THE ROLE OF AIP AND CDKN1B/p27Kip1 IN ENDOCRINE NEOPLASIA

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS ........................................................................... 5
ABBREVIATIONS ............................................................................................................ 6
ABSTRACT ........................................................................................................................ 8

1. INTRODUCTION ....................................................................................................... 11

2. REVIEW OF THE LITERATURE ............................................................................... 12
   2.1. Tumor genes .......................................................................................................... 12
       2.1.1. Tumor suppressor genes ................................................................................ 12
       2.1.2. Oncogenes ....................................................................................................... 13
   2.2. Endocrine system .................................................................................................. 13
       2.2.1. The pituitary gland ......................................................................................... 16
   2.3. Endocrine neoplasia ............................................................................................. 16
       2.3.1. Pituitary adenomas ...................................................................................... 17
           2.3.1.1. Classification of pituitary adenomas ..................................................... 18
   2.4. Disorders of pituitary adenomas ........................................................................ 20
       2.4.1. Sporadic pituitary adenomas ........................................................................ 20
       2.4.2. Familial pituitary adenomas ........................................................................ 21
           2.4.2.1 Multiple Endocrine Neoplasia type 1 (MEN1) ....................................... 21
           2.4.2.2. Carney Complex (CNC) .......................................................................... 22
           2.4.2.3. Pituitary Adenoma Predisposition (PAP) ............................................. 24
           2.4.2.4. Isolated Familial Somatotropinomas (IFS) ............................................ 25
           2.4.2.5. Familial Isolated Pituitary Adenomas (FIPA) ....................................... 25
           2.4.2.6. Multiple Endocrine Neoplasia type 4 (MEN4) ...................................... 26

3. AIMS OF THE STUDY ............................................................................................... 29

4. MATERIALS AND METHODS ................................................................................ 30
   4.1. Subjects .................................................................................................................. 30
       4.1.1. Pituitary adenoma patient samples (I, II, IV) ............................................... 30
       4.1.2. Sporadic endocrine tumors (II) ..................................................................... 31
       4.1.3. Familial thyroid cancer patient cohort (III) .................................................. 31
       4.1.4. Healthy control samples (I, II, III, IV) ........................................................... 31
   4.2. Analysis methods ................................................................................................. 31
       4.2.1. Direct sequencing (I, II, III, IV) ...................................................................... 31
       4.2.2. Immunohistochemistry (IHC) (I, III, IV) ....................................................... 32
       4.2.3. In silico analysis (I, III, IV) .............................................................................. 32

5. RESULTS ...................................................................................................................... 33
   5.1. Molecular analysis of PAP (I) ............................................................................. 33
   5.2. Somatic AIP mutation screening in sporadic endocrine neoplasia (II) .............. 34
   5.3. Screening of AIP in familial non-medullary thyroid cancer (NMTC) cases (III) ................................................................................................................................ 35
   5.4. The analysis of CDKN1B/p27kip1 mutations in endocrine neoplasia (IV) ...... 36
6. DISCUSSION .................................................................................................................. 37
6.1. The PAP phenotype ..................................................................................................... 37
  6.1.1. AIP mutation frequencies in diverse clinical settings (I) .................................... 37
  6.1.2. The IHC in identification of PAP (I) .................................................................. 39
6.2. Somatic AIP mutations are rare or non-existent in sporadic endocrine neoplasia (II) ............................................................. 40
6.3. AIP mutations seem not to be involved in familial non-medullary thyroid cancer (III) ................................................................. 41
6.4. AIP in tumorigenesis ............................................................................................... 41
6.5. The role of CDKN1B/p27kip1 in multiple endocrine neoplasia (IV) .................. 43

7. CONCLUSIONS AND FUTURE PROSPECTS .......................................................... 45

8. ACKNOWLEDGEMENTS ............................................................................................ 47

9. REFERENCES .............................................................................................................. 49
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by Roman numerals I-IV.


* Equal contribution

Publication I was included in the thesis of Marianthi Georgitsi (Genetic basis of pituitary adenoma predisposition, Helsinki 2008)

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ABBREVIATIONS

A
aad
ACTH
AIP
AHR
ALK
ARA9
ARNT
bp
BIRC5
BRCA1
BRCA2
C
CNC
cAMP
CDK4
CDKN1B/p27Kip1
DNA
EBNA3
FH
FIPA
FKBP
FSH
guanine
GADD45γ
growth arrest and DNA-damage-inducible gamma
GH
growth hormone
GNAS
guanine nucleotide-binding protein, alpha stimulating activity polypeptide
Gsα
guanosine triphosphate-binding protein
GTP
guanosine triphosphate
HIF1-α
hypoxia inducible factor 1, alpha subunit
HNPPC
hereditary non-polyposis colorectal cancer
HSP90
heat-shock protein 90
IFS
isolated familial somatotropinoma
IGF-I
insulin-like growth factor 1
IHC
immunohistochemistry
KIT
v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LH
luteinizing hormone
LOH
loss of heterozygocity
MAS
McCune-Albright syndrome
MEN1
multiple endocrine neoplasia type 1
MEN4
multiple endocrine neoplasia type 4
MET
met proto-oncogene (hepatocyte growth factor receptor)
MIM  Mendelian Inheritance in Man
MLH1  MutL *E.coli* homologue 1
MSH2  MutS *E.coli* homologue 2
MRI   magnetic resonance imaging
NFPA  non-functioning pituitary adenoma
NMTC  non-medullary thyroid cancer
PAP   pituitary adenoma predisposition
PCR   polymerase chain reaction
PDE2A phosphodiesterase 2 A
PDE4A5 phosphodiesterase 4 A5
PKA   protein kinase A
PPAR-α peroxisome proliferation-activated receptor alfa
PPNAD pigmented nodular adrenocortical disease
PRKAR1A protein kinase A (PKA) regulatory subunit 1 alfa
PRL   prolactin
PTC   parathyroid cancer
ptg-FGFR4 pituitary tumor-derived fibroblast growth factor receptor 4
RET   rearranged during transfection proto-oncogene
SNP   single nucleotide polymorphism
T     thymine
THRβ1 thyroid receptor beta 1
TOMM20 translocase of the outer membrane of mitochondria 20
TPR   tetratricopeptide repeat
TSH   thyrothrophin-releasing hormone
XAP2  hepatitis B virus x-associated protein 2 (or AIP, ARA9)

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

Identification of genes predisposing to tumor syndromes has raised general awareness of tumorigenesis. Genetic testing of tumor susceptibility genes aids the recognition of individuals at increased risk of tumors. Identification of novel predisposing genes enables further studies concerning the classification of potential associated tumors and the definition of target patient group.

Pituitary adenomas are common, benign neoplasms accounting for approximately 15% of all intracranial tumors. Accurate incidence estimation is challenging since a great portion of these adenomas are small and asymptomatic. Clinically relevant adenomas, that cause symptoms due to the expansion of the cell mass or the over-secretion of normally produced hormones, occur in approximately one of 1000 individuals. Although the majority of pituitary adenomas are sporadic, a minority occur as components of familial syndromes, such as Multiple Endocrine Neoplasia type 1 (MEN1) and Carney complex (CNC). MEN1 syndrome is caused by germ-line mutations in the \textit{MEN1} gene, whereas most of the CNC patients carry the mutated \textit{protein kinase A (PKA) regulatory subunit-1-a (PRKAR1A)} gene.

Recently, other conditions predisposing to endocrine tumors have been identified: Pituitary Adenoma Predisposition (PAP) and MEN type 4 (MEN4). PAP was originally identified in a genetically homogeneous Finnish population. In a population based cohort from Northern Finland, \textit{aryl hydrocarbon receptor-interacting protein (AIP)} gene mutations were found in 16% of all patients diagnosed with growth hormone (GH) producing pituitary adenoma, and in 40% of the subset of patients who were diagnosed under the age of 35 years.

Since \textit{AIP} mutations were originally described in a defined, homogeneous population from Northern Finland, it was relevant to study whether mutations also occur in more heterogeneous populations. In patient cohorts with different ethnic origins and variable clinical phenotypes, germ-line \textit{AIP} mutations were detectable at low frequencies (range 0.8-7.4%). \textit{AIP} mutation-positive patients were often diagnosed with a GH-producing adenoma at a young age, and usually had no family history of endocrine tumors. The low frequency of \textit{AIP} mutations in randomly selected patients, and the lack of any family history of pituitary adenomas create a challenge for the identification of PAP patients. Our preliminary study suggests that AIP immunohistochemistry may serve as a pre-screening tool to distinguish between the \textit{AIP} mutation-negative and the mutation-positive tumors.

Tumors of various endocrine glands are components of MEN1 and CNC syndromes. Somatic \textit{MEN1} and \textit{PRKAR1A} mutations in sporadic pituitary adenomas are rare, but occur in some of the other tumors related to these syndromes. The role of \textit{AIP} mutations in endocrine neoplasia was studied and
our results indicated that somatic AIP mutations are rare or non-existent in sporadic tumors of endocrine glands (0 of 111). Furthermore, germ-line AIP mutations in prolactin producing adenomas (2 of 9) confirmed the role of this pituitary tumor type in the PAP phenotype.

Thyroid disorders are common in the general population, and the majority of them are sporadic. Interestingly, it has been suggested that thyroid disorders might be more common in PAP families. For this reason we studied germ-line AIP mutations in 93 index cases from familial non-medullary thyroid cancer (NMTC) families. The underlying gene or genes for familial NMTC have not been identified yet. None of the patients had any potentially pathogenic AIP mutation. This suggests that AIP is unlikely to play a role in familial NMTCs.

A novel multiple endocrine syndrome was originally described in rats with phenotypic features of human MEN type 1 and 2. Germ-line mutations of cyclin-dependent kinase inhibitor 1B (CDKN1B also known as p27kip1) gene were reported later in these rats and a germ-line mutation was also identified in one human family with MEN1-like phenotype (later named MEN4). To confirm the importance of this gene’s mutations in humans, we performed a mutation screening in MEN-like patients and in patients with pituitary adenoma. Our results indicate that CDKN1B/p27kip1 mutations appear in a small portion of MEN1-like patients (one of 36), and that such mutations are rare or non-existent in both familial (0 of 19) and sporadic pituitary adenoma patients (0 of 50).

In conclusion, this work strengthens the tumor susceptibility role of AIP and CDKN1B/p27kip1 in endocrine neoplasia. Clarifying the PAP phenotype facilitates the identification of potential AIP mutation carriers. Genetic counseling can be offered to the relatives and follow-up of the mutation carriers can be organized, hence an earlier diagnosis is feasible.
1. INTRODUCTION

Tumors arise from normal tissue when a cell transforms into a malfunctioning one. The growth advantage of the cell is achieved by accumulation of genetic alterations. Variety of environmental and lifestyle factors can affect tumor formation due to their ability to mutate genes. These factors correlate with the incidence of certain cancers such as exposure to ultraviolet radiation with skin cancer, tobacco smoking with lung cancer, and inadequate diet with stomach cancer (Weinberg 2007).

An individual with an inherited mutation in a crucial gene is predisposed to tumor formation at a higher risk than the general population. However, a single mutated gene alone is seldom sufficient to trigger tumor formation but does contribute to it. For example, it has been estimated that 15 mutations are involved in the initiation, progression, and maintenance of colorectal and breast cancer (Wood et al. 2007). Approximately 1.5% of human genome carries the sequence information that encodes the structures of protein and occurrence of somatic mutations in human tissues is estimated to be less than $10^{-8}$ per base pair (Bielas et al. 2006; Weinberg 2007). Thus, sporadic tumor formation is a slow process. Mutation patterns vary between different tumor types but all of them display features uncommon to normal cells. These include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replication potential, sustained angiogenesis, and the capability of invasion and metastasis (Hanahan and Weinberg 2000).

Tumors can be divided into two categories according to their growth pattern. Benign tumors grow locally without invading adjacent tissues, whereas malignant tumors are able to invade and send metastases. The majority of the primary tumors are actually benign and thus cause no symptoms. In rare cases, however, the over-secretion of hormones and/or excessive tumor expansion causing pressure on the adjacent tissues, lead to clinical symptoms. For example growth hormone (GH) producing pituitary adenomas lead to acromegaly. Even though benign tumors only rarely cause deaths, those tumors can be clinically important.

This work focuses on endocrine neoplasia caused by mutations in two genes, *aryl hydrocarbon receptor-interacting protein* (AIP) and *cyclin-dependent kinase inhibitor 1B* (CDKN1B also known as *p27*Kip1). Both genes predispose to pituitary adenomas and the latter gene also to other endocrine tumors. The contribution of these genes to tumorigenesis in endocrine neoplasia is not completely clarified. This work aims to study the role of AIP and CDKN1B/p27*Kip1* in endocrine neoplasia.
2. REVIEW OF THE LITERATURE

2.1. Tumor genes

As mentioned above, the transformation from a normal cell to a tumor lesion requires several cellular events. These include mutations in the genome leading to loss of function of tumor suppressor genes or to gain of function of oncogenes.

2.1.1. Tumor suppressor genes

Tumor suppressor genes can be divided into three groups according to their role in cellular processes: gatekeepers, landscapers, and caretakers (Kinzler and Vogelstein 1997; Kinzler and Vogelstein 1998). Inhibitors of the cell cycle progression and promoters of the apoptosis of abnormal cells are called gatekeepers. Landscaper genes are involved in the cellular microenvironment (stromal cells). Caretaker genes repair DNA errors and therefore mutations in these genes leads to the accumulation of alterations in the genome (Kinzler and Vogelstein 1997; Kinzler and Vogelstein 1998).

Tumor suppressor genes are mainly inactivated when the activity of both alleles is lost (Knudson’s two hit hypothesis) (Knudson 1971). In heritable neoplastic syndromes the “first hit” is in the germ-line and the “second hit” occurs somatically to the wild-type allele. Often the inherited mutation is small, such as a point mutation or a small deletion/insertion, and the somatically occurring one is larger, such as loss of a particular chromosomal area. Loss of the tumor suppressor function may also be due to haploinsufficiency or dominant-negative effect when only a single allele inactivation is enough to promote tumorigenesis (Payne and Kemp 2005).

Predisposing mutations in tumor suppressor genes have been identified in several well known syndromes. These genes include BRCA1 and BRCA2 in hereditary breast and ovarian cancer (Futreal et al. 1994; Miki et al. 1994; Wooster et al. 1995), MEN1 in multiple endocrine neoplasia type 1 (MEN1) (Chandrasekharappa et al. 1997), FH mutations in hereditary leiomyomatosis and renal cell cancer (Tomlinson et al. 2002), and mismatch-repair genes (e.g. MLH1 and MSH2) in hereditary nonpolyposis colorectal cancer (HNPCC) (Bronner et al. 1994; Fishel et al. 1993; Leach et al. 1993; Papadopoulos et al. 1994).
2.1.2. Oncogenes

Oncogenes are abnormally activated normal genes, called proto-oncogenes. These normal genes are involved in several cellular processes, such as cell proliferation, differentiation, and apoptosis. Oncogene activation is achieved by activating point mutations in the proto-oncogene, chromosomal translocations, or gene amplifications. This leads to either increased expression of the normal protein or to aberrant activation of the gene in unfamiliar conditions. Activating point mutations and translocations are normally initiating events in the tumor formation or occur during tumor progression, whereas amplifications mainly occur later in tumorigenesis (Croce 2008).

Inherited oncogene mutations are rare but some have been identified such as RET in multiple endocrine neoplasia type 2 (Mulligan et al. 1993), MET in hereditary papillary renal cell carcinoma (Schmidt et al. 1997), KIT in familial gastrointestinal stromal tumors (Nishida et al. 1998), CDK4 in familial malignant melanoma (Zuo et al. 1996), and ALK in familial neuroblastoma (Mosse et al. 2008).

2.2. Endocrine system

Major endocrine glands consist of pituitary, pineal, thyroid, parathyroids, adrenals, endocrine pancreas, thymus, testes (males), and ovaries (female) (Fig. 1). These tissues release signaling molecules, known as hormones, mainly into the bloodstream to affect the functions of the target tissues such as growth, development, and metabolism. Through the bloodstream the hormones can be circulated to every part of the body. This is called endocrine signaling. Some hormones are secreted straight from the neural cells to the endocrine gland (neuroendocrine signaling), for example the hypothalamus secretes hormones that regulate the function of the anterior pituitary gland. The target of hormones can also be the cells nearby (paracrine signaling), the cell’s own receptors in the outer membrane (autocrine signaling) or the cell’s own receptors in the nucleus (intracrine signaling) (Valimaki et al. 2009).
Cooperation of the endocrine systems is composed of feedback mechanisms in both hormonal and neural communication networks. Positive and negative feedback control the hormone secretion. For instance, releasing and inhibiting factors secreted by the hypothalamus affect the function of the pituitary gland (Fig. 2). The pituitary hormones induce the hormone secretion of target organs and tissues. These peripheral hormones inhibit the hormone secretion of the hypothalamus and the pituitary gland. Also, para- and autocrine signaling are involved in the feedback system (Fig. 2) (Valimaki et al. 2009).
Figure 2. Diagram of the feedback control in the hypothalamic-pituitary-target tissue axis. Secretion of the pituitary hormone-releasing hormone induces the target tissue-stimulating hormone secretion from the pituitary gland. Pituitary hormones stimulate the synthesis and release of the hormones from the target tissue. Inhibition of the hormone secretion is achieved by negative feedback from the target tissues to the pituitary gland and the hypothalamus. Also autocrine and paracrine signaling affect the function of the pituitary gland and the hypothalamus. Stimulation is indicated by a plus sign, whereas inhibition is by a minus sign. The direction of the feedback is indicated by arrows. (Valimaki et al. 2009)

In this study, pituitary adenomas relate to both of the studied genes (AIP and CDKN1B/p27Kip1) and therefore pituitary gland (section 2.2.1.) and its adenomas (section 2.3.1.) are introduced in more details.
2.2.1. The pituitary gland

The pituitary gland is located behind the eyes, at the base of the brain, and on top of the sphenoid bone (Fig. 1). The gland is a bean-shaped tissue and it is divided into the anterior and the posterior lobes (Fig. 3). Hypothalamic antidiuretic hormone (arginine vasopressin) and oxytocin are stored and secreted to the blood-stream from the posterior lobe when needed. Six different pituitary hormones are produced and released from the anterior lobe (Fig. 3, Table 1). These pituitary hormones regulate growth and development in general and as well as the function of three other endocrine glands: the thyroid, the adrenals, and the gonads (Valimaki et al. 2009).

Figure 3. A schematic presentation of the pituitary gland, the secreted hormones, and the target tissues of the hormones.

2.3. Endocrine neoplasia

Neoplasias of the endocrine system are normally benign. Even thought malignancies are rare, thyroid cancers are the most common type, accounting for 1% of all cancers in the developed countries (Steward and Kleihues 2003). Tumors are prevalent in some endocrine tissues such as in the pituitary and thyroid glands. However, adrenal and pancreatic endocrine tumors are rare (DeLellis et al. 2004). Most of the endocrine tumors are sporadic but some arise as
components of familial syndromes such as multiple endocrine neoplasias, and Carney complex (introduced in section 2.4. below).

Clinical symptoms can vary in different endocrine disorders according to hormone activity. Decreased or lack of hormone synthesis can be due to a missing endocrine gland, dysfunction of hormone synthesis or destruction of the endocrine gland e.g. after infection or radiation. A defective response to hormones can be due to the incorrect structure of the hormone or malfunction of the receptor. Increased hormone synthesis is a consequence of a hyperplasia, a tumor or extra stimulation of the endocrine gland. (Valimaki et al. 2009)

Table 1. The pituitary hormones secreted by the anterior lobe. The target tissues and functions are also presented (Valimaki et al. 2009).

<table>
<thead>
<tr>
<th>Pituitary hormone</th>
<th>Target tissue</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolactin (PRL)</td>
<td>Mammary glands</td>
<td>Initiation and maintenance of milk production</td>
</tr>
<tr>
<td>Growth hormone (GH)</td>
<td>Liver and other tissues</td>
<td>Metabolic and anabolic effects, growth stimulation, and induction of particularly hepatic IGF-I production</td>
</tr>
<tr>
<td>Adrenocorticotropic hormone (ACTH)</td>
<td>Adrenal glands (cortex)</td>
<td>Stimulation and maintenance of steroid synthesis</td>
</tr>
<tr>
<td>Thyroid stimulating hormone (TSH)</td>
<td>Thyroid gland</td>
<td>Stimulation of thyroid hormone (T3 and T4) production</td>
</tr>
<tr>
<td>Follicle stimulating hormone (FSH) and luteinizing hormone (LH)</td>
<td>Gonads (ovaries and testis)</td>
<td>Germ cell development and stimulation of sex steroid hormone production</td>
</tr>
</tbody>
</table>

2.3.1. Pituitary adenomas

Tumors, mostly benign, arise mainly from the anterior lobe of the gland. These adenomas are common accounting for ~15% of all intracranial tumors (Karhu and Aaltonen 2007). Hormonally inactive, small tumors with a slow growth rate do not necessarily cause symptoms, and therefore those are not detected until radiographic examination or post-mortem examination. In autopsy studies and in radiological imaging done for other indications, the prevalence of incidentally discovered pituitary adenomas is estimated to be 5-20% (Burrow et al. 1981; Hall et al. 1994; Molitch and Russell 1990). This was supported recently by a meta-analysis where pituitary adenomas were reported to occur with a frequency of 16.7% (Ezzat et al. 2004).

The majority of the clinically relevant adenomas produce hormones excessively, which can affect many organ systems, thus causing severe symptoms or even
death. In addition to the over-secretion of hormones, the expansion of the tumor mass causes symptoms, such as headache, and visual-field disturbance. Furthermore, tumor compression of the normal pituitary tissue can lead to loss of normal hormone production i.e. hypopituitarism (Arafah and Nasrallah 2001). These clinically relevant adenomas are rare, but more common than previously estimated, as indicated by recent cross-sectional study conducted in Belgium (1/1064 population) and an international study (average 0.75/1000 population) (Daly et al. 2006b; Daly et al. 2007a).

Pituitary adenomas appear to have reversible plasticity, varying from hypoplasia to hyperplasia (Melmed 2003). X-chromosome inactivation in human pituitary adenomas suggests that these lesions arise from a single pituitary cell that has acquired growth advantage due to genetic or epigenetic alterations (Alexander et al. 1990; Herman et al. 1990). According to animal models, long-term pituitary hyperplasia predisposes to tumor progression (Asa et al. 1992; Heaney et al. 1999). In humans, however, pituitary hyperplasia due to pregnancy or lactation, its enlargement due to estrogen administration, or untreated primary hypothyroidism does not seem to enhance tumor formation (Coogan et al. 1995; Ghannam et al. 1999; Horvath et al. 1999; Kovacs et al. 1994).

2.3.1.1. Classification of pituitary adenomas

The World Health Organization’s classification of pituitary adenomas is based on clinical and biochemical features. These include imaging, operative findings, histology, immunocytochemistry, and electron microscopy (Kovacs et al. 1996). Adenomas can also be classified according to the size of the tumor where microadenomas are equal or less than 10 mm in diameter, macroadenomas greater than 10 mm but less than 4 cm, and tumors over 4 cm in diameter are giant adenomas (DeLellis et al. 2004). Classification can also be based on the endocrine activity as detailed in the following paragraphs and summarized in Table 2.

The majority of the pituitary adenomas over-secrete prolactin (PRL; 40-45%; Table 2) (Arafah and Nasrallah 2001). Prevalence of these adenomas, also called prolactinomas, is estimated to be 60-100 cases per million and their incidence is around 6-10 new cases per million per year (Ciccarelli et al. 2005; DeLellis et al. 2004). Besides symptoms due to the expansion of the tumor mass and variable degrees of hypopituitarism, over-secretion of PRL can lead to menstrual irregularities (amenorrhea or oligomenorrhea) or lactation (galactorrhea) in females, and to sexual impotence or decreased libido in males (DeLellis et al. 2004).

The second largest group of pituitary adenomas over-secrete growth hormone (GH; 20%; Table 2) (Arafah and Nasrallah 2001). These adenomas, also called somatotropinomas, can simultaneously over-secrete PRL. The clinical
manifestation depends on the age of occurrence of the adenoma and the time of exposure. GH over-secretion during childhood or adolescence, before epiphyseal fusion is completed, leads to accelerated linear growth termed “gigantism” (Eugster and Pescovitz 1999). Adulthood exposure to high GH-levels causes acromegaly with clinical features such as coarse facial features, broadened nose, enlarged extremities, obesity, organomegaly, sweating, and nausea (Chanson and Salenave 2008; Melmed 2006). Often adenomas causing acromegaly are large and therefore headaches and visual disturbances are common symptoms (Laws et al. 1985). Incidence of acromegaly is estimated to be 3-4 new cases per million per year and the prevalence 40-60 cases per million people (Alexander et al. 1980; Bengtsson et al. 1988; Kauppinen-Makelin et al. 2005). Morbidity of acromegaly depends on the exposure time of high GH and IGF-I levels (GH induces IGF-I production which controls linear and organ growth). Furthermore, untreated patients face an increased mortality risk caused by e.g. diabetes, cardiovascular or cerebrovascular disease (Colao et al. 2004; Erfurth and Hagmar 2005). Due to the slow progression and insidious onset of this disease, the diagnosis may take from four to more than ten years (Chanson and Salenave 2008).

Adrenocorticotrophin (ACTH) producing adenomas, also known as adenocorticotropinomas, account for 10-12% of all pituitary adenomas (Table 2) (Arafah and Nasrallah 2001). These tumors are mostly benign, but they show more invasive features than other pituitary adenomas (Arafah and Nasrallah 2001). ACTH over-secretion causes increased glucocorticoid production i.e. hypercortisolism with clinical features of central obesity, hypertension, proximal myopathy, striae, hirsutism, easy bruisability, mood changes, poor wound healing, menstrual irregularity, osteoporosis, hyperglycemia, increased supraclavicular, and dorso-cervical fat pads (Arafah and Nasrallah 2001). This clinical manifestation caused by the excess pituitary secretion of ATCH is called Cushing’s disease.

Thyrotropin (TSH) producing adenomas, i.e. thyrotropinomas, are rare among pituitary adenomas (1-2%; Table 2) (Arafah and Nasrallah 2001). Diagnosis is often delayed and tumors tend to be macroadenomas with invasive and aggressive nature (DeLellis et al. 2004). Patients with these adenomas often present a goiter and hyperthyroidism but co-secretion of GH and/or PRL can occur in a minority of cases leading to acromegaly and/or amenorrhea/galactorrhea (Arafah and Nasrallah 2001; Beck-Peccoz et al. 1996; Beckers et al. 1991; Sanno et al. 2000).
Table 2. Classification of pituitary adenomas according to their hormone activity. (modified from Arafah and Nasrallah 2001; DeLellis et al. 2004)

<table>
<thead>
<tr>
<th>Name</th>
<th>Prevalence</th>
<th>Clinical signs and symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolactinomas (PRL-producing)</td>
<td>40-45%</td>
<td>Reproductive and sexual dysfunction</td>
</tr>
<tr>
<td>Somatotropinomas (GH-producing)</td>
<td>20%</td>
<td>Pre-pubertal: unrestrained somatic growth <em>i.e.</em> gigantism</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Post-pubertal: enlargement of acral parts of body <em>i.e.</em> acromegaly</td>
</tr>
<tr>
<td>Adenocorticotropinomas (ACTH-producing)</td>
<td>10-12%</td>
<td>Cushing’s disease</td>
</tr>
<tr>
<td>Gonadotropinomas (NFPA)</td>
<td>10-15%</td>
<td>Mass effects causing headaches, visual disturbance, and hypopituitarism</td>
</tr>
<tr>
<td>Null-sell adenomas (NFPA)</td>
<td>5-10%</td>
<td>Mass effects (see gonadotropinomas)</td>
</tr>
<tr>
<td>Thyrotropinomas (TSH-producing)</td>
<td>1-2%</td>
<td>Goiter and mild hyperthyroidism</td>
</tr>
</tbody>
</table>

Endocrinically silent adenomas are non-functioning pituitary adenomas (NFPA) including both gonadotropinomas and null-cell adenomas (Table 2). The majority of NFPAs, accounting for 10-15% of all adenomas, are gonadotropinomas producing follicle-stimulating hormone (FSH) and/or luteinizing hormone (LH) (Arafah and Nasrallah 2001). However, the hormone secretion is mainly minimal or inefficient and therefore clinical behavior is due to the tumor mass expansion (Samuels and Ridgway 1995; Snyder 1995). Null-cell adenomas are truly endocrinically non-functioning and those account for 5-10% of all pituitary adenomas (Table 2) (Arafah and Nasrallah 2001). Null-cell adenomas grow slowly and their diagnosis is often made in the 6th decade of life (Kontogeorgos et al. 1993; Yamada et al. 1988). Because of the late diagnosis, these adenomas are often macroadenomas with cavernous sinus invasion and suprasellar extension occasionally reaching to the hypothalamus (DeLellis et al. 2004).

2.4. Disorders of pituitary adenomas

2.4.1. Sporadic pituitary adenomas

The majority of the pituitary adenomas (~95%) are sporadic tumors. Microarray studies have shown that the gene expression patterns vary, not only between pituitary adenomas and normal pituitary, but also between different pituitary adenoma subtypes (Fernandez-Ranvier et al. 2008; Moreno et al. 2005; Morris et al. 2004).
These studies, among other approaches, have indicated potential factors in pituitary tumorigenesis such as pituitary tumor-derived fibroblast growth factor receptor 4 (ptg-FGFR4) (Ezzat et al. 2002), growth arrest and DNA damage-inducible gamma (GADD45γ) (Zhang et al. 2002), and guanine nucleotide-binding protein, alpha stimulating activity polypeptide (GNAS) (Vallar et al. 1987). However, in many cases it is still unclear which of these alterations (reviewed in Asa and Ezzat 2005) are involved in the initiation of sporadic pituitary adenoma formation, which of them promote the progression of the tumor growth or which are bystanders.

GNAS encodes guanosine triphosphate (GTP)-binding protein Gsα. This protein activates adenylyl cyclase that increases the cellular levels of cyclic adenosine monophosphate (cAMP). In sporadic GH-producing adenomas activating mutations of GNAS, also known as gsp oncogene, cause constitutive activation of Gsα, overactivity of adenylyl cyclase, and increased cAMP levels (Vallar et al. 1987). Approximately 40% of human GH-producing adenomas carry activating mutations in GNAS gene (Lyons et al. 1990). Furthermore, the expression of Gsα can be high in the GH-producing adenomas without these mutations (Picard et al. 2007).

McCune-Albright syndrome (MAS; MIM 174800) is a genetic, but not an inherited, congenital syndrome due to a post-zygotic somatic mutation in the GNAS gene (Schwindinger et al. 1992; Weinstein et al. 1991). These mutations lead to over-secretion of hormones in endocrine cells and also to excessive bone matrix formation. MAS is characterized by polyostotic fibrous dysplasia, café-au-lait spots, endocrine abnormalities such as excess of GH and PRL, and in rare cases by other tumors.

2.4.2. Familial pituitary adenomas

Approximately 5% of pituitary adenomas arise as components of familial syndromes. These include Multiple Endocrine Neoplasia type 1 (MEN1), Carney’s Complex (CNC), and recently identified conditions: Pituitary Adenoma Predisposition (PAP) and Multiple Endocrine Neoplasia type 4 (MEN4). These familial syndromes and other conditions involving pituitary adenomas are described below and summarized in Table 3.

2.4.2.1 Multiple Endocrine Neoplasia type 1 (MEN1)

MEN1 (MIM131100; Table 3) is an autosomal dominant syndrome characterized by a predisposition to different combinations of tumors of the endocrine system. These tumors occur most commonly in parathyroids (~95%), pancreatic islet cells (~40%), and the anterior pituitary (~30%) (Lemos and Thakker 2008). Less common tumors include adrenocortical tumors, angiomylipomas, foregut lipomas, angiofibromas, thyroid adenomas, carcinoids, and spinal cord

21
ependymomas (Chandrasekharappa et al. 1997; Thakker 1998). MEN1 is inherited with a high degree of penetrance; <95% of the patients over 40 year of age develop clinical manifestations of the syndrome (Bassett et al. 1998). Clinical symptoms are mainly due to hormone over-secretion, and without treatments MEN1 tumors are mortal (Lemos and Thakker 2008). Furthermore, relatives of MEN1 patients are at 50% risk of developing the disease (Benson et al. 1987; Calender et al. 1995; Marx et al. 1998; Trump et al. 1996).

The predisposing gene for MEN1 locates at 11q13 and codes for a protein called “menin” (Chandrasekharappa et al. 1997; Larsson et al. 1988). MEN1 gene is comprised of ten exons, and encodes ten transcripts resulting in 555 aa to 615 aa long proteins (Ensembl 54). This protein is mainly nuclear and involved in transcriptional regulation, genome stability, cell division, and cell cycle control (reviewed in Lemos and Thakker 2008). In sporadic pituitary adenomas MEN1 is rarely somatically mutated (e.g. Schmidt et al. 1999; Wenbin et al. 1999; Zhuang et al. 1997a) and also menin expression is rarely altered (e.g. McCabe et al. 1999; Theodoropoulou et al. 2004; Wrocklage et al. 2002). However, somatic MEN1 mutations are found in other lesions related to this syndrome such as in pancreatic islet cell tumors (e.g. insulinomas ~15% and glucagonomas ~60%) (Hessman et al. 1998; Shan et al. 1998; Zhuang et al. 1997b), lipomas (~30%) (Pannett and Thakker 2001; Vortmeyer et al. 1998), and parathyroid tumors (~10-20%) (e.g. Carling et al. 1998; Farnebo et al. 1998; Heppner et al. 1997).

It has been estimated that 5-10% of MEN1 patients do not carry MEN1 mutations in the coding region or adjacent splice sites (Lemos and Thakker 2008). These patients, however, might carry predisposing mutations in untranslated regions, in the promoter area, or in introns. It is also possible that they may present phenocopies of the disorder or occasionally belong to the multiple endocrine neoplasia type 4 (MEN4) (described in section 2.4.2.6.).

2.4.2.2. Carney Complex (CNC)

CNC (MIM160980; Table 3) is a rare, autosomal dominant, multiple endocrine neoplasia syndrome with spotty skin pigmentation, myxomatosis, schwannomas, and endocrine tumors (Carney et al. 1985). Endocrine tumors include e.g. testicular neoplasm (33% of male patients), pigmented nodular adrenocortical disease (PPNAD) (26% of patients), GH- or PRL-producing pituitary adenomas (~10%), and thyroid adenoma or carcinoma (5%) (Stratakis et al. 2001). The manifestation of CNC can be diverse and occur in different ages. For instance, PPNAD or cardiac myxomas are normally seen in the second or third decade of life but also already in the first couple of years of life (Boikos and Stratakis 2007a). Over half of the disease-specific deaths among CNC patients are due to cardiac myxomas (Boikos and Stratakis 2007a).
More than 60% of the CNC patients that meet diagnostic criteria have mutations in protein kinase A (PKA) regulatory subunit-1-α (PRKAR1A) gene (Kirschner et al. 2000). This gene locates at 17q24 and comprises of 11 exons and three possible transcripts each encoding for identical 381 aa long protein (Ensembl 54). PKA, also known as cAMP-dependent kinase, is involved in transcription, metabolism, cell cycle progression, and apoptosis. Loss of PRKAR1A function leads to enhanced intracellular signaling by PKA, thus causing elevated cAMP levels in CNC tumors (Kirschner et al. 2000). Somatic PRKAR1A mutations are infrequent in sporadic pituitary adenomas (Kaltsas et al. 2002; Sandrini et al. 2002a; Yamasaki et al. 2003) and in cardiac myxomas (Fogt et al. 2002; Mantovani et al. 2009). However, somatic mutations are detected in sporadic PPNAD (three out of five) (Groussin et al. 2002), adrenocortical tumors (3;44) (Bertherat et al. 2003), thyroid carcinomas (2;17) (Sandrini et al. 2002b), and odontogenic myxomas (2;21) (Perdigao et al. 2005).

Apart from the 17q14 locus, linkage to 2p16 has also been reported but a predisposing gene or genes have not been identified (Stratakis et al. 1996). Not all the CNC families map to predisposed areas and it is possible that a third so far unidentified region exist.

Table 3. Familial pituitary adenoma syndromes.

<table>
<thead>
<tr>
<th>Clinical condition</th>
<th>Tissues most often affected by endocrine tumors</th>
<th>Chromosomal locus</th>
<th>Predisposing gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple Endocrine Neoplasia type 1</td>
<td>parathyroid glands, pancreas, pituitary gland</td>
<td>11q13</td>
<td>MEN1</td>
</tr>
<tr>
<td>Carney Complex</td>
<td>adrenal glands, testicles, pituitary gland, thyroid glands</td>
<td>17q23-4 2p16 2p16 other locus?</td>
<td>PRKAR1A unidentified unidentified</td>
</tr>
<tr>
<td>Pituitary Adenoma Predisposition</td>
<td>pituitary gland</td>
<td>11q13</td>
<td>AIP</td>
</tr>
<tr>
<td>Isolated Familial Somatotropinomas</td>
<td>pituitary gland (^a)</td>
<td>11q13 11q13 other locus?</td>
<td>AIP unidentified</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>unidentified</td>
</tr>
<tr>
<td>Familial Isolated Pituitary Adenomas</td>
<td>pituitary gland</td>
<td>11q13</td>
<td>AIP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>unidentified</td>
</tr>
<tr>
<td>Multiple Endocrine Neoplasia type 4</td>
<td>parathyroid glands, pituitary gland, kidney (^b)</td>
<td>12p13</td>
<td>CDKN1B/p27Kip1</td>
</tr>
</tbody>
</table>

\(^a\) Only GH-producing adenomas.

\(^b\) One family reported.
2.4.2.3. Pituitary Adenoma Predisposition (PAP)

PAP is a recently characterized condition with familial pituitary adenomas (MIM102200; Table 3) (Vierimaa et al. 2006). The occurrence of PAP was suspected when three small clusters of familial pituitary adenomas were detected in Northern Finland. Two of these families were related through common ancestor couple born in the 1700s, which was found by additional genealogical studies (Vierimaa et al. 2006). The putative predisposing locus at 11q12-13 was identified using whole-genome single nucleotide polymorphism (SNP) genotyping and narrowed down with fine-mapping (Vierimaa et al. 2006). Previous studies had already suggested that a gene other than MEN1 in 11q13 would predispose to GH-producing pituitary adenomas as allelic loss of the region had indicated (Gadelha et al. 1999; Soares et al. 2005; Thakker et al. 1993; Yamada et al. 1997).

With the combined information from linkage data and whole transcriptome expression data the primary candidate predisposing gene *aryl hydrocarbon receptor-interacting protein* (*AIP*) was defined (Vierimaa et al. 2006). And indeed, pituitary adenoma clusters detected from Northern Finland harbored a nonsense germ-line mutation c.40C>T (p.Gln14X). This mutation segregated perfectly with GH- and GH/PRL-producing adenomas. *AIP* mutation screening was also performed for 45 acromegaly patients belonging to a population based cohort from Northern Finland (Kauppinen-Makelin et al. 2005), and six c.40C>T and one c.469-1G>A mutations were detected (Vierimaa et al. 2006). Thus, *AIP* mutations accounted for 16% (7 out of 45) of all acromegaly patients, and 40% of those diagnosed under the age of 35 years (6/15). *AIP* as a novel pituitary adenoma predisposing gene was further confirmed with the detection of a nonsense mutation c.910C>T (p.Arg304X) in two Italian acromegalic siblings (Vierimaa et al. 2006).

Loss of the wild-type allele was seen in all studied GH-, PRL, and GH/PRL-producing tumors from *AIP* mutation carriers (Vierimaa et al. 2006). Authors concluded that the mutations predispose to at least these tumor types, that the gene is likely a tumor suppressor, and that the penetrance of PAP appears to be low. The tumor suppressor role of *AIP* was further confirmed with functional studies (Heliovaara et al. 2009; Leontiou et al. 2008). In general, a typical PAP patient (*i.e.* *AIP* mutation carrier) is diagnosed at a young age and the family history of pituitary adenomas might not be convincing (Vierimaa et al. 2006).

*AIP* gene, also known as *XAP2* or *ARA9*, locates at 11q13 and the six exons code for a 330 aa long protein (Ensembl 54). An FKBP-homology domain is located in the amino-terminus of *AIP* and three tetratricopeptide repeats (TPR), which mediate protein-protein interactions, in the carboxy-terminal region. *AIP* is known to form a complex with aryl hydrocarbon receptor (AHR), with two 90
kDa heat-shock proteins (HSP90), and with p23 (Carver and Bradfield 1997; Chen and Perdew 1994; Kazlauskas et al. 1999). Interactions have been shown at least with PDE4A5, PDE2A, PPAR-α, BIRC5, THRβ1, TOMM20, EBNA3, and RET (Bolger et al. 2003; de Oliveira et al. 2007; Froidevaux et al. 2006; Kang and Altieri 2006; Kashuba et al. 2000; Sumanasekera et al. 2003; Vargiolu et al. 2009; Yano et al. 2003). Many of these proteins can be linked to pituitary tumorigenesis such as AHR that is involved in xenobiotic-induced metabolism and in the absence of xenobiotics in e.g. cell proliferation, cell adhesion, and migration (Barouki et al. 2007). Interestingly, a recent study showed significantly reduced immunoreactivity for AHR translocator (ARNT) and somewhat increased localization of AHR in the nucleus in the AIP mutation-positive adenomas compared to the mutation-negative ones (Heliovaara et al. 2009). This indicated that these molecules are potentially involved in tumorigenesis of AIP mutation-positive pituitary tumors.

Many interaction partners of AIP are known but the mechanism between how the loss of AIP tumor suppressor gene leads to the pituitary tumorigenesis remains to be elucidated. Hopefully the ongoing functional studies, both in vitro and in vivo, will clarify this issue.

2.4.2.4. Isolated Familial Somatotropinomas (IFS)

IFS (Table 3) is defined as follows: at least two individuals of acromegaly or gigantism in a familial setting in the absence of MEN1 and CNC (Gadelha et al. 1999). These patients are often diagnosed with macroadenomas at a young age (Soares and Frohman 2004). Efforts to identify the predisposing locus have shown loss of heterozygocity (LOH) in chromosome 11q13 but MEN1, locating in this area, was not mutated (Gadelha et al. 1999; Tanaka et al. 1998; Teh et al. 1998; Yamada et al. 1997). At the same locus Vierimaa et al. (2006) reported truncating mutations in AIP in three families with at least two cases of pituitary adenomas. Two of these families harbored only GH-producing adenomas e.i. IFS. Later on, the following mutation screening studies have identified additional IFS cases with AIP mutations (e.g. Daly et al. 2007b; Iwata et al. 2007; Leontiou et al. 2008; Toledo et al. 2007).

2.4.2.5. Familial Isolated Pituitary Adenomas (FIPA)

Compared to IFS, FIPA is a wider clinical entity with familial cases of pituitary adenomas not restricted only to GH-producing adenomas (Table 3). These families can be divided into subcategories according to tumor phenotype between the family members. Homogeneous are those families where only one tumor type exists and heterogeneous those with varying tumor types (Daly et al. 2006a). All subtypes of pituitary adenomas have been identified in heterogeneous kindreds but often at least one GH-producing or PRL-producing adenoma is present (Beckers and Daly 2007). The majority of the pituitary adenomas
(approximately 75%) in the FIPA cohort are PRL-producing, GH-producing, or GH/PRL-producing adenomas (Beckers and Daly 2007). Moreover, FIPA patients are often diagnosed at a younger age and with larger pituitary adenomas compared to corresponding sporadic pituitary counterparts (Beckers and Daly 2007).

Pituitary adenomas in the FIPA families were reported to be significantly larger in the AIP mutation-positive cases when compared to the AIP mutation-negative ones (Daly et al. 2007b). Authors concluded that the pituitary adenomas in the AIP mutation-positive patients were of an aggressive type. Indeed, preliminary results have indicated a poor response to therapy in the AIP mutation-positive patients (Daly et al. 2008; Leontiou et al. 2008). However, the majority of the FIPA patients do not harbor AIP mutations and therefore the underlying genetic component(s) of FIPA remains to be elucidated.

2.4.2.6. Multiple Endocrine Neoplasia type 4 (MEN4)

MEN4 (MIM610755; Table 3) is an autosomal recessive condition with MEN1-related tumors. Identification of this novel syndrome was based on a naturally occurring rat strain with features overlapping with both human MEN type 1 and type 2 (Fritz et al. 2002). These included bilateral pheochromocytomas, parathyroid adenomas, medullary thyroid cell neoplasia, parathyroid hyperplasia, pituitary adenomas, and endocrine pancreas hyperplasia (Fritz et al. 2002; Pellegata et al. 2006). A recent study identified a homozygous germ-line mutation in Cdkn1b/p27<sup>Kip1</sup> gene in these rats (Pellegata et al. 2006). In the same study, a germ-line mutation in humans was reported in a family with two individuals diagnosed with MEN1-related tumors: the proband was diagnosed with primary hyperparathyroidism and pituitary adenoma and her sister with renal angiomyolipoma. Although the wild-type allele was retained in the tumor tissue, immunohistochemical staining of the CDKN1B/p27<sup>Kip1</sup> gene protein product, called p27, showed no protein in the tumor tissue (Pellegata et al. 2006).

The CDKN1B/p27<sup>Kip1</sup> gene at 12q13, has three protein coding transcripts containing two (205 aa) or three (104 aa and 198 aa) exons (Ensembl 54). Nuclear p27 negatively regulate the cell cycle by inhibiting cyclin and cyclin-dependent kinase complexes. Previous knockout mouse models had already indicated that this gene is involved in multiple endocrine neoplasia (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). Despite the efforts to screen human pituitary adenomas, neither pathogenic mutations nor loss of heterozygocity was found (e.g. Ikeda et al. 1997; Tanaka et al. 1997). However, lowered or even absent expression of p27 has been reported in most human pituitary adenomas (Bamberger et al. 1999; Komatsubara et al. 2001; Lidhar et al. 1999). Interestingly, the regulation of the expression of p27 includes both menin (Karnik et al. 2005; Milne et al. 2005; Scacheri et al. 2006) as well as AIP through AHR (Kolluri et al. 1999).
Additional \textit{CDKN1B/p27^{kip1}} mutations in humans would strengthen the role of this gene in endocrine neoplasia.
3. AIMS OF THE STUDY

I  Clarify the clinical and molecular identification of PAP patients

II  Analyze somatic \textit{AIP} mutations in sporadic endocrine neoplasia

III Analyze germ-line \textit{AIP} mutations in familial non-medullary thyroid cancer

IV Study the role of \textit{CDKN1B/p27^{kip1}} in MEN-like phenotype
4. MATERIALS AND METHODS

4.1. Subjects

This study was approved by the Ministry of Social Affairs and Health, the Ethics Review Committees of the Hospital District of Helsinki and Uusimaa, and the Department of Medical Genetics of the University of Helsinki. The permission to use patient samples was obtained either with appropriate informed consent or with the permission of the National Authority for Medicolegal Affairs. Detailed information of samples can be found from the corresponding publications or from their supplementary material.

4.1.1. Pituitary adenoma patient samples (I, II, IV)

Altogether 460 samples were analyzed in study I. This cohort consisted of heterogeneous pituitary adenoma patients originating from several populations from Europe and the United States. The samples can be divided into the following subcategories: a) young acromegaly patients consisting of 27 samples from German patients (<40 years at the time of surgery) and 36 from Finnish patients (<45 years at the time of diagnosis), b) unselected acromegaly patients including 71 samples from Italian patients, c) unselected pituitary adenoma patients consisting of 113 samples from American patients and 122 from Polish patients, and d) MEN1 suspected patients without MEN1-gene mutations including 55 samples from Spanish patients and 36 from Dutch patients.

Fifty pituitary adenoma samples were available to study AIP protein expression (study I). Two patients with PRL-producing and seven with GH-producing adenomas had c.40C>T mutation. Three GH-producing adenomas were from patients with c.280-1G>C, c.824_825insA, or c.469-1G>A mutation. AIP mutation-negative tumors accounted for 32 GH-producing and five PRL-producing adenomas, and one GH- and PRL-negative adenoma.

In study II, thirty-two sporadic Finnish pituitary adenoma samples were analyzed: nine were PRL-producing and 23 GH-producing adenomas. Twenty-one of the GH-producing adenomas originated from the population-based cohort of 54 acromegaly patients diagnosed in five Finnish university hospitals during 1980-1999 (Kauppinen-Makelin et al. 2005; Vierimaa et al. 2006). As previously reported, 20 samples from this cohort of 54 patients are AIP mutation-negative (Vierimaa et al. 2006).

Thirty-five out of 36 suspected MEN1 patients (also in study I) without MEN1- or AIP-mutation from Netherlands and one additional suspected MEN1 patient from Germany were included in study IV. Furthermore, sequence analysis consisted of 34 out of 36 young Finnish acromegaly patients (also in study I), 16
AIP-mutation negative sporadic pituitary adenoma patients (Oulu University hospital, also in study I), and 19 familial acromegaly/pituitary adenoma patients originating from various nationalities. All of the samples were AIP-mutation negative.

4.1.2. Sporadic endocrine tumors (II)

The tumors analyzed from tissues of the endocrine system other than the pituitary gland, included 61 Finnish and 18 Danish samples. The samples were from the thyroid gland (26 tumors), the adrenal gland (19 tumors), lung, cecum, appendix and small intestine (16 neuroendocrine tumors), and the parathyroid gland (8 tumors). Four paragangliomas, four pancreatic endocrine tumors, and two mixed endocrine-exocrine tumors were also included in study II.

4.1.3. Familial thyroid cancer patient cohort (III)

Up to 7% of all thyroid tumors belong to the clinical entity of familial non-medullary thyroid cancer (NMTC). Eighty percent of a familial NMTC cohort, including 261 families, did not connect to previously identified predisposing locus 2q21 and 19p13.2 (Canzian et al. 1998; McKay et al. 2001; McKay et al. 2004). AIP was sequenced in 93 index cases of these non-linked families. Eighty-five patients were diagnosed with papillary thyroid cancer (PTC), one with PTC and Hashimoto thyroiditis, one with PTC and hypothyroidism, and one with PTC and colloidal adenoma. Two patients had micropapillary thyroid cancer, two had multinodular goiter, and one had thyroid adenoma.

4.1.4. Healthy control samples (I, II, III, IV)

Control DNA samples were received from 749 unrelated, anonymous, healthy individuals. These samples consisted of 288 Caucasians from UK (Human Random Control DNA panel, Porton Doen, Salisbury, Wiltshire, UK), 110 Caucasians from the Centre d’Étude du Polymorphisme Humain (Fondation Jean Dausset-CEPH, Paris, France), 209 Finnish Red Cross blood donors, 90 Germans (Leipzig University, Germany), and 52 Italians (Treviso General Hospital, Italy). The analyzed control samples in each study are described in corresponding publications.

4.2. Analysis methods

4.2.1. Direct sequencing (I, II, III, IV)

Genomic DNA was extracted from blood or paraffin-embedded tissue according to standard protocols (Kannio et al. 1996; Lahiri and Nurnberger 1991; Shibata et al. 1988). Primer pairs used for sequencing the coding region of AIP and the flanking intronic areas have been previously described (Vierimaa et al. 2006).
CDKN1B/p27<sub>kip1</sub> primer pairs were designed by using Primer3 (http://frodo.wi.mit.edu/primer3), and are presented in the supplementary material of the original publication (study IV). In both genes, tumor-derived DNA was amplified in shorter fragments compared to normal DNA.

PCR products were analyzed by electrophoresis using 2% agarose gel and the specifically amplified products were purified using ExoSAP-IT purification kit (USB Corporation). Purified PCR-fragments were sequenced using the Big Dye 3.1 Termination chemistry on an ABI3730 DNA sequencer (Applied Biosystems).

LOH analysis was performed on the tumor-derived DNA. From the sequence chromatograms the peak heights/areas between the wild-type and the mutant allele were compared. LOH was detected when the wild-type allele was either completely or nearly completely disappeared, when compared to the mutant allele.

4.2.2. Immunohistochemistry (IHC) (I, III, IV)

Immunoreactions against AIP (1:4000, AIP SP5213P; Acris Antibodies) and p27 (1:500, p27<sub>Kip1</sub>, clone 57; BD Biosciences) were done according to standard IHC procedures. The AIP IHC is introduced shortly. Five-micrometer-thick sections were cut from the paraffin blocks and pre-warmed from 30 minutes up to one hour at 55°C. After deparaffinization and rehydration in graded alcohol series, antigen retrieval was carried through with either 0.01 M citrate (pH 6.0) buffer in a microwave oven at 800 W for 2 min and at 300 W for 10 min or in 0.01 M Tris-EDTA (pH 6.0) buffer in a microwave oven at 800 W for 2 min and at 300 W for 15 min. The slides were allowed to cool for 20 min and rinsed with PBS. Thereafter, primary antibody was incubated for 30 min at room temperature, followed by rinsing in PBS, after which the bound antibodies were detected using diaminobenzidine (DAKO, Copenhagen, Denmark) with hematoxylin counter stain. The slides were dehydrated in graded alcohol series, embedded in xylene, and covered.

4.2.3. In silico analysis (I, III, IV)

Computational programs were used to predict whether the detected intronic, silent, or missense changes affect splicing. The used in silico splice site prediction programs were Berkeley Drosophila Genome Project, ESEfinder 2.0, Splice Scan, Alternative Splice Site Predictor, and NetGene2 (Brunak et al. 1991; Cartegni et al. 2003; Hebsgaard et al. 1996; Reese et al. 1997; Smith et al. 2006; Tchourbanov and Ali 2005; Wang and Marin 2006).
5. RESULTS

5.1. Molecular analysis of PAP (I)

The identification of \(AIP\) as a predisposing gene was originally described in a homogeneous population (Vierimaa et al. 2006). To clarify the clinical features of this condition in a more heterogeneous pituitary adenoma setting, \(AIP\) was sequenced in 460 pituitary adenoma patients from the European and North American populations. Potentially pathogenic mutations were observed at low frequencies (Table 4). \(AIP\) mutations were the most common in young acromegaly patients from Germany (2/27 or 7.4%) and Finland (2/36 or 5.5%). Mutations were less common in unselected pituitary adenoma patients from North America (2/113 or 1.8%) and Poland (1/122 or 0.8%). Also, \(AIP\) mutations were identified in suspected MEN1 patients without \(MEN1\) mutations from Spain (1/55 or 1.8%) and the Netherlands (1/36 or 2.8%). None of the studied Italian unselected acromegaly patients harbored \(AIP\) mutations.

Table 4. Detected \(AIP\) mutations from pituitary adenoma patients from European and North American populations. Only the potentially pathogenic mutations are presented. None of these variations were present in the control samples.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Detected (AIP) mutation</th>
<th>LOH</th>
<th>Pituitary adenoma type</th>
<th>Age at the diagnosis</th>
<th>Family history of pituitary adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young acromegaly</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>German</td>
<td>c.66_71del</td>
<td>Yes</td>
<td>GH</td>
<td>20 yr</td>
<td>Yes (acromegaly)</td>
</tr>
<tr>
<td>German</td>
<td>c.[878_879delinsGT; c.880_891del]</td>
<td>Yes</td>
<td>GH</td>
<td>29 yr(^a)</td>
<td>NA</td>
</tr>
<tr>
<td>Finnish</td>
<td>c.40C&gt;T</td>
<td>NA</td>
<td>GH</td>
<td>36 yr</td>
<td>No</td>
</tr>
<tr>
<td>Finnish</td>
<td>c.40C&gt;T</td>
<td>NA</td>
<td>GH</td>
<td>41 yr</td>
<td>No</td>
</tr>
<tr>
<td>Unselected pituitary adenoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>American</td>
<td>c.280-1G&gt;C</td>
<td>NA</td>
<td>GH</td>
<td>20 yr</td>
<td>No</td>
</tr>
<tr>
<td>American</td>
<td>c.824_825insA</td>
<td>Yes</td>
<td>GH</td>
<td>8 yr</td>
<td>No</td>
</tr>
<tr>
<td>Polish</td>
<td>c.911G&gt;A</td>
<td>NA</td>
<td>ACTH</td>
<td>26 yr</td>
<td>No</td>
</tr>
<tr>
<td>(MEN1)-negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spanish</td>
<td>c.542del</td>
<td>NA</td>
<td>GH</td>
<td>18 yr</td>
<td>Yes (acromegaly)</td>
</tr>
<tr>
<td>Dutch</td>
<td>c.896C&gt;A</td>
<td>NA</td>
<td>GH</td>
<td>16 yr</td>
<td>No</td>
</tr>
</tbody>
</table>

LOH, loss of heterozygocity e.g. loss of wild-type allele; NA, not available

\(^a\) Age at the time of operation, the age at the time of diagnosis is unknown.
Other detected *AIP* variations were c.100-18C>T in one German sample, c.47G>A (p.Arg16His) in one Italian, in one American with Polish roots, and in three Polish samples, c.906G>A (p.=) in three American samples, and c.696G>C (p.=) in one Polish sample. These variations were considered as a rare polymorphism according to *in silico* prediction (c.100-18C>T, c.906G>A, c.696G>C), negative LOH analyze (c.47G>A) and/or detection of variations also in the healthy controls (c.100-18C>T, c.47G>A, c.906G>A).

To help in identifying the *AIP* mutation-positive patients, the possibility to use immunohistochemistry as a pre-screening tool was studied. *AIP* mutation-positive tumors were from patients with c.40C>T, c.280-1G>C, c.824_825insA, or c.469-1G>A mutation. Most of *AIP* mutation-positive tumors (9/12 or 75%) showed complete loss of AIP in the cytoplasm and nucleus (Fisher’s Exact test, p=0.000004; Fig. 4). Positive AIP immunoreactions in these tumors may be due to e.g. unspecific staining or presence of nonfunctional yet immunoreactive AIP protein. Positive immunoreactions of AIP were detected in most of the *AIP* mutation-negative tumors (36/38 or 95%). Therefore, AIP IHC showed 75% sensitivity and 95% specificity for truncating *AIP* germ-line mutations. This indicates the potentiality of AIP IHC as a pre-screening tool.

![AIP IHC stainings with 40x magnification.](image)

**Figure 4.** AIP IHC stainings with 40x magnification. (A) *AIP* mutation-negative adenoma shows positive immunoreaction against AIP. (B) Pituitary adenoma from a patient with c.40C>T mutation shows the lack of AIP protein whereas peripheral blood leukocytes, indicated by black arrows, display positive immunoreaction.

5.2. Somatic *AIP* mutation screening in sporadic endocrine neoplasia (II)

To study the possible somatic mutations of *AIP* in sporadic endocrine tumors, thirty-two pituitary adenomas and 79 other tumors of the endocrine system were analyzed. No somatic mutations were identified in the studied tumors. However, two PRL-producing adenoma patients harbored the Finnish founder mutation c.40C>T (p.Gln14X) and one thyroid adenoma patient a potential polymorphism c.69A>G (p.Gly23Glu) in the *AIP* gene.
c.40C>T was detected in two patients with the PRL-producing pituitary adenoma. These patients were diagnosed at the age of 35 years and both of the tumor tissues showed the complete loss of the wild-type allele. One mutation-positive patient was a male with no family history of the endocrine tumors. The mutation was also present in his germ-line. The other mutation carrier was a female whose normal tissue and family history information were not available.

The other detected germ-line variation was c.69A>G in a female patient with thyroid adenoma diagnosed at the age of 60 years. This variation was present in the corresponding normal tissue and wild-type allele was retained in the tumor tissue. Because of this and previous observations of c.69A>G in the healthy controls (five out of 532) and in Finnish colorectal cancer samples (6/873), this variation was considered as a germ-line polymorphism (study I; Georgitsi et al. 2007; Vierimaa et al. 2006).

5.3. Screening of AIP in familial non-medullary thyroid cancer (NMTC) cases (III)

The possible germ-line mutations of AIP were studied in index patients from 93 NMTC families. Two previously reported AIP variations were detected. One patient, with PTC and colloidal adenoma diagnosed at the age of 44 years, harbored c.47G>A (p.Arg16His). The other patient, with unilateral PTC diagnosed at the age of 36 years, harbored c.36G>A (p.=). These variations did not segregate in the corresponding families. According to in silico analysis, neither of the variants had predicted effects on splicing. Tumor tissues were not available for LOH analysis.

In the previous studies, c.47G>A has been identified in fourteen pituitary adenoma patients, two Finnish colorectal cancer patients, and three healthy controls (study I; Buchbinder et al. 2008; Cazabat et al. 2007; Daly et al. 2007b; Georgitsi et al. 2007; Yaneva et al. 2008). Whereas c.36G>A has been detected in one Finnish prostate cancer patient and one sporadic acromegaly patient, but all of the studied 802 healthy controls have been negative for this change (Cazabat et al. 2007; Georgitsi et al. 2007). Both variants were considered as rare polymorphisms, although the pathogenic potential of c.36G>A cannot be totally excluded.

In addition, one follicular thyroid adenoma from an AIP mutation-positive (c.469-1G>A) patient from an additional cohort was available to the study. No LOH was seen in the tumor tissue and the AIP IHC was positive in the adenoma and the normal cells. This suggested the presence of functional AIP protein in the tumor tissue.
5.4. The analysis of CDKN1B/p27\textsuperscript{Kip1} mutations in endocrine neoplasia (IV)

The germ-line mutation of CDKN1B/p27\textsuperscript{Kip1} was reported in one patient with multiple endocrine neoplasia (Pellegata et al. 2006). To further study the role of CDKN1B/p27\textsuperscript{Kip1} in multiple endocrine neoplasia, mutation screening was performed in 36 clinically suspected MEN1 patients without MEN1 gene mutation. Furthermore, nineteen familial acromegaly or familial pituitary adenoma patients, and 50 sporadic acromegaly patients were included in the study. One suspected MEN1 patient carried 19-bp duplication (c.59_77dup) in the coding region of CDKN1B/p27\textsuperscript{Kip1} gene. This mutation causes frame shifting change at Serine-27. It creates a new reading frame ending in a stop codon at 69 residues earlier than the wild-type allele. The sequencing of DNA extracted from the neuroendocrine cervical carcinoma revealed the loss of the wild-type allele, and the IHC staining confirmed the absence of p27 in this tumor. This CDKN1B/p27\textsuperscript{Kip1} mutation was the second reported mutation in humans.

The other detected CDKN1B/p27\textsuperscript{Kip1} gene variation was a silent change c.426G>A (p.=) in one young sporadic acromegaly patient. According to in silico analysis, this change has no predicted effect on splicing and thus it was considered as a rare polymorphism.
6. DISCUSSION

6.1. The PAP phenotype

The Finnish population is overall genetically homogeneous due to the relatively small founder populations, the small genetic drift, and the isolated location resulting from geographical, linguistic, and religious reasons (Peltonen 1997). Thus, rare conditions with low-penetrance can be found as clusters. This facilitates the identification of the predisposing gene(s) as was the case in the identification of the AIP mutations in PAP (Vierimaa et al. 2006). In the initial study, AIP mutations were relatively frequent in a population-based cohort from Northern Finland: 16% in GH-producing pituitary adenomas, and 40% in a subset of patients diagnosed under the age of 35 years (Vierimaa et al. 2006). To gain insight into the PAP phenotype and the mutation frequencies it was relevant to study more heterogeneous sample materials as well.

6.1.1. AIP mutation frequencies in diverse clinical settings (I)

In study I, the contribution of AIP mutations was studied in pituitary adenoma patients from genetically heterogeneous populations from Europe and North America. If taking into account the previously reported two patients belonging to the same Italian cohort of which 71 were analyzed in study I (Vierimaa et al. 2006), AIP mutations were found from all of the studied clinical subcategories (0.8-7.4%). The highest mutation frequencies were detected in young acromegaly/gigantism patients from Germany (2.27 or 7.4%) and Finland (2.86 or 5.5%). Among all studied AIP mutation-positive patients the average age at diagnosis was 23.8 years. Additional studies have detected similar ages in the AIP mutation-positive patients (average ~25 years) which are lower than in the mutation-negative ones (average ~38 years) (Daly et al. 2007b; Leontiou et al. 2008). Besides the young age of the AIP mutation-positive patients, the majority of these patients did not have family history of pituitary adenomas. These observations are in concordance with the original study by Vierimaa et al. (2006) where the young age of onset and the low penetrance of this condition were reported. In the following studies, even up to 66% penetrance has been suggested (Daly et al. 2007b; Iwata et al. 2007; Khoo et al. 2009; Leontiou et al. 2008; Naves et al. 2007; Toledo et al. 2007). However, this high frequency may reflect a bias caused by the incomplete data from some of the families. More accurate and age-related penetrance estimation requires additional AIP mutation-positive families to be studied and extended time period of surveillance.

After the initial study of Vierimaa et al. (2006) and followed by study I, AIP mutation status has been studied in familial and sporadic pituitary adenoma patients. Daly et al. (2007b) performed AIP mutation screening in 73 FIPA families. Mutations accounted for 15% of these families and for 50% of those
families with only GH-producing adenomas *e.g.* IFS (Daly et al. 2007b). A similar trend was seen in 26 FIPA families where 35% of the families were AIP mutation-positive (Leontiou et al. 2008). Additional familial cases with AIP mutations have also been reported (Georgitsi et al. 2008b; Iwata et al. 2007; Jennings et al. 2009; Khoo et al. 2009; Toledo et al. 2007). AIP mutations seem to account for less than 2% of the sporadic pituitary adenomas (study II; Barlier et al. 2007; Buchbinder et al. 2008; Cazabat et al. 2007; Digiovanni et al. 2007; Georgitsi et al. 2008a; Igreja et al. 2009; Iwata et al. 2007; Leontiou et al. 2008; Vierimaa et al. 2006; Yaneva et al. 2008; Yu et al. 2006). The majority of the AIP mutation-positive patients have been diagnosed with GH-producing adenomas, but cases with PRL-, ACTH-producing adenomas or NFPAs have also been reported (study I; study II; Daly et al. 2007b; Georgitsi et al. 2008b; Leontiou et al. 2008). Recently, male predominance in PAP was suggested: 70% of the reported AIP mutation-positive patients were males (*p* < 0.001) (Cazabat et al. 2009). This association remains statistically significant in an update of the AIP mutation-positive patients (62%, 71 M/44 F; *p* = 0.014, Chi-square test). Additional AIP mutation-positive patients and careful clinical examinations will show if males are truly at a higher risk of developing pituitary adenomas in PAP.

Approximately 40 different AIP mutations, scattered throughout the coding region, have so far been identified (Fig. 5). Interestingly, most of the pathogenic missense mutations occur in a region between codons 241-304 in the two last exons of AIP. Two of the TPR motifs, which are critical for protein-protein interactions, are located in this area. In addition, the five last amino acids (codons 325-330) of the protein are required for binding to AHR (Bell and Poland 2000).

**Figure 5.** A schematic figure of AIP with all pathogenic germ-line mutations reported in the literature by November 2009. The exon boundaries of the AIP gene are indicated by dashed lines. The pink box shows the location of FKBP domain and the violet boxes the locations of TPRs.
A large portion of the familial and sporadic pituitary adenoma cases are not explained by the known genes predisposing to pituitary adenomas. LOH of 11q13 is observed in the pituitary adenomas without detectable MEN1 or AIP mutation (e.g. Gadelha et al. 1999; Tanaka et al. 1998; Teh et al. 1998; Yamada et al. 1997). One explanation might be the limitations of the current sequencing methods, as seen in a recent study where large genomic deletions in the AIP area were detected in patient samples previously sequenced to be AIP mutation-negative (Georgitsi et al. 2008b). However, it is possible that unidentified predisposing gene(s) still lays in the 11q13.

6.1.2. The IHC in identification of PAP (I)

The lack of family history and the low frequency of AIP mutations in randomly selected pituitary adenoma patients create a challenge for the identification of the PAP patients. Routine screening of AIP in all pituitary adenoma patients seems unreasonable. Since the pituitary adenomas are stained against hormones for diagnostic purpose in many laboratories, we wanted to test the specificity and sensitivity of the AIP IHC.

In study I, the AIP IHC using a polyclonal antibody against mouse recombinant AIP showed high specificity (95%), but weaker sensitivity (75%). The AIP IHC has also been tested in later studies. A monoclonal antibody against the human AIP with amino-terminal epitope (FKBP region) showed decreased immunostaining in a pituitary adenoma sample with early truncating AIP mutation (p.Glu174fs) (Naves et al. 2007). In another study, the AIP IHC with the same antibody showed weak immunoreactivity in the pituitary adenomas with AIP mutation (e.g. p.Gln82fs, p.Arg304X, and p.Gln285fs), albeit immunoreactivity was also diminished in the adenomas with probably non-pathogenic variations (e.g. c.279+23C>T and c.468+16G>T) (Jaffrain-Rea et al. 2009). Moreover, immunostaining with the same AIP antibody in the pituitary adenoma samples with p.Arg304X or p.His274fs mutation showed positive staining of the protein (Leontiou et al. 2008). In some of these AIP mutation-positive adenomas, the positive immunoreaction may be due to carboxyl-terminal location of the mutation and amino-terminal location of the antibody epitope.

Discrepancies in the sensitivities between the AIP IHC studies may arise from different reasons. For example, the IHC assays may differ between the laboratories. It is also possible that carboxyl-terminal or missense mutation leads to a stable and immunoreactive, yet possibly dysfunctional, protein. Thus, the nature and position of the mutation and the selection of antibody are critical. Improving the AIP IHC requires further optimization with new carboxyl-terminal AIP antibodies. Functional AIP IHC could be useful at least as a pre-screening tool to identify the potential PAP patients. For instance, IHC screening
of tumors in HNPCC, also known as Lynch syndrome, has been successfully used even as a diagnostic tool (Hampel et al. 2005).

6.2. Somatic AIP mutations are rare or non-existent in sporadic endocrine neoplasia (II)

As already mentioned, germ-line AIP mutations predispose mainly to GH-producing adenomas and less frequently to PRL-, ACTH-producing adenomas, and NFPAs. Pituitary adenomas also occur in MEN1 and CNC syndromes. Somatic mutations in MEN1 and PRKAR1A are rare in sporadic pituitary adenomas (e.g. Wenbin et al. 1999; Yamasaki et al. 2003). Such mutations are seen in some other tumors related to these syndromes (e.g. Farnebo et al. 1998; Sandrini et al. 2002b). Thus, we studied the occurrence of somatic AIP mutations in the pituitary adenomas and also, for the first time, in non-pituitary endocrine tumors.

None of the studied sporadic pituitary adenomas harbored purely somatic mutations. However, the Finnish founder mutation was detected in two PRL-producing adenomas from individuals diagnosed at a young age (two out of nine, 22%). For one of the patients the mutation was confirmed to be in the germ-line. This patient, from whom information was available, had no family history of endocrine tumors. These results supported the previous observations that AIP mutations also predispose to PRL-producing adenomas (Daly et al. 2007b; Vierimaa et al. 2006).

Thus far the number of PRL-producing adenomas, in which somatic AIP mutations has been studied, is limited. This may be because of the infrequent surgery of the PRL-producing adenomas; tumors have a slow growth rate and good response to the therapy (Spada et al. 2005). Therefore, further studies are required to clarify the possible contribution of somatic AIP mutations in the PRL-producing adenomas.

In study II, somatic AIP mutations were also examined in the sporadic GH-producing adenomas, but all samples were mutation-negative. This is in concordance with the previous studies which have not identified somatic mutations in that particular tumor type (Barlier et al. 2007; Iwata et al. 2007). These results indicate that somatic AIP mutations do not have a major contribution to the formation of the sporadic GH-producing adenomas. Actually, many tumor susceptibility genes are only rarely somatically mutated in the respective sporadic tumors such as BRCA1/2 in the sporadic breast tumors (Khoo et al. 1999; Yang et al. 2002).

In this study, the occurrence of somatic AIP mutations were analyzed for the first time in non-pituitary endocrine tumors. No somatic AIP mutations were detected. Results suggest that AIP mutations do not seem to be strongly involved
in the development of these tumors. In previous studies, LOH of 11q13 has been reported in adrenal carcinomas from two AIP mutation-positive patients (Leontiou et al. 2008; Toledo et al. 2008). However, a larger number of PAP families and additional evidence is needed to clarify whether AIP mutations are predisposing to adrenal tumors since LOH of 11q13 is a frequent event in this tumor type (Luccio-Camelo et al. 2004).

6.3. AIP mutations seem not to be involved in familial non-medullary thyroid cancer (III)

NMTC occurs with a greater frequency than expected in familial syndromes such as CNC (Malchoff and Malchoff 2006). Familial NMTC is a distinct clinical condition characterized by a higher degree of tumor aggressiveness and an increased mortality (Grossman et al. 1995). Several susceptibility loci for familial NMTC have been suggested (e.g. Cavaco et al. 2008; McKay et al. 2001), but the main genetic components are still unknown. Thyroid disorders have also been identified in FIPA, and PAP families (O Vierimaa and PI Salmela, unpublished observations; Beckers and Daly 2007). Thus, the possible role of AIP in familial NMTC was examined in study III. Sequencing of AIP did not reveal potentially pathogenic AIP mutations indicating that germ-line AIP mutations have little or no role in the genesis of familial NMTC.

The possible loss of AIP in thyroid tumors was studied in one tumor sample from a Finnish AIP mutation-positive patient. LOH and immunohistochemical analyses suggested that the immunoreactive protein seems to be present in this particular tumor tissue. Interestingly, patients with GH-producing adenomas are at a higher risk of developing non-toxic nodular goiters. Also, these patients have a somewhat elevated risk of thyroid cancer compared to the general population (e.g. Herrmann et al. 2004; Kurimoto et al. 2008). This seems to be linked to the increased plasma levels of IGF-I in patients with GH-producing adenomas (Siegel and Tomer 2005). Thus, at least some of the thyroid disorders seen in the AIP mutation-positive patients may be due to the elevated IGF-I levels. On the other hand, thyroid disorders are quite common in the general population (up to 7%) (Roman 2003) and those tumors may occur just by chance in the AIP mutation-positive patients.

6.4. AIP in tumorigenesis

So far the number of endocrine tumors screened for AIP mutations, other than pituitary adenomas, is limited. Thus, AIP mutations may be identified by screening additional non-pituitary endocrine tumors. Besides endocrine tumors, AIP mutations have been studied in common cancer types, including colorectal cancer, breast cancer, and prostate tumors, with the negative results (Georgitsi et al. 2007). Even though AIP is ubiquitously expressed in all human tissues, with current knowledge AIP mutations seem to predispose only to pituitary
adenomas. Thus, AIP could be associated to tissue selective tumorigenesis. This was supported in a recent study where the silencing of Aip resulted in increased cell proliferation in a rat pituitary cell line (GH3), but not in two other studied cell lines (HEK293 and HeLa) (Heliovaara et al. 2009). In the same study, IHC analysis showed that ARNT was less frequently expressed in the AIP mutation-positive tumors compared to the mutation-negative ones. Again the tissue-selectivity was indicated since the reduction of Arnt was only seen in the GH3 cell line after Aip-silencing (Heliovaara et al. 2009). Heliovaara et al. (2009) also noticed that the expression of the nuclear AHR was somewhat increased in the AIP mutation-positive adenomas compared to the mutation-negative ones. Thus, ARNT and AHR were suggested to be involved in the pituitary tumorigenesis in the AIP mutation-positive tumors.

Aberrant cAMP signaling is often detected in pituitary tumorigenesis (Boikos and Stratakis 2007b). This signaling is also possibly involved in the pituitary tumorigenesis of the AIP mutation-positive tumors. AIP targets PDE2A to the AHR complex (de Oliveira et al. 2007). This binding ensures AHR retention in the cytoplasm by lowering the local cAMP concentrations (de Oliveira et al. 2007). Thus, the lack of functional AIP may lead to elevated local cAMP levels and cause the translocation of AHR to the nucleus (Heliovaara et al. 2009). Furthermore, the cAMP-mediated translocation of AHR prevents the formation of the AHR/ARNT complex (Oesch-Bartlomowicz et al. 2005). Heliovaara et al. (2009) suggested that the disturbances in the formation of AHR/ARNT, possibly also HIF1-α/ARNT, complex may unbalance the transcription of target genes of this complex leading to pituitary tumorigenesis.

Aip knock-out mice (Aip+/−) were reported to show embryonic lethality accompanied by cardiac malformations (Lin et al. 2007). The phenotype of these Aip+/− mice was different from those of Ahr+/− and Ppara+/− mice. Thus, Lin et al. (2007) suggested that Aip seems to have a role in another pathway distinct from xenobiotic-induced metabolism. In the same study, heterozygous Aip+/− mice appeared phenotypically normal and fertile, but the possibility of endocrine tumor formation was not studied.

In a recent study, a tendency towards a higher risk of pituitary adenomas was seen after massive exposure to dioxin after the Seveso accident, Italy, in 1976 (Pesatori et al. 2008). The tumors that occurred did not include GH-producing adenomas, which is the main tumor type in PAP. Moreover, it should be noted that the prevalence of pituitary tumors was not statistically significant (Pesatori et al. 2008). Thus, the direct activation of AHR, leading to xenobiotic-induced metabolism, did not cause increased risk of pituitary adenoma development.

Certainly the mechanism how the loss of functional AIP leads to tumor formation requires further studies. Functional studies and work with animal models should help to resolve this issue in the future.
6.5. The role of CDKN1B/p27Kip1 in multiple endocrine neoplasia (IV)

Recently, a germ-line CDKN1B/p27Kip1 mutation was reported in one family with multiple endocrine neoplasia (Pellegata et al. 2006). In this family, the MEN1-related tumors included GH-producing pituitary adenoma, primary hyperparathyroidism, and angiomyolipoma. To further study the role of CDKN1B/p27Kip1 gene in endocrine neoplasia, we performed a mutation screening of CDKN1B/p27Kip1 in suspected MEN1 patients without MEN1 gene mutation. Also the contribution of CDKN1B/p27Kip1 mutations in the familial and sporadic pituitary adenomas was studied.

In this study, we were able to confirm the finding of Pellegata et al. (2006) by publishing the second germ-line CDKN1B/p27Kip1 mutation in a clinically suspected MEN1 patient. This patient had three MEN1-related tumors: small-cell neuroendocrine cervical carcinoma, ACTH-producing adenoma, and hyperparathyroidism. The neuroendocrine cervical carcinoma showed a loss of the wild-type allele in tumor-derived DNA. Furthermore, the p27 IHC analysis showed negative immunoreactivity of p27 protein in the particular tumor tissue. These results strengthened the tumor suppressor role of the CDKN1B/p27Kip1 in endocrine neoplasia (Pellegata et al. 2006). The previous knock-out mouse models had suggested the role of CDKN1B/p27Kip1 in endocrine neoplasia (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996), but our finding confirmed the role of CDKN1B/p27Kip1 in human endocrine neoplasia.

So far, five mutation-positive index cases have been identified among approximately 340 studied cases (study IV; Agarwal et al. 2009; Igreja et al. 2009; Owens et al. 2009; Ozawa et al. 2007). Two of these cases are familial and three apparently sporadic. These results indicate that CDKN1B/p27Kip1 mutations occur at a low frequency in the suspected MEN1 patients without MEN1 mutation.

In study IV, none of the familial and sporadic pituitary adenoma patients harbored potentially pathogenic CDKN1B/p27Kip1 mutations. This is in concordance with the previous negative mutation screenings of CDKN1B/p27Kip1 in the pituitary adenomas (e.g. Ikeda et al. 1997; Tanaka et al. 1997). Thus, it seems that CDKN1B/p27Kip1 mutations are rare or non-existent in the familial or sporadic pituitary adenoma patients.

Hyperparathyroidism has been diagnosed in all CDKN1B/p27Kip1 mutation-positive index cases (study IV; Agarwal et al. 2009; Pellegata et al. 2006). In a recent study, potentially pathogenic CDKN1B/p27Kip1 mutations were not detected in presumably familial hyperparathyroidism patients (Vierimaa et al. 2009). Furthermore, screening of somatic CDKN1B/p27Kip1 mutations in sporadic secondary/tertiary hyperparathyroidism was also negative (Lauter and Arnold...
2008). These results indicate that $CDKN1B/p27^{kip1}$ does not have a major contribution to familial or sporadic hyperparathyroidism.

The rarity of mutations among the suspected MEN1 patients and the variable phenotype of $CDKN1B/p27^{kip1}$ mutation carriers complicate the identification of these patients. Nonetheless, the clinicians should be aware that at least some of the suspected MEN1 patients without $MEN1$ mutation may harbor $CDKN1B/p27^{kip1}$ mutation.
7. CONCLUSIONS AND FUTURE PROSPECTS

This work studied the role of AIP and CDKN1B/p27Kip1 in endocrine neoplasia. Conclusions of this work are summarized as follows:

I) Germ-line AIP mutations are found at low frequencies (0.8-7.4%) in diverse settings of the pituitary adenoma patients. Most of the AIP mutation-positive patients have GH-producing adenomas. Often these patients are young and they may not display a family history of pituitary adenomas. With further optimization, the AIP IHC could be used as a pre-screening tool for the identification of potential PAP patients.

II) Somatic mutations of AIP are rare or do not exist in sporadic endocrine tumors.

III) AIP is unlikely to be a predisposing gene for familial NMTC.

IV) The second CDKN1B/p27Kip1 mutation was identified in suspected MEN1 patients without MEN1 mutations.

Identification of the predisposing genes for familial syndromes has raised general awareness. The recognition of the mutation-positive families enables regular follow-up of the mutation carriers followed by early diagnosis. With the appropriate treatments, the morbidity of these patients can be diminished. This way the quality of the patient's life can be improved and premature death can even be avoided. In addition, the family members without mutation can be relieved from the unnecessary follow-ups.

So far, mutations in MEN1, PRKAR1A, CDKN1B/p27Kip1, and AIP are known to predispose to endocrine neoplasia with pituitary adenomas. However, the majority of the familial, isolated pituitary adenomas do not harbor mutations in these genes and thus new predisposing gene(s) remain to be identified.

The diagnosis of gigantism is unambiguous whereas acromegaly develops insidiously often leading to delayed diagnosis. For this reason, the identification of the AIP mutation carriers is important. This is challenging due to the rarity of AIP mutations in sporadic cases and often the lack of family history of pituitary adenomas. However, the AIP mutation screening should be considered for patients with familial pituitary adenomas, particularly if affected patients have GH-producing adenomas. Furthermore, the contribution of AIP mutations should be suspected if a seemingly sporadic patient is diagnosed with GH-producing adenoma at a young age.
For the *AIP* mutation carriers it seems reasonable to offer a non-invasive clinical follow-up, such as a biochemical screening for markers (e.g. hormones) from the blood and possibly the pituitary MRI scanning. For instance, in the *MEN1* mutation-positive patients the early marks of neoplasia can be detected biochemically on average 10 years prior to the clinically evident disease (Lairmore *et al.* 2004).

Clinical follow-up of the *CDKN1B/p27<sup>Kip1</sup>* mutation carriers is recommended. However, the mutation screening of *CDKN1B/p27<sup>Kip1</sup>* gene seems unreasonable due to the rarity of mutations. Nonetheless, the clinicians should be aware that small portion of suspected MEN1 patients without *MEN1* gene mutations may have *CDKN1B/p27<sup>Kip1</sup>* gene mutation.

During the last decades, factors contributing to the pituitary tumorigenesis have been identified but the underlying processes are largely unknown. To gain insight into this issue, rigorous *in vitro* and *in vivo* studies are needed. The lack of functional human pituitary cell line requires the use of animal cell lines for these experiments. Currently, there is a great interest towards the possible tumor spectrum of the *Aip<sup>+</sup>* mice: is the human PAP phenotype reproduced or not?
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