterior pituitary tumors, schwannomas
Cowden syndrome (CS)	*PTEN*	TSG	Breast, thyroid, endometrial, CNS cancers, GI polyps
Familial adenomatous polyposis (FAP)	*APC*	TSG	Adenomatous colonic polyps, increased risk of colorectal cancer
Familial malignant melanoma (FMM)	*CDK4*	Oncogene	Malignant melanoma
	CDKN2A	TSG	Malignant melanoma, pancreatic cancer
Familial neuroblastoma	*ALK*	Oncogene	Pediatric neuroblastomas
Familial retinoblastoma	*PRB*	TSG	Pediatric retinoblastoma, osteosarcoma
Familial gastrointestinal stromal tumors	*KIT*	Oncogene	GI stromal tumors
Hereditary breast and ovarian cancer (BRCA)	*BRCA1*	TSG	Breast and ovarian cancer
	BRCA2	TSG	Breast and prostate cancer
Hereditary leiomyomatosis and renal cell cancer (HLRCC)	*FH*	TSG	Skin and uterine leiomyomas, renal carcinoma
Hereditary papillary renal cell cancer (HPRCC)	*MET*	Oncogene	Papillary renal cell carcinoma
Hereditary nonpolyposis colorectal cancer (HNPCC)	*MLH1, MSH2, MSH6, PMS1, PMS2, MSH3,*	TSGs	Colorectal, endometrial adenocarcinoma, gastric, ovarian, kidney cancer
Juvenile polyposis (JP)	*SMAD4/DPC4, BMPR1A*	TSGs	Juvenile polyps of the GI tract, increased risk of colorectal, GI, and pancreatic cancer
Li-Fraumeni	*TP53, CHEK2*	TSGs	Breast cancer, sarcomas, leukaemia, brain tumors
Multiple endocrine neoplasia type 1 (MEN1)	*MEN1*	TSG	Parathyroid hyperplasia/adenomas, enteropancreatic neuroendocrine tumors, anterior pituitary adenomas
Multiple endocrine neoplasia type 2 (MEN2)	*RET*	Oncogene	Medullary thyroid cancer, pheochromocytoma, parathyroid hyperplasia
Neurofibromatosis type 1 (NF1)	*NF1*	TSG	Neurofibromas, brain and skin tumors
Neurofibromatosis type 2 (NF2)	*NF2*	TSG	Neurofibromas, schwannomas, brain, spinal, and skin tumors
Peutz-Jeghers syndrome (PJS)	*STK11/LKB1*	TSG	GI tract carcinoma, breast, pancreatic, ovarian, testicular cancers
von Hippel-Lindau (VHL)	*VHL*	TSG	Renal cell carcinoma, hemangioblastomas, pheochromocytomas
Autosomal recessive pattern of inheritance			
Ataxia-telangiectasia (AT)	*ATM*	TSG	Lymphoma, leukaemia, breast cancer
Bloom syndrome	*BLM*	TSG	Solid tumors, leukemia
Fanconi's anemia	*FANCA, B, C, D2, E, F, G BRCA2*	TSGs	Leukemia, squamous carcinomas, hepatoma
Xeroderma pigmentosum (XP)	*XPA, B, C, D, E, F, G*	TSGs	Skin cancers (squamous cell carcinoma, malignant melanoma), leukemia

1.5 Identification of tumor predisposing genes

Most tumor predisposing genes have been identified by positional cloning, without pre-existing hypotheses on their biological function. The primary positional clues can be diverse, including chromosomal rearrangements visible in metaphase spreads of cancer cells, DNA copy number changes identified by, for instance, comparative genomic hybridisation (CGH) and fluorescence *in situ* hybridization (FISH), or clues after genetic linkage analysis (Futreal *et al*., 2004; Shih & Wang, 2005).

Powerful linkage analysis on large, multigenerational pedigrees has been a traditional disease gene identification approach, successfully applied also in the quest for tumor predisposing genes. Heredity contributes to the etiology of tumor development by a small fraction; yet, the crucial identification of large families segregating particular tumor phenotypes revealed an important number of tumor susceptibility genes. Before the era of the Human Genome Project, one approach for the identification of such genes was positional cloning. This term describes the approach of cloning a disease predisposing gene first by identifying its chromosomal location, followed by laborious efforts in identifying and cloning the gene itself.

The key element in positional cloning is the collection of a sufficient number of families segregating a particular tumor phenotype, who are then analysed by genome-wide linkage analysis, using polymorphic DNA microsatellite markers. A genetic locus is established and a physical map of the region is constructed by utilizing the informativity obtained by key meiotic recombinations. When searching for TSGs in tumor predisposition syndromes, tumor LOH analysis can greatly assist the candidate locus fine-mapping effort; LOH is the most common type of somatic gene inactivation, and tumors arising in the context of the syndrome frequently exhibit somatic loss of the wild-type allele of markers in the vicinity of the susceptibility gene. Next, sequencing of the minimal candidate locus aims at identifying presumably pathogenic mutations harbored in a candidate gene. Alternatively, if the number of genes in the linked region is very high, the most likely candidates are selected based on their functional relevance to the disease, an approach designated as “positional candidate gene” strategy (Collins, 1995). Identification of the MEN2, HPRCC, FMM, and HNPCC genes are some examples of the success of the approach (reviewed in Collins, 1995; Fearon, 1997).

With the completion of the Human Genome and HapMap projects (Lander *et al*., 2001; Venter *et al*., 2001; International Human Genome Sequencing Consortium, 2004; International HapMap Consortium, 2005; 2007), the complete human DNA sequence, and many of its common genetic variants, such as the single nucleotide polymorphisms (SNPs), have become known and globally available. The era of modern human genetics offers new possibilities in identifying novel tumor predisposing genes. Current approaches may still include positional cloning; yet, nowadays, pure gene cloning has been replaced by retrieving relevant genomic information from DNA sequence databases by utilizing bioinformatic tools. Yet, the powerful sequencing platforms and the high-throughput chip-based molecular methodologies, such as high-density SNP and gene expression microarrays, have aided in the identification of novel tumor susceptibility genes: TSGs involved in autosomal dominantly inherited predisposition to conditions associated with tumor

formation (Horvath *et al*., 2006), or autosomal recessive tumor syndromes (Vahteristo *et al*., 2007), or identification of oncogenes predisposing to autosomal dominant familial cancers with incomplete penetrance (George *et al*., 2007; Mosse *et al*., 2008). Despite the advances in the methodological and experimental front, one major challenge that remains in tumor gene identification is the recognition and collection of suitable family materials segregating rare tumor phenotypes. Genetically homogeneous populations, such as the Finns, or isolated inbred populations may serve as ideal material towards this end.

The identification of tumor susceptibility genes necessitates subsequent efforts in establishing the associated tumor risk (i.e. the disease penetrance) in familial settings, as well as in the general population. Moreover, the elucidation of histologic, immunohistochemical, and molecular features, which characterize the component tumors, are essential for the functional and biological validation of a newly identified tumor gene. These approaches eventually aim at developing effective strategies for surveillance, prevention, disease management, and even targeted molecular-based intervention (Nagy *et al*., 2004; Garber & Offit, 2005).

Unravelling the role of tumor susceptibility genes can be greatly assisted by genetically engineered animal models by manners impossible to perform in humans. Even though animal models are not always successful in depicting human phenotypes (Antonarakis & Beckmann, 2006), they are expected to aid in the elucidation of certain mechanisms underlying human tumorigenesis. Genetic engineering of mouse *Msh6* and *Brca2/p53* resulted in the successful recapitulation of human hereditary non-polyposis colorectal carcinoma and breast ductal carcinoma, respectively (reviewed in Frese & Tuveson, 2007). However, the disease outcome in model organisms may largely depend on the approaches used; germline knockout of a mouse TSG might result in a different tumor phenotype than its conditional (i.e. tissue-specific) biallelic deletion. This was, for instance, the case with the *neurofibromatosis 2* gene, where *Nf2* heterozygous mice developed primarily osteosarcomas, among other malignancies (McClatchey *et al*., 1998), whereas conditional ablation of *Nf2* in Schwann cells resulted primarily in the development of benign schwannomas, as in human NF2 patients (Giovannini *et al*., 2000). Differences in tumor spectrum and incidence may be attributable to the specific genetic backgrounds of mice strains or may truly reflect species-specific differencies (Fearon, 1997).

2. The pituitary gland

The pituitary gland, also known as hypophysis, is one of the most important glands of the mammalian endocrine system. Through its secreted hormones (see 2.1.1 and 2.1.2), it controls the growth and activity of three other endocrine glands: The thyroid, the adrenals, and the gonads. The pituitary is not, however, acting independently, but it is under the continuous control of the nervous system through the hypothalamus. A wide range of external stimuli – varying from the supply of nutrients, the ambient temperature, or exercise, to physical or psychological stress – causes secretion of hypothalamic hormones. As a response to hypothalamic control, the pituitary secretes the hypophyseal hormones, which maintain crucial homeostatic functions, including metabolism, growth, and reproduction (Fig. 1). These two glands compose the “hypothalamopituitary axis”

(Goodman, 2003). Apart from the hypothalamic inputs, pituitary hormone secretion is also regulated by the feedback effects of the circulating hormones, as well as the autocrine and paracrine secretions of the pituitary cells (Fig. 1) (Bilezikjian *et al*., 2004; Mechenthaler, 2008).

2.1 Morphology and histology

The pituitary gland is situated at the base of the brain, under the optic chiasm, inside a bony cavity called "sella turcica". It is connected to the hypothalamus by the pituitary stalk, the latter consisting of blood vessels and the axons of the hypothalamic neuronal cell bodies that reach the posterior pituitary gland. The hypophysis is composed of the neurohypophysis (or posterior lobe) and the adenohypophysis (or anterior lobe); the latter is comprised of distinct types of differentially distributed, hormone-secreting cells (Brook & Marshall, 2001). These secretory cells release the hormonal peptides of the secretory storage granules to the blood vessels by exocytosis (Goodman, 2003).

2.1.1 Posterior lobe

The posterior lobe consists of cells secreting the antidiuretic hormone (ADH) or vasopressin. ADH exerts its physiological role mainly on the kidneys as a response to increased plasma osmolality or decreased plasma volume, thus mediating the regulation of water levels in the body. ADH also acts on arterioles by controlling blood pressure. Posterior lobe cells also produce oxytocin, the hormone that causes the uterus to undergo contractions during delivery (Brook & Marshall, 2001).

2.1.2 Anterior lobe

The anterior lobe has three distinct anatomical areas, namely the central wedge and the two lateral wings, and is composed of six distinct cell types (Heaney & Melmed, 2004). Three main pathways of cell differentiation have been elucidated in the anterior pituitary: Differentiation to adrenocorticotrophs, bihormonal gonadotrophs, and cells that mature either into somatotrophs, mammosomatotrophs, lactotrophs, or thyrotrophs (Al-Shraim & Asa, 2006).

Approximately 50% of all anterior lobe cells are growth hormone (GH)-secreting cells, also known as somatotrophs, and occupy the largest part of both lateral wings (Heaney & Melmed, 2004). GH has a crucial role in controlling body growth and metabolism, by acting either directly on multiple tissues or indirectly, via the hepatic production of insulin-like growth factors (IGFs, mainly IGF-I) (Brook & Marshall, 2001) (Table 3).

Prolactin (PRL)-secreting cells, also known as lactotrophs, reside in both lateral wings. In men and nulliparous women they may account for approximately 10% of the anterior pituitary cells, whereas in multiparous women the number can be up to three times higher (Heaney & Melmed, 2004). PRL inhibits the function of the gonads, and stimulates breast enlargement, and milk production during and after pregnancy (Table 3). GH- and PRL-secreting cells derive from progenitor mammosomatotrophs, which are bihormonal cells that can differentiate into either somatotrophs or lactotrophs depending on the needs of each phase the body is in (i.e. growth, or pregnancy and lactation) (Asa & Ezzat, 2002). During pregnancy, for instance, many PRL-secreting cells are recruited from the population of mammosomatotrophs. This phenomenon is called "reverse transdifferentiation", since the

normal state is gradually re-established following delivery and lactation (Horvath *et al*., 1999).

Adrenocorticotrophin (ACTH)-secreting cells, also known as corticotrophs, are localized in the central wedge and account for approximately 10-20% of all anterior lobe cells (Heaney & Melmed, 2004). Apart from ACTH, they secrete endorphins, γ-lipotrophin and other pro-opiomelanocortin derivatives. ACTH stimulates the secretion of glucocorticoid hormone (cortisol) from the adrenal gland cortex, while cortisol, in turn, concerts metabolic and anti-inflammatory effects (Goodman, 2003) (Table 3).

Follicle stimulating hormone (FSH) and luteinizing hormone (LH)-secreting cells, or gonadotroph cells, account for roughly equal numbers as corticotrophs, but are scattered throughout the anterior lobe (Heaney & Melmed, 2004). These hormones regulate the sex steroid hormone production in the gonads, as well as the development and maturation of the germ cells (Table 3).

Lastly, a small percentage of thyrotroph cells (5%), concentrated at the central wedge, secrete the thyroid stimulating hormone (TSH) (Heaney & Melmed, 2004). TSH is the stimulus for thyroid hormone (T3/T4) production from the thyroid gland. Thyroid hormone mainly controls GH synthesis and secretion, metabolism and thermogenesis, as well as fetal skeletal maturation, and central nervous system development and maturation (Goodman, 2003) (Table 3).

Table 3. Hypothalamic and adenohypophyseal hormones and their functions (adapted from Brook & Marshall, 2001; Goodman, 2003; Heaney & Melmed, 2004).

Hypothalamic Hormones	Function	Target Organ	
Stimulatory			
GHRH	Induction of GH secretion	Anterior pituitary lobe	
CRH	Induction of ACTH secretion	Anterior pituitary lobe	
TRH	Induction of TSH secretion	Anterior pituitary lobe	
GnRH	Induction of FSH/LH secretion	Anterior pituitary lobe	
Inhibitory			
Somatostatin	Inhibition of GH secretion	Anterior pituitary lobe	
Dopamine	Inhibition of PRL secretion	Anterior pituitary lobe	
Adenohypophyseal Hormones	**Function**	**Target Organ**	**Homeostatic functions**
GH	Indirect action through the induction of hepatic IGF-I Direct action on several tissues	Bones and cartilage, soft tissues, adipose tissue, organs	• Promotion of body growth • Control of body mass • Blood glucose regulation
PRL	Mammary gland development Lactation stimulation	Mammary glands	• Milk production
ACTH	Induction of glucocorticoid hormone (cortisol) secretion	Adrenal glands (cortex)	• Metabolism (gluconeogenesis in liver, catabolism in adipose tissue, muscles, bone) • Blood glucose increase • Anti-inflammatory effects
TSH	Induction of thyroid hormone (T3/T4) secretion	Thyroid gland	• Metabolism and thermogenesis • Skeletal growth and maturation • CNS growth and maturation
FSH / LH	Induction of sex steroid hormone production: Ovaries: Estradiol, progesterone Testes: Testosterone	Gonads (ovaries and testes)	• Germ cell development (ovulation and spermatogenesis)

2.1.3 Regulation of hormone secretion

2.1.3.1 Positive regulation

The positive regulation of the anterior pituitary hormones' expression and secretion, with the exception of PRL, is controlled by hypothalamic hormones that reach the corresponding adenohypophyseal cells through the pituitary stalk and exert their regulatory action via the recognition of the corresponding receptors (Fig. 1, Table 3). Hypothalamic GH-releasing hormone (GHRH) induces GH secretion; GH reaches the liver and other tissues, through the systemic circulation, and mediates IGF-I production, which primarily controls the post-natal linear and organ growth, after the first 8-10 months of life (Brook & Marshall, 2001). Hypothalamic corticotropin-releasing hormone (CRH) stimulates ACTH secretion, which in turn stimulates glycocorticoid secretion from the adrenal glands. TSH is positively regulated by the hypothalamic thyrotropin-releasing hormone (TRH), which stimulates the thyroid hormone secretion (T3/T4). Finally, hypothalamic gonadotropin-releasing hormone (GnRH) induces gonadotrophs to produce FSH and LH, which regulate the production of the sex steroid hormones (estradiol, progesterone, testosterone) in the ovaries and testes (Table 3). PRL secretion is mainly mediated by the direct action of estrogens on the prolactin gene transcription level (Heaney & Melmed, 2004).

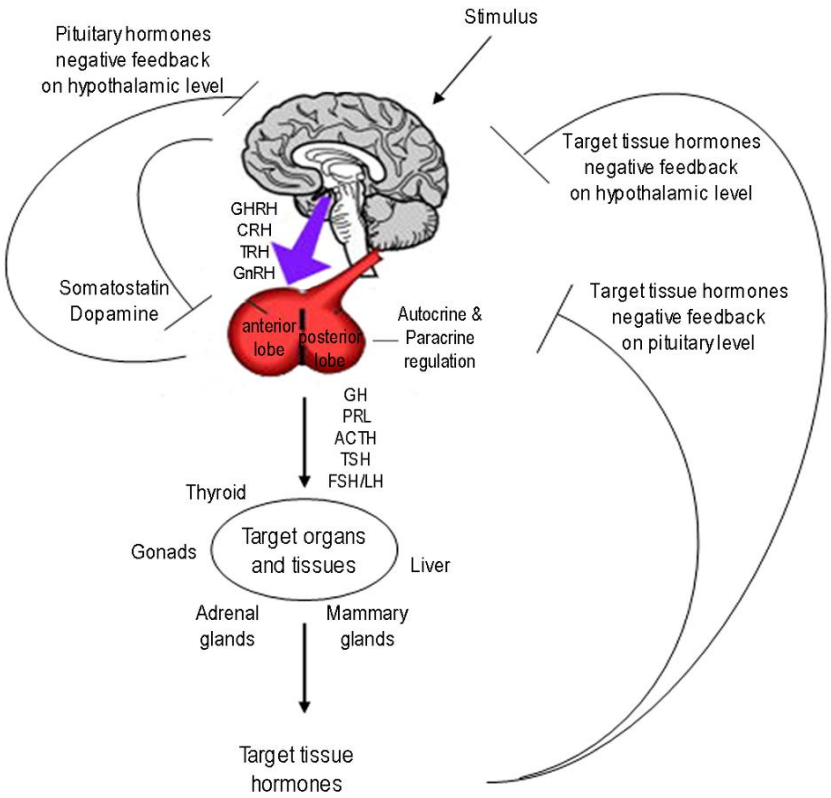

Figure 1. Schematic representation of the mechanisms regulating the anterior pituitary hormone secretion at the pituitary and the hypothalamic level. Positive regulation is indicated by straight black arrows, whereas negative regulation is shown by curved lines (The brain and pituitary images are modified from the website www.tiscali.co.uk).

2.1.3.2 Negative regulation

The negative regulation of the pituitary hormones is conferred by hypothalamic hormones, such as somatostatin in the case of GH, and dopamine in the case of PRL (Table 3). Secretion of pituitary hormones is subject to a negative feedback by the secreted products from the target glands: GH (and GHRH) secretion is inhibited by IGF-I, the thyroid hormone, or

cortisol. Both ACTH and CRH are under the negative feedback control of the peripheral glycocorticoid hormones. The negative regulation of TSH (and TRH) is exerted by the thyroid hormone. FSH/LH (and GnRH) are negatively regulated by the sex steroid hormones (Fig. 1) (Goodman, 2003; Heaney & Melmed, 2004).

2.2 Benign tumors of the anterior pituitary lobe and pathological features

Pituitary adenomas are benign adenohypophyseal tumors that may arise *de novo* or due to lack of suppression by the hypothalamic hormones (Brook & Marshall, 2001). They account for approximately 15% of all intracranial tumors (Heaney & Melmed, 2004) and are the third most common intracranial tumor type after meningiomas and gliomas (Scheithauer *et al*., 2006). Although classified as benign, many of these lesions are locally invasive and can cause major effects on a patient's quality of life, due to aberrant hormone secretion, as well as compressive effects on nearby tissues or the healthy pituitary (hypopituitarism). Irregular hormone hypersecretion can lead to a number of well recognised clinical conditions, such as acromegaly or Cushing's disease (see section 2.2.3 below).

2.2.1 Incidence and prevalence

Pituitary adenomas occur at an approximately equal incidence in both sexes (Asa & Ezzat, 2002). Their annual incidence is estimated at 19-28 new cases per million people (Davis *et al*., 2001; Soares & Frohman, 2004). However, their small size, and their insidious, often asymptomatic, nature pose a challenge in accurate prevalence estimation (Monson, 2000; Ezzat *et al*., 2004). Observations from autopsy series, as well as imaging studies in healthy individuals, incidentally revealed pituitary lesions (usually microadenomas) in 5-20% of the examined cases (Burrow *et al*., 1981; Molitch & Russell, 1990; Hall *et al*., 1994). In a thorough meta-analysis of post-mortem and radiological studies, Ezzat *et al*. (2004) estimated an overall prevalence of unsuspected pituitary adenomas of 16.7%. The prevalence of clinically relevant pituitary adenomas is not as high, but it is higher than previously thought, as observed in a cross-sectional study in Belgium (1/1000 population) (Daly *et al*., 2006a). A subsequent study undertaken by 18 centers on three continents has confirmed the high prevalence of clinically-relevant pituitary adenomas, identified among >700,000 individuals (average 0.75/1000 population) (Daly *et al*., 2007a).

2.2.2 Tumor characteristics and classification

Pituitary adenomas are believed to develop by monoclonal expansion of a single neoplastic cell, due to an acquired intrinsic primary cell defect (genetic or epigenetic) that confers growth advantage (Asa & Ezzat, 2002). X-chromosome inactivation studies on pituitary tumors from female patients confirmed monoclonality in all types of adenomas (Herman *et al*., 1990; Alexander *et al*., 1990; Schulte *et al*., 1991; Gicquel *et al*., 1992).

Pituitary tumors are most often benign and can grow both slowly and expansively. Enclosed adenomas have a clear delineation to the rest of pituitary tissue and the sinuses; yet, if the tumor increases in size, it may invade surrounding structures. Although defined as benign, nearly 50% of pituitary adenomas invade surrounding tissues, but invasiveness rate differs between various pituitary adenoma types (Brook & Marshall, 2001; Saeger *et al*., 2007). Very rarely do pituitary adenomas become metastatic, and are then referred to as pituitary carcinomas (see section 2.4).

Pituitary adenomas are generally classified as "functioning", when the corresponding hormones are oversecreted and, thus, cause clinical manifestations of the disease, and "non-functioning", when there is no hormone hypersecretion and no aberrant blood hormone levels observed. The main proportion of non-functioning adenomas produces, however, enough hormones to be detected by immunohistochemical staining. According to their size, they can be macroadenomas (i.e. tumors greater than 10 mm in diameter), or microadenomas (i.e. tumors less than 10 mm in diameter) (DeLellis *et al*., 2004). The complete classification of pituitary adenomas is based on functional, imaging/surgical, histopathological, immunohistochemical, and ultrastructural features (Kovacs *et al*., 1996).

2.2.3 Clinical features

The clinical manifestations of pituitary adenomas could be briefly divided into three categories: a) signs and symptoms due to excessive hormone secretion (i.e. acromegaly/gigantism in patients with GH-secreting adenomas, or galactorrhea and/or reproductive dysfunction in PRL-secreting adenoma patients), b) signs and symptoms due to mechanical effects of an expanding tumor mass – ranging from headaches and diminished visual acuity to severe visual disturbances, due to the compression of the optic chiasm – and c) impairment of the normal pituitary function in the case of large adenomas causing partial or panhypopituitarism due to compression (Arafah & Nasrallah, 2001). The major characteristics and clinical manifestations of each pituitary adenoma type are detailed below and summarized in Table 4.

2.2.3.1 Prolactinomas

PRL-secreting adenomas, also known as prolactinomas, account for the majority of pituitary tumors (40-45%) (Table 4) (Arafah & Nasrallah, 2001). Their estimated incidence is 6-10 new cases per million per year and a prevalence of about 60-100 cases per million (Davis *et al*., 2001; Ciccarelli *et al*., 2005). They are reported to occur much more frequently in women than in men, in particular between the second and third decades of life (Mindermann & Wilson, 1994), presumably because of the belated recognition of symptoms in men. Elevated serum PRL concentrations are diagnostic of prolactinomas. Hyperprolactinemia in premenopausal women causes oligomenorrhea or amenorrhea, in addition to galactorrhea, because of decreased estogen levels. Due to the early manifestations in young adult women, tumors are diagnosed as microadenomas. The main presenting symptom in men is sexual impotence and diminished libido, due to the decrease in testosterone levels, and by the time of diagnosis tumors are usually macroadenomas (DeLellis *et al*., 2004; Ciccarelli *et al*., 2005). In both sexes fertility is compromised. Other symptoms include headaches and visual disturbances, as well as variable degrees of hypopituitarism, all manifested in the presence of macroadenomas (Arafah & Nasrallah, 2001).

2.2.3.2 Somatotropinomas

GH-secreting adenomas, also known as somatotropinomas, account for approximately 20% of all pituitary tumors (Table 4). These tumors hypersecrete GH, whereas in about a quarter of them GH hypersecretion is synchronous to PRL hypersecretion. This event may be either due to the co-presence of somatotroph and lactotroph cells in the tumor ('dimorphous'), or due to a mammosomatotroph adenoma ('monomorphous'), with the same cells secreting

both GH and PRL. GH hypersecretion leads to acromegaly or gigantism, depending on the age of occurrence of a GH-secreting adenoma.

Table 4. Classification of pituitary adenomas (adapted from Arafah and Nasrallah, 2001; Jaffe, 2006)

Adenoma type	Prevalence	Principal hormone immunoreactivity	Clinical manifestations
PRL-secreting (prolactinomas)	40-45%	PRL	Signs of hyperprolactinemia
GH-secreting (somatotropinomas)	20%	GH ± PRL	Acromegaly/Gigantism
ACTH-secreting (adrenocorticotropinomas)	10-20%	ACTH	Hypercortisolism Cushing's disease
Non-functioning (gonadotropinomas)	15%	FSH, LH, αSU, βSU	Compression effects
Non-functioning (null-cell adenomas)	5-10%	None	Compression effects
TSH-secreting (thyrotropinomas)	1-2%	TSH	Mild hyperthyroidism Compression effects
FSH/LH-secreting (gonadotropinomas)	Rare	FSH, LH, αSU, βSU	Ovarian hyperstimulation in women, gonadal hyperplasia and elevated testosterone levels in men Compression effects

Acromegaly

The medical term "acromegaly" originates from the Greek words '*akro'*, which means 'extreme' or 'extremities', and '*megas'*, which means 'large', indicating the enlargement of the extremities. However, in reality, a large number of organs and tissues are affected. More than 98% of the acromegaly cases are attributed to GH oversecretion due to a somatotroph adenoma (Melmed, 1990), whereas the rest are the result of rare excessive hypothalamic or ectopic GHRH secretion, or even more rare ectopic GH secretion, from a neuroendocrine tumor (Melmed, 2006). The incidence is estimated to be three to four new cases per million per year, and the prevalence is about 40-60 cases per million people (Alexander *et al*., 1980; Bengtsson *et al*., 1988; Kauppinen-Makelin *et al*., 2005). Due to the insidious nature of the disease, it may take years (~4-10 years) before a definitive diagnosis of acromegaly is made, usually during the forth or fifth decade of life (Chanson & Salenave, 2008). Prolonged bodily exposure to increased levels of GH and IGF-I results in increased morbidity in acromegaly. Table 5 summarizes some of the most prominent clinical features observed in patients with acromegaly. If left untreated, acromegaly may lead to increased mortality, due to more severe complications (i.e. cardiovascular, cerebrovascular disease, and diabetes) (reviewed in Colao *et al*., 2004 and Erfurth & Hagmar, 2005). The current overall mortality for acromegaly patients in whom treatment targets are reached, is not estimated to be very different from that of the general age- and gender-matched population; however, patients reaching suboptimal post-treatment serum GH levels, and possibly patients having received irradiation, face an increased mortality risk (Orme *et al*., 1998; Ayuk *et al*., 2004; Holdaway *et al*., 2004; Kauppinen-Makelin *et al*., 2005; reviewed in Ayuk & Sheppard, 2008).

Early studies supported that malignancies, such as colorectal, breast, and prostate cancer, may arise at a higher risk in the context of acromegaly (Nabarro, 1987; Colao *et al*., 1998; Jenkins, 2004; reviewed in Colao *et al*., 2004). Acromegaly patients have increased risk for

developing premalignant adenomatous colonic polyps and colorectal cancer (Ezzat *et al*., 1991; Renehan *et al*., 2000; Jenkins & Besser, 2001; Kurimoto *et al*., 2008). A possible explanation for this might be the trophic (mitogenic, antiapoptotic) effects of excessive IGF-I on the colonic epithelium, but theoretically on other epithelia, such as the breast and prostate as well (Jenkins & Besser, 2001). The exact magnitude of the neoplasia risk remains the subject of much debate, since other epidemiological studies do not support an increased incidence of *de novo* malignancy in acromegaly (Orme *et al*., 1998; Renehan *et al*., 2000; reviewed in Melmed, 2001). Lastly, thyroid disorders, including goitre and benign or malignant tumors, have been detected in several series of acromegaly patients (Gasperi *et al*., 2002; Tita *et al*., 2005; Kurimoto *et al*., 2008).

Table 5. Clinical features and complications of acromegaly (adapted from: Melmed, 2006; Chanson & Salenave, 2008; Orphanet, 2008 at www.orpha.net).

Signs and symptoms
1. Enlarged upper and lower extremities (enlarged toes, fingers)
2. Coarsening of facial features (brows, ears, nose, lips)
3. Hyperhydrosis (excessive sweating)
4. Tall stature / Gigantism
5. Fatigue and myopathy (muscle weakness)
6. Goitre (thyroid hyperplasia)
7. Visceromegaly (enlarged salivary glands, heart, liver, spleen, kidneys, prostate)
8. Macroglossia, soft tissue swelling
9. Jaw malocclusion, tooth gaps, prognathism
10. Headaches and visual disturbances
11. Severe snoring, sleep disturbancies
12. Arthralgia (joint pain) and arthritis (limited joint mobility)
13. Carpal tunnel syndrome (wrist neuropathy)
14. Thick, coarse, oily skin and skin tags (skin tissue outgrowths)
15. Menstrual irregularities in women (amenorrhea, galactorrhea)
16. Sexual impotence in men
Complications
1. Hypertension
2. Diabetes mellitus
3. Sleep apnea (due to obstruction of the upper airway)
4. Colorectal polyposis and increased risk for colorectal cancer
5. Cerebrovascular disease
6. Congestive heart failure

Gigantism

Approximately 10% of adult patients with acromegaly exhibit tall stature; however, when a GH-secreting adenoma develops during childhood or adolescence, before the closure of the epiphyseal plates of the long bones, it results in accelerated linear growth, a condition called "gigantism" (Eugster & Pescovitz, 1999). The diagnosis is fairly straightforward, compared to acromegaly patients who may remain undiagnosed for many years. The majority of giants eventually develop acromegalic features, but the number of affected cases is not sufficient to draw any precise figures regarding the prevalence of other signs and symptoms in children with gigantism (Eugster & Pescovitz, 1999).

2.2.3.3 Adrenocorticotropinomas

The ACTH-secreting adenomas, also known as adrenocorticotropinomas, account for approximately 10-20% of all pituitary tumors (Table 4). These adenomas are typically microadenomas and occur more frequently in women than men (Mindermann & Wilson, 1994). ACTH hypersecretion causes excessive corticosteroid (cortisol) secretion from the adrenal gland cortex. Cushing's disease is the condition in which patients exhibit hypercortisolism almost exclusively due to the presence of an adrenocorticotropinoma, and very rarely due to ectopic ACTH or CRH secretion. Among the typical signs and symptoms of hypercortisolism in Cushing's disease are: Central obesity, easy bruisability, hyperpigmentation, myopathy, striae, hypertension, hirsutism, menstrual irregularities, mood changes, osteoporosis, poor wound healing, and hyperglycemia, due to insulin resistance (Arafah & Nasrallah, 2001).

2.2.3.4 Thyrotropinomas and gonadotropinomas

TSH-secreting adenomas, or thyrotropinomas, are very rare among all pituitary adenomas (1-2%) (Table 4), and their clinical manifestations may be mistaken for primary thyroid dysfunction, as the TSH hypersecretion typically results in clinically mild hyperthyroidism. Thyrotropinomas may grow to macroadenomas (DeLellis *et al*., 2004). Symptoms are primarily caused by the hormonal hypersecretion (i.e. mild hyperthyroidism), but also due to tumor size (i.e. hypopituitarism, headaches, visual field impairment) (Arafah & Nasrallah, 2001).

Functioning gonadotrophic tumors, or gonadotropinomas, producing FSH and/or LH, or their respective alpha and beta subunits (αSU or βSU) are really rare. Symptoms caused by excessive FSH/LH secretion include ovarian hyperstimulation in women, and gonadal hyperplasia and elevated serum testosterone levels in men (Arafah & Nasrallah, 2001).

2.2.3.5 Clinically non-functioning pituitary adenomas (NFPA)

Roughly one third of all pituitary adenomas are endocrinologically silent; they produce hormones that can be detected by immunostaining, but do not cause elevation of the blood hormone levels, and, thus, no manifestations typical of a hormone oversecretion syndrome (Heaney & Melmed, 2004). NFPAs may grow insidiously for years and by the time of diagnosis they are large (>10 mm); thus, their clinical presentation is related to the mechanical effects of an expanding macroadenoma (hypopituitarism, headaches, visual defects) (Jaffe, 2006). A subset of NFPAs, accounting for approximately 10-15% of all pituitary adenomas, produce the gonadotrophic hormones FSH and/or LH, or their respective alpha and beta subunits (αSU and βSU). The true NFPAs (i.e. no immmunoreactive hormone found by immunostaining) are less common (5-10%) and are referred to as "null-cell" adenomas (Table 4) (Arafah & Nasrallah, 2001).

2.3 Pediatric pituitary adenomas

Pituitary adenomas occur rarely in childhood and adolescence. According to several series, approximately 2-6% of all surgically treated pituitary adenomas occur in young patients (Haddad *et al*., 1991; Partington *et al*., 1994; Dyer *et al*., 1994; Mindermann & Wilson, 1995), whereas approximately 3% of all diagnosed intracranial tumors in childhood are pituitary adenomas (Keil & Stratakis, 2008). PRL-secreting adenomas are the most common type

among pubertal and post-pubertal patients (Mindermann & Wilson, 1995), and represent approximately 50% of pediatric pituitary tumors in several series (Partington *et al*., 1994; Kane et al., 1994; Mindermann & Wilson, 1995; Artese *et al*., 1998; reviewed in Kunwar & Wilson, 1999). ACTH-secreting adenomas are the second most common adenomas and the most frequently encountered in early childhood, even though they can occur at all pediatric ages (Mindermann & Wilson, 1995). The higher frequency of ACTH-secreting adenomas (29-50%) than GH-secreting adenomas (5-15%) in pediatric patients contrasts observations in adults (Partington *et al*., 1994; Dyer *et al*., 1994; Kane et al., 1994; Mindermann & Wilson, 1995; Colao *et al*., 2007). NFPAs are rare in children and adolescents (3-6%), despite representing roughly one third of all pituitary adenomas diagnosed in adults (Partington *et al*., 1994; Dyer *et al*., 1994; Mindermann & Wilson, 1995; Abe *et al*., 1998). Pediatric TSH- or FSH/LH-secreting adenomas are extremely rare (Kunwar & Wilson, 1999).

The early onset of pituitary adenomas suggests that tumorigenesis in children and adolescents may in part be explained by genetic factors, as observed in pediatric onset pituitary adenomas occurring in the context of Multiple Endocrine Neoplasia type 1 (MEN1) and CNC (O'Brien *et al*., 1996; Stratakis *et al*., 2000; Brandi *et al.*, 2001; Stratakis *et al*., 2001; Keil & Stratakis, 2008).

2.4 Carcinomas of the anterior pituitary lobe

Occasionally, invasive pituitary tumors can become aggressive and metastasize to distant locations in the central nervous system, or systemically reach lymph nodes and other sites throughout the body, including the liver, lungs, and bones (DeLellis *et al*., 2004; Scheithauer *et al*., 2006). These tumors are referred to as pituitary carcinomas and are characterized as such only after metastases are identified (Saeger *et al*., 2007). These carcinomas are extremely rare, with about 140 cases reported in the literature thus far (Kaltsas *et al.*, 2005; Manahan *et al*., 2007). Their incidence has been suggested to be 0.2% of symptomatic pituitary tumors (Pernicone *et al*., 1997), with almost equal frequency in both sexes (DeLellis *et al*., 2004; Kaltsas *et al.*, 2005). Most are ACTH- or PRL-secreting tumors; GH-, TSH-secreting or NFPAs rarely develop into carcinomas (Saeger *et al*., 2007). The time interval between initial adenoma diagnosis and carcinoma development may vary greatly, depending on the tumor subtype, with a mean of seven years (Pernicone *et al*., 1997; Sidibe, 2007). The prognosis is poor, with a mean survival rate of less than four years (Pernicone *et al*., 1997; Kaltsas *et al.*, 2005). The initial clinical, biochemical and histopathological characteristics are of minimal utility in distinguishing benign adenomas from those that will develop into carcinomas (Kaltsas *et al*., 2005; Kars *et al*., 2006).

3. Genetic features of pituitary tumorigenesis

The pathogenesis of pituitary adenomas has attracted great interest and controversy. It is established that pituitary adenomas develop by monoclonal expansion of a single cell that has acquired intrinsic primary genetic (i.e. activation of oncogenes or inactivation of TSGs) or epigenetic (i.e. methylation) defects that confer a growth advantage. This section shortly reviews only a handful of possible etiologic genetic features of sporadic pituitary tumor development. Yet, despite the broad genetic background underlying pituitary

tumorigenesis, genetic abnormalities are encountered not only as somatic events in sporadic pituitary tumors, but also in the context of inherited pituitary tumor susceptibility. A more extensive review is presented later in this chapter, regarding the genetic background of pituitary adenomas occurring in the context of familial endocrine-related tumor syndromes.

3.1 Sporadic pituitary adenomas

3.1.1 *GNAS* / *gsp* oncogene

GNAS (20q13) is a ubiquitously expressed gene that codes for the stimulatory guanosine triphosphate (GTP)-binding protein $G_s\alpha$. $G_s\alpha$ activates adenylyl cyclase, which in turn increases the cyclic adenosine monophosphate (cAMP) cellular levels; cAMP is a secondary signal transduction messenger that mediates a signalling cascade through the activation of protein kinases in many cell types, including pituitary cells. Vallar *et al*. (1987) found that two "hot spots" (codons 201 and 227) of *GNAS* – or *gsp* oncogene – were frequently mutated in sporadic GH-secreting adenomas; these mutations caused constitutive activation of $G_s\alpha$, resulting in high adenylyl cyclase activity and increased cAMP levels (Vallar *et al*., 1987). It is now established that approximately 40% of GH-secreting adenomas harbor somatic mutations in *GNAS* (Lyons *et al*., 1990). Interestingly, Hayward *et al*. (2001) found that, contrasted to biallelic G_sa expression in all human tissues, the G_sa expression in the pituitary is monoallelic – subject to imprinting – and is derived from the maternal allele. Activating mutations, occuring almost exclusively on the maternal allele, partly explain the underlying background of somatotroph tumorigenesis (Hayward *et al*., 2001). Subsequent studies failed to consistently replicate the finding of *GNAS* mutations in other types of pituitary adenomas (reviewed in Lania *et al*., 2003); data concerning mutations in other G proteins, such as the stimulatory Gαq and Gα11, or the α-subunit of GIP2 – a protein coupled to the inhibitory $G_i2\alpha$ – were also discordant (Lyons *et al*., 1990; Petersenn *et al*., 2000). *Gsp* activating mutations in GH-secreting adenomas remain the only unequivocally identified pathogenic mutations thus far. However, the lack of clinical differences between patients with and without *GNAS* mutations is intriguing, suggesting the existence of additional pathogenic mechanisms (Spada *et al*., 1990; Adams *et al*., 1993).

A postzygotic gain-of-function mutation in the *GNAS* gene is the genetic defect in McCune-Albright syndrome (MAS) (MIM 174800) (Weinstein *et al*., 1991; Schwindinger *et al*., 1992). MAS is a congenital syndrome characterized by polyostotic fibrous dysplasia, multiple café-au-lait spots, precocious puberty, and often endocrinopathies, including hyperthyroidism and GH and PRL excess (Albright *et al*., 1937; Dumitrescu & Collins, 2008). *GNAS* mutations result in constitutive $G_s\alpha$ activation and elevated cAMP levels, which leads to excessive bone matrix production in the skeleton and hormonal oveproduction in endocrine cells.

3.1.2 Other features of sporadic pituitary tumorigenesis

Other oncogenes that have been analyzed include proteins involved in signal transduction, growth factors and their receptors, and cell cycle-related proteins. These, together with loss-of-function events in TSGs and other players, are summarized in Table 6. A possible model of pituitary tumorigenesis is presented in Figure 2.

Regarding the gross chromosomal rearrangements observed in pituitary adenomas, the published reports are relatively few and the results rather inconclusive. Overall, CGH, FISH, and traditional cytogenetic studies have shown losses and gains of almost all chromosomes, without definite trends (Bettio *et al*., 1997; Larsen *et al*., 1999; Kontogeorgos *et al*., 1999; Finelli *et al*., 2000; Bello *et al*., 2001). Such aberrations are more frequent among secreting than NFPAs, with higher incidence in invasive/recurrent tumors than the non-invasive ones. The rarity of pituitary carcinomas does not facilitate valid conclusions concerning somatic gross chromosomal defects and the carcinoma pathogenesis (DeLellis *et al*., 2004).

Finally, late advances in molecular biotechnology have facilitated the search for novel candidate genes involved in pituitary tumorigenesis. Microarray-based differential gene expression profiles have been recently generated for different types of human pituitary adenomas (Evans *et al*., 2001; Morris *et al*., 2005; Moreno *et al*., 2005; Ruebel *et al*., 2006).

Table 6. Genetic alterations in sporadic pituitary tumorigenesis (modified from Asa & Ezzat, 2002; Lania *et al*., 2003; DeLellis *et al*., 2004; Boikos & Stratakis, 2007).

Gene	Alteration in the tumors	Type of pituitary adenoma
Gain-of-function events		
Signal Transduction		
$G_{s}\alpha$ (gsp)	Activating somatic mutations	GH-secreting
$G_{i}2\alpha$	Activating somatic mutations	NFPAs (i)
RAS	Activating somatic mutations	Pituitary carcinomas (metastatic)
PKC	Activating somatic mutations	GH-secreting, invasive tumors (i)
PKA	Loss of function of *PRKAR1A* due to germline inactivating mutations	GH-secreting
Growth factors and their receptors		
FGFR4	Alternative transcription initiation	All types
ptd-FGFR4	Constitutive activation	All types
TGF-α	Overexpression	PRL-, GH/PRL-, ACTH-secreting
Cell cycle		
Cyclin D1	Overexpression	Aggressive adenomas, NFPAs
Cyclin E	Overexpression	ACTH-secreting
PTTG	Overexpression	All types
HMGA2	Overexpression	GH- and PRL-secreting
Loss-of-function events		
TSGs		
PRB	LOH of 13q Underexpression due to promoter methylation	Aggressive adenomas, carcinomas
CDKN2A/$p16^{INK4A}$	Underexpression due to promoter methylation	All types, GH-secreting (i)
CDKN1B/$p27^{Kip1}$	Underexpression due to protein degradation	ACTH-secreting, recurrent tumors and carcinomas
MEN1	Somatic inactivating mutations (rare) and LOH	PRL-, GH- PRL/GH-, ACTH-secreting and NFPAs
CDKN2C/$p18^{INK4C}$	Underexpression	NFPAs, PRL- and GH-secreting
Other players		
D2R	Underexpression	Resistant PRL-secreting (i)
SSTR2/SSTR5	Underexpression	Resistant GH-secreting
GADD45G	Underexpression due to methylation	NFPAs, PRL- and GH-secreting
MEG3A	Underexpression due to methylation	NFPAs

RAS, rat sarcoma oncogene; PKA/C, protein kinase A/C, FGFR4, fibroblast growth factor receptor 4; ptd-FGFR4, pituitary-derived FGFR4; TGF-α, transforming growth factor α; PTTG, pituitary tumor transforming gene; HMGA2, high mobility group at-hook 2; PRB, retinoblastoma protein; CDKN, cyclin-dependent kinase inhibitor; D2R, dopamine D2 receptor; SSTR, somatostatin receptor; GADD45γ, growth arrest and DNA-damage-inducible protein gamma; MEG3A, maternally expressed protein 3A; (i): infrequently.

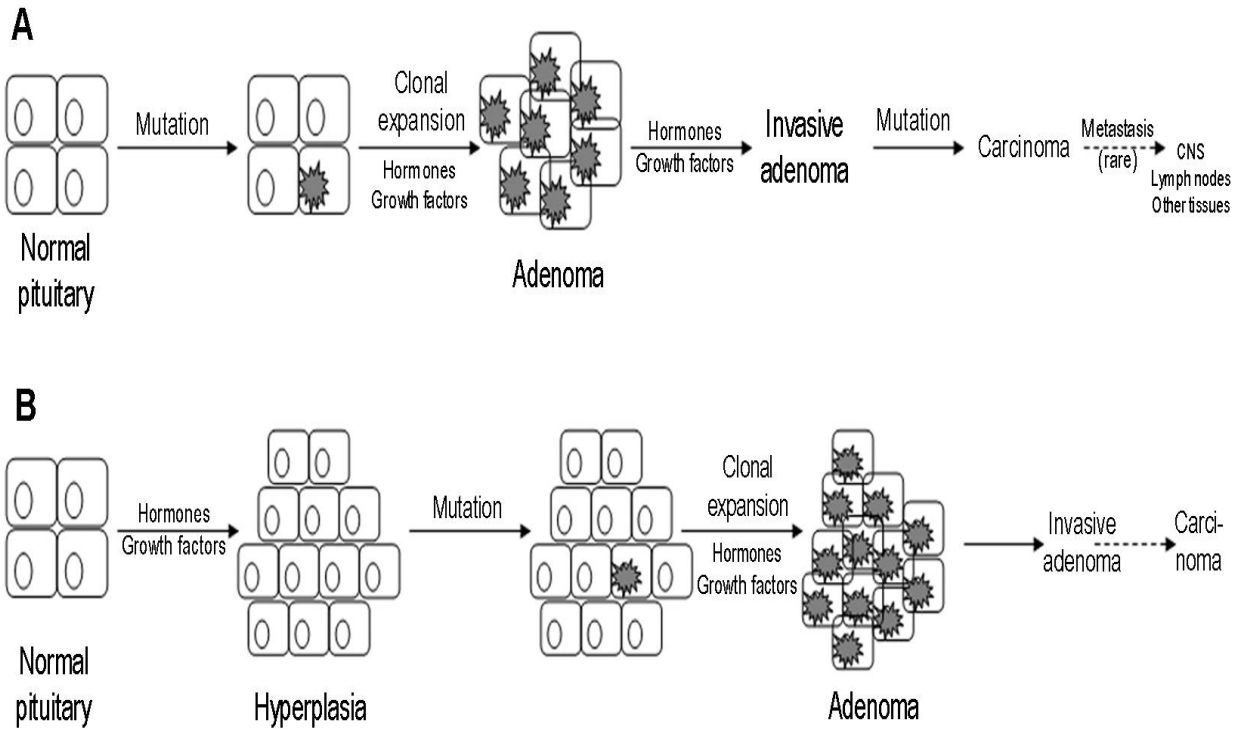

Figure 2. Possible pathways of pituitary tumorigenesis. A) If genetic or epigenetic alterations occurring in a pituitary cell confer growth advantage (e.g. *PRKAR1A* mutations or constitutive activation of *gsp* leading to increased cAMP levels, loss of menin, methylation of p16 or p18), clonal expansion in a permissive environment (stimulatory hormones or growth factors) can lead to adenoma development. Additional genomic changes (e.g. loss of pRB, loss of p27, *PTTG* overexpression) can lead to more aggressive (invasive) adenomas, or rarely (e.g. *RAS* mutations) to metastatic carcinomas. B) Deregulated pituitary exposure to hypothalamic hormones (e.g. increase of the GHRH/CRH/GnRH stimulatory effect or decrease of the dopamine/somatostatin inhibitory effect) may lead to mild hyperplasia, followed by a series of possible events as described in A). Both pathways are presented, despite the lack of clarity as to whether hyperplasia necessarily preceeds pituitary adenoma formation (modified from Asa & Ezzat, 1998; Boikos & Stratakis, 2007).

3.2 Pituitary adenomas in familial endocrine-related tumor syndromes

The vast majority of pituitary adenomas occur sporadically (95%). Familial pituitary tumors account for approximately 5% of all pituitary adenomas (Marx and Simonds, 2005; Daly *et al*., 2007b). These tumors arise as a component of endocrine-related tumor syndromes, namely Multiple Endocrine Neoplasia type I (MEN1), Carney complex (CNC), Isolated Familial Somatotropinomas (IFS), and Familial Isolated Pituitary Adenomas (FIPA), which are detailed below.

3.2.1 Multiple Endocrine Neoplasias

3.2.1.1 Multiple Endocrine Neoplasia type I (MEN1)

Multiple Endocrine Neoplasia Type 1 (MEN1) (MIM 131100) is a rare (~1:30.000) autosomal dominant tumor susceptibility syndrome, characterized by varying combinations of

parathyroid hyperplasia or adenomas (90-95%), tumors of the enteropancreatic neuroendocrine tissues (30-75%), and adenomas of the anterior pituitary gland (10-60%) (Brandi *et al*., 2001). Primary hyperparathyroidism is the first clinical manifestation of the disease, present in more than 90% of the cases. Gastrinomas and insulinomas are the typical enteropancreatic neuroendocrine tumors, encountered in 40% and 10% of MEN1 cases, respectively. PRL-secreting adenoma is the most common pituitary adenoma type (20% of cases), whereas GH-secreting adenomas are less frequent (~10% of cases) (Thakker, 1998; Brandi *et al*., 2001). Other less common phenotypic features include lipomas, angiomyolipomas, angiofibromas, colalgenomas, carcinoid tumors, and adrenocortical adenomas (Chandrasekharappa *et al*., 1997; Thakker, 1998). Symptoms in MEN1 are mainly caused by the overproduction of specific hormones, tumor mass effects, malignancy, or any combination of these (Lemos & Thakker, 2008).

Familial MEN1 is defined as the occurrence of at least two of the three main MEN1-related lesions in an individual, with at least one first-degree relative having clinical, radiological, and/or surgical evidence, or repeated biochemical evidence of at least one of the MEN1-related lesions (Brandi *et al*., 2001). MEN1 exhibits an equal sex distribution (Teh *et al*., 1998) and a high age-related penetrance, with more than 95% of the patients being symptomatic by the fifth decade of life (Trump *et al*., 1996).

In 1988, the *MEN1* susceptibility gene was mapped on chromosomal region 11q13 (Larsson *et al*., 1988) and positionally cloned nearly ten years after (Chandrasekharappa *et al*., 1997). *MEN1* spans a region of approximately 9 kb, has 10 exons, and encodes a 610 aa protein termed "menin" (Marx, 2005). The overwhelming number of loss-of-function mutations, together with LOH as the second mutational hit in MEN1-related tumors, leaves little doubt that *MEN1* represents a classical TSG, according to Knudson's two-hit hypothesis.

Approximately 70-90% of typical MEN1 families carry pathogenic *MEN1* mutations (Agarwal *et al*., 1997; Teh *et al*., 1998; Giraud *et al*., 1998; Poncin *et al*., 1999a; Bergman *et al*., 2000a; Cebrian *et al*., 2003, Klein *et al*., 2005; Ellard *et al*., 2005). No phenotype-genotype correlations have been clearly recognized thus far (Agarwal *et al*., 1997; Tanaka *et al*., 1998a; Teh *et al*., 1998; Giraud *et al*., 1998; Lemos & Thakker, 2008). Founder *MEN1* mutations have been identified (Agarwal *et al*., 1997) in families from Finland, Sweden, and France (Teh *et al*., 1998; Giraud *et al*., 1998; Vierimaa *et al*., 2007). Bassett *et al*. (1998) estimated the age-related penetrance from 47 unrelated MEN1 probands and their families as: Nonexistent in individuals <5 years of age, 52% by the age of 20 years, >95% after the age of 40 years, and complete at 60 years of age (Bassett *et al*., 1998). Such disease risk assessments have a great impact on clinical evaluation, counseling, and management of at-risk individuals.

Sporadic MEN1 is defined as a MEN1 patient without known family history of any endocrine manifestation (Brandi *et al*., 2001). Mutations in sporadic MEN1 cases have been reported in 45-69% of the cases (Agarwal *et al*., 1997; Teh *et al*., 1998; Giraud *et al*., 1998; Bassett *et al*., 1998; Tanaka *et al*., 1998b; Poncin *et al*., 1999b; Hai *et al*., 2000; Cebrian *et al*., 2003; Ellard *et al*., 2005). Somatic *MEN1* mutations are rather common among MEN1-related sporadic tumors, such as parathyroid adenomas and enteropancreatic tumors (reviewed in Lemos & Thakker, 2008), but are rare among non-MEN1 sporadic pituitary adenomas (Zhuang *et al*., 1997a; Tanaka *et al*., 1998b; Prezant *et al*., 1998; Schmidt *et al*., 1999; Poncin *et*

al., 1999b; Bergman *et al*., 2000b). It also seems that *menin* gene expression remains intact in most sporadic pituitary adenomas, indicative of a lack of promoter mutations or hypermethylation (Asa *et al*., 1998).

To date more than 560 unique germline or somatic mutations have been described, scattered throughout the genomic *MEN1* sequence, with only a few potential "hot spots". The majority or these mutations (80%) cause menin truncation, leading to lack of interaction domains and the nuclear localization signals (NLSs), or absence of a translated product because of nonsense-mediated mRNA decay (NMD) (Lemos & Thakker, 2008; The Human Gene Mutation Database, May 2008 at www.hgmd.org).

Menin is a 67 kDa ubiquitously expressed protein, located in the nucleus due to three NLSs (La *et al*., 2006), and binds directly or indirectly to at least 21 candidate molecules or complexes (reviewed in Agarwal *et al*., 2004). Recent data support a functional role of menin at cell division and proliferation, apoptosis, transcriptional regulation, or genomic stability (reviewed in Marx & Simonds, 2005). *Men1*$^{-/-}$ mice are embryonic lethal or have severe developmental abnormalities (Crabtree *et al*., 2001; Bertolino *et al*., 2003a; Loffler *et al*., 2007), whereas *Men1*$^{+/-}$ mice have proved an excellent model of MEN1 disease; these mice develop major MEN1-related lesions, albeit with certain differences from human MEN1 (Crabtree *et al*., 2001; Crabtree *et al*., 2003; Bertolino *et al*., 2003b; Loffler *et al*., 2007). Pituitary- and pancreas-specific *Men1*-knockout mice exhibit normal pancreatic and pituitary development, but pancreatic hyperplasia and prolactinomas develop eventually, as in human MEN1 (Biondi *et al*., 2004).

3.2.1.2 MEN1-like (MEN4)

The identification of the *MEN1* gene has made genetic diagnosis possible in a great number of patients suspected for this syndrome. Yet, a rather intriguing subset of suspected familial cases, varying between 10-25%, test negative for mutations in the *MEN1* coding region (Agarwal *et al*., 1997; Bassett *et al*., 1998; Giraud et al., 1998; Hai *et al*., 2000; Cebrian *et al*., 2003; Ellard *et al*., 2005; Klein *et al*., 2005). Interestingly, these families do not exhibit significant phenotypic differences when compared to *MEN1* mutation-positive families (Bassett *et al*., 1998). It is likely that mutations outside the coding region (i.e. promoter, untranslated regions, and intronic regions) or possible disease-associated SNPs of yet undetermined significance escape identification. Disease phenocopies, not caused by *MEN1* mutations, have also been reported (Burgess *et al*., 2000; Hai *et al*., 2000; Klein *et al*., 2005). Nonetheless, theoretically, MEN1 may exhibit genetic heterogeneity, with other predisposing genes harboring pathogenic mutations.

Recently, Pellegata *et al*. (2006) identified *Cdkn1b*, which encodes the cyclin-dependent kinase inhibitor (Cdk) p27^{Kip1}, as the gene predisposing to a MEN-like phenotype (MENX) in a rat model (Pellegata *et al*., 2006). The animals exhibited phenotypic overlap of both MEN1 and MEN2 (bilateral pheochromocytomas, parathyroid adenomas, thyroid hyperplasia, paragangliomas, and endocrine pancreas hyperplasia) in an autosomal recessive pattern of inheritance. Affected rats were found homozygous for a tandem *Cdkn1b* duplication of 8 bp that resulted in frameshift and a premature termination codon. p27^{Kip1}–deficient rats showed increased body weight compared to their wild type littermates (Fritz *et al*., 2002; Pellegata *et*

al., 2006). This rat model was very similar to *Cdkn1b*-knockout mice: These mice displayed a 20-30% increase in body weight, as well as multiple organ hyperplasia, and pituitary intermediate lobe adenomas as the sole tumor phenotype (Nakayama *et al*., 1996; Kiyokawa *et al*., 1996; Fero *et al*., 1996).

Based on the rat MENX model, a heterozygous germline nonsense mutation was subsequently identified in the human MEN1-like (MEN4) (MIM 610755) predisposing gene, namely *CDKN1B* (12p13), which spans a 5 kb region, is composed of 3 exons, and codes for a 198 aa protein. The mutation was identified in a patient suspected for MEN1 (acromegaly, pituitary adenoma, and primary hyperparathyroidism), but tested negative for *MEN1* mutations. The mutation was segregating in the patient's family, with several family members exhibiting endocrine neoplasia. Functional studies clearly established an association between *CDKN1B* as a novel putative TSG and this heritable endocrine-related neoplasia syndrome (Pellegata *et al*., 2006).

Since *CDKN1B* gene identification, a heterozygous germline duplication of 19 bp has been detected in a patient clinically suspected for MEN1 (hyperparathyroidism, Cushing's disease, and small-cell neuroendocrine cervical carcinoma). First-degree relatives were free of MEN1-related lesions, but due to lack of extensive family history, it was not possible to establish whether this was a truly familial or sporadic case (Georgitsi *et al*., 2007). *CDKN1B* mutations have not been found in cases of familial acromegaly, familial isolated pituitary tumors, familial hyperparathyroidism, or familial MEN1 (Ozawa *et al*., 2007; Georgitsi *et al*., 2007; Owens *et al*., 2008; Igreja *et al*., 2008; Vierimaa *et al*., submitted manuscript). Recently, three novel mutations (P95S, 5'UTR -7G>C, and stop>Q) were reported in three suspected MEN1 families with parathyroid and other endocrine lesions, but, interestingly, without pituitary involvement (Dr. Agarwal, oral communication at the 11th International Workshop on MEN, Delphi, Greece, 2008).

Finally, regarding sporadic endocrine neoplasia, germline *CDKN1B* mutations are a very rare cause of MEN1 (Owens *et al*., 2008; Igreja *et al*., 2008), whereas the MEN1 variant of parathyroid/pituitary tumors (Hai *et al*., 2000) seems to occur due to genetic causes other than *CDKN1B* (Ozawa *et al*., 2007). This is likely also the case for sporadic GH-secreting adenomas (Georgitsi *et al*., 2007), and other types of pituitary tumors (Takeuchi *et al*., 1998; Dahia *et al*., 1998). Yet, it should be noted that CDKN1B/p27^{Kip1} protein expression levels, and not mRNA levels, are significantly reduced during progression from normal to neoplastic pituitaries, suggesting a contribution of CDKN1B/p27^{Kip1} in sporadic pituitary tumorigenesis by posttranslational mechanisms (Takeuchi *et al*., 1998; Dahia *et al*., 1998; Lidhar *et al*., 1999; Bamberger *et al*., 1999; Pellegata *et al*., 2006).

CDKN1B/p27^{Kip1} protein plays an important role in the cell cycle regulation, through the binding and inhibition of cyclin/CDK complexes during the cellular G1 to S phase transition (Sherr & Roberts, 1999); thus, CDKN1B/p27^{Kip1} participates in determining several cell fate decisions, including proliferation, differentiation, apoptosis, cell density, and even cell migration (Besson *et al*., 2004; Chu *et al*., 2008). Interestingly, it has been shown that *CDKN1B* is a transcriptional gene target of menin (Karnik *et al*., 2005), a possible target of the oncogenic RET protein in endocrine cells (Drosten *et al*., 2004), as well as a direct transcriptional target of aryl hydrocarbon receptor (AHR), as detailed later (Kolluri *et al*.,

1999) (see Discussion, section 6.2). These data indicate that functionally disrupted CDKN1B/p27^{Kip1} is likely to play a role in endocrine tumorigenesis.

3.2.2 Carney Complex (CNC)

Carney complex (CNC) (MIM 160980) is a rare autosomal dominant disease manifested by spotty-skin pigmentation, cardiac and other myxomas (tumors of the connective tissue), endocrine tumors (mainly GH-secreting adenomas), and schwannomas (benign tumors of the myelin sheath) (Carney *et al*., 1985). The pituitary presentation of CNC is essentially limited to mammosomatotroph hyperplasia that may progress to adenoma (Pack *et al*., 2000). Clinically manifested acromegaly is encountered in approximately 10% of CNC patients, despite the fact that up to 75% of them have altered GH/IGF-I and PRL levels (Pack *et al*., 2000; Stratakis *et al*., 2004).

In 70% of CNC cases a genetic causation has been identified, with two susceptibility loci, one on chromosome 17q24 and the second on chromosome 2p16 (Stratakis *et al*., 1996; Kirschner *et al*., 2000a). The former locus was found to harbor the predisposing gene *protein kinase A type I-alpha regulatory subunit* (*PRKAR1A*), which covers a genomic region of approximately 21 kb, is comprised of 11 exons, and encodes a 381 aa protein. *PRKAR1A* codes for a serine/threonine protein kinase A (PKA) regulatory subunit that is the main mediator in cAMP signalling. Inactivating *PRKAR1A* mutations have been identified in up to 60% of CNC patients meeting the diagnostic criteria (Kirschner *et al*., 2000a). CNC is a highly penetrant disease, with expression typically manifested by the age of 20 years. No gene has been identified in the second susceptibility locus (2p16), which has been restricted to a 100 kb region (Stratakis *et al*., 1996).

Almost all 40 distinct germline *PRKAR1A* mutations reported thus far, lead to mRNA instability and NMD, and thus, decreased or absent expression of the mutant protein (Kirschner *et al*., 2000a; 2000b; Groussin *et al.*, 2002). Mutations that escape NMD result in the retention of abnormal PRKAR1A and increased PKA activity, leading to typical manifestations of CNC (Greene *et al*., 2008). These alterations do not appear to occur on mutation “hot spots” (Kirschner *et al*., 2000b; Sandrini & Stratakis, 2003). LOH at 17q22-q24 has been demonstrated in CNC-associated pituitary tumors (Bossis *et al*., 2004). Somatic *PRKAR1A* mutations have not been detected in sporadic pituitary tumors, indicating that *PRKAR1A* is not prominently involved in sporadic pituitary tumorigenesis (Kaltsas *et al*., 2002; Sandrini *et al*., 2002; Yamasaki *et al*., 2003).

The functional inactivation of PRKAR1A protein results in excess PKA signalling and elevated cAMP levels in the affected tissues (Groussin *et al.*, 2002). Ablation of both *Prkar1a* copies in mice results in embryonic lethality (Amieux *et al*., 2002). Contrary, *Prkar1a*$^{+/-}$ mice and transgenic mice with an antisense *Prkar1a* exon 2 construct develop features compatible with the CNC phenotype, without, however, marked pituitary disease (Griffin *et al*., 2004a; Griffin *et al*., 2004b; Kirschner *et al*., 2005). The recently reported pituitary-specific knockout mice (pitKO), in which *Prkar1a* is deleted from the GH/PRL/TSH cell lineage, develop GH-secreting tumors by 18 months of age, with moderate frequency. Many pitKO mice show marked serum GH elevation, despite the lack of frank tumors, which is analogous to human CNC (Yin *et al*., 2008).

3.2.3 Isolated Familial Somatotropinomas (IFS)

By definition, IFS (MIM 102200) describes the occurrence of two or more cases of acromegaly or gigantism in a family in the absence of MEN1 or CNC (Gadelha *et al.*, 1999). Reports on familial acromegaly/gigantism date back to the early 1970s and 1980s (Levin *et al*., 1974; Kurisaka *et al*., 1981; Jones *et al*., 1984; Abbassioun *et al*., 1986). The notion that familial acromegaly/gigantism represents a rare entity, distinct from MEN1, became clearer with the accumulation of reports on first-degree relatives diagnosed with GH-secreting adenomas without mutations in *MEN1* or *GNAS* (Pestell *et al*., 1989; McCarthy *et al.*, 1990; Tamburrano *et al*., 1992; Links *et al*., 1993; Matsuno *et al*., 1994; Benlian *et al*., 1995; Verloes *et al*., 1999; Ackermann *et al*., 1999). An autosomal dominant inheritance pattern with incomplete penetrance was proposed (Pestell *et al*., 1989; Tamburrano *et al*., 1992; Benlian *et al*., 1995; Verloes *et al*., 1999; Gadelha *et al*., 1999).

In sporadic GH-secreting adenomas, LOH of 11q13 has been detected in as many as 10-40% of tumors (Thakker *et al*., 1993; Boggild *et al*., 1994; Zhuang *et al*., 1997a; Simpson *et al*., 2003); however, such allelic imbalance was very rarely seen in concert with somatic inactivating *MEN1* mutations (Boggild *et al*., 1994; Zhuang *et al*., 1997a; Prezant *et al*., 1998; Tanaka *et al*., 1998b; Schmidt *et al*., 1999). The same phenomenon had been observed in many studies that aimed at identifying the predisposing locus for familial GH-secreting adenomas in families with acromegaly and gigantism: Genome-wide LOH studies on tumor DNA confirmed loss of one allele on 11q13, but patients did not carry germline *MEN1* mutations, whereas the *menin* expression in the tumors was normal (Yamada *et al*., 1997; Kakiya *et al*., 1997; Teh *et al*., 1998; Tanaka *et al*., 1998a; Gadelha *et al*., 1999; Ackermann *et al*., 1999). In 2000, Gadelha *et al*. established linkage of the IFS locus on 11q13. This and subsequent studies restricted the candidate IFS 11q13 locus, still without making the exclusion of *MEN1* possible. At the time, mutations in the promoter region, introns, untranslated regions, or hypermethylation of promoter CpG islands of *MEN1* could not be excluded (Gadelha *et al*., 2000; De Menis & Prezant, 2002). Also linkage of IFS to chromosomes 2 and 17 (*PRKAR1A* locus) was excluded (Gadelha *et al*., 2000; Frohman & Eguchi, 2004). The number of reported IFS families continued to increase (Jorge *et al*., 2001; De Menis & Prezant, 2002; Tamura *et al*., 2002; Luccio-Camelo *et al*., 2004; Tiryakioglu *et al*., 2004; Soares *et al*., 2005), with approximately 50 IFS families reported by 2006 (Daly *et al*., 2006b).

Based on reports on IFS families by the year 2004, a number of important conclusions could be drawn regarding the IFS characteristics: The clinical manifestations of IFS were similar to those seen in patients with sporadic GH-secreting adenomas; macroadenomas surpassed microadenomas, gigantism was reported, and half of all GH-secreting adenomas were also immunopositive for PRL. Most of the families were represented by two affected cases. On the other hand, the median age at diagnosis in IFS was 25 years, and the age at onset was <30 years in roughly three quarters of the patients. This was contrasted to sporadic acromegaly, with an age of onset at the forth or fifth decade of life. Lastly, a slight preponderance of male versus female patients (1.5:1) was noted (Soares & Frohman, 2004; Frohman & Eguchi, 2004).

It was eventually concluded that loss-of-function of a TSG distinct from *MEN1* was responsible for IFS. Apart from *MEN1*, *GHRH receptor*, *GNAS*, and *PRKAR1A*, other genes residing in 11q13 had been excluded as candidates in patients with IFS (Soares *et al*., 2005).

3.2.4 Familial Isolated Pituitary Adenomas (FIPA)

Until recently, literature on families encompassing different types of pituitary adenomas, outside the contexts of MEN1, CNC, or IFS, had been scarce (Himuro *et al*., 1976; Yuasa *et al*., 1990; Stock *et al*., 1997). Familial prolactinoma was reported by Berezin and Karasik (1995), whereas hereditary early-onset prolactinoma was already known in a rat model (Chedid *et al*., 1988), the only animal model exhibiting genetic susceptibility to pituitary adenomas at the time.

Recently, a distinct clinical entity, namely Familial Isolated Pituitary Adenomas (FIPA) (MIM 102200), was reported in order to characterize families with isolated pituitary adenomas outside the clinical and genetic contexts of MEN1 and CNC (Daly *et al*., 2005). The initiative – undertaken by the Department of Endocrinology, University of Liège, in Belgium, as an international, collaborative effort – resulted in a collection of 64 families that exhibited different patterns of pituitary adenomas among 138 affected individuals (Daly *et al*., 2006b). Families with up to four affected cases were reported (Daly *et al*., 2006b; Beckers & Daly, 2007); they were characterized either as "homogeneous" (same adenoma phenotype among affected cases) – including 12 IFS families – or "heterogeneous" (different adenoma phenotypes among affected members). FIPA cases were significantly younger at diagnosis than population-matched sporadic cases. Prolactinomas were the predominant adenoma type, but not as frequent as in familial MEN1; females were affected more often than males, reflecting the fact that prolactinomas are encountered more often in females; GH-secreting adenomas were seen much more often in FIPA than MEN1 or sporadic pituitary adenomas (Daly *et al*., 2006b).

This initial study indicated that FIPA may account for 2.5% of pituitary adenomas (Daly *et al*., 2006b), similar to the estimate that 2.7% of pituitary adenomas are due to familial MEN1 (Scheithauer *et al*., 1987). Thus, and including the few CNC and IFS cases known worldwide, it was suggested that hereditary tumor susceptibility may contribute to pituitary tumorigenesis by approximately 5% (Marx & Simonds, 2005; Daly *et al*., 2007c).

A first-degree relationship between affected members was observed in the majority of the FIPA kindreds. Thus, based on pedigree analysis, an autosomal dominant inheritance pattern with incomplete penetrance was suggested for FIPA, as previously hypothesized for IFS (Daly *et al*., 2006b). However, the exact genetic cause remained elusive.

AIMS OF THE STUDY

The primary aim of the present work was

1. To localize and identify a novel tumor susceptibility gene that causes Pituitary Adenoma Predisposition (PAP) in familial and sporadic cases from Northern Finland (I).

Subsequently from gene identification, we envisaged the following aims:

2. To gain insights into the genetic basis of PAP in an effort to establish molecular diagnosis (II) by:
    a) Studying the contribution of PAP in pituitary adenoma patients of various ethnic origins,
    b) Further elucidating the PAP phenotype, and
    c) Testing the potential of immunohistochemistry as a diagnostic tool.

3. To examine whether and to what extent the PAP gene is implicated in the tumorigenesis of common tumor types (III).

4. To examine whether pediatric onset pituitary tumorigenesis is attributed to tumor susceptibility caused by mutations in the PAP gene (IV).

SUBJECTS AND METHODS

1. Subjects

1.1 Familial cases (I)

Three familial clusters of pituitary adenoma patients had been detected in Northern Finland. Genealogy analysis established linkage between two of these clusters, whereas the third appeared separate. Altogether, 11 affected individuals were identified in an impressively large pedigree (Fig. 3A), hereafter referred to as 'family 1'. In the second pedigree, hereafter referred to as 'family 2', two individuals were affected with gigantism (Fig. 3B). Overall, DNA samples from 13 affected individuals (Table 7), seven obligatory carriers, and four key unaffected relatives were obtained from both families. Paraffin blocks of embedded pituitary tumor tissue were available from six of 13 affected cases. Apart from the two Finnish families, three foreign families with two affected individuals each were included in the study: Blood-extracted DNA was available from two Italian siblings with acromegaly/gigantism, as well as from one Turkish and one German familial somatotropinoma case (Table 7).

1.2 Other pituitary adenoma patient cohorts (I, II, IV)

Study I: A population-based cohort of 54 acromegaly patients, diagnosed with GH-secreting adenomas between 1980 and 1999 in Oulu University Hospital, Northern Finland, had been previously characterized (Kauppinen-Makelin *et al*., 2005). Of these, material from 45 patients became available, either as blood sample or paraffin-embedded normal or tumor tissue blocks. In addition to the population-based cohort, 10 unselected Finnish sporadic acromegaly patients were included in the study (Table 7).

Study II: Four hundred and sixty pituitary adenoma patients were analyzed. This cohort consisted of: a) 63 young acromegaly patients from Finland (different from study I) and Germany; b) 71 unselected Italian acromegaly patients; c) 235 unselected pituitary adenoma patients from USA and Poland; and d) 91 Dutch and Spanish patients counseled and examined for MEN1, but negative for *MEN1* mutations. A detailed description of these cohorts is provided in publication II and Table 7. Genetic analysis of the young acromegaly German samples was carried out at tumor DNA level; for all other samples, analysis was performed on blood-extracted DNA. Familial history was revealed in two cases, one from the German and one from the Spanish cohort. Tumor samples from the identified mutation carrier patients were utilized if available.

Study IV: The material of this study consisted of blood-extracted DNA samples from a population-based cohort of 36 pediatric Italian, sporadic pituitary adenoma patients from Italy, referred to two medical centers since 1988. Inclusion criteria for the cases were: 1) either age at diagnosis less than 18 years, or clinical evidence of adenoma development before the age of 18 years, and 2) no evidence of familial pituitary adenomas (Table 7).

Table 7. Pituitary adenoma patients recruited in the studies of the present thesis work.

Subjects	Number of affected cases	Pituitary adenoma type of affected cases (number of cases)	Selection criteria	Study
Familial cases				
Finland				
Family 1	11	GH- (n=4), GH/PRL- (n=2), PRL-secreting (n=5)	Family history	I
Family 2	2	GH-secreting (n=2)		
Italy	2	GH-secreting (n=2)	Family history	I
Germany	1	GH-secreting	Family history	I
Turkey	1	GH-secreting	Family history	I
Other cohorts				
Finland	45	GH-secreting (n=45)	Population-based acromegaly patients	I
Finland	10	GH-secreting (n=10)	Sporadic acromegaly patients	I
Finland	36	GH-secreting (n=36)	Young (<45 yrs at dg) sporadic acromegaly patients	II
Germany	27	GH-secreting (tumor samples) (n=27)	Young (<40 at op) acromegaly patients	II
Italy	71	GH-secreting (n=71)	Sporadic acromegaly patients	II
USA	113	GH- (n=13), PRL- (n=11), ACTH-secreting (n=13), NFPA (n=76)	None (all types of pituitary adenomas)	II
Poland	122	GH- (n=30), ACTH-secreting (n=74), NS (n=18)	None (all types of pituitary adenomas)	II
Spain	55	GH- (n=18), GH/PRL- (n=2), PRL- (n=5), ACTH-secreting (n=4), NFPA (n=5)	Suspicion for MEN1 (all types of pituitary adenomas)	II
The Netherlands	36	GH- (n=6), PRL-secreting (n=6), NFPA (n=1), NS (n=9)	Suspicion for MEN1 (all types of pituitary adenomas)	II
Italy	36	GH- (n=5), GH/PRL- (n=2), PRL- (n=19), ACTH-secreting (n=3), NFPA (n=7)	Pediatric onset (all types of pituitary adenomas), sporadic	IV

yrs, years; dg, diagnosis; op, operation; NS, not specified

1.3 Other tumor samples (III)

A total of 499 samples from three common tumor types were utilized: 373 fresh-frozen colorectal tumor samples, chosen from a series collected between 1994 and 1998 (Aaltonen *et al*., 1998, Salovaara *et al*., 2000); these samples displayed at least 50% tumor tissue, according to a pathologist's histological evaluation. Corresponding normal tissue had been previously extracted from blood or normal colonic epithelium, distant from the tumor margins. A series of 82 breast (Winqvist *et al*., 1995) and 44 prostate tumor DNA samples (Waltering *et al*., 2006) were also analyzed.

1.4 Healthy controls (I, II, III, IV)

Overall, 749 unrelated, healthy individuals were available as population-matched healthy controls. In detail, the cohort consisted of: 209 anonymous Finnish Red Cross blood donors, 288 Caucasians from United Kingdom (Human Random Control DNA panel, Porton Down, Salisbury, Wiltshire, UK), 110 Caucasians from Centre d'Étude du Polymorphisme Humain (Fondation Jean Dausset-CEPH, Paris, France), 52 Italians (Treviso General Hospital, Italy), and 90 German individuals (Leipzig University, Germany).

2. DNA/RNA extraction (I, II, III, IV)

DNA was extracted from peripheral EDTA-blood samples by a standard non-enzymatic procedure (Lahiri & Nurnberger, 1991). Tumor DNA from fresh-frozen tissue samples [colorectal, breast, and prostate tumors (study III)] had been previously extracted (Aaltonen *et al*., 1998, Salovaara *et al*., 2000, Winqvist *et al*., 1995, Waltering *et al*., 2006). DNA from paraffin-embedded pituitary adenomas and paraffin-embedded normal tissue was isolated using a standard protocol (Kannio *et al*., 1996), or alternatively, a quick-extraction protocol (Shibata *et al*., 1988). Total cellular RNA was extracted using the RNeasy kit (Qiagen Inc., Valencia, CA) and used for cDNA synthesis by reverse transcription PCR (RT-PCR), according to a standard protocol (Promega Corporation, Madison, WI) (study I).

3. Disease locus identification (I)

3.1 SNP arrays

Whole-genome SNP genotyping was performed on peripheral blood-extracted DNA samples from 16 individuals from family 1 (Fig. 3A, individuals A2, A5, A6, A9, A10, A11, A13, A14, A16, A18, A20, A21, A31, A32, A33, and one healthy spouse). DNA was extracted with the PureGene DNA isolation kit (PureGene, Gentra Systems, Minneapolis, MN). Genotyping was carried out on an Affymetrix Human Mapping 50K *Xba* 240 SNP array (Affymetrix Inc., Santa Clara, CA). Signal intensities were analyzed by the GeneChip DNA analysis software (GDAS), version 3.0.2.8 (Affymetrix).

3.2 Linkage analysis

The SNP genotyping data were converted to appropriate linkage format by the ALOHOMORA software (Ruschendorf & Nurnberg, 2005) and were subjected to quality control routines, including gender check, graphical representation of relationship errors (GRR) (Abecasis *et al*., 2001), and Mendelian errors (O'Connell & Weeks, 1998). All non-informative markers were deleted before further analyses. Two alternative affected-only analyses were performed: One with "high-stringency" criteria, considering only acromegaly/gigantism as the disease phenotype (GH- or mixed GH/PRL-secreting adenomas), and one with "low-stringency" criteria, considering all pituitary adenoma types (GH-, GH/PRL-, and PRL-secreting adenomas) as the affected phenotype. This approach was undertaken, because acromegaly/gigantism is a rare phenotype and phenocopies are expected to be much more rare than the number of phenocopies for PRL-secreting adenomas. Linkage calculations were performed with the Allegro and SimWalk2 softwares (Gudbjartsson *et al*., 2000; Sobel & Lange, 1996; Sobel *et al*., 2001; Gudbjartsson *et al*., 2005) and the results were visualized with HaploPainter V.024beta (Thiele & Nurnberg, 2005).

3.3 Fine mapping and haplotype analysis

Fine mapping studies of the candidate disease locus were performed on genomic DNA samples isolated from blood or paraffin-embedded normal tissue from the affected familial cases and the unaffected obligatory carriers, as well as from sporadic acromegaly cases. A total of 30 published (Ensembl Genome Browser and NCBI) and novel microsatellite

markers from chromosomal region 11q12.2-11q13.3 (physical location 61.4-69.0 Mb) were analysed. The novel markers were identified using the Tandem Repeats Finder program (Benson, 1999) (http://tandem.bu.edu/trf/trf.html). In addition, six informative SNP-markers from the Human Mapping 50K Xba 240 SNP array mapping to the region of interest were utilized (Table S1 in the supporting material of publication I). These SNPs were selected on the basis of the significance of the difference in genotype frequency between the affected cases versus 42 unrelated Finnish samples previously genotyped with the same platform but for a different project. Microsatellite and SNP marker polymerase chain reaction (PCR) products were run on an ABI3730 sequencer (Applied Biosystems, Foster City, CA). Microsatellite alleles were viewed and scored with the GeneMapper v.3.7 software (Applied Biosystems). The haplotypes were constructed manually. Two unrelated CEPH individuals (1347-2 and 1347-13) were used as internal controls in all fine-mapping experiments.

The most informative markers in the shared region (Table S1 in the supporting material of publication I) were selected for the overall parametric linkage calculations in families 1 and 2, using the SimWalk2 software. Linkage analysis was again performed for "high-stringency" and "low-stringency" criteria. With the exception of the affected individuals, all the others were considered of unknown phenotype. The disease model parameters were: Disease allele population frequency 0.001, penetrance for heterozygous and homozygous disease genotypes 0.1, phenocopies for acromegaly/gigantism 0.0002, and phenocopies for any pituitary adenoma 0.01.

3.4 Gene expression profiling

3.4.1 Gene expression microarrays

Expression profiles were generated by the Human Genome U133 Plus 2.0 expression arrays (Affymetrix). Blood-derived total RNA samples were obtained from 16 individuals: Nine affected/obligatory carriers, segregating a disease haplotype, (Fig. 3A and 3B, A2, A6, A8, A14, A16, A18, A20, A21, A22 from families 1 and 2) and seven controls: Five unrelated spouses from families 1 and 2 and two identically and simultaneously processed samples from another project. All experimental procedures were performed according to the manufacturer's recommendations (Affymetrix).

3.4.2 Data analysis

The quantitative expression data were normalized by scaling all chips to the average gene expression data of all 16 chips. The expression of each probe set was divided by the mean expression of that probe across all the samples, so that the resulting mean expression for every probe was 1. The normalized expression data were filtered using the Affymetrix Detection Algorithm which assigned flag calls (as 'present', 'absent', or 'marginal'), in order to remove expression values below detectable levels. Subsequent analyses were restricted to probe sets with detectable expression in a sufficient number of samples to allow a Student's *t*-test between the affected/obligatory carriers and control groups. Data normalization and filtering analyses were performed using GeneSpring 7.0 software (Silicon Genetics, Redwood City, CA).

4. Genetic analysis (I, II, III, IV)

4.1 Mutation screening by direct sequencing

Genetic analyses were performed by PCR and direct genomic DNA sequencing. For primer design, genomic sequences were retrieved from the University of California Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway, version May 2004) and primers were designed by the use of the publicly available softwares ExonPrimer (http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html) and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer sequences are provided in Table S4 in the supporting material of publication I.

PCR reactions were performed at optimized conditions, according to standard procedures. The basic protocol was modified depending on the DNA sample (blood- or paraffin-extracted DNA), the primer annealing temperatures, and the amplification outcome of the template. PCR products were purified from residual primer oligonucleotides and unincorporated dNTPs by ExoSAP-IT (USB Corporation, Cleveland, OH), according to the manufacturer's instructions. Sequencing was performed using the BigDye3.1 termination chemistry on an ABI3730 sequencer (Applied Biosystems). Sequencing chromatograms were visualised and aligned to the wild type reference sequence(s) using the BioEdit Sequence Alignment Editor software (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) (Ibis Biosciences, Carlsbad, CA). All observed sequence changes were confirmed on independent experiments. All findings on pituitary tumor DNA level were examined on the corresponding normal tissue DNA, if available, and vice versa.

All mutations and variants have been annotated according to the nomenclature for human genomic sequence variations (den Dunnen & Antonarakis, 2001; The Human Genome Variation Society 2008, at www.hgvs.org).

4.2 Loss of heterozygosity (LOH) study

Before gene identification, LOH was partially assessed by tumor-DNA microsatellite marker analysis (selected from the panel of 30 markers used for fine-mapping purposes), in four familial cases bearing the Finnish founder haplotype and two sporadic cases. For each tumor individually, the most polymorphic markers were selected. LOH was concluded when the marker alleles segregating with the affected haplotype were retained, whereas the corresponding wild type alleles were significantly reduced or lost (unpublished data). LOH on tumor-derived DNA was assessed by visual observation of the sequence chromatograms by comparing the peak heights/areas between the wild type and mutant allele. Allelic loss was concluded when the wild type allele was either completely or nearly completely invisible, or significantly reduced when compared to the mutant allele.

5. Immunohistochemistry (IHC) (I, II, IV)

IHC staining of familial and sporadic pituitary adenomas for hypophyseal hormones (GH, PRL, ACTH, FSH, LH, and TSH) was performed according to standard procedures (Table S5 in the supporting material of publication I). For AIP detection (studies II and IV), the mouse polyclonal antibody SP5213P (Acris Antibodies, Hiddenhausen, Germany) was used at a 1:4000 dilution. All IHC laboratory procedures are detailed in the original publications. IHC stainings were performed at the Department of Pathology, University of Oulu, and evaluation was carried out by a pathologist.

6. In silico analysis (II, III, IV)

The potential effect on splicing of previously unreported *AIP* variants of undetermined significance (silent or missense changes, intronic variants) was tested *in silico* by computational methods with freely available softwares (NetGene2, the Berkley Drosophila Genome Project, Alternative Splice Site Predictor, and SpliceScan; for the corresponding websites see list of references on page 93). For protein alignments, the human AIP protein sequence and its homologues in other species were retrieved from the UCSC Genome Bioinformatics database (version March 2006) and from the Ensembl Genome Browser (version 38, April 2006). Alignments were performed by the BioEdit Sequence Alignment Editor software.

7. Ethical issues

The study was approved by the Ministry of Social Affairs and Health and the Ethics Review Committees of the Hospital District of Helsinki and Uusimaa, and the Department of Medical Genetics of the University of Helsinki. Samples were used after informed consent of the patients or their parents. Relatives of patients were contacted only through the patients. Tumor samples were obtained with authorization by the National Authority for Medicolegal Affairs.

RESULTS

1. Pituitary Adenoma Predisposition (PAP) gene identification (I)

1.1 PAP locus maps on chromosome 11q13

Previously, three familial clusters of pituitary adenoma cases had been detected in Northern Finland (Oulu region) (Fig. 3). The first cluster displayed three cases of acromegaly or gigantism (Fig. 3A, individual A13, affected by a mixed GH/PRL-secreting adenoma, and individuals A13 and A31 affected by GH-secreting adenomas). The second cluster consisted of two patients; one had been diagnosed with mixed GH/PRL-secreting adenoma, and the other with PRL-secreting adenoma (Fig. 3A, individuals A14 and A21). The third cluster consisted of two distantly related patients with gigantism (Fig. 3B, individuals A8 and A34). In 2004, a third individual from the first cluster was diagnosed with gigantism (Fig. 3A, individual A33). This realization prompted the hypothesis that, at least in this cluster, genetic pituitary adenoma predisposition (PAP) is likely to be present, since acromegaly is a rare disease (Alexander *et al*., 1980; Bengtsson *et al*., 1988; Kauppinen-Makelin *et al*., 2005). Later, the first and second clusters were linked by genealogy (family 1), whereas the third appeared separate (family 2). For PAP gene identification purposes, the relevant members of families 1 and 2 were invited to participate in the study.

For the identification of the putative disease predisposition locus, segregating in family 1, we performed linkage analysis on SNP chip genotyping data from 16 individuals. For the genome-wide parametric and non-parametric multipoint linkage calculations, family 1 had to be divided into three separate branches, since linkage programs were not suitable for performing analyses of large number of markers in such a large pedigree. LOD scores were calculated separately for each branch and then added together by loci. In the genome-wide search, the highest parametric LOD score of 3.9 was detected for the “high-strigency” criteria (acromegaly or gigantism, 6 individuals) for chromosome 11 at 68.4-73.6 cM. This region, which also includes the *MEN1* gene, had been previously suggested to harbor a putative TSG for IFS. In addition to chromosome 11, other regions displaying significant linkage were on chromosomes 4 (3.15), 5 (3.21), and 8 (3.08). Non-parametric linkage results were compatible with the parametric analysis. Next, the whole family 1 was reanalyzed as one pedigree, in the regions displaying LOD scores over 3.

In order to evaluate the effect of linkage disequilibrium (LD) in regions with LOD scores over 3, the data were further analyzed with reduced marker density (< 0.1 cM). Prior to LD removal, LOD scores over 3 were observed for chromosomes 4 (5.61) and 11 (7.5) for the “high-stringency” criteria, and for chromosomes 4 (2.99), 11 (6.3), and 14 (3.59) for the “low-stringency” criteria. The only significant LOD score after LD removal was obtained for chromosome 11 in the parametric linkage analysis. Haplotype construction of family 1 individuals finally placed the PAP locus between SNPs rs174449 (i.e. last non-linked SNP upstream) and rs1938685 (i.e. first non-linked SNP downstream) (11q12.2-q13.3, 61.4-69.0 Mb, Ensembl, release 36-Dec2005).

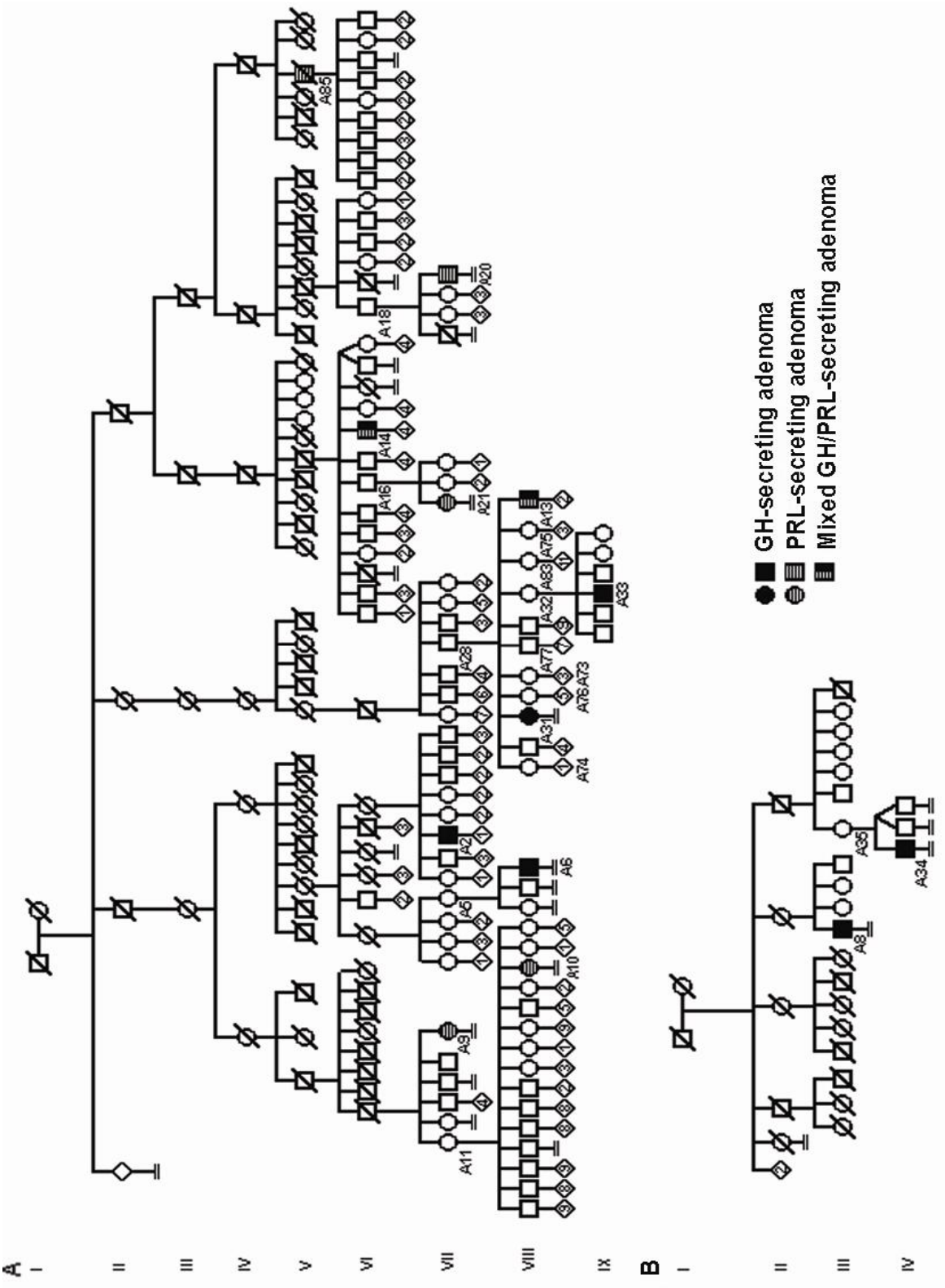

Figure 3. Pedigrees of the two Finnish families with pituitary adenomas that facilitated the identification of the *AIP* gene. (A) Family 1, (B) Family 2. The pedigrees have been modified to ensure confidentiality. Individuals are indicated with their coded names (A2, A5, A6, etc). Number of offspring is indicated by Arabic numerals, enclosed in diamond symbols. Generations are indicated by Roman numerals, on the left side.

1.2 Candidate locus fine-mapping reveals a founder haplotype of ~7 Mb

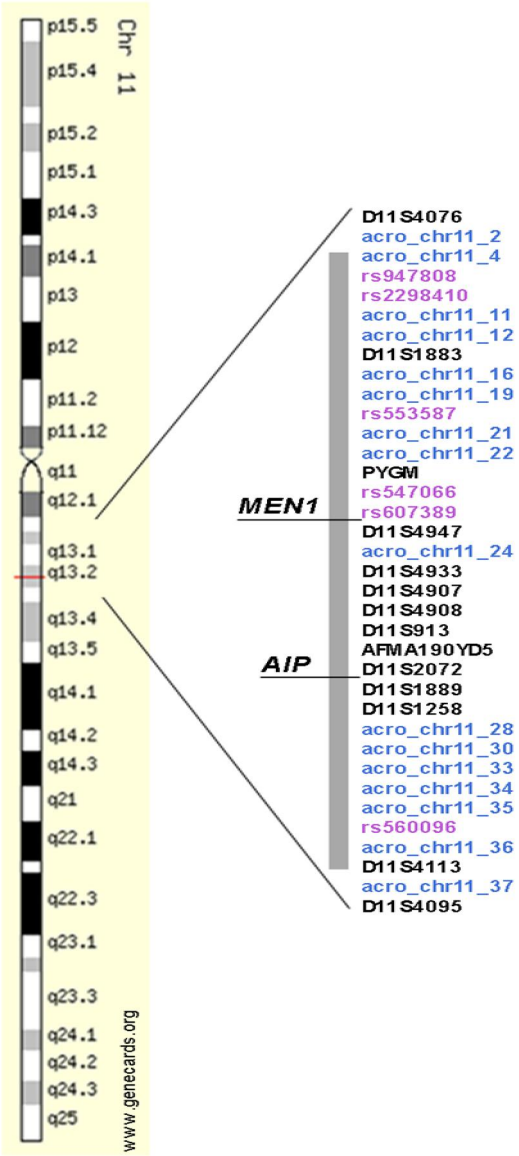

Figure 4. Founder haplotype spanning ~7 Mb. Fine-mapping placed the candidate locus between markers acro_chr11_2 and acro_chr11_37 (grey vertical bar). Known microsatellite markers are indicated in black color, whereas novel microsatellite markers are shown in blue. Linked SNPs are shown in purple. The *MEN1* and *AIP* loci are indicated in relation to neighboring markers.

Further characterization of the linked haplotype was performed with 30 microsatellite and six SNP markers. The most informative markers in the shared region were used for parametric linkage calculations in families 1 and 2. The added maximum LOD scores were 8.3 (7.5 for family 1 and 0.8 for family 2) for the "high-stringency" criteria, and 7.1 (6.3 for family 1 and 0.8 for family 2) for the "low-stringency" criteria. The linked founder haplotype (Fig. 4) segregated perfectly in families 1 and 2 with the GH- and GH/PRL-secreting adenoma phenotype (acromegaly). Although significant linkage was obtained with the "low-strigency" criteria, two PRL-secreting adenoma cases in family 1 appeared to represent phenocopies (Fig. 3A, individuals A9 and A10). We, then, used the identified founder haplotype in order to detect additional haplotype carriers among sporadic patients from Northern Finland, but we were not able to significantly narrow down the linked region. Because this chromosomal region contained approximately 295 protein-coding and non-coding genes, an alternative approach was needed to help simplify selection of candidate genes for mutation screening. For this purpose, we opted to perform gene expression profiling, seeking for genes with aberrant expression in the germline.

1.3 Gene expression profiling reveals *AIP* as the prime candidate gene for PAP

Gene expression profiles were generated from a total of 16 blood samples of patients and obligatory carriers, as well as control individuals. A total of 23096 probe sets fulfilled the analysis criteria at the whole-transcriptome level. Of these, 1735 probe sets reached a p-value of ≤ 0.05 in the *t*-test. The 50 probe sets most differentially expressed between

patients/obligatory carriers versus controls are presented in Table S3 in the supplemental material of publication I.

Next, we combined the genetic mapping with the gene expression array data, in order to select positional candidate genes for mutation analyses, based on decreased expression. In the linked region (11q12-11q13), 172 probe sets fulfilled the criteria; of these, 27 reached a p-value of ≤0.05. Two probe sets representing the *AIP* (*aryl hydrocarbon receptor interacting protein*) gene occupied the first two positions, with p-values of 0.00026 and 0.00114 (Table S2 in the supplemental material of publication I). Removal of the two additional control samples from a different project did not change the overall result, as *AIP* remained the best candidate gene with the two probe sets at the 1st and 4th position (p=0.002 and 0.007, respectively) (data not shown in Table S2). Therefore, *AIP* was chosen as the primary positional candidate gene for mutation analysis.

1.4 Candidate gene mutation analysis establishes *AIP* as the predisposing gene

Apart from *AIP*, a second positional candidate gene, namely *LGALS12* or galectin-12 (Yang *et al*., 2001), was chosen; this was based on decreased *LGALS12* expression and the previous association of another member of the same protein family of lectins (*LGALS3* or galectin-3) to pituitary tumorigenesis (Riss *et al*., 2003). *LGAL12* sequencing did not reveal germline mutations in its coding region and exon-intron boundaries.

The *AIP* gene (NCBI NM_003977) resides on 11q13.3, spanning a region of 8 kb, and consists of six exons transcribed in an mRNA transcript of 1212 bp that is translated to a 330 aa protein. Direct genomic DNA sequencing in families 1 and 2 revealed an early stop codon mutation, c.40C>T/Q14X, in the first exon of *AIP*. The mutation segregated perfectly with the GH-secreting, GH/PRL-secreting, and three of five cases with the pure PRL-secreting adenoma phenotype in both families. Q14X was absent in 209 population-matched healthy controls. The number of affected cases in the giant pedigree (nine patients with the Q14X mutation of a total of 11 affected cases) (Fig. 3A) is small compared to the number of unaffected obligatory carriers identified (64 individuals at 50% risk) (Dr. Outi Vierimaa, unpublished data); this indicates that *AIP* is a low penetrance tumor susceptibility gene.

Mutation screening of the Finnish population-based material of 45 acromegaly patients, including four cases from families 1 and 2, revealed the Finnish founder mutation Q14X in six patients and a splice-acceptor site (IVS3-1G>A) mutation in one patient. IVS3-1G>A was not detected among 219 healthy, population-matched controls. cDNA analysis revealed that IVS3-1G>A results in the recognition of a cryptic splice-acceptor site, 260 bp upstream of the constitutive splice-acceptor site, at intron 3-exon 4 boundaries; this change leads to an altered reading frame and, subsequently, to a premature termination codon. Overall, these two *AIP* mutations accounted for 16% (7/45) of the population-based cases.

In this cohort, no statistically significant differences were observed between PAP (i.e. *AIP* mutation-positive) patients (n=7) and mutation-negative (n=38) patients in terms of gender or tumor size. Conversely, PAP patients were significantly younger at diagnosis (24.7±10.7 versus 43.6±11.9 years, p=0.0003). Six *AIP* mutation positive patients represented 40% of the

15 patients that were younger than 35 years at diagnosis, indicating that young age at onset is a useful indicator for identification of PAP cases.

In addition, Q14X was identified in two of ten (20%) unselected Finnish acromegaly patients from Northern Finland, both diagnosed before the age of 35 years. This figure was very close to the occurrence of *AIP* mutations among the 45 selected acromegaly cases.

To strengthen the association of *AIP* and PAP, it was relevant to identify additional pathogenic mutations. For this purpose, we performed mutation screening in the index cases of three families with two GH-secreting adenoma patients each; families originated from Germany, Turkey, and Italy. We did not detect mutations in the German or Turkish probands. On the contrary, a late stop codon mutation in exon 6 (c.910C>T/R304X) was identified in the two Italian siblings; R304X was absent from 203 Caucasian controls and 52 healthy, population-matched controls. The Italian phenotype was very similar to that observed in the Finnish PAP patients; both cases had been diagnosed at a very young age (18 years) with acromegaly/gigantism and without visible evidence of dominant transmission, which was compatible with incomplete penetrance.

Pituitary tumor samples were available from eight PAP cases with the Finnish founder mutation (six familial and two sporadic). LOH analysis in these adenomas revealed the loss of the wild type allele, compatible with the classical two-hit hypothesis for TSG inactivation. Prior to gene identification, microsatellite marker-based LOH analysis, performed in six out of these eight tumors, revealed LOH on the 11q12.2-q13.3 locus (unpublished data). No tumor samples were available from the Italian siblings with the R304X mutation. These findings, i.e. truncating mutations and biallelic inactivation of *AIP* in all tumors studied, suggested that *AIP* is likely to act as a novel tumor suppressor gene.

2. Molecular diagnosis of PAP (II)

2.1 The contribution of *AIP* in heterogeneous pituitary adenoma patient cohorts of different ethnic origins

Gene identification was facilitated given the genetically homogeneous Finnish population. To gain insight into the clinical features of the condition, and to provide clues for molecular identification of PAP, it was relevant to examine the contribution of *AIP* in other patient materials and of different ethnic origin. For this purpose, the whole coding region and exon-intron boundaries of *AIP* were screened in a total of 460 pituitary adenoma cases, representing the various cohorts detailed in Subjects and Methods, section 1.2 and Table 7.

The analysis revealed nine presumably pathogenic *AIP* mutations and mutations were identified in all cohorts studied: The Finnish founder mutation Q14X; two in-frame deletions (c.66-71delAGGAGA/G23_E24del, and c.880-891delCTGGACCCAGCC/L294_A297del); one bp insertion (c.824-825insA/H275Qfs) and one bp deletion (c.542delT/L181fs), both leading to frameshift and premature stop codons; a splice-acceptor site substitution (IVS2-1G>C); and three missense changes (c.878-879AG>GT/E293G, c.896C>T/A299V, and c.911G>A/R304Q). All changes were absent in about 500 healthy control samples analyzed.

The prevalence of *AIP* mutations varied between different cohorts and clinical settings: The frequency was very low among apparently sporadic pituitary adenoma patients from Poland (0.8%) and the USA (1.8%), recruited irrespectively of age at diagnosis or pituitary tumor type; in addition, there were no significant findings among 71 unselected acromegaly patients from Italy. On the other hand, in the selected cohorts of young acromegaly patients from Finland and Germany, two mutations in each series accounted for 5.5% and 7.4%, respectively.

Of interest is a missense variant in exon 1, R16H (c.47G>A), which was detected in one Italian, one American, and three Polish pituitary adenoma patients, but also in one of 90 (1.1%) healthy control of German origin. In addition, two previously unreported silent variants were found: c.696G>C/P232P in one Polish patient and c.906G>A/V302V in three American patients. Even though neither of the silent variants was detected among more than 200 Caucasian controls, they were not predicted to alter the transcript's splicing pattern, as tested *in silico*.

Finally, we opted to include in the analysis cases clinically suggestive of MEN1, but without mutations in the *menin* gene, and found two mutations in a total of 91 (2.2%) Spanish and Dutch cases of unexplained endocrine neoplasia. The findings are summarized in Table 1 of the original publication (II) and also in Appendix Table 1; silent variants detected in study II are excluded from the tables.

2.2 Clues into the phenotypic presentation of PAP patients

AIP mutations were enriched in patients diagnosed mainly with GH-secreting adenomas (acromegaly) and at a young age, supporting the notion that *AIP* is a major GH-secreting adenoma susceptibility gene. Despite the very young age at onset of the mutation carriers in this study, family history of acromegaly became known for two PAP cases after mutation identification: The father of the German patient with the G23_E24del also had acromegaly, whereas his grandfather was suspected to have acromegaly (no biochemical tests available); the Spanish patient with the L181fs mutation had two maternal uncles diagnosed with acromegaly. Family history of endocrine neoplasia was not evident for the remaining PAP cases. In addition, a patient with an ACTH-secreting tumor and Cushing's disease was for the first time identified to carry an *AIP* mutation (R304Q). These results pose a challenging question regarding patient selection for possible genetic testing for PAP. Medical history of the identified PAP patients was negative for tumor types other than pituitary adenomas.

2.3 Immunohistochemical detection of AIP protein

The identified germline *AIP* mutations in study I were truncating, associated with loss of the wild-type allele in tumors; in other words, those pituitary tumors were null with respect to the AIP protein. For this reason, in study II we examined the feasibility of AIP IHC, as a molecular tool for possible PAP identification. By using a polyclonal AIP antibody, we observed the subcellular localization of AIP in normal anterior pituitary tissue; the protein is present in both the cytoplasm and the nucleus. Next, we examined 50 pituitary adenoma specimens, of which 38 were mutation-negative and 12 were mutation-positive. The latter

cohort consisted of nine tumors bearing the Finnish founder Q14X mutation, one tumor bearing the Finnish IVS3-1G>A mutation, and two tumors from two US patients, one with the IVS2-1G>C and the other with the H275Qfs. Most *AIP*-mutation negative adenomas (36/38 or 95%) (i.e. AIP-proficient tumors) had preserved cytoplasmic and nuclear immunoreaction, whereas most AIP-mutation positive adenomas (9/12 or 75%) (i.e. AIP-deficient tumors) showed complete loss of both cytoplasmic and nuclear staining (Fisher's Exact test, $p=4x10^{-6}$). Peripheral leukocytes captured in AIP-deficient tumor tissue sections served as internal positive controls. Overall, AIP IHC showed 75% sensitivity and 95% specificity for germline truncating mutations, indicating that it could be considered as predictor for PAP.

3. The role of *AIP* in tumorigenesis of common cancers (III)

Following identification of *AIP* as a candidate TSG, it was relevant to examine its role in tumorigenesis of common neoplasias, such as colorectal, breast, and prostate tumors. These are the most prevalent cancers worldwide (Parkin *et al*., 2005). Moreover, acromegaly patients have an increased risk for developing colonic polyps and, subsequently, colorectal cancer. Circumstantial, but controversial, evidence supported that breast and prostate hyperplasia and malignancy may arise more often in the context of acromegaly compared to the general population (Jenkins, 2004). For this purpose, a total of 499 Finnish colorectal, breast, and prostate tumor samples were utilized for somatic *AIP* mutation screening; no presumably pathogenic mutations were identified.

Of interest was the previously mentioned missense variant R16H, detected in two of 373 colon cancer specimens, one being a microsatellite stable (MSS) and the other being a microsatellite unstable (MSI) tumor. R16H was also present in the germline of both colon cancer patients, but was absent in 209 healthy Finnish controls. No LOH was observed in the tumors. The MSS patient had been diagnosed with cancer of the rectum at 64 years of age, but due to unilateral breast enlargement, an occult pituitary adenoma was suspected; results of a nuclear magnetic resonance examination undertaken in a private clinic were not available and the patient is deceased. The MSI patient had been diagnosed with colon cancer at the age of 81 years, but several other cancer patients with colorectal, cervical, and/or carcinoid tumors clustered in the family. None of these patients' samples were available for further studies.

Apart from R16H, two silent changes (G12G and C238C), two rare polymorphisms (G23E and 3'UTR +60G>C), a 5'UTR single bp substitution (-5G>C), and an intronic variant (IVS3+15C>T) were also observed; the intronic and silent changes were not predicted to affect the transcript's splicing pattern, as tested *in silico*. The findings are summarized in Table 1 of publication III.

4. The role of *AIP* in pediatric pituitary tumorigenesis (IV)

Pituitary adenomas occur rarely in childhood and adolescence. Such early disease onset may in part be explained by genetic factors, since pediatric pituitary adenomas are observed in the context of MEN1 and CNC (O'Brien *et al*., 1996; Stratakis *et al*., 2000; Brandi *et al*., 2001;

Stratakis *et al*., 2001; Keil & Stratakis, 2008). In the adult sporadic pituitary adenoma patient groups from studies I and II, germline *AIP* mutations had been identified among those diagnosed mainly with GH-secreting tumors and at earlier age at onset than their non-mutated sporadic counterparts. To gain more insight regarding the PAP phenotype, we examined the prevalence of *AIP* mutations in a tailored pediatric series encompassing different types of pituitary adenomas, in a unique population-based cohort of 36 unrelated, sporadic patients from Italy: PRL-secreting adenomas accounted for 19 cases (53%), GH-secreting adenomas for seven cases (19%), of which two cases were mixed GH/PRL-secreting tumors, ACTH-secreting for three cases (8%), and NFPAs for seven cases (19%). MEN1 had been excluded in all cases, after medical and biochemical investigation, and *MEN1* sequencing.

One heterozygous germline in-frame deletion of one amino acid (Y248del/c. 742-744delTAC) was identified in a male adolescent giant patient, who was operated on for his GH-secreting macroadenoma at the age of 19 years. IHC staining displayed expression of the mutant protein, since LOH analysis of the tumor DNA clearly revealed that the wild type allele was lost. The mutation was absent in 253 healthy Caucasian individuals, including 52 population-matched controls (Italy). The patient did not have a family history of pituitary adenomas or any other endocrine-related disease. Mutation analysis among his first degree relatives (both parents and the two siblings) revealed that the father was the carrier of the mutant allele, which was transmitted to his three children. Except for the proband, all other mutation carriers remain unaffected. Detailed clinical examination and biochemical evaluation (IGF-I) of the unaffected carriers were normal, but other endocrine and imaging studies had not been performed at the time of the preparation of the present work. The brief medical history obtained from the relatives of the paternal side was negative for endocrine disease, but none has been genetically tested nor clinically examined for pituitary lesions. Overall, this adolescent PAP patient accounted for 1/36 or 2.8% of pediatric pituitary adenomas and for 1/7 or 14.3% of all GH-secreting tumors in the series.

DISCUSSION

1. *AIP* is a novel, low penetrance tumor susceptibility gene that causes Pituitary Adenoma Predisposition (PAP) (I)

1.1 Insights into the hereditary predisposition to pituitary adenoma development (I)

The occurrence of multiple cases of pituitary tumors, and in particular GH-secreting adenomas, among first-degree relatives within single families is very uncommon (Soares & Frohman, 2005). Acromegaly is rare in the general population, with merely three to four new cases per million per year (Alexander *et al*., 1980; Bengtsson *et al*., 1988; Kauppinen-Makelin *et al*., 2005). Therefore, the occurrence of pituitary adenomas in Finnish familial settings, which did not depict the MEN1 and CNC syndromes, prompted consideration of an inherited disorder. Efforts by other groups had previously concentrated on the identification of a TSG predisposing to acromegaly (IFS); this gene was thought to reside on 11q13, very close to, yet distinct from, *MEN1* (Yamada *et al*., 1997; Gadelha *et al*., 1999; Gadelha *et al*., 2000).

The identification of three familial pituitary adenoma clusters in Northern Finland in 2004, two of which were linked by genealogy to a giant pedigree, led to the hypothesis that a previously uncharacterized form of low penetrance pituitary adenoma predisposition (PAP) would contribute to the disease burden in the area. These families had been excluded from MEN1 and CNC, due to the lack of clinical, biochemical, and genetic (in the case of MEN1) evidence, but also due to the unique low penetrance of the PAP phenotype. Family 1 was excluded from the IFS phenotype, since several affected members had been diagnosed with a PRL-secreting adenoma.

We named the condition caused by germline *AIP* mutations as “Pituitary Adenoma Predisposition” (PAP). Because susceptibility to pituitary adenomas is likely to be genetically heterogeneous, and each defective gene might confer to particular clinical features, it was useful that the condition attributed to *AIP* had a name. Thus, PAP refers to a person with an *AIP* mutation, independent of the affection status; predisposition exists also for the unaffected germline mutation carriers. Germline mutations in tumor susceptibility genes cause tumor predisposition, not tumors *per se* (Vogelstein & Kinzler, 2004), since additional genetic changes are needed to convert a predisposed cell to a neoplastic one.

The approach for PAP gene identification is summarized as follows (Fig. 5): First, identification of the putative disease predisposition locus segregating in family 1, through genome-wide SNP chip-based genotyping and linkage analysis; second, characterization of the linked haplotype with 36 microsatellite and SNP markers; third, utilization of the founder haplotype for detection of additional haplotype carriers among sporadic patients from Northern Finland, in an effort to further narrow down the disease locus; fourth, generation of gene expression profiles from the blood samples of individuals segregating the disease-associated haplotype versus controls; and fifth, the combination of genetic mapping

data and whole-transcriptome expression array data to pinpoint positional candidate genes for germline mutation analyses.

The successful identification of *AIP* as the PAP gene is owing to the innovative approach of combining traditional gene mapping methods with modern chip-based technologies. Despite the large size of family 1, informative meiotic recombinations that would help us restrict the predisposition locus below ~7 Mb were still lacking. In genetically homogeneous populations, such as the Finnish, haplotypes spanning as much as 10 cM have been observed to surround ancestral founder mutations with strong LD (de la Chapelle, 1993), as in the identification of the HNPCC predisposition loci (Nystrom-Lahti *et al*., 1994).

Luccio-Camelo *et al*. (2004) mapped the locus for familial acromegaly (IFS) to 10 Mb on 11q13; in this family, an 18-year old member had a critical recombinant chromosome at 11q13, but remained unaffected. If this person eventually developed acromegaly, it would have helped narrow the IFS candidate region from 10 Mb to 3.9 Mb (including the *AIP*, but not the *MEN1*, locus) (Luccio-Camelo *et al*., 2004). This case highlights that the quest for meiotic recombinations in such rare pedigrees, in concert with the slowly progressing acromegaly phenotype, may had been a time-consuming approach for susceptibility gene identification, if based solely on linkage analysis and screening of potentially attractive positional candidate genes.

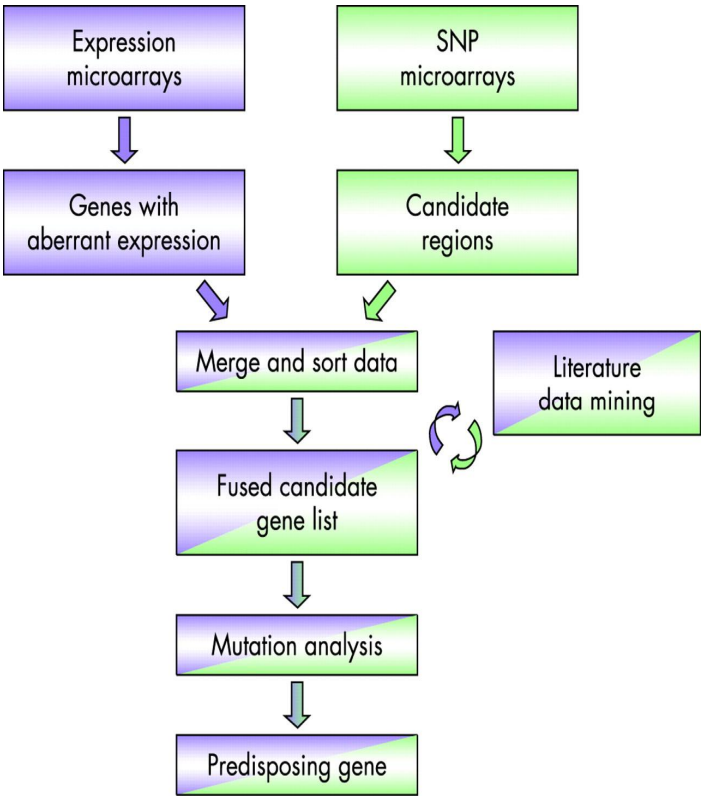

Figure 5. Flowchart describing the strategy for PAP gene identification (reproduced from Vahteristo *et al*., 2007 with the copyright holder's permission).

With the advent of gene expression profiling, an alternative approach to candidate gene identification was made possible. Here, we tested for aberrant gene expression in the germline of pituitary adenoma patients/obligatory carriers versus healthy controls. In our gene expression profiling experiments, we were seeking for genes that would be underexpressed, based on the literature supporting the loss of a putative TSG. The success of this experiment was due to two determining factors: Firstly, the fact that human *AIP* is rather ubiquitously expressed (SymAtlas, 2008 at http://symatlas.gnf.org; unpublished observations); if its expression had been pituitary-specific, or restricted in endocrine tissues, it would have been impossible to detect aberrant expression from blood derived mRNA. Secondly, gene underexpression would require that the predisposing mutations are severe enough to cause loss of the expression of the mutant allele, such as: a) (early) nonsense

mutations and small insertions or deletions that cause frameshift, all leading to premature stop codons and degradation of the transcripts by NMD, b) promoter aberrations impairing gene transcription, or c) copy number variations, such as partial or whole-gene deletions. Indeed, the germline mutation analysis revealed a very early termination codon, in exon 1 of *AIP* (Q14X), segregating with the GH-, mixed GH/PRL, and partially with the PRL-secreting adenoma phenotypes, in families 1 and 2.

The power of this approach, combining peripheral blood genome and transcriptome (BGT) analysis (Vahteristo *et al*., 2007), had been previously tested for two other site-specific tumor susceptibility genes, *MLH1* and *fumarase* (*FH*), causing HNPCC and HLRCC respectively; in both cases, genes displayed significantly reduced expression in mutation carriers (unpublished data). More recently, Vahteristo *et al*. (2007) reported the success of the method in identifying the genetic etiology of autosomal recessive xeroderma pigmentosum type E (XPE) in a patient who had negative results in tests routinely undertaken in XP diagnostics. The striking feature of this study was that only the samples from the patient and his healthy carrier parents, versus healthy controls, suffized to reveal the genetic defect in this skin cancer case. Thus, this approach may also assist in elucidating genetic defects in disorders with high locus heterogeneity.

The low PAP penetrance contrasts with the two major familial endocrine-related tumor syndromes MEN1 and CNC where penetrance is nearly complete (Bassett *et al*., 1998; Stratakis *et al*., 2001). In fact, only one affected sibling pair was identified among the Finnish patients. Low penetrance alleles may be much more frequent in the general population than high penetrance susceptibility alleles that are typically uncommon. It may be that environmental factors, additional random somatic mutations in the pituitary, or genetic – perhaps pituitary-specific – modifiers affect the clinical expression of PAP. Despite the plausibility of these hypotheses, the issue of the true PAP penetrance in Finland remains the subject of study. Clearly, much larger pedigrees and thorough medical investigation is needed.

Here, it is interesting to note that a subcluster from the Finnish family 1, with two female prolactinoma cases (daughter and a maternal sister) did not harbor an *AIP* mutation, as analyzed by direct sequencing (Fig 3A, A9 and A10). Since prolactinomas are the most common type of pituitary adenomas (40-45%) and occur more frequently in women (Arafah & Nasrallah, 2001), these cases may represent disease phenocopies. Therefore, the occurrence of two sporadic cases within the same family, independent of *AIP* predisposition, is possible.

In a subsequent report of a large collection of FIPA families, Daly *et al*. (2007b) found that 15% of the families screened for germline *AIP* mutations were positive; among these, the IFS families represented 50% of all IFS families recruited in the study. It is important to highlight that a large number of candidate families screened for *AIP* mutations were negative (Table 8). It may be that these patients carry different types of mutations, not detected by conventional sequencing, such as large genomic deletions or other rearrangements. Very recently, 21 familial pituitary adenoma index cases, previously tested negative for intragenic germline *AIP* mutations, were analysed for *AIP* copy number changes by the Multiplex Ligation-dependent Probe Amplification (MLPA) assay; two of 21

(9.5%) families were found to carry large genomic deletions, presumably occurring due to *Alu*-mediated unequal homologous recombination (Georgitsi *et al*., 2008). Nevertheless, the lack of intragenic *AIP* mutations in pedigrees with strong familiality for pituitary adenomas (Daly *et al*., 2007b) makes it likely that other genes, yet to be identified, may confer genetic susceptibility to pituitary tumorigenesis.

2. Molecular diagnosis of PAP

2.1 Germline *AIP* mutations in pituitary adenoma patients of various ethnic origins and clinical settings (II)

In order to gain more insight regarding the spectrum of the PAP phenotype, a large cohort of pituitary adenoma patients from genetically heterogeneous populations from Europe and the USA was screened for germline *AIP* mutations. Interestingly, presumably pathogenic mutations were identified in all populations and clinical settings studied, albeit with low frequencies. The highest mutation frequencies (5.5% and 7.5%) were observed among patients with a very young age at onset, diagnosed with acromegaly/gigantism. The mean age at diagnosis among all *AIP* mutation carriers was 23.8 years, supporting the findings of study I that PAP is associated with young age at onset (Karhu & Aaltonen, 2007). In some young PAP patients a family history of acromegaly was unravelled after mutation detection, but, overall, positive family history was a weak indicator of PAP, supporting the notion of a low penetrance condition. The contribution of *de novo* mutations in PAP remains to be examined, but it was not possible in the context of study II. These findings indicate that due to the low mutation frequencies, routine molecular screening is not currently justified for all pituitary adenoma patients, as discussed below (see section 5).

The clinical relevance of the three missense variants observed in this study (E293G, A299V, R304Q) remains undetermined and functional studies are necessary to clearly establish a connection to the disease; yet, the absence of these variants in the hundreds of healthy controls screened, as well as the conservation of the AIP amino acid sequence among several species, argue for an association with the condition. In addition, these missense mutations occur on exon 6, which codes for the C-terminal part of the AIP protein that harbors crucial protein-protein interaction domains, as detailed later (see section 6); thus, a potential pathogenic effect cannot be excluded at present.

The R16H variant detected in four sporadic pituitary adenoma patients from study II, has also been found in a French FIPA family (Daly *et al*., 2007b), in two French sporadic acromegaly patients (Cazabat *et al*., 2007), in two German sporadic NFPA patients (Buchbinder *et al*., 2008), in a young Bulgarian sporadic PRL-secreting adenoma case (Yaneva *et al*., 2008), in one of two affected cases of an Italian non-medullary thyroid cancer family (Raitila *et al*., submitted manuscript), in two Finnish colorectal cancer patients (study III), as well as in three healthy controls (study II; Cazabat *et al*., 2007). The following lines of evidence now argue in favor of R16H being a rare polymorphism: Firstly, its presence among healthy individuals; secondly, its presence among different pituitary adenoma types studied, as well as in thyroid and colorectal cancer patients without pituitary adenomas; thirdly, the lack of LOH in all R16H-positive tumors examined, and lastly the possible lack

of an effect on splicing as tested *in silico*. On the other hand, because of the low disease penetrance observed in PAP, at this point we cannot exclude any pathogenic association.

2.2 The PAP phenotype

By reviewing the PAP cases reported in study II, it emerges that PAP patients are typically diagnosed at a young age, have mainly GH-secreting adenomas and lack a strong family history of pituitary adenomas or other endocrine disease. Yet, other pituitary adenoma types in PAP cannot be excluded, since in the overall literature *AIP* mutations have been detected among mixed GH/PRL and PRL-secreting adenoma patients (study I; Daly *et al*., 2007b; Raitila *et al*., 2007; Naves *et al*., 2007; Leontiou *et al*., 2008), NFPA patients (Daly *et al*., 2007b; Leontiou *et al*., 2008; Georgitsi *et al*., 2008), and two ACTH-secreting adenoma (Cushing's disease) cases (study II; Beckers *et al*., 2008). It has also become evident that the same *AIP* mutation can be associated with a variable pituitary tumor phenotype, even within the same family: For instance, Q14X carriers in the Finnish family 1 had GH-,PRL-, or mixed GH/PRL-secreting adenomas (study I), E174fs carriers in a Brazilian family had GH- and mixed GH/PRL-secreting adenomas (Naves *et al*., 2007), R304X carriers in several families had mostly GH-secreting, but also PRL- and mixed GH/PRL-secreting adenomas (Daly *et al*., 2007b; Leontiou *et al*., 2008), whereas among the two siblings with the in-frame Ex2del, one had been diagnosed with NFPA and the other with a GH-secreting adenoma (Georgitsi *et al*., 2008) (Appendix Table 1). The lack of phenotype-genotype correlation among patients with the same mutations has been observed in other hereditary tumor syndromes (reviewed in Kinzler & Vogelstein, 1996; Vierimaa *et al*., 2007) and points towards the involvement of additional genetic/epigenetic factors or modifier genes (Antonarakis & Beckmann, 2006).

Whether *AIP* mutations are associated with larger, and perhaps more aggressive, pituitary tumors warrants further investigation. In the analysis of sporadic PAP cases in study I no significant difference was observed in the tumor size between mutation-positive (n=7) and mutation-negative (n=38) cases. Contrary, Daly *et al*. (2007b) observed that tumor diameter was significantly larger among familial *AIP* mutation-positive (n=26) versus *AIP* mutation-negative cases (n=130) (p=0.0005). Perhaps our numbers in study I were small and could not reach statistical significance.

To date, the only tumors that have been found to unambiguously associate with *AIP* mutations are pituitary adenomas, contrasted with MEN1 and CNC, where several other tumor types are among the typical manifestations. Interestingly, several cases of thyroid disorders, including nodular goitres, follicular adenomas, and follicular and papillary thyroid carcinomas, have been observed in Finnish *AIP* mutation-positive families (Drs Outi Vierimaa and Pasi Salmela, unpublished observations). Whether there is an association between such a spectrum of thyroid disorders and predisposition caused by *AIP* remains unclear; the identification of more PAP families presenting with thyroid disease is clearly needed to address this issue. In a recent study, no evidence for such an association was found (Raitila *et al*., submitted manuscript). Thyroid abnormalities, such as goitre, have been detected among acromegalic patients (Gasperi *et al*., 2002; Tita *et al*., 2005; Kurimoto *et al*., 2008), but thyroid cancer is more rare, despite being the most common endocrine malignancy (Grubbs *et al*., 2008). The occurence of such abnormalities in acromegaly patients may be attributed to the prolonged thyroid tissue exposure to high serum IGF-I (Tita *et al*.,

2005; Siegel & Tomer, 2005). Moreover, adrenal carcinoma and lipomas have presented with pituitary adenomas in *AIP* mutation-positive families (Leontiou *et al*., 2008; Toledo *et al*., 2008a). What is of particular interest here is that the GH-secreting adenoma and the adrenal carcinoma tissues of two patients reported by Leontiou *et al*. (2008) and Toledo *et al*. (2008b) showed LOH. Because adrenal carcinomas often exhibit chromosomal instability (LOH) in 11q13 (Leontiou *et al*., 2008), the analysis of more adrenal gland tumors in the context of PAP is now warranted.

2.3 The potential of AIP immunohistochemistry as a diagnostic tool (II)

In study II, AIP IHC staining in 50 pituitary adenoma specimens proved to be a useful predictor for PAP, with 75% sensitivity and 95% specificity for germline truncating mutations. In two cases that were negative for an *AIP* mutation, negative staining was observed; this may be attributed to technical reasons, or to the presence of a different type of germline mutation that remains undetected by conventional sequencing (i.e. a large genomic deletion). Three tumors were positive for an *AIP* mutation (two with the Q14X and one with the IVS3-1G>A), but also positive for AIP staining. In the case of the Q14X positive tumors LOH had been detected previously, thus unspecific staining cannot be ruled out. LOH analysis for IVS3-1G>A was equivocal; a plausible explanation could be that the "second hit" is not loss of the wild type allele, but a missense-type of mutation or a small in-frame deletion that results in non-functional, yet stable and immunoreactive, protein. However, with the exception of half of exon 3 that repeatedly failed to amplify, the rest of the coding *AIP* sequence was negative for a second point mutation.

Here, it should be noted that IHC detection of mutant AIP largely depends on the type of germline mutation and second hit mutation in the tumor tissue, but also on the antibody used. In study II, a polyclonal mouse antibody was utilized as optimal at the time. IHC staining showed 75% sensitivity for germline truncating *AIP* mutations and in these cases tumors clearly exhibited LOH as the second hit. Others found partial immunoreactivity in a germline truncating mutation-positive pituitary tumor, by using a different monoclonal antibody against human AIP (Naves *et al*., 2007). In this study, cytoplasmic AIP staining was observed in approximately 30% of tumor cells when compared to adjacent normal pituitary tissue; results could not be correlated to LOH analysis, because the latter was equivocal. In another study, AIP immunostaining in adenomas from two *AIP*-mutation positive families, bearing the R304X and the in-frame duplication c.794-823dup30 (A274_H275ins10), was positive (Leontiou *et al*., 2008). Yet, this may not be completely unexpected, since R304X and A274_H275ins10 occur late in the *AIP* sequence (both in exon 6) and may allow for the expression of a stable and immunoreactive, yet possibly dysfunctional, protein.

2.4 Overview of the molecular genetics of *AIP*

Since identification of the *AIP* gene, tens of germline mutations have been found in pituitary adenoma patients of various ethnic origins, either in familial or sporadic settings (summarized in Tables 8, 9, and Appendix Table 1). The mutation spectrum includes single-point mutations (missense or nonsense), small insertions and deletions, in-frame deletions and one in-frame duplication, splice-site mutations, promoter changes, and even large genomic deletions (Fig. 6, Appendix Table 1, Appendix Fig. 1). These pathogenic changes

are predicted to either hinder the expression of normal AIP protein, or result in altered protein structure and/or protein function. Interestingly, the majority of germline *AIP* mutations are truncating, supporting a tumor suppressive role. This is reminiscent of the other two endocrine-related tumor susceptibility TSGs, *MEN1* and *PRKAR1A*, with the vast majority of reported mutations being truncating (Stratakis *et al*., 2001; Marx, 2005; The Human Gene Mutation Database 2008 at www.hgmd.org).

Mutations appear to be scattered quite evenly in the coding region of *AIP*, despite a notable, yet possibly coincidental, clustering of missense changes at the 3' end (exons 5 and 6), between codons 238-304. These missense mutations occur within the regions coding for the crucial interaction domains of AIP, the tetratricopeptide (TPR) repeats (Fig. 6). Mutational analyses of the TPR domains, before anything was known about the implication of the gene in pituitary tumorigenesis, showed how essential they are for the interactions of AIP with its cellular partners (Bell & Poland, 2000; Melville *et al*., 2000; Bolger *et al*., 2003).

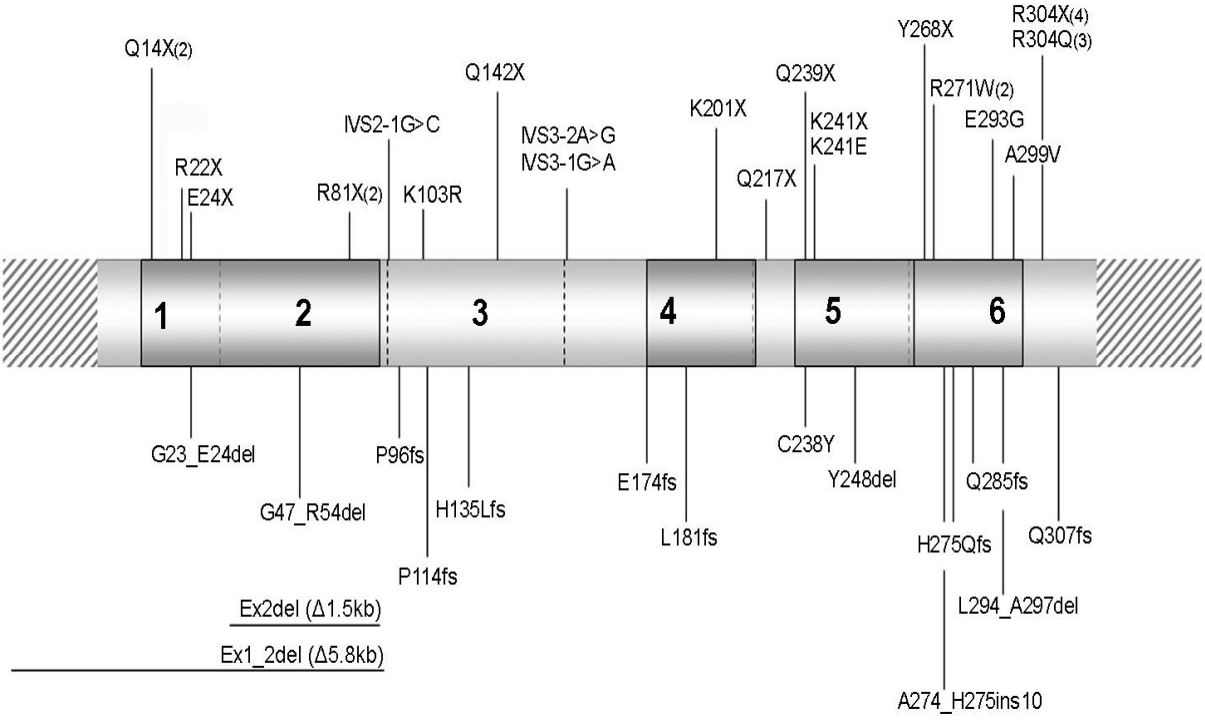

Figure 6. Schematic representation of the *AIP* transcript with all germline mutations reported in the literature by June 2008 (see also Appendix Table 1). The numbers in parentheses indicate the number of families with the same mutation. Numbers 1-6 denote the six exons of the transcript, separated by vertical dashed lines. The grey-lined boxes represent the 5' and 3' UTRs. The dark grey box partly overlying exon 1 and almost completely exon 2 designates the FKBP domain. The smaller dark grey boxes overlying parts of exons 4, 5, and 6 represent the three TPR repeats (see also Fig. 7).

It is not currently clear whether mutations causing NMD (i.e. early stop codons and frameshift mutations causing premature stop codons) cause a less severe phenotype than smaller intragenic mutations that possibly escape transcript degradation (i.e. missense mutations, small in-frame insertions or deletions, including single exon deletions). In the future, detailed clinical and biochemical examinations of patients carrying these different types of mutations may help reveal information on disease penetrance and severity, response to drug therapy, or tumor relapse.

Table 8. Germline *AIP* mutation analysis conducted in familial pituitary adenoma cases as reported in the literature by June 2008.

Reference	Total No of analyzed families	Total No of mutation-positive families	No of kindreds with familial acromegaly in the series	No of mutation-positive kindreds with familial acromegaly in the series
Study I	5	3	4	2/4 (50%)
Daly *et al*., 2007b	73	10	16	8/16 (50%)
Iwata *et al*., 2007	1	1	1	1/1 (100%)
Toledo *et al*., 2007	1	1	1	1/1 (100%)
Raverot *et al*., 2007	1	0	0	0
Leontiou *et al*., 2008	26	9	21	9/21 (43%)
Georgitsi *et al*., 2008	23*	2	7	1/7 (14%)
Fajardo Montanana *et al*., 2008	1	0	1	0/1
Yaneva *et al*., 2008	2	0	0	0
Total	133	26	51	22/51 (43%)

No, number

* 21/23 probands were successfully analyzed by the MLPA assay.

Table 9. *AIP* mutation analysis carried out in sporadic pituitary adenoma cases as reported in the literature by June 2008§.

Reference	Total No of analyzed cases	Total No of mutation-positive cases	No of cases diagnosed with GH-secreting adenoma in the series	No of mutation-positive cases with GH-secreting adenoma in the series
Germline screening				
Study I	41	5	41	5/41 (12%)
Study II	433	7/433 (1.6%)	176	6/176 (3.4%)
Yu *et al*., 2006*	63	0/63	35	0/35
Barlier *et al*., 2007	107	0/107	26	0/26
Cazabat *et al*., 2007	154	5/154 (3%)	154	5/154 (3%)
DiGiovanni *et al*., 2007**	78	0/78	62	0/62
Leontiou *et al*., 2008	37	0/37	37	0/37
Study IV	36	1/36 (2.8%)	7	1/7 (14%)
Yaneva *et al*., 2008	8	1/8 (12.5%)	5	1/5 (20%)
Somatic screening	Total No of tumors analyzed	Total No of mutation-positive tumors	No of GH-secreting tumors in the series	No of mutation-positive GH-secreting tumors in the series
Study I	10	0	10	0
Study II	27	2/27 (7.4%)	27	2/27 (7.4%)
Iwata *et al*., 2007	40	0/40	40	0/40
Barlier *et al*., 2007	41	2/41 (5%)	23	2/23 (8.7%)
Raitila *et al*., 2007	32	2/32 (6%)	23	0/23
Leontiou *et al*., 2008	48	0	NA	0

No, number; NA, information not available

§ Cohorts are presented irrespectively of the selection criteria based on which they were published.

* *AIP* mutation analysis was incomplete, performed only for exons 1, 4, and 6.

** *AIP* mutation analysis was incomplete, performed only for exons 1, 4, 5 and 6.

All germline *AIP* mutations reported in the literature to date have been seen in heterozygosity, which implies that homozygous germline *AIP* mutations and AIP deficiency are most likely not compatible with life. This is in agreement with the recent observations

that *Aip*$^{-/-}$ mice die *in utero* at various time points, but already around E10, due to cardiovascular defects, suggesting an essential role for Aip protein in cardiac development (Lin *et al*., 2007). In addition, homozygous hypomorphic *Aip* mice are born without cardiac defects; instead, they show defective closure of the hepatovascular shunt, called *ductus venosus*, and have decreased liver weight due to reduced blood supply (Lin *et al*., 2008). Aip is expressed early in embryonic development, as early as E9.5, well before some of its known interaction partners (Lin *et al*., 2007). These observations speak for a broader role of Aip in mammalial biology. It remains to be seen whether human AIP has yet unidentified, and perhaps pituitary-specific, interaction partners and, thus, additional biological functions.

3. *AIP* does not appear to contribute to tumorigenesis of common cancers (III)

The biallelic inactivation of *AIP*, based on LOH and IHC data obtained from pituitary tumors (studies I, II; Raitila *et al*., 2007), in concert with recent *in vitro* functional data on the reduced ability of mutant AIP to inhibit cell proliferation (Leontiou *et al*., 2008), argue in favor of a tumor suppressive role. Following gene identification, it was relevant to examine whether *AIP* is involved in the tumorigenesis of other tissues. For this purpose, we performed somatic mutation analysis in a total of 499 colorectal, breast, and prostate tumor samples. Apart from being very prevalent worldwide, these tumors – colorectal neoplasia in particular – have been previously associated with acromegaly (Jenkins, 2004).

The only interesting variant observed was the missense change R16H, detected in one MSS and one MSI colorectal tumor without LOH, as well as in the corresponding normal tissues. The possibility of an occult pituitary GH- or PRL-secreting adenoma in the MSS patient (no data on serum PRL levels available) could have promoted the breast enlargement and colorectal cancer, but the patient passed away and no further studies were performed. The presence of a carcinoid tumor in the brother of the MSI patient with the R16H was interesting, since carcinoids are features of MEN1. However, the lack of material for segregation analysis in this family hindered further investigation of a possible association.

No other presumably pathogenic mutations were detected, indicating lack of evidence for an immediate role of *AIP* in the initiation or progression of these tumor types. Recently, Sjoblom *et al*. (2006) and Wood *et al*. (2007) published the genomic landscapes of human breast and colorectal cancers in two massive screening works; *AIP* was not found among the genes that are somatically mutated at significant frequency, adding support to the notion that *AIP* is unlikely to be involved in these two cancer types (Sjoblom *et al*., 2006; Wood *et al*., 2007).

These findings did not rule out the possibility that *AIP* could be somatically mutated in other tumor types, perhaps more closely related to endocrine neoplasia. To address this issue, sporadic endocrine-related tumors (total n=111; 32 pituitary adenomas and 79 other endocrine tumors) were analyzed by Raitila *et al*. (2007); somatic *AIP* mutations appeared to be very rare in this setting as well. The only finding was the Finnish founder mutation Q14X, identified in two PRL-secreting adenomas showing LOH. Both patients were

diagnosed at the age of 35 years. The first patient did not have a family history of endocrine tumors, whereas relevant information was not available from the second. These results were in accordance with previous observations regarding the early age of onset and the lack of strong family history among PAP patients (study I, II). In addition, this study further confirmed that PRL-secreting adenomas are part of the PAP phenotype.

Since gene identification, other groups have sought for somatic *AIP* mutations in sporadic pituitary adenomas of various types (Table 9), with mostly negative results (Iwata *et al*., 2007; Barlier *et al*., 2007; Leontiou *et al*., 2008). Interestingly, the other two major endocrine tumor susceptibility genes, *MEN1* and *PRKAR1A*, are only rarely found somatically mutated in sporadic pituitary adenomas (reviewed in Thakker, 1998; Sandrini *et al*., 2002; Kaltsas *et al*., 2002; Yamasaki *et al*., 2003) or other sporadic endocrine tumors (Zhuang *et al*., 1997b; Toliat *et al*., 1997; Heppner *et al*., 1997; Vortmeyer *et al*., 1998). Sporadic pituitary tumors have been also screened for somatic *CDKN1B* mutations, with negative results (Takeuchi *et al*., 1998; Dahia *et al*., 1998; Ozawa *et al*., 2007).

Recent data show that other mechanisms, such as epigenetic inactivation of TSGs, may also account for a subset of the sporadic form of the disease (Esteller *et al*., 2000). Gene silencing caused by epigenetic mechanisms, through for instance promoter hypermethylation, is an event encountered in as many as 50% of TSGs in sporadic tumors (Jones & Baylin, 2002), such as *BRCA1* in sporadic breast tumors, *PRB* in sporadic retinoblastomas, and *MLH1* in sporadic colorectal and endometrial cancers (reviewed in Weinberg, 2007). Epigenetic silencing of *AIP* has not been reported in pituitary adenomas thus far, but little time has elapsed since gene identification and relevant studies may be under way.

4. Pediatric GH-secreting tumors may arise due to *AIP* mutations (IV)

The occurrence of pituitary tumors among children and adolescent patients is very rare. Approximately 2-6% of all surgically treated pituitary adenomas occur in young patients; PRL- and ACTH-secreting adenomas are the most common types. It is conceivable that such early disease onset may be in part explained by an underlying genetic predisposition, as observed in very young MEN1 and CNC patients with pituitary adenomas (O'Brien *et al*., 1996; Stratakis *et al*., 2000; Brandi *et al*., 2001; Stratakis *et al*., 2001; Keil & Stratakis, 2008). In order to examine whether PAP could partly explain the pediatric disease onset, and given the typically young age at onset among the adult PAP patients, we obtained a pediatric series encompassing all pituitary adenoma types encountered at this age group.

Among 36 sporadic pediatric cases analyzed for germline *AIP* mutations, one male patient was found to carry the in-frame deletion Y248del. This patient was operated on at the age of 19 years for a GH-secreting adenoma causing gigantism during adolescence. Loss of the wild type allele on tumor DNA level was clearly observed; thus, the positive IHC staining was indicative of the retention of the mutant protein, assuming that the presumably defective mRNA escaped degradation. It may be that non-functional, yet immunoreactive, protein is present in these GH-secreting adenoma cells. It is not straightforward whether Y248del compromises the normal AIP function in the pituitary tissue. Several reasons argue in favor of a presumably pathogenic effect of the mutation on the protein's normal function:

Firstly, Y248del occurs on the second of three TPR repeats, which mediate the crucial interactions between AIP and its cytoplasmic partners. Secondly, tyrosine 248 is highly conserved among several species. Thirdly, the change was not observed in 253 healthy individuals, including 52 population-matched controls, thus providing little evidence for Y248del being a rare polymorphism among the Italian population.

Interestingly, all of the patient's first-degree relatives that are mutation-carriers (the father and the two other siblings) remain to date unaffected and no family history of endocrine disease has been reported. Clinical investigation and genetic testing among the paternal relatives would help clarify whether this is a truly sporadic case or yet another family with very low disease penetrance.

Data from study IV showed that *AIP* mutations are an important factor underlying GH-secreting adenomas (1/7 or 14.3%) in children and adolescents. Therefore, it was relevant to examine the prevalence of *AIP* mutations among young (≤25 years) GH-secreting adenoma patients, in order to also gain insight into a patient group that could benefit from genetic counseling and genetic testing. Along these lines, relevant data were reviewed from our previous reports and the work of others (studies I, II, and Iwata *et al*., 2007). It was concluded that PAP patients account for 40% of patients diagnosed at ≤25 years, in comparison to 1.6% of patients diagnosed after 25 years ($p=5x10^{-9}$) (Table 3 of publication IV). If the cases from the Finnish population, known to harbor a founder mutation, were excluded, then young (≤25 years) PAP patients would account for 14.3%, in comparison to 0.5% of cases diagnosed >25 years (p=0.013) (Table 4 of publication IV). These figures are very similar to the numbers reported by Cazabat *et al*. (2007), where, in a similar approach, *AIP* mutation-positive patients accounted for 12.5% of patients <30 years at diagnosis versus 0.8% of patients diagnosed at >30 years of age.

Obviously, study IV was conducted in a cohort of pediatric patients of a single ethnic origin, and more studies are needed to further address the role of *AIP* in pediatric pituitary tumorigenesis. Yet, the size of the cohort, the detailed clinical documentation, and the fact that most pituitary adenoma types are represented despite the extreme rarity of the disease in children and adolescents, add further value to these findings. After study IV was accepted for publication, a cohort consisting of seven sporadic giants of various ethnic origins (Australia=1, USA=1, UK=3, Brazil=2) with childhood-onset GH-secreting pituitary adenomas was published; no germline *AIP* mutations were detected (Leontiou *et al*., 2008). On the contrary, a novel *AIP* mutation (K103R) was identified in one Cushing's disease patient from a cohort of 76 pediatric-onset cases, and three novel mutations (Q307fs, P114fs, and K241X) were detected in three patients of a series of 11 pediatric-onset pituitary adenoma cases with syndromic features (Beckers *et al*., 2008). These results add further support to the notion that mutant *AIP* predisposes to pediatric-onset pituitary tumorigenesis.

5. Implications for genetic counseling and follow-up in PAP

Whether genetic testing of *AIP* could facilitate identification of at-risk individuals has attracted interest and controversy. Years of research may be still required before *AIP*

mutation screening has an impact on the patients' clinical management and treatment. Yet, implementation of predictive genetic testing for the relatives of mutation-positive patients can identify asymptomatic mutation carriers and result in early tumor detection (Cazabat *et al*., 2007). On the other hand, predictive testing raises psychological and ethical considerations, which may be of great concern in the case of low penetrance conditions, such as PAP.

It has been proposed that genetic screening of *AIP* is rather premature, given the rarity of the familial pituitary adenomas, the small size of the affected families, and the infrequent occurrence of *AIP* mutations (Melmed, 2007; Melmed, 2008). Instead, biochemical screening tests (i.e. serum IGF-I, GH, and PRL measurements), should be preferred in the clinic as the primary mode of action for monitoring at-risk individuals, since they are universally available, easily affordable, accurate, and superior to imaging screening (Melmed, 2008). Indeed, it is true that genetic counseling demands resources, DNA testing typically requires prior genetic counseling, and DNA sequencing might still be more costly that biochemical tests. However, it seems futile to offer lifelong biochemical screening to non-carriers; on the contrary, predictive genetic testing can help identify the family branches and individuals who segregate an *AIP* mutation and truly are at an increased risk. Therefore, biochemical and imaging screening, following *AIP* mutation testing, could be focused on the actual carriers, which is clearly superior to biochemical screening alone. In a similar approach, *MEN1*-negative individuals have been spared regular biochemical evaluations, whereas biochemical and radiological monitoring has been provided to *MEN1* mutation carriers only, with beneficial effects on the disease morbidity and long-term outcome (Bassett *et al*., 1998; Brandi *et al*., 2001; Klein *et al*., 2005; Pieterman *et al*., 2008).

No concensus guidelines exist yet regarding the criteria that should be fulfilled to justify *AIP* genetic testing. Perhaps the widespread use of genetic screening in unselected patients with sporadic pituitary adenomas is not currently warranted. However, genetic testing could focus on a targeted group, such as the familial acromegaly cases with young age at onset and their relatives. Among all pedigrees screened for *AIP* mutations thus far (n=133), familial acromegaly kindreds account for about one third (n=51), of which almost half have PAP (22/51 or 43%) (Table 8). This highlights the fact that *AIP* remains the major known GH-secreting adenoma susceptibility gene outside the context of MEN1, CNC, or MAS. Lastly, diagnostic genetic testing could be considered for the young patients with aggressive pituitary tumors (Beckers & Daly, 2007).

Undoubtably, in order to provide informative counseling about PAP to patients and their family members, there is the primary need to unravel its penetrance. Because PAP is a low penetrance condition, it is expected that most mutation carriers are healthy. Among the giant Finnish pedigree (study I), 64 individuals at 50% risk have been identified (Dr. Outi Vierimaa, unpublished data); however, the estimated disease penetrance has not been established yet. In such families, detailed clinical and biochemical investigations of mutation-carriers are expected to contribute significantly to defining the disease penetrance, but this has proven a time-consuming effort.

Direct DNA sequencing and MLPA analysis unambiguously remain the most appropriate tools for PAP indetification on genomic level. One consideration may be, though, that

genetic testing, as the primary molecular tool for diagnosis, remains a costly procedure that requires genetic counseling and resources. On the other hand, surgically removed pituitary adenomas are routinely examined by IHC for tumor classification and diagnostic purposes; in United States alone, thousands of paraffin-embedded GH-secreting adenoma samples are available for prescreening of PAP by IHC, pending patients' consent. For this reason, in study II we proposed that, in addition to signs such as young age at onset or positive family history, and with the appropriate methodological improvements (e.g. new specific antibodies), negative AIP IHC in pituitary adenomas may serve as a useful tool for PAP identification in the future. This approach would be similar to tumor-based approaches now in routine use in the diagnosis of hereditary colon cancer (Hampel *et al*., 2005).

6. The AIP protein and its cellular functions

6.1 Features of the AIP protein

Originally, AIP was identified through its interaction with and transcriptional suppression of the hepatitis B virus (HBV) X protein; HBV X protein is possibly required for the viral replication *in vivo*, and may play a role in HBV-induced carcinogenesis (Petrulis & Perdew, 2002). Hence, AIP is also known as HBV X-associated protein 2 (XAP2) (Kuzhandaivelu *et al*., 1996). Carver & Bradfield (1997) independently identified AIP, to which they attributed the name Aryl Hydrocarbon Receptor (AHR)-associated protein 9 (hence 'ARA9').

AHR, also known as dioxin receptor, is a ligand-inducible transcription factor that plays central role in regulating the cellular response to polycyclic aromatic compounds, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Meyer *et al*., 1998); AHR induces the transcription of the xenobiotic metabolizing enzymes, such as cytochrome P450. In 1996, in a yeast two-hybrid experiment aiming to identify novel interaction partners to AHR, AHR-interacting protein (hence 'AIP') emerged as a previously unknown AHR partner in mouse hepatoma cells (Ma & Whitlock, 1997).

Ma & Whitlock (1997) found that the mouse *Aip* cDNA contained an open reading frame that coded for a protein of 330 aa, with a molecular mass of ~37 kDa. A region of mouse Aip exhibits sequence homology with human and mouse immunophilin FKBP proteins, which are molecular chaperones involved in steroid receptor signalling and nuclear targeting, protein folding, heat shock responses, and drug-induced immunosuppression (reviewed in Ma & Whitlock, 1997). In addition, AIP harbors three tetratricopeptide repeats (TPRs), a 34 aa motif mediating various protein-protein interactions (Lamb *et al*., 1995), one of which is the interaction with AHR (Fig. 7). TPR motifs have been identified in a diverse group of proteins involved in mitosis, protein import, RNA splicing, neurogenesis, transcription, and serine/threonine phosphorylation, and are essential for the assembly of multi-protein complexes (Goebl & Yanagida, 1991; Lamb *et al*., 1995).

In uninduced cells, AIP exists in a cytoplasmic complex with AHR and HSP90, the latter required for the proper folding of AHR (Carver *et al*., 1998; Petrulis & Perdew, 2002). The presence of ligands, such as dioxin and dioxin-like chemicals, results in the dissociation of the complex and the translocation of AHR into the nucleus, where it forms a heterodimer transcription complex with AHR nuclear translocator (ARNT). The AHR/ARNT complex

mediates the xenobiotic metabolizing response, by binding to specific DNA elements, known as dioxin responsive elements (DREs) and initiating transcription of detoxification enzymes (Fig. 8). AIP- AHR and AIP-HSP90 interactions are mediated through the AIP TPR domains (Carver *et al*., 1998). Bell & Poland (2000) also showed that the last five amino acids of the protein are of crucial importance for its interaction with AHR (Fig. 7).

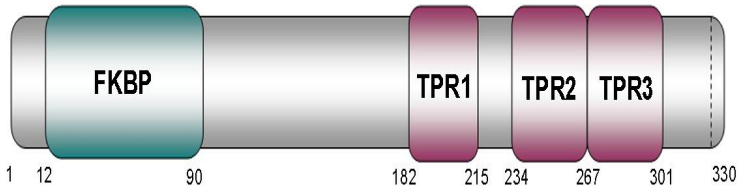

Figure 7. Schematic representation of the AIP protein with its FKBP and TPR domains (adapted from van der Spuy, 2006). The vertical dashed line indicates the last 5 aa that are crucial for the interaction of AIP with AHR.

Overall, it is now known that AIP maintains the cytoplasmic localization of AHR, via its association with HSP90, thus preventing the nuclear accumulation of the ligand-activated receptor. In addition, AIP increases the ligand binding capacity of AHR, alters the ability of AHR to be recognized by importin β (Fig. 8), and decreases the receptor's proteosomal degradation by protecting it against ubiquitination (Kazlauskas *et al*., 2000; Petrulis & Perdew, 2002; Pollenz & Dougherty, 2005).

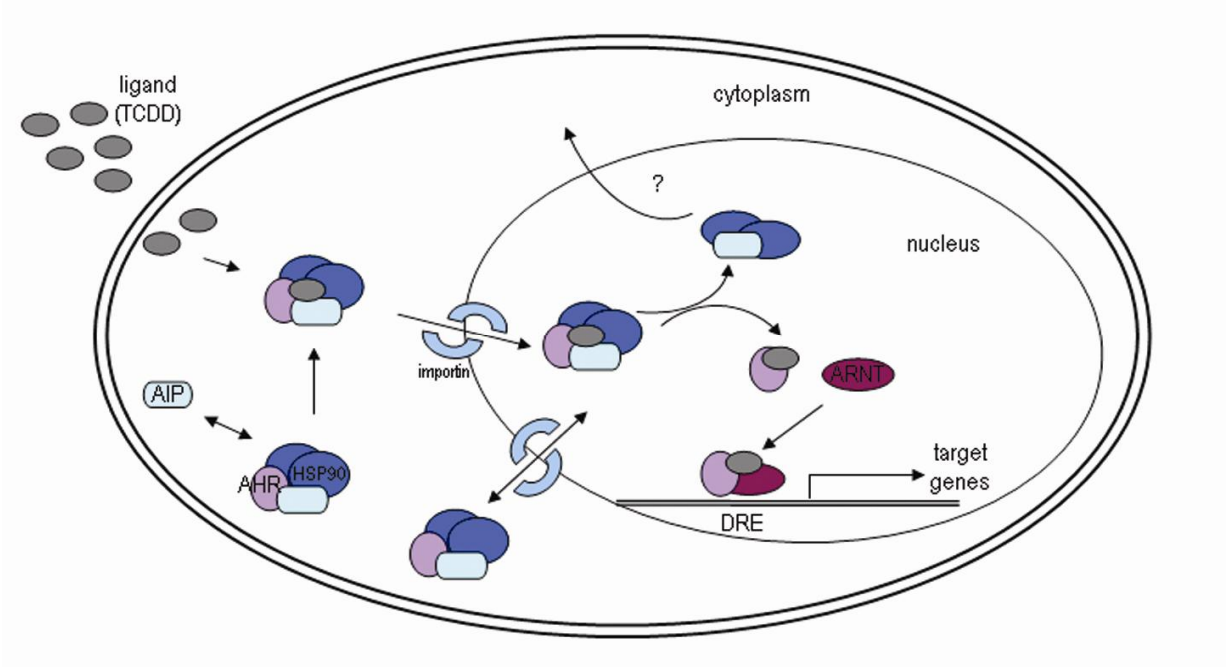

Figure 8. A simplified model of the human cytoplasmic AHR multi-protein complex and its translocation into the nucleus upon ligand binding (modified from Meyer & Perdew, 1999; Petrulis & Perdew, 2002; Ramadoss *et al*., 2004). The AIP/AHR/HSP90 complex can translocate into the nucleus irrespectively of the ligand-bound or ligand-free state of AHR.

In general, the interaction of AIP with AHR and HSP90 appears to be conserved across mammalian species (Meyer *et al*., 1998; Meyer & Perdew, 1999; Bell & Poland, 2000); yet, some differences have been observed between the mouse and human AIP/AHR interaction: Mouse Aip increases the cytoplasmic levels of Ahr by inhibiting its nucleocytoplasmic shuttling and its binding to importin β, whereas human AIP does not hinder the latter interaction; as a result human AIP seems to translocate into the nucleus with the AHR

complex, either in the presence or absence of ligand (Fig. 8). In contrast, mouse Aip seems to dissociate from Ahr prior to its nuclear translocation (Ramadoss *et al*., 2004). The biological effect of these differences is not known, but some caution is needed when extrapolating results from the murine model to humans.

6.2 Possible implications of AIP/AHR pathway in AIP-mediated tumorigenesis

Until recently, environmental factors had not been rigorously implicated in pituitary tumorigenesis (DeLellis *et al*., 2004); however, since AIP is part of the AHR-mediated xenobiotic metabolic pathway, the role of dioxin and dioxin-like chemicals warrants further investigation. Prolonged exposure of cells to chemical carcinogens can render them vulnerable to tumorigenesis (Weinberg, 2007). To date, definitive correlation between dioxin exposure and pituitary adenoma development is lacking. Yet, Pesatori *et al*. (2008) recently analyzed the occurence of pituitary adenomas in the Seveso population (Italy), after a dioxin exposure accident in 1976; despite the lack of statistically significant increase in the prevalence of pituitary adenomas, a tendency toward a higher risk of pituitary tumorigenesis was observed in subjects that had been exposed to high or intermediate dioxin concentrations compared to low-exposed and non-contaminated reference population. Similar detailed analyses may be a laborious task with human subjects, yet, animal models of PAP, such as *Aip*$^{+/-}$ mice, may facilitate testing this hypothesis in a more controllable fashion. If this is the correct pathway, it remains unknown why AIP-mediated chemical-induced tumorigenesis seems to exclusively affect the anterior pituitary tissue, despite the ubiquitous expression of AIP and AHR in all human tissues.

Some clues in AIP-associated tumorigenesis may lie in the interaction of AHR with cell cycle regulators, such as p27^{Kip1} and pRB. AHR was shown to inhibit cell cycle progression in dioxin-induced rat hepatoma cells, by directly and specifically inducing the cell cycle inhibitor p27^{Kip1}. This induction occurs at the mRNA expression level and not on p27^{Kip1} protein stability or the rate of p27^{Kip1} mRNA translation (Kolluri *et al*., 1999). In pituitary adenomas, p27^{Kip1} protein expression levels are significantly reduced (Table 6) (Takeuchi *et al*., 1998; Dahia *et al*., 1998; Lidhar *et al*., 1999; Bamberger *et al*., 1999), thus neoplastic pituitary cells appear to have lost a crucial cell proliferation brake.

Another mechanism by which AHR seems to exert anti-proliferative activity, upon dioxin induction of rat hepatoma cells, is through the direct interaction with the retinoblastoma protein pRB. pRB is a negative regulator of the cell cycle G1/S phase transition via the direct binding and inhibition of E2F transcription factors (Ge & Elferink, 1998; Puga *et al*., 2000). Puga *et al*. (2000) demonstrated that, under the presence of mitogenic signals, AHR directly binds pRB and cooperates to the repression of E2F-dependent transcription of target genes that lead to G1/S phase transition. Moreover, Marlowe *et al*. (2008) showed that ligand-induced AHR activation leads to direct formation of nuclear AHR-E2F1 complexes, resulting in inhibition of E2F1-dependent expression of proapoptotic genes and, thus, inhibition of apoptosis. Thus, it seems that AHR, pRB, and E2Fs may synergistically activate an environmental checkpoint that regulates the balance between cell cycle progression and arrest. It may be that loss of AIP is linked to these pathways, by perturbing the role of AHR and compromising the delicate cellular balance. Aggressive pituitary adenomas and

carcinomas appear to have lost the *PRB* chromosomal locus or the gene is rendered silent due to promoter hypermethylation (Table 6) (Boikos & Stratakis, 2007).

6.3 Other AIP interaction partners and possible implications in AIP-mediated tumorigenesis

Aip is ubiquitously expressed during mouse embryonic development, as early as E9.5, preceding expression of other Aip-associated proteins, such as Ahr (Abbott *et al*., 1995); this suggests that Aip functions in other signal transduction pathways apart from the Ahr pathway (Carver *et al*., 1998; Lin *et al*., 2007).

Indeed, AIP has been reported to bind a number of other proteins, such as the Epstein-Barr virus nuclear antigen 3 (EBNA-3), a transcriptional transactivator with an unclear functional role in viral pathogenesis (Krauer *et al*., 1996; Kashuba *et al*., 2000). Later, AIP was found to interact via its TPR domains with the TPR motif present in the C-terminal domain of Tom20, a main mitochondrial import receptor. AIP bound specifically also to mitochondrial preproteins, maintained their unfolded status and suppressed their aggregation in the cytoplasm; thus, AIP exhibits a chaperone-like activity (Yano *et al*., 2003). At the same time, it was demonstrated that AIP interacts with a nuclear receptor of the steroid receptor superfamily, the peroxisome proliferator-activated receptor α (PPARα), in a complex with HSP90 (Sumanasekera *et al*., 2003). In humans, PPARα regulates energy homeostasis via control of lipid metabolism. Sumanasekera *et al*. (2003) showed that AIP represses the transcriptional activity of PPARα, but this interaction has not been addressed in the pituitary tissue.

Kang & Altieri (2006) demonstrated the role of AIP in the stabilization of survivin, an inhibitor of apoptosis and regulator of cell division, a pathway that could be theoretically associated with the possible progression of adenomas to more aggressive tumors (Altieri, 2003; Kang & Altieri, 2006). Survivin, which is present during fetal development, but undetectable in terminally differentiated normal adult tissues, is overexpressed in many human tumors, including pituitary adenomas (Wasko *et al*., 2005; Hassounah *et al*., 2005), and this interaction warrants further investigation. Recently, AIP was also shown to specifically interact with TRβ1, one of the two nuclear thyroid hormone receptors (TRβ1 and TRβ2) that upon T3 thyroid hormone binding, they modulate the hypothalamic TRH transcription (Froidevaux *et al*., 2006). Interestingly, this group demonstrated that AIP is necessary for a T3-independent TRβ1-mediated TRH transcription.

Moreover, AIP specifically interacts with cAMP-specific phosphodiesterase PDE4A5 and directly inhibits its enzymatic activity and attenuates the ability of PDE4A5 to be phosphorylated by the cAMP-dependent PKA (Bolger *et al*., 2003). Functional validation of the effect of five germline *AIP* mutations detected in pituitary adenoma patients, including two missense (C238Y, R271W) and three nonsense (R81X, Q217X, R304X) mutations, revealed that all changes abolish the interaction of AIP with PDE4A5. Because the same observation was made for three different cell lines, including the rat mixed GH/PRL-secreting adenoma cell line (GH3), a human embryonic kidney cell line (HEK293), and a human embryonic lung fibroblast cell line (TIG3), these results pose additional questions

regarding the tumorigenic effects of AIP loss in the human pituitary specifically (Leontiou *et al*., 2008).

Interestingly, human AIP functionally interacts with a different phosphodiesterase isotype, PDE2A, involved in the hydrolysis of cAMP (de Oliveira *et al*., 2007). It had been shown earlier that elevation of cAMP levels induces the nuclear translocation of AHR, in the absence of exogenous ligands; cAMP-induced AHR adopts a structure that hinders interaction with ARNT and, thus, acting as a repressor rather than an activator of AHR-dependent gene expression (Oesch-Bartlomowicz *et al*., 2005). Later, de Oliveira *et al*. (2007) provided further evidence that PDE2A mediates the cytosolic sequestration of AHR, presumably via locally reducing the cAMP levels. It may be that in the absence of functional AIP, PDE2A promotes increase in cAMP levels, and nuclear translocation of cAMP-induced AHR, which in turn orchestrates a different gene expression pattern compared to the one activated by dioxin-induced AHR/ARNT complexes. Whether these facts create a permissive environment for pituitary tumorigenesis deserves further investigation.

It is of great interest that genes involved in cAMP-dependent signalling have been previously found causative for tumorigenesis in endocrine tissues: *GNAS*, which codes for $G_s\alpha$, harbors somatic mutations in as many as 40% of sporadic GH-secreting adenomas and germline mutations in MAS patients with GH-secreting adenomas. $G_s\alpha$ is required for the activation of adenylyl cyclase, which in turn increases cAMP levels, leading to a signalling cascade through the activation of protein kinases in many cell types, including pituitary cells (see Introduction, section 3.1.1). Moreover, *PRKAR1A* carries inactivating mutations in the majority of CNC patients (see Introduction, section 3.2.2). In this case, unconstrained phosphorylation of cAMP by PKA, results in elevated mitogenic signalling, as explained above. In addition, inactivating germline mutations in the *PDE11A* gene predispose to micronodular adrenocortical hyperplasia in a subgroup of patients with Cushing's syndrome. PDE11A also catalyzes cAMP and cGMP, and cAMP levels are found increased in *PDE11A* mutation-positive adrenal tissue samples compared to controls (Horvath *et al*., 2006). It was later shown that less severe germline *PDE11A* mutations predispose to a variety of benign and malignant adrenocortical tumor types; these *PDE11A* variants may account for the genetic predisposition of adrenocortical tumors on population level (Libe *et al*., 2008). Lastly, *PDE8B* has been also found mutated or underexpressed in adrenocortical hyperplasia (Horvath *et al*., 2008). However, possible interactions of PDE11A and PDE8B with AHR or AIP have not been reported yet. In the anterior pituitary gland tissue, deregulated cAMP signalling may act as the initiating event for hyperplasia and/or adenoma development. Additional events leading to cell cycle disregulation and genomic instability could allow for the monoclonal expansion of a pituitary tumor (Boikos & Stratakis, 2007).

Overall, AIP directly associates with a number of interaction partners (Table 10) and all these interactions are mediated by its C-terminal TPR motifs. In general, AIP acts as a molecular chaperone, whereas it represses the transcriptional activity of the transcription factors it binds. Despite the variety of these interactions, little can be told currently regarding the mechanisms by which AIP leads to pituitary tumorigenesis; further studies are needed to address its role in normal pituitary cells, before conclusions can be drawn for its implications in the adenomatous pituitary.

Only recently has the expression pattern of AIP been somewhat elucidated in the normal versus the adenomatous pituitary tissue: In the normal pituitary, AIP co-localizes only with GH and PRL in the secretory vesicles of GH and PRL producing cells, but ACTH, TSH, and FSH/LH producing cells do not express AIP (Leontiou *et al*., 2008). However, in sporadic pituitary adenomas, AIP is expressed in all adenoma types studied (GH-, PRL-, ACTH-secreting, and FSH-positive NFPAs), but remains co-localized only with GH in the secretory vesicles of the GH-secreting adenomas, as in the normal pituitary; contrary, AIP remains in the cytoplasm of PRL- and ACTH-secreting adenomas, and NFPAs (Leontiou *et al*., 2008). To date, the mechanisms that induce the expression of AIP in non-GH and non-PRL-secreting adenomas remain unknown.

Table 10. AIP partners and the functions mediated through these interactions.

AIP interaction partners	AIP Function	Reference
HBV X protein	Suppression of the transcriptional activity of HBV X protein	Kuzhandaivelu *et al*., 1996
AHR – HSP90	• Cytoplasmic stabilization of AHR • Prevention of the nucleocytoplasmic shuttling of AHR • Increase of AHR ligand binding capacity • Increase of recognition of AHR by importin β • Protection of AHR from ubiquitination and targeting for proteosomal degradation	Ma & Whitlock, 1997; Carver & Bradfield, 1997; Carver *et al*., 1998
EBNA3	Undefined role	Kashuba *et al*., 2000
Tom20 and mitochondrial preproteins	Chaperone-like activity for the maintenance of unfolded and non-aggregated mitochondrial preproteins in the cytoplasm	Yano *et al*., 2003
PPARα – HSP90	Repression of PPARα-mediated transcription	Sumanasekera *et al*., 2003
PDE4A5	Attenutation of the activity of PDE4A5 and its ability to be phosphorylated by PKA	Bolger *et al*., 2003
Survivin	Regulation of surviving stability	Kang & Altieri, 2006
TRβ1	T3-independent TRβ1-mediated TRH transcription	Froidevaux *et al*., 2006
PDE2A	Targeting of PDE2A to the AHR complex and restriction of AHR nucleo-cytoplasmic motility	de Oliveira *et al*., 2007

CONCLUSIONS AND FUTURE PROSPECTS

The recent studies addressing the prevalence of clinically relevant pituitary adenomas indicate that these tumors occur more often than previously thought (Daly *et al.*, 2006a; Daly *et al*., 2007a). Thus, the need to comprehend the mechanisms underlying this type of tumorigenesis is important, in order to provide improved diagnosis and treatment. The contribution of genetics towards this end has been the localization and identification of four, so far, endocrine-related tumor predisposing genes, namely *MEN1*, *PRKAR1A*, *CDKN1B,* and *AIP,* in MEN1, CNC, MEN1-like (MEN4), and PAP, respectively.

This work describes the methodological approach that led to the elucidation of the genetic component underlying a form of pituitary adenoma predisposition (PAP) with incomplete penetrance, caused by dominantly inherited germline mutations in *AIP* (I). The conclusions drawn from this and the subsequent studies are summarized as follows:

I) The Finnish founder *AIP* mutation, Q14X, was originally identified in two affected families from Northern Finland, with multiple cases of pituitary adenomas of the GH/PRL-secreting cell lineage. Q14X was also detected among sporadic Finnish acromegaly patients: Two *AIP* mutations, Q14X and IVS3-1G>A, account for 16% of the Nordic population-based cohort, and for 40% of these patients diagnosed before the age of 35 years. Genetic data presented herein – i.e. inactivating germline mutations segregating among the affected individuals, and biallelic inactivation of *AIP* in the studied tumors – provide support to the notion that *AIP* is likely to act as a tumor suppressor gene.

II) Germline *AIP* mutations are found in populations of various ethnic origins; the mutation prevalence is low (0.8-7.4%), varying in different clinical settings. Overall, it emerges that *AIP* mutations are enriched among patients with very young age at onset and/or a positive family history of acromegaly. These patients are diagnosed mainly with GH-secreting adenomas (II). AIP IHC was tested as a pre-screening molecular tool and was found to be a useful predictor of PAP, with 75% sensitivity and 95% specificity for germline truncating *AIP* mutations. Further investigation is required before its clinical application, since little is known concerning normal and aberrant AIP localization in normal and adenomatous pituitaries, respectively.

III) The study of common tumor types, including a large collection of MSI and MSS colorectal, breast, and prostate tumors, did not reveal somatic mutations in *AIP*, suggesting that this gene is not involved in such tumorigenesis.

IV) The screening of pediatric pituitary adenoma patients revealed that *AIP* mutations once again underlie the early onset of GH-secreting adenomas, without an evident family history, but seem to be very rare in non-GH-secreting pediatric tumors. This finding has implications regarding genetic testing of very young acromegaly/gigantism patients and their families.

The lack of *AIP* mutations in a large number of familial pituitary adenoma cases analyzed worldwide to date, implies that other susceptibility genes await identification, a fact that

will expand the spectrum of genetic heterogeneity in familial pituitary adenomas. On the other hand, in sporadic patients without identified intragenic *AIP* mutations, it is theoretically possible that other mechanisms result in *AIP* inactivation; methylation of genes in pituitary adenomas is not an infrequent event (reviewed in Farrell *et al*., 1999; Farrell & Clayton, 2003). Epigenetic silencing through promoter hypermethylation is a mechanism estimated to be involved in the inactivation of more than 50% of TSG in sporadic tumors of various types (Baylin & Herman, 2000); it remains to be shown whether this applies to *AIP*.

One important question that remains to be answered, which may have direct implications in disease outcome and counseling, is the functional validation of the mutations that do not cause transcript degradation. This is a question that could be addressed either by classical cell-based studies or by the more laborious generation of genetically engineered animal models. For instance, hypomorphic *Aip* mice, with reduced Aip expression in the germline, exhibit hepatovascular defects and reduced liver weights, but implications caused by the hypomorphic allele in the pituitary were not reported (Lin *et al*., 2008). Overall, it will be challenging to robustly evaluate the effect of missense, small in-frame insertions and deletions, or in-frame whole-exon deletions in human pituitary tumor predisposition.

An issue of clinical relevance is whether *AIP* mutations have an impact on the patients' response to currently available therapy (Beckers and Daly, 2007; Daly *et al*., 2007a). Preliminary results show that *AIP* mutation-positive tumors are large and often invasive; therapeutic response is often poor, with infrequent surgical cure, significant resistance to somatostatin analogs, and frequent radiotherapy (Daly *et al*., 2008). Further work on the functional role of AIP will hopefully reveal novel cellular mechanisms of pituitary tumorigenesis, including potential drug targets. Other issues of clinical relevance to be addressed in the future will be the study of the phenotypic variation(s) in PAP, the possible existence of genotype-phenotype correlations, as well as the estimation of the disease penetrance. These issues will have direct implications in genetic counseling; yet, the overall benefits of predictive genetic testing should be assessed.

Despite the limitations, such as lack of functional human cell lines, appropriate animal models, and the critical location of the pituitary gland, significant progress has been made in understanding the genetic and molecular basis of pituitary tumorigenesis. Rodent pituitary cell lines are of course available, but it is not certain that they faithfully depict the human pituitary gene expression profile and biology. Among the primary future prospects of this project would be the elucidation of the role of AIP in pituitary tumorigenesis. One means of doing so will undoubtedly be the development of appropriate animal models. A knock-out mouse model has already been engineered (Lin *et al*., 2007). Despite the lack of viability, constitutional knockout mouse models could provide the material for the development of cellular models, such as embryonic fibroblast *Aip* knockout cell lines, in order to further experiment on. A conditional mouse model (i.e. pituitary-specific), similar to the pituitary and pancreas-specific *Men1* knockout model (Biondi *et al*., 2004) or the pituitary-specific *Prkar1a* knockout model (Yin *et al*., 2008), will hopefully facilitate future studies addressing the role of *AIP* in pituitary tumorigenesis.

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ELECTRONIC DATABASE INFORMATION

Human Genome	Ensembl Genome Browser: http://www.ensembl.org GeneCards: http://www.genecards.org NCBI: http://www.ncbi.nlm.nih.gov The Human Gene Mutation Database: http://www.hgmd.org The Human Genome Variation Society: http://www.hgvs.org/mutnomen/ UCSC: http://genome.ucsc.edu/cgi-bin/hgGateway Wellcome Trust Sanger Institute: http://www.sanger.ac.uk/genetics/CGP/Census
Primer Design	ExonPrimer: http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html Primer3: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
In silico analysis	Alternative Splice Site Predictor: http://www.es.embnet.org/~mwang/assp.html Berkeley Drosophila Genome Project: http://www.fruitfly.org/seq_tools/splice.html BioEdit: http://www.mbio.ncsu.edu/BioEdit/bioedit.html NetGen2: http://www.cbs.dtu.dk/services/NetGene2 Tandem Repeats Finder: http://tandem.bu.edu/trf/trf.html SpliceScan: http://bioinformatics.ist.unomaha.edu/~achurban/SpliceScan/ScoreAcceptor.html and http://bioinformatics.ist.unomaha.edu/~achurban/SpliceScan/ScoreDonor.html
Other references	GNF SymAtlas: http://symatlas.gnf.org/SymAtlas OMIM: http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM Orphanet: http://www.orpha.net

APPENDIX

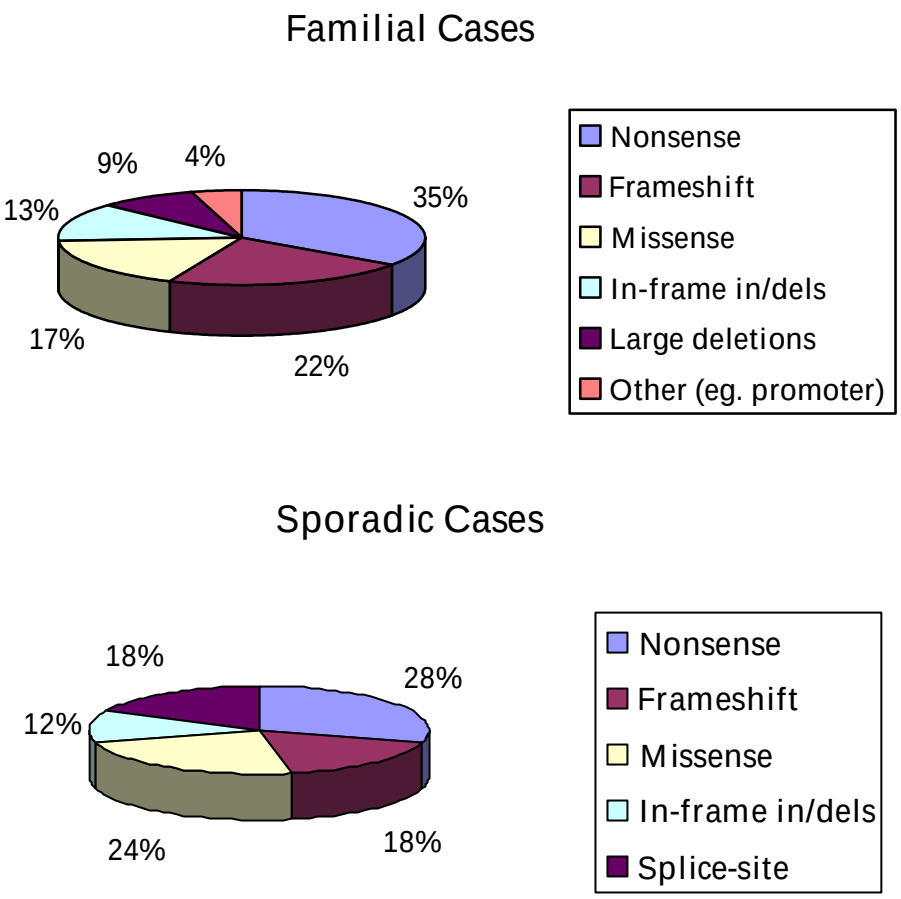

Figure 1. The distribution of different types of germline *AIP* mutations identified in familial and sporadic pituitary adenoma patients of European, American, and Asian origin, as reported in the literature between May 2006 and June 2008.

Table 1. Germline *AIP* mutations identified in pituitary adenoma patients of European, American, and Asian origin, as reported in the literature between May 2006 and June 2008.

Mutation (Protein level)	Mutation (Transcript level)	*AIP* Fragment	Patient origin*	Pituitary adenoma phenotype of *AIP* mutation-positive patients	Tumor analysis (second hit mutation)	Family history	Reference
Familial							
Nonsense							
Q14X	c.40C>T	Exon 1	Finland (11 cases)	GH- (4), GH/PRL- (2), PRL-secreting (3)	LOH		study I
			Finland (2 cases)	GH-secreting (2)	LOH		study I
E24X	c.70G>T	Exon 1	Brazil (7 cases)	GH- (6), GH/PRL-secreting (1)	LOH		Leontiou *et al*., 2008
R81X	c.241C>T	Exon 2	USA (2 cases)	GH/PRL-secreting (2)	LOH		Leontiou *et al*., 2008
			Brazil (2 cases)	GH-secreting (2)	LOH		Toledo *et al*., 2008
Q142X	c.424C>T	Exon 3	Italy (4 cases)	GH- (3), PRL-secreting (1)	NA		Daly *et al*., 2007b
Q217X	c.649C>T	Exon 5	Belgium (2 cases)	GH- (1), GH/PRL-secreting (1)	NA		Daly *et al*., 2007b
Q239X	c.715C>T	Exon 5	France (2 cases)	GH-secreting (2)	NA		Daly *et al*., 2007b
Y268X	c.804A>C	Exon 6	Brazil (3 cases)	GH-secreting (2), NFPA** (1)	NA		Toledo *et al*., 2007
R304X	c.910C>T	Exon 6	Italy (2 cases)	GH-secreting (2)	NA		study I
			Italy (3 cases)	GH- (2), GH/PRL-secreting (1)	NA		Daly *et al*., 2007b
			Romania (2 cases)	NA (↑ serum GH and PRL) (2)	NA		Leontiou *et al*., 2008
			UK (8 cases)	GH- (6), PRL- (1), GH/PRL-secreting (1)	NA		Leontiou *et al*., 2008
Frameshift							
P96fs	c.286-287delGT	Exon 3	Japan (3 cases)	GH- (2), GH/PRL-secreting (1)	LOH		Iwata *et al*., 2007
E174fs	c.517-521delGAAGA	Exon 4	Brazil (3 cases)	GH- (2), GH/PRL-secreting (1)	NA LOH equivocal		Daly *et al*., 2007b and Naves *et al*., 2007
L181fs	c.542delT	Exon 4	Spain (1 case)	GH-secreting	NA	Acromegaly	study II
Q285fs	c.854-857delAGGC	Exon 6	Italy (2 cases)	GH- (1), GH/PRL-secreting (1)	NA		Daly *et al*., 2007b
Q307fs	c.919insC	Exon 6	NA (1 case)	GH-secreting	NA	Prolactinoma	Beckers *et al*., 2008
In-frame deletions							
G23_E24	c.66-71delAGGAGA	Exon 1	Germany (1 case)	GH-secreting	LOH	Acromegaly	study II
G47_R54	c.138-161del24	Exon 2	Argentina (2 cases)	GH-secreting (2)	NA		Daly *et al*., 2007b
In-frame insertions							
A274_H275ins10	c.794-823dup30	Exon 6	UK (3 cases)	GH-secreting (3)	LOH		Leontiou *et al*., 2008
Large genomic deletions							
A34_K93	Ex2del (Δ1562bp)	Exon 2	UK (2 cases)	NFPA (1), GH-secreting (1)	NA	NFPA	Georgitsi *et al*., 2008
na	Ex1_2del (Δ5818kb)	Exons 1-2	Germany (2 cases)	GH-secreting (2)	NA		Georgitsi *et al*., 2008
Missense							
C238Y	c.713G>A	Exon 5	Mexico (3 cases)	GH-secreting (3)	LOH		Leontiou *et al*., 2008
K241E	c.721A>G	Exon 5	Belgium (2 cases)	PRL-secreting (1), NFPA (αLH/SU+) (1)	NA		Daly *et al*., 2007b
R271W		Exon 6	France (2 cases)	GH-secreting (2)	NA		Daly *et al*., 2007b
			France (2 cases)	GH- (1), PRL-secreting (1)	NA		Daly *et al*., 2007b

R304Q	c.911G>A	Exon 6	Romania (2 cases)	GH-secreting (2)	NA		Leontiou *et al*., 2008
			Italy (1 case)	GH-secreting	NA	Prolactinoma	Unpublished data
			Canada (2 cases)	GH-secreting (1)	NA		Vallette *et al*., (ENDO2007, oral communication)
Promoter							
na	na (c.-270-269CG>AA and c.-220G>A)	na	Japan (2 cases)	GH- (1), GH/PRL-secreting (1)	LOH		Leontiou *et al*., 2008
Sporadic							
Nonsense							
Q14X	c.40C>T	Exon 1	Finland (4 cases)	GH- (3), GH/PRL-secreting (1)	LOH		study I
			Finland (2 cases)	GH-secreting (2)	LOH		study II
			Finland (2 cases)	GH/PRL- (1), PRL-secreting (1)	LOH		Raitila *et al*., 2006
R22X	c. 64C>T	Exon 1	France	GH-secreting	LOH		Barlier *et al*., 2007
K201X	c. 601A>T	Exon 4	France (2 cases)	GH-secreting (2)	NA		Cazabat *et al*., 2007
K241X	c.721A>T	Exon 5	NA	PRL-secreting	NA		Beckers *et al*., 2008
R304X	c.910C>T	Exon 6	France	GH-secreting	NA		Cazabat *et al*., 2007
Frameshift							
P114fs	NA	Exon 3	NA	GH-secreting	NA		Beckers *et al*., 2008
H135Lfs	c.404delA	Exon 3	France	GH-secreting	NA		Cazabat *et al*., 2007
H275Qfs	c.824-825insA	Exon 6	USA	GH-secreting	LOH		study II
In-frame deletions							
Y248del	c.742-744delTAC	Exon 5	Italy	GH-secreting	LOH		study IV
L294_A297del	c.880-891del12	Exon 6	Germany	GH-secreting	LOH		study II
Splice-site bp substitution							
na	IVS2-1G>C	IVS2-Exon 3	USA	GH-secreting	NA		study II
na	IVS3-1G>A	IVS3-Exon 4	Finland	GH/PRL-secreting	LOH equivocal		study I
na	IVS3-2A>G	IVS3-Exon 4	France	GH/PRL-secreting	NA		Cazabat *et al*., 2007
Missense							
K103R	NA	Exon 3	NA	ACTH-secreting (Cushing's disease)	NA		Beckers *et al*., 2008
A299V	c.896C>T	Exon 6	The Netherlands	GH-secreting	NA		study II
E293G	c.878-879AG>GT	Exon 6	Germany	GH-secreting	LOH		study II
R304Q	c.911G>A	Exon 6	Poland	ACTH-secreting (Cushing's disease)	NA		study II
			France	GH-secreting	NA		Cazabat *et al*., 2007

NA, information not available; IVS, intron variable sequence (i.e. intron); na, not applicable

* In sporadic cases only one case with each mutation was identified, unless otherwise stated in parenthesis.

** Pituitary nodule, likely a NFPA microadenoma (3 mm)