Thesis for doctoral degree (Ph.D.) 2016

GENETIC AND MOLECULAR BACKGROUND OF PHEOCHROMOCYTOMA AND PARAGANGLIOMA

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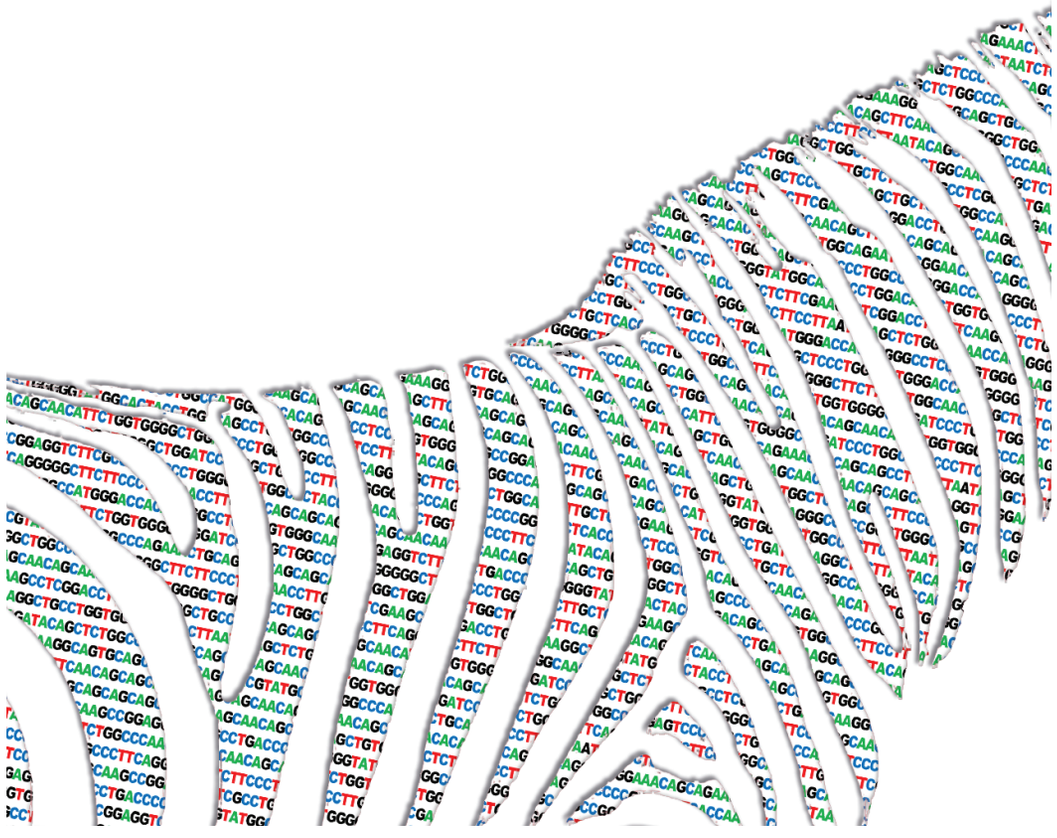

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GENETIC AND MOLECULAR BACKGROUND OF PHEOCHROMOCYTOMA AND PARAGANGLIOMA

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Stockholm 2016

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Karolinska Institutet

Department of Oncology-Pathology

Genetic and Molecular Background of Pheochromocytoma and Paraganglioma

AKADEMISK AVHANDLING som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Leksellsalen, Karolinska Universitetssjukhuset, Solna.

Fredagen den 22 januari 2016, kl 10:00

av

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Stockholm 2016

THESIS DEFENSE

Leksell auditorium, Eugeniahemmet
Karolinska University Hospital, Solna, T3:02
Friday January 22nd 2016, 10:00

The zebra striped NET Cancer Awareness Day ribbon.
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Why a zebra?

The phrase ”when you hear hoof beats, think horses, not zebras” is often told to medical students during their clinical training. In this context, the term “zebra” is used to explain a rare disease or condition, such as a pheochromocytoma or a paraganglioma. Doctors *in spe* are often taught to assume that the simplest explanation usually is the best and that common diseases are what physicians should expect to encounter. Therefore, patients can experience a delay obtaining the correct diagnosis and proper treatment for a rare disease if doctors forget that zebras exist and assume horses every time hoof beats are heard. In order to increase neuroendocrine tumor (NET) awareness, the Worldwide NET Cancer Awareness Day (Nov 10th) has chosen the zebra and the zebra striped ribbon.

Dedicated to cancer patients

ABSTRACT

Pheochromocytomas (PCC) and abdominal paragangliomas (PGL), collectively denoted PPGL, are neuroendocrine tumors (NET) with a highly diverse genetic and molecular etiology, arisen in the adrenal medulla and abdominal paraganglia respectively. The tumors often cause cardiovascular symptoms due to the high production of catecholamines, and malignancy occurs in 10% of the PCCs and 30% of the PGLs. The genetic background of the PPGL disease constitutes of hereditary mutations in a growing list of susceptibility genes, although a large subset of the sporadic tumors still has an unknown etiology. This thesis work aimed to further characterize the genetic and molecular background of PPGL, in order to contribute to better diagnosis, prognosis and treatment for the patients.

In the first study we investigated the role of susceptibility gene promoter methylation in PPGL. By studying the gene expression and assessing the promoter methylation levels, it was found that the *VHL* gene is epigenetically inactivated in PPGL. Subsequently, in order to investigate the established susceptibility genes in a large cohort, we used a targeted sequencing approach in the second study and found that next-generation sequencing is fast and cost-effective method for mutational screening of PPGL. Additionally, the *NF1* gene was found to be the most frequently mutated gene, and in the third study we used the *NF1* mutational status obtained in the second study to investigate if immunohistochemistry could be used as a screening tool for *NF1* mutations. We found a strong sensitivity but poor specificity for the method and therefore recommend genetic screening as the most efficient tool to identify NF1 patients. The list of PPGL susceptibility genes is constantly growing, and one of the latest genes verified is the *HRAS* gene. In the fourth study we screened a large cohort of PPGL for mutations in the *HRAS* gene and compared with the overall gene expression obtained using a mRNA microarray approach. Taken together with *HRAS* mutations in the literature, the overall *HRAS* mutation frequency was calculated to 5.2% in PPGL. The gene expression profiling showed that the *HRAS* mutated tumors clustered together with the *NF1*- and *RET*-mutated tumors that are associated with activation of kinase pathways. With the mutational information obtained in the second study, we were able to screen PCCs without known genetic drivers. In the fifth study, these tumors underwent whole-exome sequencing, detecting recurrent mutations in the *KMT2D* gene. After screening of a verification cohort, a total *KMT2D* mutation frequency of 14% was established. In the sixth study we screened PGLs for mutations in the *KMT2D* gene. All PGLs exhibited wild-type however *KMT2D* gene over-expression was observed in PGLs compared to normal adrenal samples. These results would imply dysregulation of methyltransferase as a novel disease mechanism in PPGL. In summary, the studies included in the thesis have increased the knowledge of the genetic and molecular background of PPGL. The results may therefore in the long run contribute to better diagnosis, prognosis and development of future treatment options for the patients.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Feokromocytom (PCC) och paragangliom (PGL) (tillsammans förkortade PPGL) är ovanliga, så kallade neuroendokrina tumörer som har en synnerligen varierad genetisk bakgrund där ungefär en tredjedel av tumörerna har visats bero på ärftliga mutationer i kända gener. Orsaken till tumöruppkomst i den resterande andelen patienter vet man fortfarande mycket lite om. PCC uppkommer i binjuremärgen och PGL i paraganglierna som är ansamlingar av neuroendokrina celler i anslutning till det autonoma nervsystemet. Tumörerna orsakar ofta hjärt- och kärlrelaterade symtom på grund av att de ofta producerar stora mängder av stressrelaterade hormoner, mestadels adrenalin och noradrenalin. De flesta tumörerna är godartade, men ungefär 5% av PCC och ca 30% av PGL är elakartade i den meningen att de ger upphov till spridd sjukdom (metastaser). Syftet med avhandlingen i sin helhet har varit att vidare klarlägga den genetiska och molekulära bakgrunden till PPGL, och detta genom de sex ingående delarbetena.

I den första studien undersökte vi vilken roll epigenetik och närmare bestämt promotor-metylering har i tumörutvecklingen av PPGL. Graden av promotor-metylering bestämdes med hjälp av pyrosekvensering för 11 av de sjukdomsorsakande PPGL-generna, och av dessa var särskilt *VHL*-genen högt metylerad. Efter att ha korrelerat uttrycket av *VHL*-genen med dess metyleringsnivåer kunde vi konstatera att genen är inaktiverad av denna metylering och att det därför kan vara en nyupptäckt orsak till tumörutveckling i PPGL.

Genom att därefter använda oss av en ny typ av riktad sekvenseringsteknik (nästa generationens sekvensering) kunde vi i den andra studien i avhandlingen undersöka alla då kända sjukdomssorsakande gener för PPGL med en och samma metod. Vi kunde konstatera att *NF1* var den gen som var mest frekvent muterad och att metoden vi designat var en snabb och kostnadseffektiv teknik att använda för att eftersöka mutationer i denna tumörgrupp.

I den tredje studien undersökte vi om immunhistokemi kunde användas för att hitta mutationer i *NF1*-genen, då denna gen är mycket svårsekvenserad på grund av sin längd. *NF1*-mutationer ger upphov till NF1-syndromet som är ett tumörsyndrom i vilket PCC ingår som delfenomen. Eftersom vi sedan studie II kände till vilka tumörer som hade *NF1*-mutation kunde denna data jämföras med immunhistokemiska färgningar. Det visade sig att sensitiviteten var god men då specificiteten inte var godtagbar rekommenderar vi i denna studie att sekvensering tillsammans med klinisk undersökning trots allt är den mest pålitliga metoden för att identifiera patienter med NF1-syndromet.

Listan på sjukdomsorsakande gener som är involverade i PPGL växer ständigt då nya gener upptäcks allt eftersom. En av de gener som senast verifierades som en orsak till PPGL är *HRAS*-genen som vi därför undersökte i en stor kohort i studie fyra. Vi använde också en metod där uttrycket av ca 29000 gener kunde mätas och jämförde detta med det mutationsdata vi hade fått fram. Den totala frekvensen av *HRAS*-mutationer i vår och alla tidigare publicerade artiklar som berört ämnet, beräknades till 5,2%. Det konstaterades också att de PPGL som hade *HRAS*-mutation samlades tillsammans med *NF1*- och *RET*-mutanterna i ett kluster med liknande genuttrycksprofil associerade med aktiv kinas-signalering.

Då vi i studie två analyserat alla då kända sjukdomsorsakande gener, kunde vi välja ut 15 PCC där ingen mutation kunnat identifieras för att undersöka alla geners kodande regioner (hel-exomsekvensering) i dessa tumörer. Syftet med den femte studien var att upptäcka eventuella nya gener som är involverade i utvecklingen av PCC. Vi fann att *KMT2D,* en gen som kodar för ett så kallat metyltranferas var upprepat muterad i tumörerna, och tillsammans med resultaten av en riktad *KMT2D*-screening bland PCC, upptäckte vi en mutationsfrekvens på 14%. I uppföljande experiment kunde vi därefter konstatera att en störd reglering av metyltransferaser kan vara en ny potentiell sjukdomsmekanism för PPGL.

I den sjätte studien undersökte vi om det fanns PGL som också hade mutationer i *KMT2D*-genen. Med hjälp av både hel-exomsekvensering och riktad *KMT2D*-sekvensering konstaterade vi att inget av de 13 undersökta PGL hade någon mutation. Däremot fann vi att genen var överuttryckt i PGL jämfört med normala binjurar och föreslår därför en onormal reglering av metyltransferaser även i PGL.

Sammanfattningsvis har avhandlingens sex ingående studier givit en ökad förståelse för den genetiska och molekylära bakgrunden till PPGL. Resultaten kan därför i framtiden bidra till bättre diagnosticering, prognos och utveckling av behandlingsalternativ för patienterna som drabbats av denna sjukdom.

LIST OF PUBLICATIONS

This thesis is based on the following papers which are referred to in the text by their Roman numerals (I-VI).

I **Andreasson A***, Kiss NB, Caramuta S, Sulaiman L, Svahn F, Bäckdahl M, Höög A, Juhlin CC, Larsson C. (2013) The *VHL* gene is epigenetically inactivated in pheochromocytomas and abdominal paragangliomas.
Epigenetics. **8,** 1347–54.

II Welander J#*, **Andreasson A#**, Juhlin CC, Wiseman RW, Bäckdahl M, Höög A, Larsson C*, Gimm O, Söderkvist P. (2014) Rare germ-line mutations identified by targeted next-generation sequencing of susceptibility genes in pheochromocytoma and paraganglioma.
Journal of Clinical Endocrinology and Metabolism. **99,** 1352-60.

III **Stenman A#**, Svahn F#, Welander J, Gustavson B, Söderkvist P, Gimm O, Juhlin CC*. (2015) Immunohistochemical NF1 analysis does not predict *NF1* gene mutation status in pheochromocytoma.
Endocrine Pathology. **26,** 9-14.

IV **Stenman A*,** Welander J, Gustavsson I, Brunaud L, Bäckdahl M, Söderkvist P, Gimm O, Juhlin CC, Larsson C. (2015) *HRAS* mutation prevalence and associated expression patterns in pheochromocytoma.
Submitted for publication.

V Juhlin CC*, **Stenman A**, Haglund F, Clark V, Brown T, Baranoski J, Bilguvar K, Goh G, Welander J, Svahn F, Rubinstein JC, Caramuta S, Yasuno K, Günel M, Bäckdahl M, Gimm O, Söderkvist P, Prasad ML, Korah R, Lifton RP, Carling T*. (2015) Whole-exome sequencing defines the mutational landscape of pheochromocytoma and identifies *KMT2D* as a recurrently mutated gene.
Genes Chromosomes and Cancer. **54,** 542-54.

VI **Stenman A**#*, Juhlin CC#, Haglund F, Brown TC, Clark VE, Svahn F, Bilguvar K, Goh G, Korah R, Lifton RP, Carling T*. (2015) Absence of *KMT2D/MLL2* mutations in abdominal paraganglioma.
Clinical Endocrinology (oxf). Accepted 19 Aug, E-pub ahead of print.

# - authors contributed equally

* - corresponding author

SELECTION OF RELATED PUBLICATIONS

This is an excerpt of related publications by the author containing supporting results.

Original articles

Haglund F, **Andreasson A**, Nilsson I-L, Höög A, Larsson C, Juhlin CC. (2010) Lack of S37A CTNNB1/β-catenin mutations in a Swedish cohort of 98 parathyroid adenomas.
Clin Endocrinol (Oxf). **73,** 552–3.

Andreasson A, Sulaiman L, do Vale S, Martins JM, Ferreira F, Miltenberger-Miltenyi G, Batista L, Haglund F, Björck E, Nilsson I-L, Höög A, Larsson C, Juhlin CC. (2012) Molecular characterization of parathyroid tumors from two patients with hereditary colorectal cancer syndromes.
Fam Cancer. **11,** 355–62.

Kiss NB, Muth A, **Andreasson A**, Juhlin CC, Geli J, Bäckdahl M, Höög A, Wängberg B, Nilsson O, Ahlman H, Larsson C. (2013) Acquired hypermethylation of the P16INK4A promoter in abdominal paraganglioma: relation to adverse tumor phenotype and predisposing mutation.
Endocr Relat Cancer. **20,** 65–78.

Andreasson A, Kiss NB, Juhlin CC, Höög A. (2013) Long-term storage of endocrine tissues At -80°C does not adversely affect RNA quality or overall histomorphology.
Biopreservation and Biobanking. **11,** 366–70.

Liu T, Brown TC, Juhlin CC, **Andreasson A**, Wang N, Bäckdahl M, Healy JM, Prasad ML, Korah R, Carling T, Xu D, Larsson C. (2014) The activating TERT promoter mutation C228T is recurrent in subsets of adrenal tumors.
Endocr Relat Cancer. **21,** 427–34.

Welander J, **Andreasson A**, Brauckhoff M, Bäckdahl M, Larsson C, Gimm O, Söderkvist P. (2014) Frequent *EPAS1/HIF2A* exon 9 and 12 mutations in non-familial pheochromocytoma.
Endocr Relat Cancer. **21,** 495-504.

Paulsson JO, Svahn FS, Welander J, Brunaud L, Söderkvist P, Gimm O, **Stenman A**, Juhlin CC. (2015) Absence of the *BRAF* V600E mutation in pheochromocytoma.
J Endocrinol Invest. Accepted for publication.

Review article

Fridegren J, **Andreasson A**, Wallin C. (2014) Pheochromocytoma can easily be missed in an unusual symptomatology. Hypotension and syncope were interpreted as orthostatism.
Läkartidningen. **111,** 340–1.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

18F-FDG	2-deoxy-2-[fluorine-18]fluoro-D-glucose
4EBP1	Eukaryotic translation initiation factor 4E binding protein 1
A	Epinephrine (adrenaline)
AAD	Aromatic amino acid decarboxylase
AC	Adrenal cortex
AM	Adrenal medulla
ABC	Avidin and biotin complex method
AFIP	Armed Forces Institute of Pathology
AKT	Protein kinase B
ALT	Alternative lengthening of telomeres
ATRX	Alpha thalassemia/mental retardation syndrome X-linked
B2M	β-2-microglobulin
BAP1	BRCA1-associated protein-1
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BRCA1	Breast cancer 1, early onset
C	Cytosine
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator gene
CGH	Comparative genomic hybridization
CH_3	Methyl group
CHASM	Cancer-specific high-throughput annotation of somatic mutations
ChrA	Chromogranin A immunostaining
CIMP	CpG island hypermethylator phenotype
CN-LOH	Copy neutral loss of heterozygosity
COMT	Catecholamine O-methyltransferase
CONDEL	Consensus Deleteriousness
COSMIC	Catalogue of Somatic Mutations in Cancer
CT	Computed tomography
CpG	Cytosine-phosphate-Guanine
DAB	3,3'-Diaminobenzidine
DBH	Dopamine beta hydroxylase
DNA	Deoxyribonucleic acid
EGLN1, EGLN2	egl-9 family hypoxia-inducible factor 1, 2
ENS@T	European Network for the Study of Adrenal Tumors
EPAS1	See HIF2α
EPO	Erythropoietin
ETC	Electron transfer chain
FGFR1	Fibroblast growth factor receptor 1

FH	Fumarate hydratase
FISH	Fluorescence *in situ* hybridization
GFR	Growth factor tyrosine receptors
GOF	Gain of function
H&E	Haematoxylin and eosin stain
H_2O_2	Hydrogen peroxide
H3K4me3	Histone 3 lysine 4 trimethylation
H3K27me3	Histone 3 lysine 27 trimethylation
HED	(11)C-hydroxyephedrine
HIF	Hypoxia-inducible factor
HIF2α	Hypoxia-inducible factor 2, subunit alfa
HRAS	Harvey rat sarcoma viral oncogene homolog
HRP	Horseradish peroxidase
HTX	Haematoxylin
IDH1	Isocitrate dehydrogenase 1
IHC	Immunohistochemistry
IMA	Inferior mesenteric artery (a. mesenterica inferior)
KI	Karolinska Institutet, Stockholm, Sweden
KIF1Bβ	Kinesin Family Member 1B subunit beta
KMT2D	Lysine (K)-specific methyltransferase 2D
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
MAX	MYC-associated factor X
MDH2	Malate dehydrogenase 2
MEN1	Multiple endocrine neoplasia type 1
MEN2	Multiple endocrine neoplasia type 2
MET	MET Proto-Oncogene
MGMT	O(6)-methylguanine-DNA methyltransferase
MIQE	Minimum Information for Publication of Quantitative Digital PCR Experiments
MLL2	Mixed-lineage leukemia protein 2
MRI	Magnetic resonance imaging
MXD1	MAD-MAX dimerization protein
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin/mammalian target of rapamycin
MYC	c-MYC
NA	Norepinephrine (noradrenaline)
NCBI	National Center for Biotechnology Information
NF1	Neurofibromatosis type 1
NGS	Next generation sequencing
PCC	Pheochromocytoma
PCR	Polymerase chain reaction

PHD	Prolyl hydroxylase
PET/CT	Positron emission tomography – computed tomography
PGL	Abdominal paraganglioma
PHD1, PHD2	Prolyl hydroxylase domain-containing protein 1, 2
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PNMT	Phenylethanolamine N-methyltransferase
PolyPhen2	Polymorphism Phenotyping version 2
PPGL	Pheochromocytoma and abdominal paraganglioma
RAF	RAF proto-oncogene
RAS	Ras superfamily
RET	Rearranged during transfection
RNA	Ribonucleic acid
qRT-PCR	Quantitative real-time polymerase chain reaction
SDH	Succinate dehydrogenase
SDHx	SDHA, SDHB, SDHC, SDHD, SDHAF1, SDHAF2/SDH5
SIFT	Sorting intolerant from tolerant
SNP	Single nucleotide polymorphism
SYP	Synaptophysin immunpstaining
T	Thymidine
TCA	Tricarboxylic acid cycle / Krebs cycle
TCGA	The Cancer Genome Atlas
TERT	Telomerase reverse transcriptase
TH	Tyrosine hydroxylase
TMEM127	Transmembrane protein 127
TMZ	Temozolomide
TSC2	Tuberous sclerosis complex 2 (Tuberin)
TSG	Tumor suppressor gene
vHL	von Hippel-Lindau disease / von Hippel-Lindau protein
WES	Whole-exome sequencing
WHO	World health organization
ZF	Zona fasciculata of the adrenal cortex
ZR	Zona reticularis of the adrenal cortex

INTRODUCTION

THE NORMAL ADRENAL GLAND AND PARAGANGLIA

Historical perspective

"Nature makes, nor does nothing in vain or inconsiderately, much less does she appoint a noble animated part, only to fill a space, which she might have filled by making the kidney a little bigger." – Thomas Bartholin 1668 in a response to Adriaan van den Spiegel who believed that the only function of the adrenal glands was to occupy the space between the kidneys and the diaphragm (1,2).

The first description of the adrenal glands may be found in the Bible. In the context of guilt offering, the King James translation of Leviticus 7:4 reads: "And the two kidneys, and the fat that is on them, which is by the flanks, and the caul that is above the liver, with the kidneys, it shall he take away". It can be argued that the fat on the kidneys described here refers to the adrenal glands, but the location - by the flanks and the word for fat make this statement somewhat dubious. However, the first detailed illustrations and less debatable description of the human adrenal glands can be found at copper plates made by Bartholomeo Eustachio in 1552 (Figure 1) ((3), reviewed in (2)). He labeled these glands "glandulae renibus incumbentes" which is translated to "glands lying on the kidneys". With this, Eustachio disputed the famous anatomist Andreas Vesalius who had neglected to describe adrenal glands in his reports of dog kidneys (4). The work "Opuscula Anatomicae" by Eustachio was forgotten in the archives of the Vatican library until 1714 when the physician of Pope Clement XI, Lancisius Giovanni Maria discovered and republished them ((5), reviewed in (1)) as he appreciated the correctness of the plates. Furthermore, Eustachio was able to describe additional adrenal tissue close to the aortic bifurcation (4). This extra-adrenal tissue is now known as the organ of Zuckerkandl and can be found close to the inferior mesenteric artery or at the aortic bifurcation (Figure 2).

In the beginning of the 19th century, Albert von Kölliker was able to make a distinction between the adrenal cortex and medulla and also described the histological and physiological features of the glands, using a microscope with achromatic lenses ((6), reviewed in (1)). In 1855, the English physician Thomas Addison observed and described a rare and fatal disease which was characterized by skin pigmentation, weakness and cardiac insufficiency. The disease was later named after him since he understood that the effects of the disease were related to the "suprarenal capsule" ((7), reviewed in (1)). The finding of norepinephrine (NA) as a secretory product from the adrenal medulla was an important break-through in the adrenomedullary research and many researchers were involved in the discovery. A notable researcher in this area was the 1970 Nobel Prize laureate, Ulf Svante von Euler at KI who was recognized for his discoveries of prostaglandin in 1935, piperidine in 1942 and norepinephrine in 1946 (2,8).

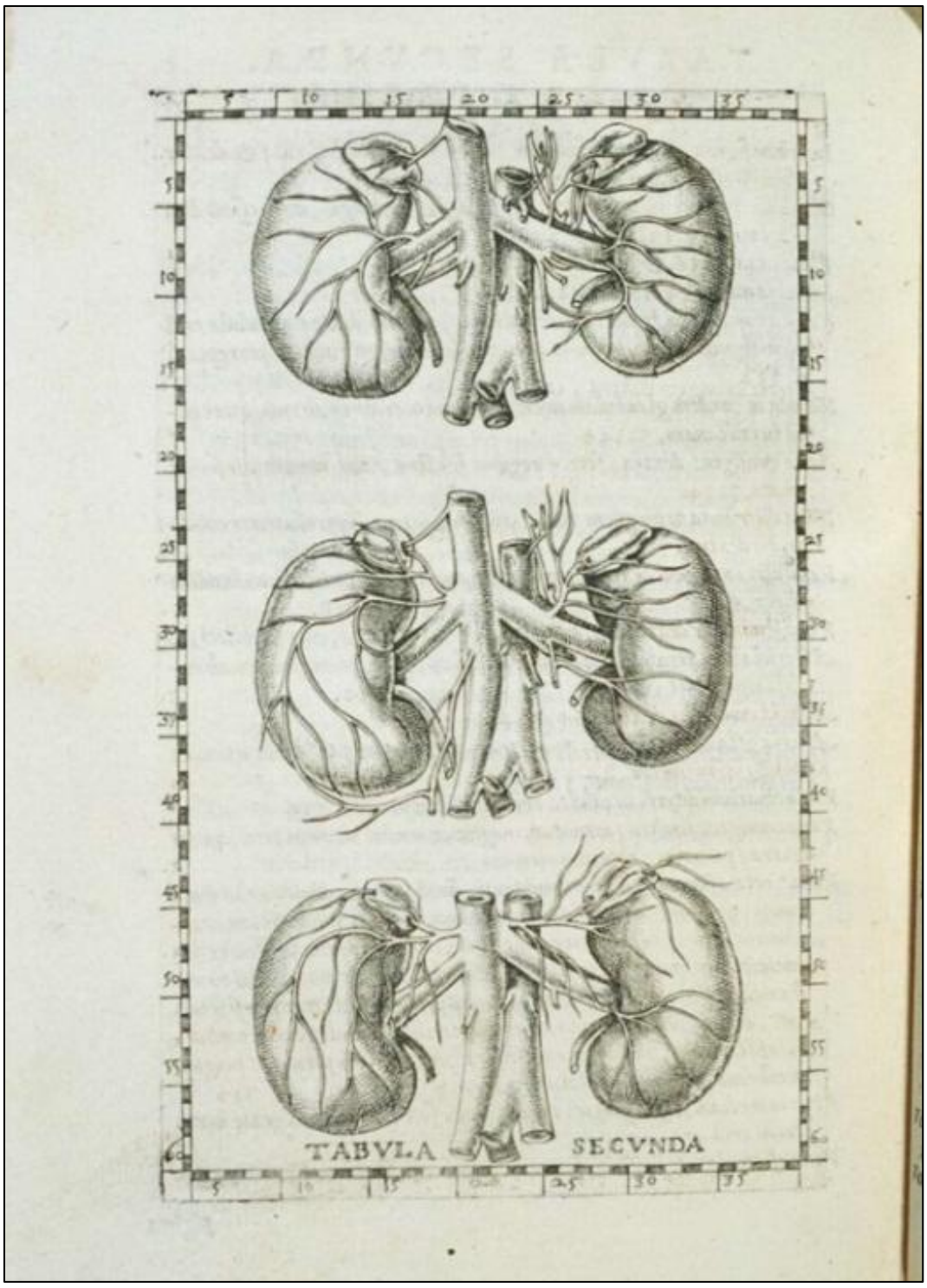

Figure 1.

The first description of the adrenal gland.

Photocopy of the original copper plate (Tabulae II) of Bartholomeo Eustachio from 1552 in Opuscula Anatomicae, published by Lancisius in 1714 and by Tagliani in 2009 (5,1). The figure is republished with permission.

Anatomy, histology and physiology of the adrenals

The adrenal glands are triangular-shaped endocrine organs, normally located on top of the kidneys in the retroperitoneal space (Figure 2). The name adrenal comes from the Latin *ad* meaning "near/close to" and *ren* meaning kidney. They usually weigh 5 grams and are composed of the adrenal cortex and the adrenal medulla (9). The cortex consists of three layers; zona glomerulosa, zona fasciculata (ZF) and zona reticularis (ZR) with important endocrine functions as they produce and secrete aldosterone, cortisol and androgens respectively (Figure 3). The adrenal medulla (AM), as of special interest in this thesis, weighs on average 0.43 grams each (9) and its main functional component is the chromaffin cells that produce the catecholamines norepinephrine/noradrenaline (NA), epinephrine/adrenaline (A) and dopamine (DA) (Figures 3 and 4). Catecholamines are peptide hormones synthesized from the amino acid tyrosine through L-dopa to dopamine. The enzyme dopamine beta hydroxylase (DBH) converts dopamine to NA and phenylethanolamine N-methyltransferase (PNMT) converts NA to A (10,11) (Figure 4). Circulating catecholamines excite fight-or-flight responses in the body with visceral manifestations including for instance affection of blood pressure through regulation of cardiac output and peripheral vessel resistance. Other manifestations are tachycardia, affection of muscle tonus, fatty acid metabolism and sweating (10,12). The adrenal medulla and the paraganglia[1] are parts of the sympathetic nervous system and are located in the adrenal glands and lengthwise the aorta (Figure 2) (13,14).

[1] The word ganglia refers to sympathetic nerve cell clusters.

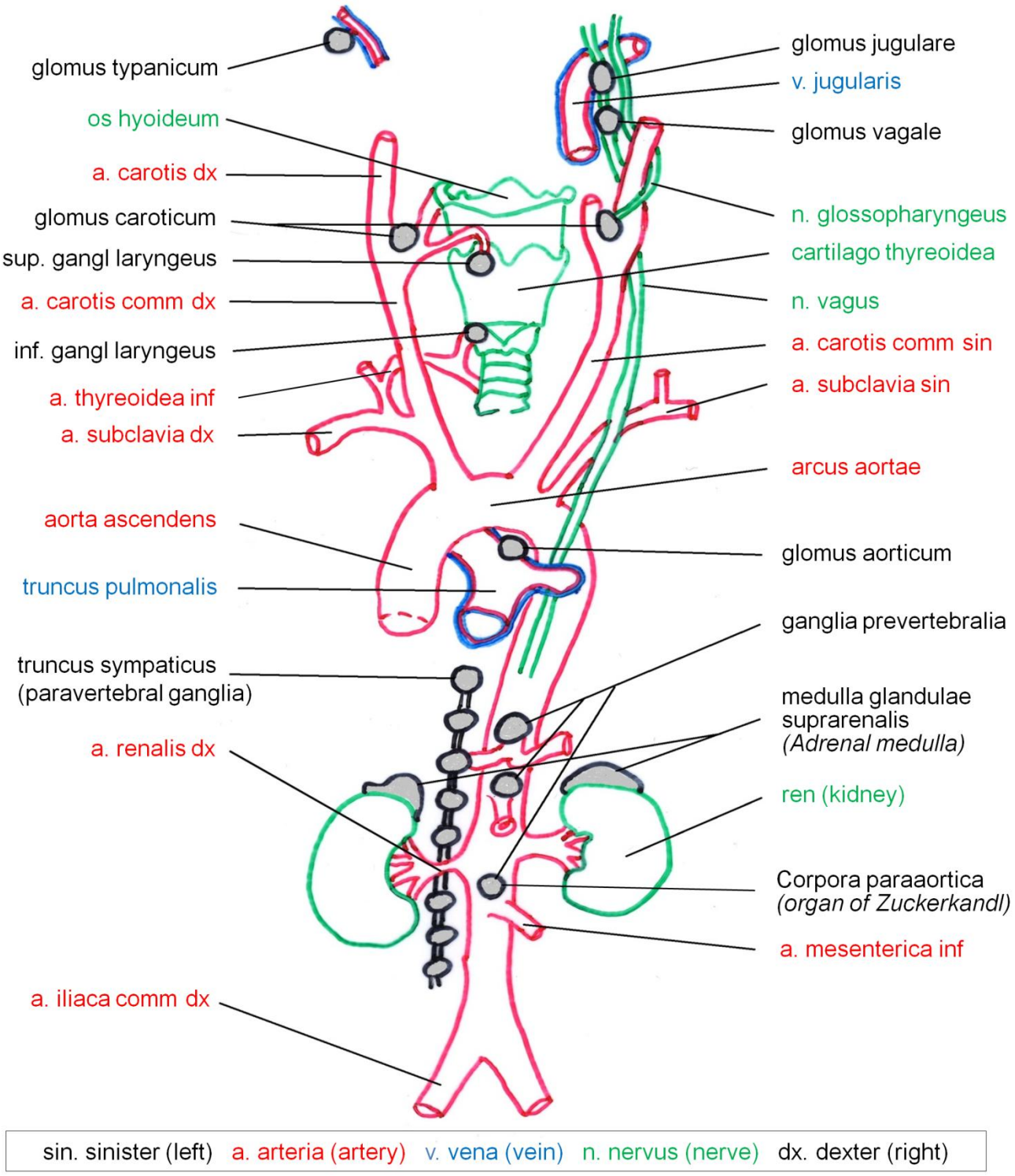

Figure 2.

Anatomical outline of human chromaffin cells and its adjacent tissues.

Chromaffin cells (black text) are derived from the embryological neuroectoderm within the paraganglia and the medulla of the adrenal glands. The organ of Zuckerkandl is also called corpora paraaortica and is one of the ganglia prevertebralia which can be located at the origin of IMA (as in this figure) or closer to the aortic bifurcation. The figure is inspired by and modified from Lips *et al*. (14).

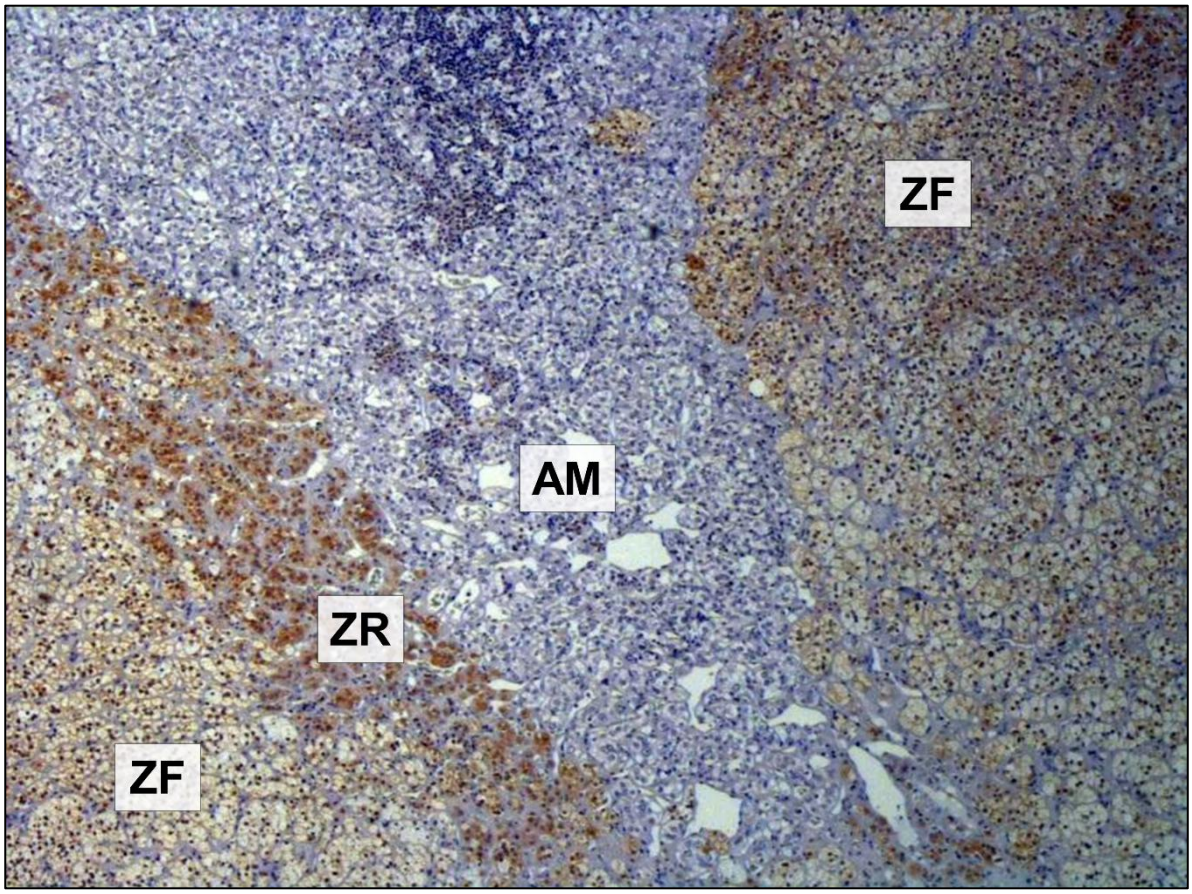

Figure 3.

Immunohistochemical picture of a normal adrenal gland.

The slide is stained with an anti-NF1 antibody (1:100) showing strong immunoreactivity in zona fasciculata (ZF) and zona reticularis (ZR) of the adrenal cortex as well as absent staining of the adrenal medulla (AM). The image is magnified x40 and adopted and modified from the supplementary material of Paper III.

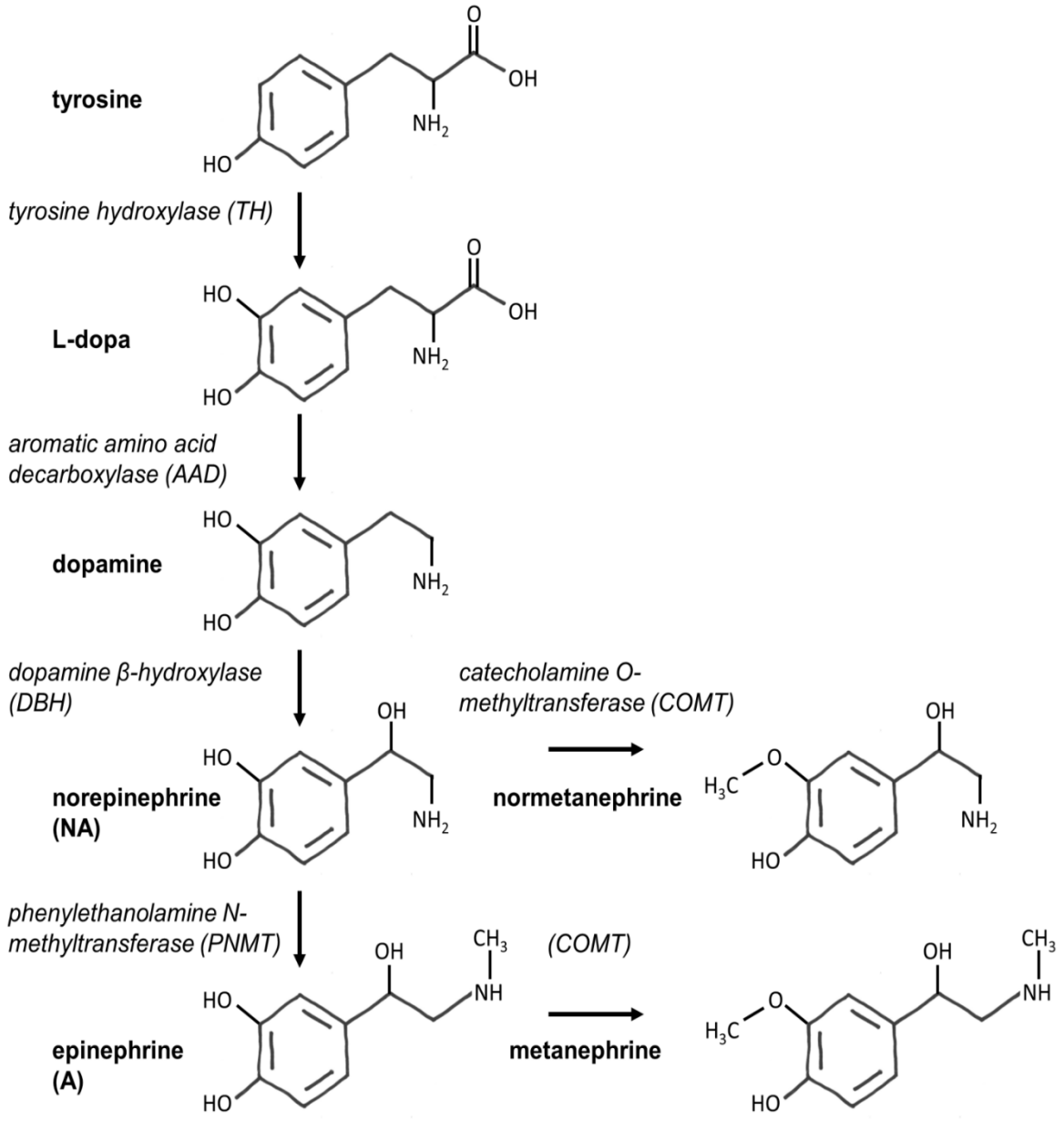

Figure 4.

The synthesis of catecholamines and metanephrines.

Arrows indicating the chemical reactions resulting in conversions between the molecules, catalyzed by the enzymes entitled in italics adjacent to the arrow. All molecules contain a phenol (C_6H_5OH) ring. Tyrosine is also a precursor for the hormone thyroxine of the thyroid gland and the pigment melanin.

CANCER AND ITS GENETIC AND MOLECULAR BACKGROUND

Nomenclature

"In reality, a mutually agreeable definition of cancer is not possible." – Douglas Green and Gerard Evan, 2002 (15).

All cancers are diseases of abnormal gene function, but only a minority (5-10%) is hereditary[2]. Abnormal gene function is due to one or more genetic and/or epigenetic aberration(s) with reference to the healthy (normal) genome. The human genome is coded by four different nucleotides[3] linked into a double stranded chain with a total length of approximately 3 billion base pairs (16) equals 2-3 meters if stretched out (17), or 3 Gigabytes of information (18). Fortunately, via histones the DNA is structured into 23 chromosomes that are located in almost[4] every cell nucleus in the human body. The information of the dynamic DNA molecule (*genome*) is divided into exons and introns where a large proportion of the DNA is transcribed to RNA (*transcriptome*). Only a small fraction of the RNA is subsequently translated into proteins and the assembly of proteins coded from DNA via mRNA is often referred to as the central dogma. Which genes that are expressed differ from cell to cell and changes over time, regularly via epigenetic variations (*methylome*). A gene often contains both exons and introns and can be of different length, function and organization.

A tumor is a cell mass that grows in an uncontrolled manner and can be either benign (grow locally within a capsule) or malignant (invade neighboring tissues and/or metastasize). The minority of malignant tumors are called cancers. Highly malignant cancers have lost many of the abilities of their fully differentiated normal precursor cell, making them "undifferentiated". (19–22).

Cancer genetics

Genetic aberrations can be acquired (sporadic tumors) and caused by environmental factors[5] that causes damage to the genes, or constitutional/germ-line (hereditary tumors) that are typically passed from a parent to a child. One single genetic abnormality alone is rarely enough for the development of malignant disease, and cancer is therefore often described as a multistep process of several alterations in one or more of different classes of genes (24).

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[2] As mentioned under "etiology"; PPGL carry the highest level of heritability of all human tumors (25-40%).

[3] Nucleobases of the human DNA: Adenine, Guanine, Cytosine and Thymidine.

[4] Some cells of the human body do not harbor a nucleus. Erythrocytes are the most common example and consequentially do not contain any DNA.

[5] The main environmental factors that can damage DNA and contribute to cancer include cigarette smoking, diet, infections and radiation (23).

During the development of a tumor, its cells progressively obtain features that give them growth-, survival- and spreading advantages over fully differentiated normal cells[6]. The multistep process can be seen as an evolutionary process within the cancer cell and often takes place during years or decades. These advantages or "hallmarks" of a full blown cancer with metastatic potential have been well described by Hanahan *et al.* (19,20) and include ten capabilities of the cancer cell as visualized in Figure 5; ability to escape cell death, independence of growth signals, desensitization of anti-growth signals, unlimited potential to replicate, continued angiogenesis and the capability to metastasize and invade other tissues (19), deregulation of cellular energetics[7] (glycolysis, aerobic oxidation), escaping immune destruction, tumor-promoting inflammation and genome instability and mutations (often in DNA repair mechanisms) (20). The genetic alterations that could give rise to these capabilities of the cancer cell (driver events) include somatic and/or constitutional mutations in key genes (oncogenes, tumor suppressor genes and DNA repair genes), chromosomal losses, gains or rearrangements (19,20,24,26), and/or epigenetic modifications (DNA methylation and/or histone alterations) (27).

In addition, during the development of a cancer cell, multiple genome aberrations occur that have no obvious driver role. These are regarded as passenger events. A passenger event has by its definition "no direct or indirect effect on the selective growth advantage of the cell" (28) but could hypothetically contribute to genetic instability which in turn could lead to other alterations of importance. Driver mutations are often defined based on the recurrence rate and existence of hotspot mutations[8]. To be able to find out whether a non-hotspot mutation is biologically relevant, functional assays often need to be performed (29). Besides functional assays, computational methods (*in silico*) can be used where different software algorithms calculate the probability of an amino acid change to have biological implications (30). In Papers II and V (31,32), three different algorithms has been used to predict the pathogenicity of the novel variants/mutations found; PolyPhen2 (33) (Paper II and V), MutationTaster (34) (Paper II) and CHASM (35) (Paper V), but several other prediction algorithms are frequently used, such as SIFT (36) and Condel (37) to name a few. The main difference between the algorithms used in Paper II and V is that MutationTaster can predict the pathogenicity of suspected splice-site mutations while the CHASM algorithm uses a class of driver mutations obtained from COSMIC and a class of cancer specific passenger mutations and gives a scored based on a statistical comparison. Finally, Polyphen2 predicts the impact of amino acid substitutions by physical and comparative considerations.

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[6] The acquiring of capabilities of a cancer cell can be likened to Darwinian evolution where an obtained capability might favor in the natural selection (22).

[7] The cancer cell characteristic of deregulation of cellular energetic has been described in the follow-up review by Hanahan et al 2011 (20), but was originally described in *Science* in 1956 by Otto Warburg (25) who found that cancer cells can limit their need of energy metabolism (referred to as the Warburg effect).

[8] A mutational hotspot is a region where mutations are observed with higher frequency.

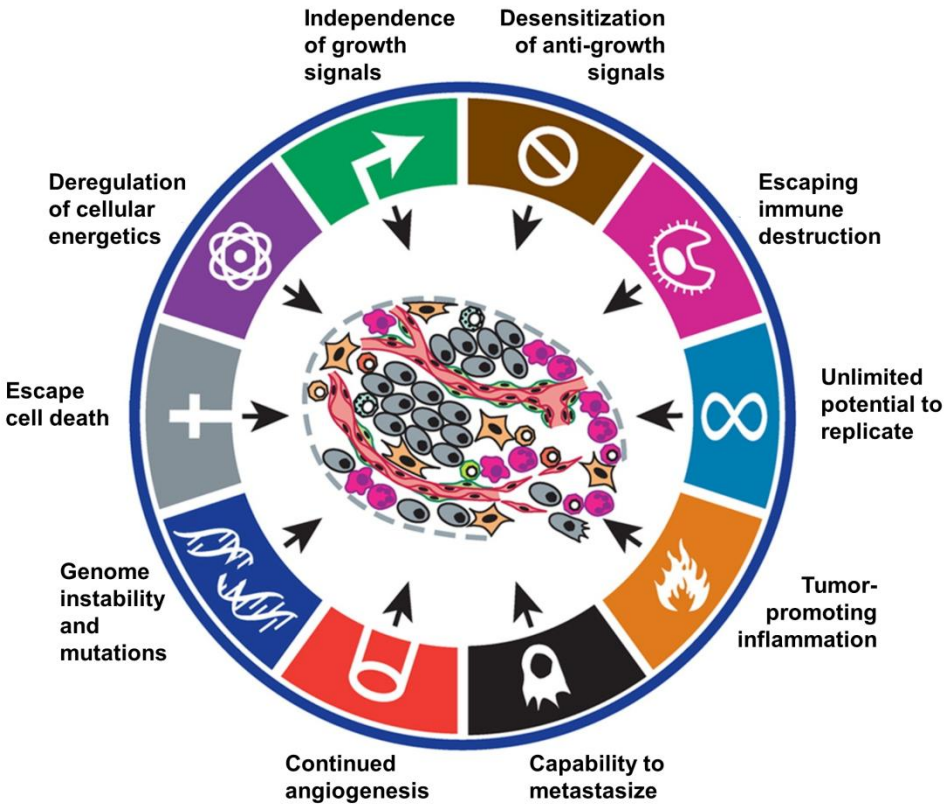

Figure 5.

Ten hallmarks of cancer.

The figure is adopted and modified from Hanahan and Weinberg 2011 (20) and reprinted with permission under personal Elsevier user license.

Tumor biology and driver mutations

The human body consists of around $4x10^{13}$ cells (38) which constantly grow, divide and die in a balanced process during the life of the individual. The life span of the cell is regulated in the cell cycle and the apoptotic process (programmed cell death) attempts equilibrium between dividing/proliferating and dying/apoptotic cells (39). Somatic cells are proliferating through mitosis when they duplicate their DNA which could be completed in a couple of hours. In the cell cycle, where these processes are controlled, a number of phases and checkpoints are present when the DNA is controlled for errors. If any inaccuracies are present, the cell will be prompted to undergo apoptosis. Any disruption in the various aspects of the cell cycle could be critical for the cell and might lead to cancer (40).

Genetic alterations can be divided into mutations at the DNA level and structural translocations and losses or gains at the chromosomal level. Loss of heterozygosity (LOH) means that one of the two constitutional DNA copies is lost which can provide the "second hit" of Knudson two-hit hypothesis[9] (42) (Figure 6). Importantly, LOH may be copy neutral (CN-LOH) meaning that no net change in the copy number[10] occurs e.g. by duplication of the remaining copy. CN-LOH cannot be detected by regular comparative genomic hybridization (CGH) techniques or qRT-PCR, instead FISH or SNP-array based techniques are required (45,46).

Mutations in its turn may be divided into single nucleotide base substitutions[11], deletions and insertions where the last two mutation types often lead to a frameshift mutation (28). Deletions and insertions can also affect a triplet of base pairs resulting in an addition or loss of one amino acid in the protein (*in-frame*). Mutations can also be silent or synonymous meaning that they do not change the amino acid sequence of the protein, or change an amino acid to another that harbor similar properties as the original. In the same way as passenger mutations can lead to genetic instability and through that cause other aberrancies, also silent or synonymous mutations have been shown to be able to act in a similar manner (47). An example of this has been shown with a synonymous mutation in the *CFTR* gene that causes an aberrant mRNA splicing in a patient with cystic fibrosis (48). The mutational driver events that contribute to tumor development most often target three main classes of genes, previously referred to as tumor suppressor genes (TSG), oncogenes and DNA repair genes. Tumor suppressors are normally antagonizing cell growth, proliferation and survival while oncogenes support the same processes. DNA repair genes are coding for proteins that are able to restore errors in replication and breaks in the DNA strand, often within the process of the cell cycle when the cell is about to proliferate. In order for an oncogene to cause a tumor-promoting effect on the cell, a gain-of-function mutation (GOF) is normally needed to cause over-activation of the gene (49). For most DNA repair genes and some tumor suppressor genes, biallelic inactivating mutations in accordance with Knudson´s two hit hypothesis is usually seen and needed for the gene to lose its tumor developmental breaking effect (26,41,42) (Figure 6).

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[9] Since a tumor suppressor gene is represented on two alleles of the chromosomes, Knudson´s two hit theory explains that both copies of the gene need to be inactivated in order for the gene to lose its function (41,42). However, some genes do need two intact alleles for proper function and those *haploinsufficient* genes can contribute to tumor development with only one allele inactivated (43,44) (Figure 6E and F). Tumor suppressor genes (TSGs) are often haploinsufficient.

[10] Copy number refers to the quantity of alleles at a specific genomic site.

[11] Further functional categorization of single nucleotide substitutions divides them into nonsense-, missense or silent mutations. Nonsense mutations code for a stop, often resulting in a truncation of the protein. Missense mutations code for an altered amino acid and silent mutations code for the same amino acid resulting in no protein alteration.

Epigenetics

The latin word *epi* means above and refers to something that happens above or on top of the DNA and without modification of the underlying nucleotides (Figure 6). Epigenetic mechanisms represent the control machinery that determines gene expression and its activity in the cell. The epigenetic machinery is highly involved in the cell differentiation processes that produce diverse subpopulations of cells from stem cells. If the machinery in some way is disrupted or dysregulated, it can contribute to tumor development in general (27), and to the development of PPGL (50–54). Epigenetic modifications can be divided into two different classes of modification mechanisms; histone regulation and DNA methylation.

Small octamer proteins called histones that provide efficient packing of DNA, also play an important role in gene regulation via acetylation, methylation and/or phosphorylation at different sites. Here, histone acetylation is the most described modification which is generally associated with activation of gene transcription. Cancers have been shown to display changes in their histone modifying enzymes compared to normal cells. (55,56)

DNA methylation is a well described mechanism for gene expression control. Anywhere throughout the genome where a cytosine nucleobase is followed by a guanine nucleobase (a CpG dinucleotide) the DNA can be methylated when a methyl group (CH_3) covalently binds to the cytosine residue. Particularly CpG rich regions along the genome is referred to as CpG islands and are often located within regulatory elements such as promoter regions of several genes. It is commonly observed that cancer cells lose their normal levels of methylation across the genome which could contribute to genetic instability and to tumor development (27). Both hypo- and hypermethylation can be seen. Hypermethylation[12] of CpG islands within TSG promoter areas can provide the second hit of Knudson two-hit hypothesis and hence contribute to tumor development (27,44,58) (Figure 6). Moreover, gene- and histone methylation is involved in the phenomenon of genomic imprinting[13], whereby the expression from a particular gene occurs from a single allele (61,63).

[12] Promoter hypermethylation is an increase in methylation, often defined as >10% of an average of CpG residue methylation at a specific site (57), but the cutoff can vary between genes dependent of the silencing effect and intrinsic methylation levels (52).

[13] All somatic cells contain two copies (alleles) of the genome. These are inherited with one copy from each parent. Through epigenetic processes, a hypothesis of "parental conflict" (59) have made approximately 1% (or more) of all genes in mammals "imprinted" (60,61), resulting in a specific mono-allelic gene expression from only one of the parents. On the other hand, loss of imprinting of specific genes could result in diseases, such as syndromes of Prader-Willi/Angelman, Beckwith-Wiedemann or Silver-Russell (62).
Two PPGL-related examples are the maternally imprinted *SDHD* and *SDHAF2* genes that give significantly different PPGL penetrance depending on if the mutations are paternally or maternally inherited (Table 1).

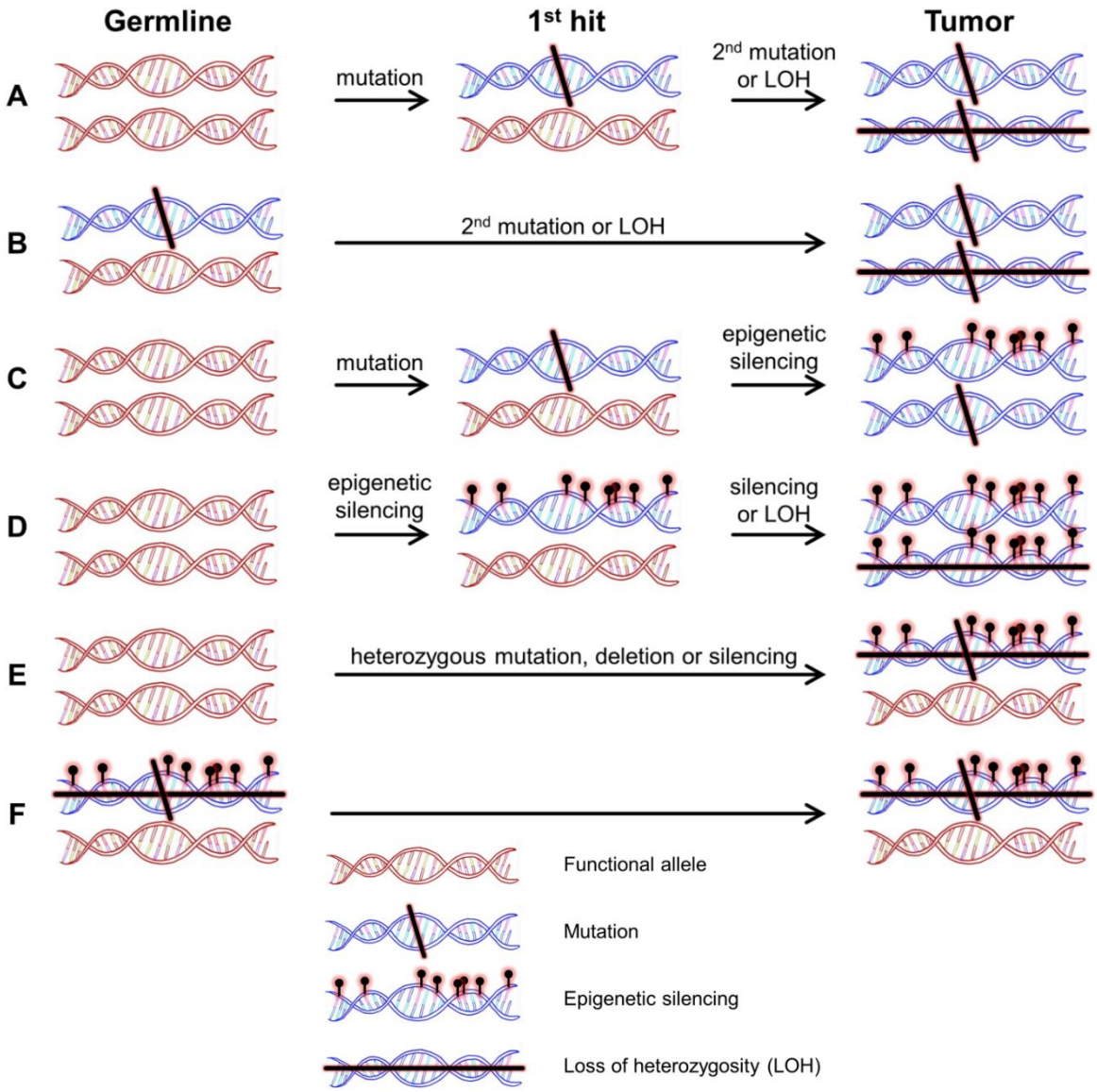

Figure 6. Genetic inactivation and the Knudson two hit model.
The left column indicates 6 germ-line allele scenarios (A-F) of a specific gene. A blue DNA spiral indicate inactivated alleles and red spirals are functional alleles. The middle column shows a 1st hit and inactivation of one allele in 3 different situations (A, C and D). The right column shows possible outcomes of the gene alleles in tumor development.
A and B: shows the Knudson two-hit model when an initial mutation leads to complete gene inactivation during tumor development.
C and D: describes two different situations of complete gene inactivation from healthy germ-line DNA when in C, the mutation is followed by gene silencing through an epigenetic event (histone acetylation or gene promoter methylation) and in D, an initial event of epigenetic silencing is followed by another event of either LOH or another epigenetically silenced allele.
E and F: describes that haploinsufficient genes (often TSGs) can contribute to tumor development with only one allele inactivated either due to a somatic (E) or a germ-line/inherited event (F). The figure is inspired by and modified from Balmain et al (44).

PHEOCHROMOCYTOMA AND PARAGANGLIOMA

"Despite being predominantly benign tumours, pheochromocytomas and paragangliomas have substantially advanced our understanding of cancer biology." – Patricia L.M. Dahia, 2014 (64).

Due to the excessive secretion of fight-and-flight hormones, the dramatic clinical presentation of pheochromocytomas and abdominal paragangliomas has fascinated physicians and researchers for ages. The disease is rare, with an annual incidence estimated to 0.8 in 100,000 person-years (65,66) but it is nevertheless a particularly well-described disease. The term pheochromocytoma was first used in 1912 by Ludwig Pick after Heinrich Poll a few years earlier had introduced the term "pheochrome" to describe the dark color produced during the "chromaffin reaction" when tumor tissue was immersed in chromium salts. The chromaffin cell got its name from this reaction by Alfred Kohn in 1902. (2)

Chromaffin cells in the adrenal medulla can give rise to pheochromocytomas (PCC). These neural-crest derived chromaffin cells can also be found extra-adrenally within the sympathetic ganglia and give rise to abdominal paragangliomas (PGL) (67) (Figure 2). Collectively PCC and PGL are abbreviated PPGL. Paragangliomas can, however, also occur in cells of non-chromaffin origin in supra-diaphragmatical chemoreceptors and in parasympathetic ganglia in the head and neck (Figure 2) (65). These neoplasms are usually unable to secrete catecholamines (68) and are often referred to in the literature as "head and neck paragangliomas". However, this entity will not be discussed further in this thesis. PPGLs occur in 2-5 patients per million per year (69,70), which gives approximately 55,000 new PPGL cases each year worldwide (71) although as many as 40% of PCC are only found at autopsy (65,66).

An outdated rule of 10 was for long time used as a traditional 'mnemonic'[14] for the disease (10% bilateral, 10% extra-adrenal, 10% familial, 10% malignant), but latter advances in genetics and diagnosis have challenged the rule and made it obsolete (73,74). The proportion of malignant cases differs between PCC (5-10%) and PGL (30-33%) and is even higher in PGLs with a mutation in the *SDHB*-gene (52,73,75). Bilateral tumors are more common within PCC-related tumor syndromes (MEN2, vHL) and the ratio of PGL probably represent at least 15% of adult and 30% of childhood chromaffin tumors (73,76). Finally, at least 25% of the tumors are hereditary (31,73,77–79). Hereditary forms of the disease are found in patients of younger age, but a vast overlap with sporadic tumors is seen in this group (74).

Clinical presentation

PPGL can be discovered in patients in several ways, *en passant* as incidentalomas (80) or due to symptoms of catecholamine release. It is also possible to discover them due to symptoms

[14] A memory technique that aims to translate information into a form that is easier for the brain to grasp and remember. Short poems, memorable phrases or acronyms are often used with favorable results regarding long-term memory (72).

of local growth (81), but they can also be asymptomatic and only be found at autopsy (66,73,82). The clinical presentation may be highly variable and can also mimic other clinical conditions. Generally PPGL produce large amounts of mainly NA and A, sometimes up to 27 times higher than normal (83). These excess levels may cause a wide array of paroxysmal symptoms with a classic triad of palpitations, headache and sweating that altogether can provide a specificity of 90% (84,85). The most common signs at presentation includes headaches, hypertension, tachycardia, diaphoresis (profuse sweating), pallor, anxiety and panic attacks (73,84). The estimated prevalence of PPGL in hypertensive patients ranges from 0.1 (86) to 0.6% (87–89). Unusual clinical manifestations of PPGL are hypotension, cardiac insufficiency and shock which could be explained by several coactive mechanisms (90–92) including less intravascular blood volume (73) sudden stop of catecholamine production due to tumor necrosis and/or desensitization of adrenergic receptors (93,94).

Diagnosis

Measurement of free metanephrines[15] in blood is today the best tool and golden standard method for both detection and exclusion of the disease (Figure 4) (96–98). PPGLs have been shown to express different catecholamine profiles dependent on their genetic background (95); epinephrine levels are often increased in patients with MEN2 (83) and NF1 syndromes as well as a mutation in the *TMEM127* gene, and decreased in tumors with *VHL* and *SDHx* mutations (tumors associated with genes in gene expression Cluster 1) (99). Dopamine levels are often increased in *SDHx* mutated tumors (100) while norepinephrine does not seem to be strongly correlated with the genetics of the tumor.

PPGL may also be detected through a range of different radiological modalities and imaging studies should be initiated when there is a biochemical evidence of PPGL (95). Computed tomography (CT) is recommended as first-choice modality for PPGL (95,101,102) and MRI is recommended if metastatic PPGL is suspected, if the patient harbor a known germ-line mutation and if the radiation exposure for the patient should be restricted (95). To detect metastatic lesions, 2-deoxy-2-[fluorine-18]fluoro-D-glucose integrated with computed tomography (18F-FDG PET/CT) may be used, preferably for suspected *SDHx*-mutated PGLs (103). The (11)C-hydroxyephedrine (HED) PET/CT has also been shown to demonstrate good sensitivity and specificity for PPGL (104).

The prevalence of malignancy in PPGL is fairly puzzling. It has long been cited as 10%, but has also been suggested to be between 5 – 26% depending on how malignancy is defined and underlying mutation (71,75,105,106) identified. As previously mentioned, 5-10% of PCC (107) and 30-33% of PGL are malignant (73). In an American cohort of 371 patients reported in 2010, 60% of PGL and 25% of PCC was found with metastatic disease (71). In addition,

[15] Metanephrines are metabolites of epinephrine/norepinephrine, specific markers of chromaffin tumors produced by the enzyme catecholamine *O*-methyltransferase (COMT). Normal sympathetic nerves lack this enzyme making these metabolites specific markers of chromaffin tumors. Moreover, the metabolites are produced and released independently of catecholamine release (Figure 4). (95)

like some other endocrine tumors, no molecular marker exists today for determining whether a PPGL is malignant. For the pathologist, the process of diagnosing PPGL therefore presents several challenges due to the difficulties of separating potentially malignant from benign cases. Since PPGL metastases have been seen up to 10-20 years after a "benign" diagnosis, lifelong follow-up is recommended. Currently, according to the WHO criteria, malignancy is defined only by the presence of (simultaneous) metastases (65). AFIP - another applied classification system for PPGL also identify metastatic disease as a diagnostic criteria for malignancy, but also recognize the prognostic value of histopathological features of the primary tumor such as local invasion, vascular invasion, mitotic activity and/or cellular atypia (13). Most often, malignant PPGL metastasize to lymph nodes, bone and lungs (64,105,108) but metastases have also been reported in the skin and breasts (105,109). Moreover, due to the location of the adrenal medulla and sympathetic trunk, adjacent organs like the liver, pancreas and kidneys may also be infiltrated (105).

Etiology

The genetic background of PPGL is remarkably diverse. Of all human tumors, PPGL carry the highest degree of heritability (25-40%) (64,73,78) and have therefore served as an important model to study general driver events for tumorigenesis. After year 2000, when Baysal *et al.* first identified an inherited mutation in a metabolic enzyme (*SDHD*) in a human tumor model (110), the list of PPGL susceptibility genes have been constantly growing. Heritable (constitutional) mutations in the *NF1*, *RET* and *VHL* genes have been known since 1990-1993 (111–114) and constituted the basis for the rule of 10% of hereditary tumors back then (full gene names are provided in the summary below). In 2006, six PPGL susceptibility genes were known (*NF1*, *RET*, *SDHB, SDHC, SDHD* and *VHL*) and the PCC researcher Patricia LM Dahia hypothesized that "*new pheochromocytoma susceptibility genes are likely to emerge in the next years.*" (115). After 2006 until today, some 10-18 more genes – hereditary and somatic - have been discovered and proved to be disease causing for PPGL (Table 1) and the list is still growing. Mutations in germ-line DNA associated with familial forms of PPGL currently include the following genes: *FH, EPAS1/HIF2α, MAX, NF1, RET, SDHA, SDHB, SDHC, SDHD, SDHAF2/SDH5, TMEM127* and *VHL*. In addition, a few other genes have been suggested to cause familial forms of PPGL including *BAP1, EGLN1/PHD2, KIF1Bβ, MDH2*, and *MEN1* (116) however these have only been reported in a few patients in a limited number of reports. Some of the above mentioned genes have also been reported to be mutated in sporadic PPGL[16], and in excess of these genes, mutations have been reported in the *ATRX, BRAF, FGFR1, HRAS, IDH1, KMT2D, MET* and *TERT* genes. In total, 25 genes are listed up to date, that in some way are associated with PPGL tumorigenesis. These genes and coupled tumor syndromes are briefly described below (alphabetical order) and summarized in Table 1.

[16] The *neurofibromin 1 (NF1)* gene is the most recurrently mutated gene in sporadic PPGL and was discovered both by Burnichon and Welander in 2012 (117,118).

Genes associated with hereditary predisposition for PPGL

BAP1

An inactivating germ-line mutation has been reported in the *BRCA1-associated protein-1 (BAP1)* gene in a Danish family with melanoma and a PGL. With only one mutation found in one single case, it remains unclear if the mutation drives the development of the PGL; however somatic loss of the wild-type allele in the PGL could support the association. (119)

BRAF

The common (in various cancers) activating missense mutation c.1799T>A in exon 15 in the *proto-oncogene B-RAF (BRAF)*, that leads to the alteration V600E has been reported in one single PCC (out of 85 screened PPGL) in one recent study (120). In PGL, a CpG island methylator phenotype (CIMP) has been shown to be associated with malignant behavior as well as young age at presentation (50). Therefore, another cohort of 53 PPGL had previously been screened for this mutation (50) since CIMP was shown to be linked to *BRAF* mutations in colorectal cancer (121). Additionally, in another study from our group that is accepted for publication (listed among the related publications), we screened 110 PCCs for the *BRAF* V600E mutation and found that all cases exhibited the wild-type sequence at that spot. Taken together, a total number of 361 PCCs have been screened for the *BRAF* V600E mutation to date, with only 1 (0.3%) single mutation found in one PCC (120). As of this, it must be concluded that the *BRAF* V600E mutation is a rare event in PCC.

EGLN1/PHD2 and *EGLN2/PHD1*

A germ-line mutation in the *egl-9 family hypoxia-inducible factor 1 gene (EGLN1)* (also called *PHD2)* was found in a patient with polycythemia and a PGL in 2008 (122). The gene encodes PHD2, a 2-oxoglutarate-dependent prolyl hydroxylase that has a key role in regulating HIFα subunits and thereby the expression of HIFα transcription factors (123), probably via accumulation of succinate that inhibits PHD activity (124). It is further hypothesized that the tumorigenesis of succinate dehydrogenase-deficient PPGL is linked to the inhibition of different prolyl hydroxylases that in turn can up-regulate HIFα targets and transcription factors. In a follow-up study that was published three years after the initial finding of an *EGLN1* mutation, the authors screened 82 PGL patients and failed to find any mutation in *EGLN1*, *EGLN2* or *EGLN3* (123). However two additional novel germ-line *EGLN1* (A228S) and *EGLN2/PHD1* (S61R) mutations were recently reported in two patients with polycythemia and PPGL (125). Additionally, these novel *EGLN1* and *EGLN2* mutations were also introduced into a vector using site-directed mutagenesis, and over-expression of the mutants *PHD1*-S61R and *PHD2*-A228S were shown to give decreased effect on HIF2α-induced transcriptional activity (125).

FH

The *fumarate hydratase (FH) gene* encodes an enzyme in the Krebs cycle (as the other PPGL susceptibility genes *IDH1, MDH2* and *SDHx* that encodes other Krebs cycle enzymes) and was found mutated in germ-line in a PCC patient for the first time in 2013 (126). Later, five additional germ-line *FH* mutations were found among 598 PPGL patients without mutations in other PPGL susceptibility genes, which equals a total frequency of 0.8% (127). Although a low mutation frequency, the authors found that multiple tumors and metastatic phenotype were significantly more frequent in patients with a germ-line *FH* mutation. Moreover, *FH*-mutated tumors were also found to display a similar pattern of epigenetic deregulation as *SDHB*-mutated PPGL using immunohistochemistry (IHC) for evaluating 5-hmC and 2SC[17] levels (127). 5-hmC was found low and 2SC was found high in *FH*-deficient samples implying a useful tool to predict *FH*-mutated tumors. The mutational results have been confirmed in subsequent studies (129) showing additional 2/71 *FH*-mutated PPGL. Mutations in the *FH* gene are associated with the Reed syndrome, a disease of multiple cutaneous and uterine leiomyomatosis, but no co-occurrence has been seen with PPGL.

EPAS1/HIF2α

Germ-line GOF mutations in the *Hypoxia-inducible factor 2 subunit alfa (HIF2α or EPAS1)* gene were found to cause a syndrome of PGL and polycythemia[18] for the first time in 2012 (130). This was also the first evidence of *EPAS1* as a *bona fide* proto-oncogene. The *EPAS1* gene encodes the transcription factor HIF-2α which is involved in several cellular interactions as a part of the hypoxic response (131). The mutations found in the original study were somatic only (130), however an exon 9 *EPAS1* mutation were subsequently found in germ-line of a polycythemic patient who developed PPGL (132). Later, it was proposed that HIF2A-related PPGL are caused by postzygotic mutations in the *EPAS1* gene causing mosaicism[19] for the mutation in the adult (135). Besides PPGL, some patients with mutations in the *EPAS1* gene have been reported with somatostatinoma (136) and it has also been found that GOF mutations in the gene cause an up-regulation of hypoxia-related genes, including *EPO* and cancer-related genes (136,137).

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[17] 5-hydroxymethylcytosine (5-hmC) is a DNA base (cytosine) containing a hydroxymethylgroup (to be compared with its precursor 5-methylcytosine which is the methylated form of cytosine) and its levels can be measured using IHC. S-(2-succinyl) cysteine (2SC) is a marker for global changes in protein succination (of fumarate) (128) and both 5-hmC and 2SC have been found altered in *SDHB*-mutated PGL (126,127).

[18] Gem-line mutations in both the *EGLN1/PHD2* and the *EPAS1/HIF2A* genes seem to cause PGL and polycythemia (122,130).

[19] Mosaicism is the presence of two or more distinct populations of cell lines within the same individual, that throughout early development can lead to genetic abnormalities (133). The Klinefelter syndrome (XXY) is an example of chromosomal mosaicism where some cells contain XY and some contain XXY chromosomes (134).

KIF1Bβ

The *Kinesin Family Member 1B (KIF1B)* gene has two splice variants; *KIF1Bα* and *KIF1Bβ* where the latter was coupled to PPGL tumorigenesis in 2008 when two constitutional mutations were found in patients who developed PCC (138). Six months after publication of the original study, another germ-line *KIF1Bβ* mutation was reported (139). The *KIF1Bβ* gene located in chromosomal region 1p36 encodes a kinesin (Kinesin-like protein) and is thought to act as a TSG via a mechanism that allows neuronal progenitor cells to escape apoptosis (138). *KIF1Bβ* mutations are linked to the Charcot-Marie-Tooth disease type 2A (140), however no co-existence with PPGL has been reported.

MAX

Until date, PPGL is the only tumor form that is associated to mutations in the *MYC-associated factor X (MAX)* gene which was found in 2011 (141). A grand follow-up study established a germ-line mutation prevalence of 1.12% based on screening of 1,694 PPGL patients from 17 centers (142) for mutations in the *MAX* gene. PC12 cells, a commonly used rat pheochromocytoma cell line (used in Paper V) was shown in 1995 to only express a mutated form of MAX (143). The protein MAX is a known transcription factor that plays a role in regulation of transcription and apoptosis as well as promotion of cell differentiation (144,145).

MEN1

The involvement of the *multiple endocrine neoplasia type 1* (*MEN1)* gene as a PPGL contributor is disputed (31,146,147). The gene was mapped to 11q13 in 1988 (148) and is known to cause multiple endocrine neoplasia type 1 (MEN 1) (149), an autosomal dominant disease involving hyperplastic and neoplastic lesions arising from the parathyroid, pituitary and pancreas with a penetrance reaching 100% with age (150). Many times, PCCs have been described in patients with MEN 1-associated tumors (146,151,152), but since the MEN 1 syndrome could not be confirmed, the connection has been unclear. Recent studies have shown that mutations in the gene can be associated with PPGL (116,150) and a PCC has been found in a known MEN 1 patient (147), however due to small sample sizes and the difficulties of dividing the MEN syndromes, it is still unknown whether a causative association exists. In Paper II, among 68 non-familial tumors, we found one recurrent *MEN1* variant (p.Arg171Gln) in two PCCs and one PGL with unknown pathogenic significance (31).

MDH2

In 2015, a 55 years old man with multiple malignant PGLs harboring a germ-line mutation in the *malate dehydrogenase 2 (MDH2)* gene was described (153). The *MDH2* gene encodes the mitochondrial malate dehydrogenase enzyme that is also involved in the Krebs cycle. The patient was endowed with a heterozygous variant (c.429+1G>T) that affects a splice site in exon 4 and the authors conclude that *MDH2* is a novel PPGL susceptibility gene (153).

NF1

The tumor suppressor neurofibromin, encoded by the *neurofibromin 1 (NF1)* gene, is highly expressed in the nervous system and mutations have been found to cause the autosomal dominant NF1 syndrome (also called von Recklinghausen's disease) (154). The gene is 58 exons long (Figure 7), located at 17q11 and is known to promote conversion of RAS to its inactive form and thereby regulating cell proliferation through the ERK/MAPK signaling pathway (155) as well as serving as a regulator of TSC2 and mTOR (156) (Figure 10). Somatic *NF1* mutations have been shown to be a frequent event in sporadic PCCs (31,117,118), and the mutations found in our cohort are shown in Figure 7 (31). Due to its length, normal Sanger sequencing is an expensive and time-consuming method to screen for *NF1* mutations. Therefore, in Paper II we evaluated NGS as a tool for efficient mutation screening of all PPGL susceptibility genes, including *NF1* (31). Patients harboring a germ-line *NF1* mutation and the NF1 syndrome often present symptoms of > 6 café au lait patches, axillary freckling, inguinal freckling, lichen eye nodules, optic gliomas, cutaneous neurofibromas (157) and 0.1- 5.7% of the patients develop unilateral PCC (158). In Paper III, we evaluated IHC as a screening tool for *NF1* mutations but found a poor specificity for the method (159).

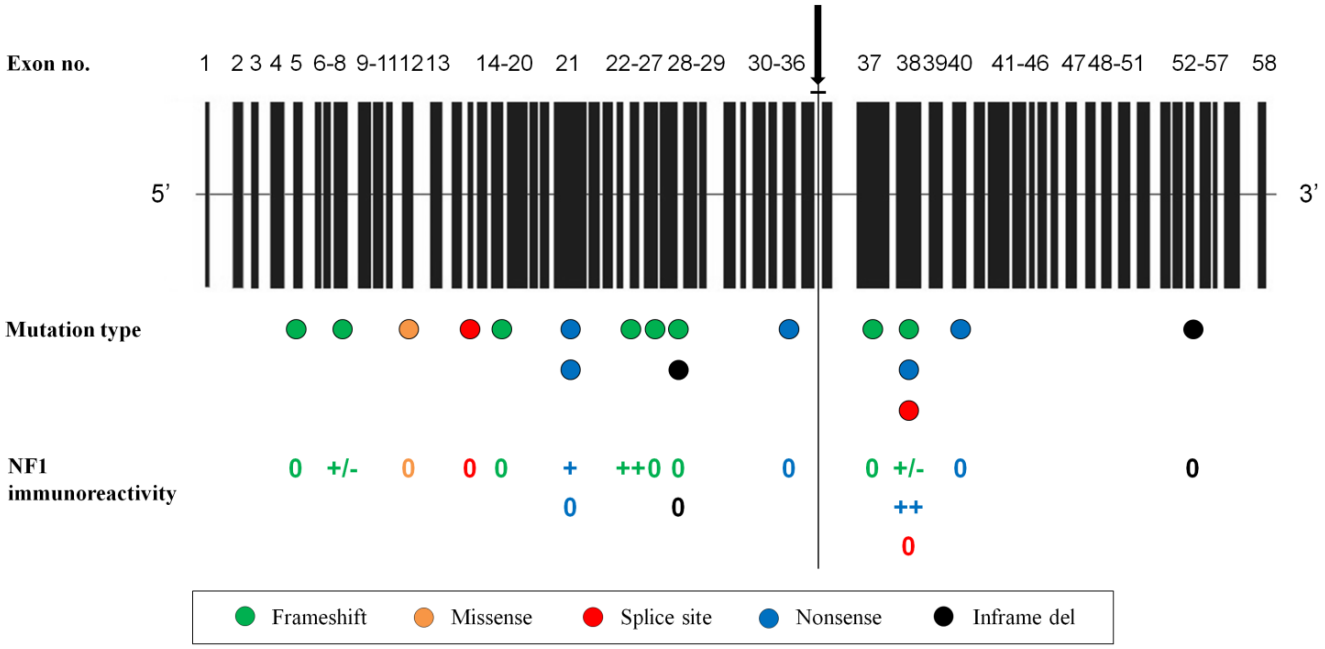


Figure 7.

The *NF1* gene.

Schematic overview of the *NF1* gene and mutational data among 86 PCCs from Paper II. The down-pointing arrow indicates the antibody (ab128054) binding position used in Paper III. Results from Immunohistochemical analysis are shown with ++ (strong/moderate immunoreactivity), + (uniformly weak immunoreactivity, +/- (focal areas with immunoreactivity or 0 (negative immunoreactivity) for each case. The picture is a modified version of a figure in Paper III.

RET

GOF-mutations in the *rearranged during transfection (RET)* proto-oncogene are known to cause the MEN 2 syndrome (111,160), which is an autosomal dominantly inherited disease characterized by the development of endocrine hyperplasias and neoplasms including PCC (with a penetrance of 50% (161,162)), parathyroid adenoma and medullary thyroid carcinoma (MTC) (163,164). The gene is located at 10q11 and encodes a transmembrane receptor tyrosine kinase, a receptor that can activate the downstream RAS/RAF/MAPK and PI3K/AKT pathway cascades (165) (Figure 10). PCCs in MEN 2 patients are usually benign, secrete both NA and A and are often bilateral (68,166,167). Due to the occurrence of bilateral PCC, MTC and parathyroid adenomas, adrenal screening and prophylactic thyroidectomy may be considered. In Paper II, we reported both constitutional and somatic *RET* mutations. In addition to activating GOF-mutations that lead to MEN 2, inactivating mutations in the *RET* gene predispose to Hirschsprung's disease, a disorder of the large intestine (168).

SDHx

Succinate dehydrogenase (SDH) is an enzyme complex that is connected to the inner membrane of the mitochondria and contains the four subunits SDHA, SDHB, SDHC and SDHD (64). SDHAF1 and SDHAF2 are also known to contribute to a functional SDH complex by regulating the SDHA subunit (169). The complex is involved in the tricarboxylic acid cycle (TCA) and in the respiratory electron transfer chain (ETC) with the roles of catalyzing the reaction from succinate to fumarate in TCA and transfer electrons to the succinate-CoQ reductase (Complex II) in the ETC (170). The proteins in the SDH complex are encoded by their respective *succinate dehydrogenase (SDHx)* genes; *SDHA, SDHB, SDHC, SDHD, SDHAF1* and *SDHAF2/SDH5.* PPGL as tumor models became paradigm shifters when an inherited mutation of a gene encoding a metabolic enzyme (*SDHD)* was reported for the first time (64,110). The syndrome caused by constitutional mutations in the *SDHD* gene is therefore called PGL1 (Table 1). The *SDHD* discovery was subsequently followed by the findings of germ-line mutations in *SDHC* (171) and *SDHB* (172) causing the PGL3 and PGL4 syndromes, respectively. Eight years later, the PGL2 syndrome was found to be caused by germ-line mutations in the *SDHAF2/SDH5* gene (173). A germ-line mutation in the *SDHA* gene is sometimes said to cause PGL5 that also include gastrointestinal stromal tumors (GIST), however this syndrome seem to have a lower PPGL penetrance compared to PGL 1-4 (174,175). Interestingly, from a clinical practice point of view, inactivation by mutations of the *SDHB, SDHC* and *SDHD* genes lead to decreased expression of SDHB that has been shown to be detectable by immunohistochemistry (IHC) (176,177). This has also been shown for SDHA IHC for *SDHA* mutated cases (178) and both SDHA- and SDHB IHC were recently verified as a reliable tools for mutation screening by the ENS@T network (179). In Paper V, we were able to verify the finding of SDHB IHC as a valuable tool for *SDHA/SDHB* mutation screening by testing 14 PCCs and 13 PGLs with known *SDHx* gene status (32). Moreover, since patients endowed with a germ-line *SDHB* mutation harbor a significantly higher risk of developing malignant disease (180) it is important to have a valid screening method to find these patients with a worse prognosis. This screening could be done

via SDHB IHC and/or sequencing of the *SDHx* genes, and maybe in the future via epigenetic screening for a hypermethylator phenotype (50,52,126).

TMEM127

Germ-line mutations in the *Transmembrane protein 127 (TMEM127)* gene were reported in PCC patients in 2010 (181) and subsequently verified as a novel PCC susceptibility gene with a prevalence of 1-2% (182,183). Mutations were found in patients with clinical criteria for inherited disease (bilateral PCC, young age at diagnosis or family history) (183). The gene is mapped to 2q11 and encodes a transmembrane protein (number 127) with TSG properties (181). PGLs seldom harbor *TMEM127* mutations (184), and the penetrance of PCC among 47 *TMEM127* mutation carriers in one study was 32% (185).

VHL

The *von Hippel-Lindau tumor suppressor (VHL)* gene is mapped to 3q25, contains 3 exons (Figure 12) and inactivating germ-line mutations cause the vHL syndrome which is characterized by numerous tumor forms including PPGL, renal carcinomas, lymphatic sac tumors, hemangioblastomas and endocrine pancreatic tumors (68,186). The PPGL penetrance is 10-30%, about half of the PCCs are bilateral and malignancy seldom occurs (186,187). As further discussed under "Hypoxic response in PPGL" in this work, *VHL* is involved in the oxygen-sensing pathway through regulation of hypoxia-inducible factors (HIFs) that are known to increase expression of factors related to angiogenesis (188,189). Moreover, since HIFs are parts of the E3 ubiquitin ligase complex, the vHL protein is thought to be able to regulate the specific ligase activity resulting in ubiquitination and degradation of specific proteins (190).

Genes associated with sporadic PPGL

As described above, several of the genes for which germ-line mutations have been associated with a PPGL predisposition may also be somatically mutated in non-familial PPGL including *EPAS1*, *MAX*, *NF1*, *RET* and *VHL* (Table 1). In addition, a set of other genes exhibit somatic mutations in PPGL in varying frequencies including *ATRX*, *FGFR1*, *HRAS*, *IDH1*, *KMT2D*, *MET* and *TERT*.

ATRX

A recent study has shown that 12.6% of the PPGL investigated had somatic truncating and missense mutations in the *alpha thalassemia/mental retardation syndrome X-linked* (*ATRX*) gene (191). Germ-line mutations are known to cause the X-linked alpha thalassemia mental retardation syndrome and somatic mutations are known as drivers in neuroblastomas and gliomas (192–194). No germ-line mutation has been found in any PPGL patient and future work will further establish the *ATRX* mutation prevalence and the role in PPGL malignancy (191).

FGFR1

Somatic mutations in the *fibroblast growth factor receptor 1 (FGFR1)* gene were recently identified via whole-exome sequencing (WES) of 16 matched PPGL and normal pairs (195). In a subsequent analysis of 80 sporadic tumors, mutations were found in three cases (3.8%) with one recurrent mutation (c.1638C>A) observed in two cases. Additionally, the tumors with mutations in the *FGFR1* gene were found to cluster with *NF1*- and *RET*-mutated PPGL using mRNA expression profiling. (195)

HRAS

A somatic mutation in the *Harvey rat sarcoma viral oncogene homolog (HRAS)* gene was first described in a PCC in 1992 (196). Before that, 10 PCC had been screened for *HRAS* mutations but no mutations were found (197). Later, somatic mutations in the *HRAS* gene have been verified in four subsequent follow-up studies and revealed additional *HRAS* mutations as shown in Table 2 (Paper IV and (120,198,199)). Four different mutations have been found in the *HRAS* gene that are thought to promote PPGL tumorigenesis including G13R in exon 2 and Q61K, Q61R and Q61L (Figure 14) in exon 3. Constitutional mutations in the gene have been coupled to the Costello syndrome (200), however no co-existence of this syndrome and PPGL has so far been reported (120,198).

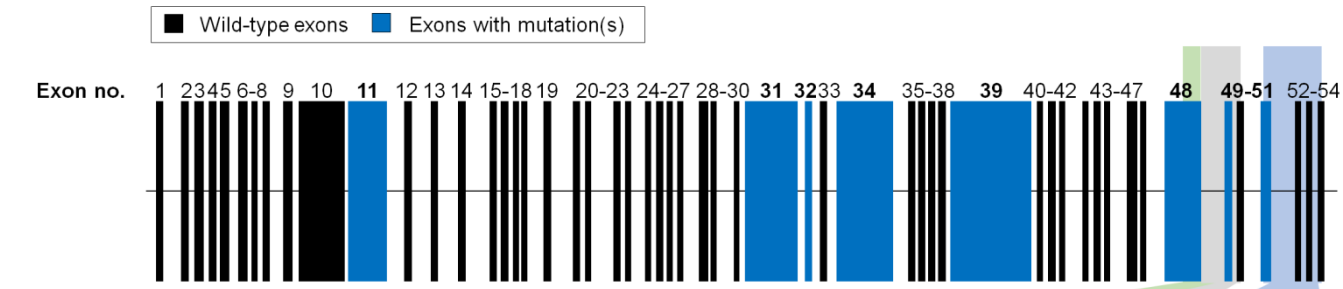
IDH1

A single somatic mutation (p.Arg132Cys) in the *isocitrate dehydrogenase 1 (IDH1/NADP+)* gene was found in a sporadic PGL in 2010 based on screening of 365 PPGL (201). The same codon 132 mutation has been described in more than 70% of grade II and III astrocytomas, oligodendrogliomas and glioblastomas (202), as well as in a few cases of prostate carcinomas and B-acute lymphoblastic leukemias (203).

KMT2D/MLL2

In Paper V, we describe missense variants of the gene *lysine (K)-specific methyltransferase 2D (KMT2D*, also called *ALR*, *MLL4*, and *MLL2)* in 14 out of 99 PCCs (32). In a follow-up study (Paper VI), we showed absence of these mutations in 13 PGLs (204). The gene encodes KMT2D – a methyltransferase that can regulate the accessibility of DNA through histone methylation (205) and it has been previously shown that histone methylation is a central mechanism of epigenetic regulation at the genome-wide level (206). The Kabuki syndrome is a developmental disorder characterized by intellectual disability, distinctive facial marks and postnatal dwarfism which is caused by mutations in the *KMT2D* gene (207,208). So far, no PPGL patient has been reported with the Kabuki syndrome and vice versa. In Paper V, we found 14 missense mutations that were spread along the 54 exon-spanning gene, and five PCCs (36%) had somatic mutations in the functional FYR or SET domains (32) (Figure 8). In non-Hodgkin lymphomas, *KMT2D* has been shown to be recurrently mutated (209) as well as in medulloblastoma (210), urinary bladder carcinoma (211), esophageal squamous cell carcinoma (212) and lung cancer (213). It has also been shown that knockdown of *KMT2D in*

vitro affects cell growth and survival (214), which are in line with our results that *KMT2D*-transfected PC12 cells exhibited a transcriptional profile enriched for genes involved in the regulation of actin cytoskeleton and focal adhesion (32).



Functional domain						FYR-N	FYR-C	SET
Exon no.	**11**	**31**	**32**	**34**	**39**	**48**	**49**	**51**
Mutation	Q1023K	F2536S	G2735S	R2922W	P4048L	N5222K	R5266H	H5420Y
		L2610P	G2735S	N2965S	L4222V	N5223S		
						N5223S		
KMT2D immunoreactivity	nd	nd	nd	+	+	+	nd	+/-
		+/-	nd	+	+	+		
						+		

Figure 8.

The *KMT2D* gene.

Schematic overview of the *KMT2D* gene and mutational data among 99 PCCs from Paper V. Exon positions are indicated for each of the 14 *KMT2D* missense mutations together with the nuclear immunostaining of KMT2D where (+) denotes positive nuclear immunostaining, (+/-) denotes partially positive nuclear staining and (nd) means no data available. Green, gray and blue shaded areas indicate the functionally important FYR and SET regions of the gene. The picture is a modified version of a figure published in Paper V (32).

MET

Five PCCs (2.5%) were found with hotspot mutations in the *MET Proto-Oncogene* (*MET*) in a preceding study (215). The gene encodes for a receptor with tyrosine kinase activity that is able to activate the RAS/MAPK signaling pathway (Figure 10). The authors suggested a role of the *MET* mutations as a potential driver. However future studies might elucidate its involvement in PPGL tumorigenesis.

TERT

In a study from 2014 (listed among the related publications in this thesis - (216)) a mutation in the *telomerase reverse transcriptase (TERT)* gene was reported in one single benign PCC operated in CT, USA and in one metastatic PGL operated in Stockholm, Sweden. The *TERT* mRNA expression was also evaluated and found up-regulated in cases with mutations which could indicate telomerase activation. However, *TERT* expression was also found in wild-type cases, which could denote additional mechanisms of *TERT* activation, including epigenetic mechanisms. After this publication, two additional C228T mutations have been found in two PGLs (217). Mutations in the *TERT* promoter was first described in melanomas (218,219) and have subsequently been described in several human cancers (220). The *TERT* gene encodes the telomerase reverse transcriptase component of telomerase, which is known to elongate telomeres and commonly activated in many cancers (221). Another mechanism that can maintain telomeres not involving telomerase is called alternative lengthening of telomeres (ALT) and is dependent on homologous recombination (220). Interestingly, it has been shown that many ALT positive cancers harbor mutations in the *ATRX* gene (a recently found PPGL susceptibility gene as previously described) which encodes a protein that interact at telomeres. (222,223)

Table 1. Summary of all genes involved in PPGL tumorigenesis up to date, their prevalence and penetrance as well as the original reference work. Data regarding prevalence and penetrance is gathered from studies cited under respective gene name as above, which for 12 of the genes are summarized in (64).

Gene	Syndrome	Discovered in PPGL (year)	Germ-line prevalence	Somatic prevalence	Penetrance of PPGL	Original reference
ATRX	-	2015	0%	12.7%	ND	(191)
BAP1	-	2012	1 case	0%	ND	(119)
BRAF	-	2015	0%	1 case	ND	(120)
EGLN1	-	2008	<1%	0%	ND	(122)
EPAS1	*	2012	<1%	7%	ND	(130)
FGFR1	-	2015	0%	3.8%	ND	(195)
FH	-	2013	1%	0%	<1%	(126)
HRAS	-	1992	0%	5.8%	ND	(196)
IDH1	-	2010	0%	1 case	ND	(201)
KIF1Bβ	-	2008	<1%	0%	ND	(138)
KMT2D	-	2015	<1%	14%	ND	(32)
MAX	-	2011	1-2%	1%	ND	(141)
MDH2	-	2015	<1%	0%	ND	(153)
MEN1	MEN1	2001	<1%	0%	<1%	(146)
MET	-	2015	0%	5 cases	ND	(215)
NF1	NF1	1990	3%	15 - 25%	1-6%	(112)
RET	MEN2	1993	5.3%	5%	50%	(111)
SDHA	PGL5	2010	1%	0%	ND	(174)
SDHAF2	PGL2	2009	<1%	0%	100/0%**	(173)
SDHB	PGL4	2001	8%	0%	77%	(172)
SDHC	PGL3	2000	<1%	0%	ND	(171)
SDHD	PGL1	2000	7.1%	0%	86/0%**	(110)
TERT	-	2014	0%	4 cases	ND	(216)
TMEM127	-	2010	1-2%	0%	32%	(181)
VHL	vHL	1993	13%	9%	10-30%	(113)

* A syndrome of paraganglioma and polycythemia has been suggested due to the finding of multiple PGLs and somatostatinomas associated with polycythemia as a result of GOF *EPAS1* mutations (136).

** PGL1 and PGL2 are commonly paternally inherited, proposing a maternally imprinting mechanism of the *SDHD* and *SDHAF2* genes (224,225). Probably due to this imprinting, the penetrance is 100%/86% for paternally inherited mutations and 0% for maternally inherited mutations.

Table 2. Summary of *HRAS* mutation studies in PPGL. The table is published in full in Paper IV.

	***HRAS* gene status**						**PPGL susceptibility gene**	
	***HRAS* mutated**	**codon 13 G13R**	**codon 61 Q61R**	**codon 61 Q61K**	**codon 61 Q61L**	**Wild-type codon 13/61**	**Known mutation**	**Unknown mutation (sporadic)**
HRAS mutations in eight studies								
- PCC (n = 814)	46 (5.7%)	5 (0.8%)	26 (4.0%)	5 (0.8%)	0	768 (94.3%)	1 / 330 (0.3%)	45 / 484 (9.3%)
- PGL (n = 136)	3 (2.2%)	0	1 (0.9%)	1 (0.9%)	1 (0.9%)	133 (97.8%)	0 / 72	3 / 64 (4.7%)
Total (n = 950)	**49 (5.2%)**	**5 (0.7%)**	**27 (3.6%)**	**6 (0.8%)**	**1 (0.1%)**	**901 (94.8%)**	**1 / 402 (0.2%)**	**48 / 548 (8.8%)**

Gene expression and epigenetics

Expressional profiling

Preceding expressional profiling studies of PPGL have shown that the tumors can be divided into two distinct clusters that are strongly correlated with known drivers; Cluster 1 with tumors characterized by hypoxic response (*EPAS1*-, *SDHx*- and *VHL*-mutated tumors) and Cluster 2 with tumors characterized by active kinase signaling (*HRAS*-, *MAX*-, *NF1*-, *RET*- and *TMEM127*-mutated tumors) (137,226–229) (Figure 9). Also when studying the role of microRNAs[20] (miRNA) in 69 PPGL, the miRNA signatures were shown to be specific to the genetic groups of PPGL (231).

[20] miRNAs are short and non-coding RNAs that have been shown to regulate gene expression in many biological processes. In cancer they regulate for instance proliferation, cell death and differentiation (230).

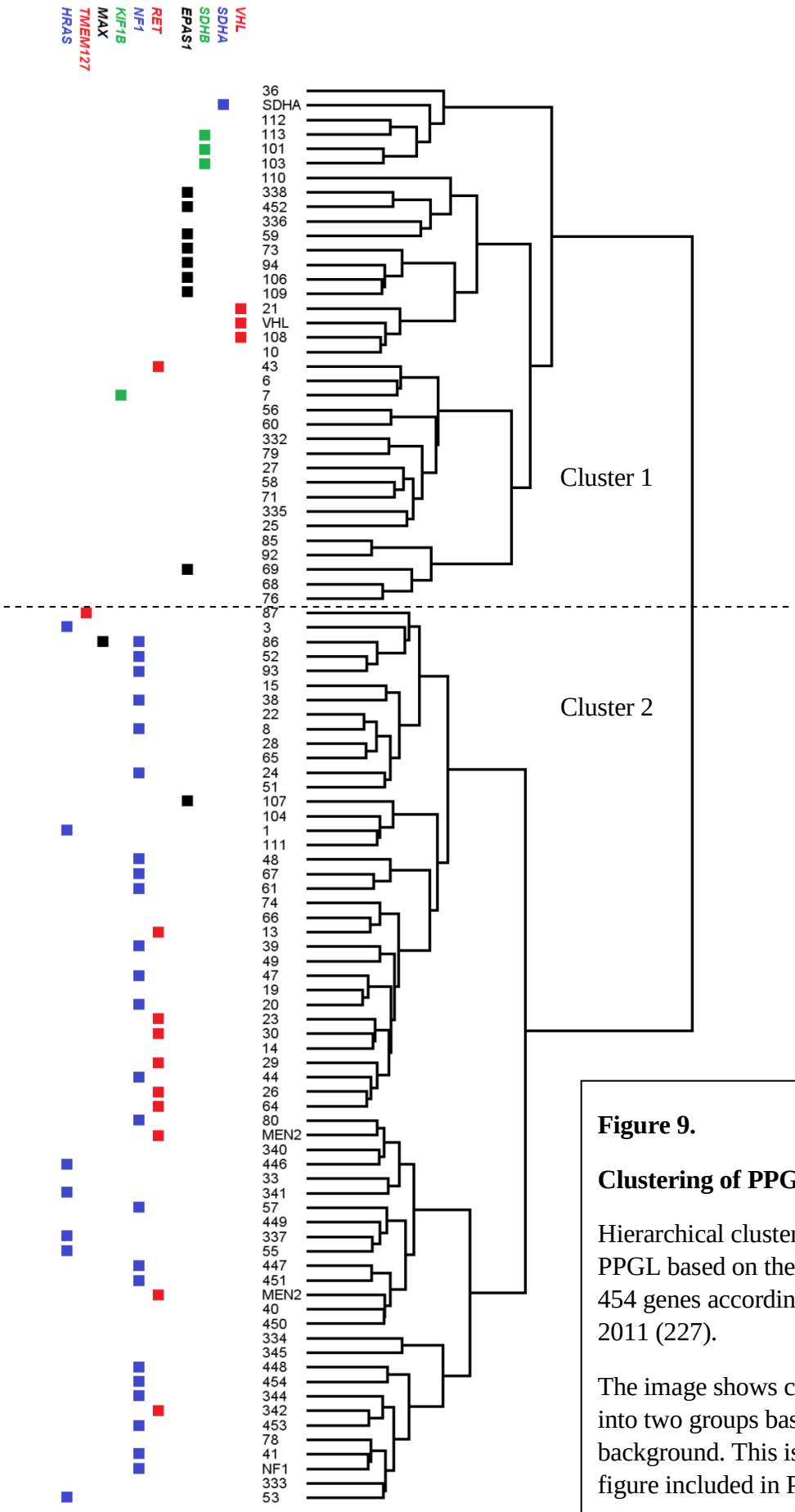


Figure 9.

Clustering of PPGL.

Hierarchical clustering in a dendrogram of PPGL based on their expression levels for 454 genes according to Burnichon *et al.* 2011 (227).

The image shows clear separation of PPGL into two groups based on their genetic background. This is a modified version of a figure included in Paper IV.

Hypoxic response in PPGL, Cluster 1

Pseudo-hypoxia driven tumors in Cluster 1 have the characteristics of activating hypoxia inducible factors (HIFs). Under conditions of low cellular oxygen levels, HIFs (HIF-1 and HIF-2) can activate genes that promote a range of functions including survival, pH regulation, cell migration and tumor growth (232,233). When the HIFs (1 and 2) are active they consist of two subunits; α and β. The HIF-1β subunit is quite stably expressed and can dimerize with (and activate) both HIF-1α and HIF-2α (the latter encoded by *EPAS1* and discussed under PPGL etiology) (232,234). The fact that the β unit is constitutively expressed makes the α units the main regulators of the HIFs and are in turn controlled by hydroxylation by prolyl hydroxylases (PHDs) (235). Two PHDs, encoded by *PHD1* and *PHD2* are further described under hereditary predisposition for PPGL since *EGLN1/PHD2* has been found with a mutation in a patient with polycythemia and PGL (122). Once a prolyl residue of a HIF is hydroxylated by a PHD, it can be ubiquitinated and degraded via the von-Hippel Lindau protein (vHL, as also discussed under PPGL etiology) containing an E3 ubiquitin ligase complex (236). The condition pseudo-hypoxia occurs when a HIF-α unit cannot be degraded due to inactivation of any molecule in the chain described, regardless of cellular oxygen levels (190). Additionally, it is currently hypothesized that inactivation of subunits of SDH lead to succinate accumulation which inhibits alpha-ketoglutarate (126), which in turn inhibits EGLN/PHD (237). When PHD is inactivated it cannot hydroxylate HIF-α (as discussed above) resulting in activation of HIF (124) that may lead to several changes in the cell including tumor growth and migration. It has been shown that inactivation of *FH* lead to the same end result of activated HIF (124,238) and possibly the other metabolic enzymes *IDH1* and *MDH2* could theoretically have a similar function in PPGL.

Activation of kinase signaling pathways, Cluster 2

Tumors in Cluster 2 are associated with activation of the kinase signaling pathways RAS/RAF/MAPK and PI3K/AKT, both well known to be aberrant in various cancers causing both cell proliferation and cell survival (239,240). In PPGL, mutations in both *NF1* and *RET* causing NF1 and MEN2 syndromes respectively, can activate both pathways although *NF1* is known as a TSG and *RET* as an oncogene (156,226,241,242). An activation of the *RET* gene through a GOF mutation can inhibit RAS (which is partly encoded by *HRAS* [in addition to *KRAS* and *NRAS*] and discussed under PPGL etiology) and also activate PI3K signaling as visualized in Figure 10. Likewise, an inactivation of the *NF1* gene can inhibit RAS which in turn can activate PI3K (64,68). *TMEM127,* another mutated gene in cluster 2 is hypothesized to inhibit mTOR activity (181) and the *MAX* gene is thought to inhibit MYC (142) which in turn can activate HIF (cluster 1) and cause cell proliferation itself through 4EBP1 (as shown in lymphomas (243)) like mTOR (Figure 10) (64,68). The cross-talk and clustering overlap mentioned for *MAX* is interesting since *MAX*-associated tumors align in cluster 2 and hence do not show a pseudo-hypoxic gene profile, but could possibly affect HIF (Figure 10) and do also show a low expression of *PNMT* and low levels of norepinephrine secretion (64,142).

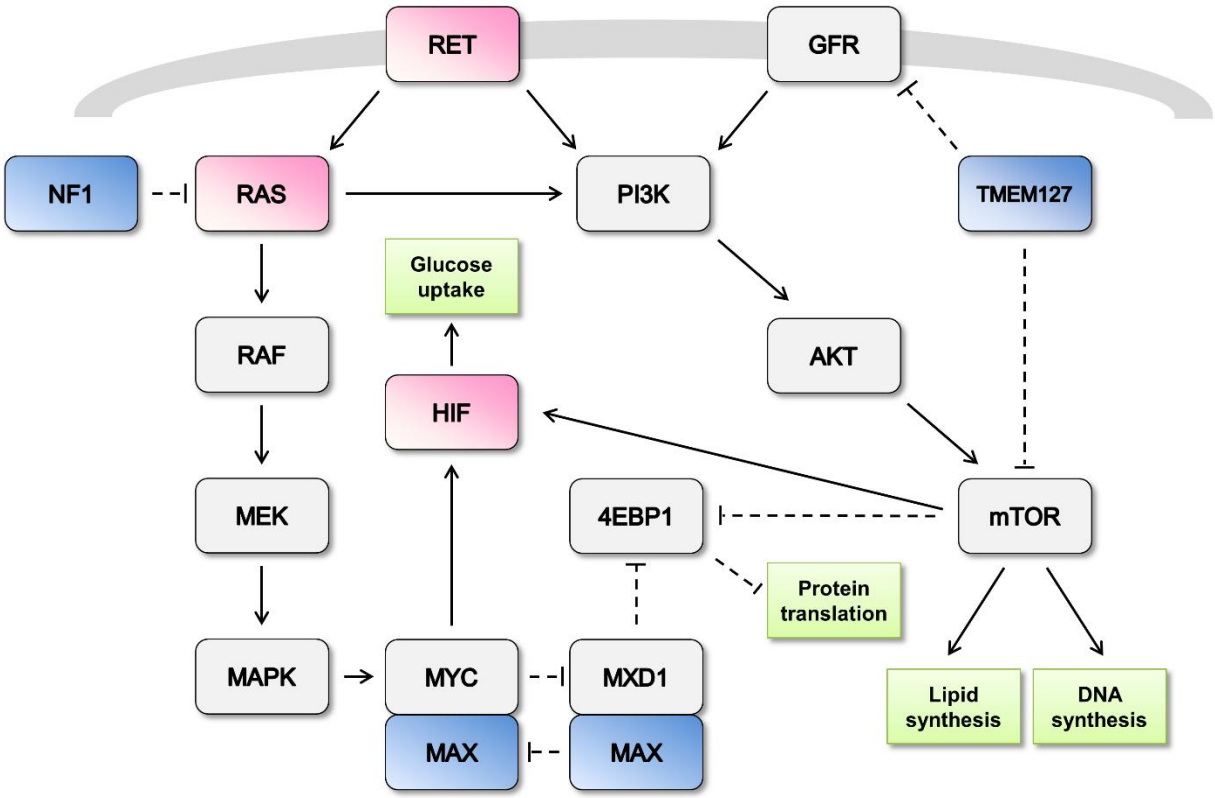


Figure 10.

PPGL susceptibility genes and their interactions in cluster 2.

The membrane bound protein RET and other membrane bound growth factor tyrosine receptors (GFR) are when activated able to initiate a cascade of downstream events that lead to activation of the RAS/RAF/MAPK and PI3K/AKT pathways including mTOR. Proteins encoded by genes that have been found with activating mutations (GOF) in PPGL are shown in red boxes and those with inactivating mutations (TSG) are shown in blue boxes. Activating pathways are shown with arrows and inhibiting ditto are shown with dashed lines.

Once mTOR is activated it can regulate cell growth through lipid-, DNA and protein synthesis (the latter through inhibition of 4EBP1 that inhibits protein translation) as well as glucose uptake through HIF. Other regulators are NF1 that inhibits RAS and TMEM127 that is thought to inhibit mTOR. The underlying mechanisms of MAX in the PPGL tumorigenesis is not yet clearly shown although the MAX-MYC dimers promote and MAX-MXD1 dimers inhibits cell proliferation. MYC in turn has also been shown to inhibit 4EBP1 and cooperates with mTOR to both increase protein translation and glucose uptake (via HIF). Cross-talk between MAPK and MYC has also been suggested. The picture is inspired from figure 3 in (64) and figure 3 in (68).

Epigenetics

Epigenetic aberrancies including DNA methylation have been shown to contribute to PPGL tumor development. In Paper V we report mutations in a gene encoding a methyltransferase that can regulate the accessibility of DNA through histone modification (32). In 2005, Dammann *et al.* showed promoter hypermethylation in subsets of PCCs for *RASSF1A, p16, MSH2, CDH1* and *PTEN* (244). This was followed by the findings by Geli *et al*. who showed hypermethylation of *NORE1* as well as coupled hypermethylation of *RASSF1A* to malignant behavior of the tumor (245). In 2008, Geli *et al*. defined a CpG island methylator phenotype (CIMP) for PPGL that exhibited hypermethylation in three or more genes in the following list of 11 genes: $p16^{INK4A}$, *CDH1, DCR2, RARB, RASSF1A, NORE1A, TP73, APC, DAPK1,* $p14^{ARF}$ and *PTEN* (246) which was first described in colon cancer (121). The patients with CIMP were mostly young patients with paraganglioma and/or malignant disease. It was found that *SDHB* mutations are associated with a CIMP phenotype (52,246) which later also was established in a mouse model (126). It was hypothesized that *SDHB* mutation predispose to CIMP and that CIMP occur before the tumor exhibit malignant features (52). It is thought that mutations in the *SDHx/FH* genes affect the Krebs cycle to succinate accumulation which leads to inhibition of DNA/histone demethylases. This inhibition leads consequently to hypermethylation which can inactivate key genes that are related to PPGL tumorigenesis and malignancy (52,126). In this way, epigenetic mechanisms contribute to the regulation of metabolic gene expression which sometimes is referred to as the "Warburg effect" (25), from the Nobel laureate Otto Warburg. To expand on this, the effect describes how cancer cells regulate their aerobic respiration and preferentially use glycolysis to generate energy, which has been shown for PPGL (247).

In Paper I, we proposed inactivation of the PPGL susceptibility gene *VHL* through promoter methylation in PPGL (51). In 2013, Letouzé *et al.* performed a genome-wide screening for altered DNA methylation with the Illumina based methylation profiling of 27k CpG sites among 145 tumors and related the findings to the gene expression clusters as described above (126). The authors were able to identify three main clusters of hypermethylation that corresponded to gene mutation, gene expression and also poorer survival for patients in the M1 cluster (characterized by hypermethylation at a large number of CpG sites) (126). Treatment of *SDHB*- related, malignant PPGL with temozolomide (TMZ) has been shown to be an effective antitumor agent, and it is hypothesized that the finding is explained by silencing of MGMT expression as a consequence of *MGMT* promoter hypermethylation in *SDHB*-mutated PPGL (248).

AIMS

A total of six studies are included in the thesis, with the overall aim to further characterize molecular and genetic alterations of importance in the development of PPGL. The overall aims, specific for each paper were:

PAPER I

- To evaluate the promoter methylation status of the PPGL susceptibility genes.

PAPER II

- To develop a next-generation sequencing method for mutational screening of the known PPGL susceptibility genes.

PAPER III

- To examine whether mutations in the *NF1* gene could be predicted by immunohistochemistry.

PAPER IV

- To establish the *HRAS* mutation frequency and investigate the expressional profiling of *HRAS* mutated PPGL.

PAPER V

- To search for novel genes involved in the PCC tumor development by whole exome sequencing.

PAPER VI

- To determine the prevalence of *KMT2D* mutations in PGL.

MATERIAL AND METHODS

"Experiment is the only means of knowledge at our disposal. Everything else is poetry, imagination." – Max Planck.

MATERIAL / STUDY POPULATION

This thesis comprises tumor and corresponding normal tissue samples mainly obtained from the endocrine biobank at Karolinska University Hospital, Sweden. In Paper IV, tumors were also collected from University de Lorraine, Vandoeuvre-les-Nancy, France, Linköping University Hospital, Sweden and Haukeland University Hospital in Bergen, Norway. In Paper V, a subset of tumors was obtained from Yale University in New Haven (CT), USA. Additionally, a PC12 rat pheochromocytoma cell line was used in Paper V.

In 1986 at the Karolinska University Hospital, the endocrine biobank project was initiated, in which surgically removed endocrine tumors and other tissues samples are stored at -80 °C after immediate freezing in liquid nitrogen (249). After operation, an experienced pathologist identified and dissected the tumor and normal tissue which were subsequently used for extraction of the specific analytes DNA, RNA and protein. Additionally, a small representational piece was cut from almost all samples (Figure 11) and was fixed in formaldehyde, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E). Subsequently the tumor and normal cell content was determined, often by two expert pathologists independently.

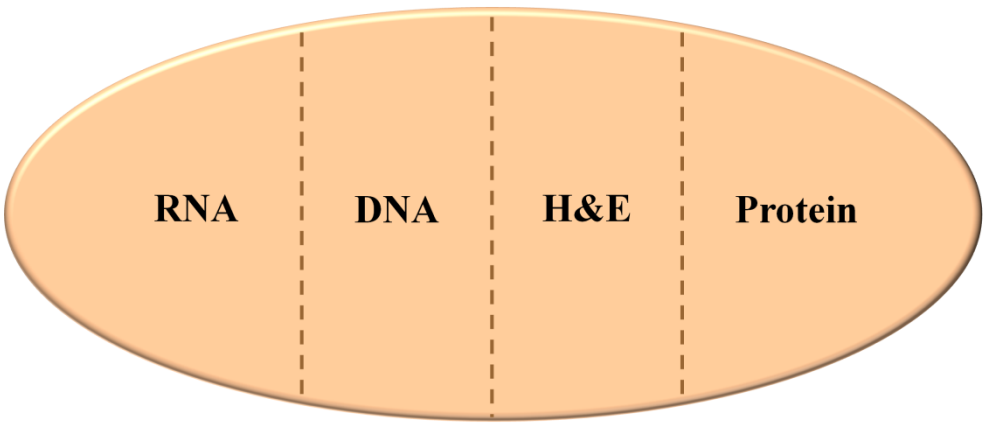


Figure 11.

Tissue sampling for extractions and representativity testing.

Schematic overview of the tissue sampling method prior to extraction of analytes (RNA, DNA and Protein) and representativity testing (H&E).

For the cases from the Karolinska biobank, genomic DNA from tumor and normal tissues was extracted with the DNeasy DNA isolation kit (QIAGEN) and validated using the Nano-Drop technology. Genomic DNA extraction from blood (used in Paper II and V) was performed with the QIAamp DNA Blood Midi Kit (QIAGEN). RNA extraction was performed using the *mir*Vana miRNA Isolation Kit (Ambion/Invitrogen) and the RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent) obtaining RIN values that have been shown to be sufficient after long-term storage (249). Protein extracted from the tumors is often used for Western Blot (250) but has not been used in any of the papers included in this thesis, although protein extracted from PC12 cells has been used for KMT2D protein validation in Paper V. DNA from the other universities was extracted using standardized methods similar to the procedure used at Karolinska; involving lysis of tissue, precipitation in ethanol, binding of the analyte to a spin column membrane and elution of the analyte with water after multiple washing. For IHC, tissue samples were fixed in 4% formaldehyde, embedded in paraffin and later sectioned and stained with the specific primary antibodies as specified in the papers and below.

METHODS

Genetic analysis

Sanger sequencing and Polymerase chain reaction (PCR)

Since the initial report in 1977, Sanger sequencing has become the golden standard method for DNA sequencing (251). The method involves the use of fluorescent dyes for each nucleotide that emit light at different wave-lengths. After amplification of the specific target with a polymerase chain reaction (PCR), the nucleotides are detected by the fluorescent emission after dye-termination sequencing. In this thesis, sequencing was carried out at the KiGene core facility at Karolinska Institutet for most of the cases. The method was used in Papers I, IV and V and the specific primers were mostly designed with the NCBI Primer Blast (http://blast.ncbi.nlm.nih.gov). The sequencing results were analyzed by visual examination of the chromatograms obtained from the dye-termination sequencing (Figure 14), using both Chromas (Technelysium) and CodonCode Aligner software.

Reverse transcription and quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) is a method used in Papers I, V and VI to quantitatively measure the amount of cDNA and specific genes during the progress of a PCR in real-time. Primarily, mRNA extracted from tumor and normal tissue was converted to cDNA via reverse transcription and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The TaqMan assays were pre-designed from the manufacturer (Applied Biosystems) and constitute of two primers and one probe that anneal to the cDNA template. In the PCR reaction, the number of the PCR cycle in which the product amount exponentially increases is used to calculate the starting amount of the specific template (252) which often is referred to as the gene expression. The expression of a specific gene is often related to one or several endogenous controls that are specific to the analyzed tissue. In Paper

I, as an effort to completely follow the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (252), 32 different endogenous control genes were tested in triplicates for 6 different PPGL and normal adrenal tissue samples. It was found that the most stable endogenous controls with the smallest variation among the samples were the *B2M, CASC3, MRPL19, PSMC4,* and *UBC* genes which subsequently were used as endogenous control genes for PPGL in the following papers (51).

Copy Number Analysis

In Paper V, genomic DNA from the tumors were analyzed for *KMT2D* gene copy number alterations using a commercially available *KMT2D* TaqMan copy number assay on a StepOnePlus system (Invitrogen/Life Technologies). The experimental procedure is essentially as briefly described above under qRT-PCR. The number of PCR cycles at which the DNA amount increases and reaches a specific threshold was used to calculate the number of *KMT2D* gene copies for each sample.

Bisulfite Pyrosequencing

Pyrosequencing was originally developed in 1996[21] for analysis of SNPs (253) but has since become a gold standard method for quantitative detection of DNA methylation. The method was used in Paper I in this thesis. When Pyrosequencing is used for assessment of DNA methylation, the methylated DNA needs to be separated from unmethylated DNA using sodium bisulfite treatment. During this treatment, methylated cytosine residues are unaffected while unmethylated cytosine residues are converted to uracil (U, complementary to T). The differences between C and T (methylated/unmethylated cytosine residues) can be quantitatified by Pyrosequencing which always is preceded by a PCR of the bisulfite treated DNA and amplification of the specific template and the CpGs of interest. Commercially available primers (PyroMark Assay Database, Geneglobe, Qiagen) and in-house designed primers (PyroMark Assay Design software, Qiagen) were used, both resulting in a biotinylated reverse primer that binds the PCR fragments on streptavidin-coated sepharose beads. After vacuum removal of the forward strand, a sequencing primer (often closely related to the forward primer strand) is added to the immobilized reverse strand on the sepharose beads and the Pyrosequencing reaction can be started. During this reaction, the nucleotides are dispensed one by one to the (reversed) template while a pyrophosphate group is simultaneously cleaved off if the nucleotide is incorporated, resulting in a flash of light that is measured and registered. The intensity of this light is proportional to the amount of nucleotides that could be incorporated and used to calculate the sequence. Since both C and T are dispensed to the specific methylated/unmethylated CpG sites, the C/T light intensity ratio is used to calculate the level of methylation at this site.

[21] The technique was invented at the Royal Institute of Technology (KTH) in Stockholm by Mostafa Ronaghi, Mathias Uhlén and Pål Nyrén (253).

Next generation sequencing (NGS)

The challenge to deliver fast, accurate and inexpensive genome information has driven the development of next-generation sequencing (NGS) technologies that allow massive parallel sequencing and production of genetic data at a low price compared to the previously described Sanger sequencing method (254). In this thesis, both targeted sequencing and whole-exome sequencing (WES) have been used – collectively known as NGS.

Targeted sequencing

In this thesis, a targeted sequencing approach was undertaken to analyze the known PPGL susceptibility genes (Paper II), and all exons of *KMT2D* (Papers V and VI) respectively. Probes were designed to allow simultaneous sequencing of all targets (all exons of the following genes: *EGLN1, EPAS1, KIF1Bβ, MAX, MEN1, NF1, RET, SDHA, SDHB, SDHC, SDHD*, *SDHAF2*, *TMEM127* and *VHL* in Paper II, and *KMT2D* in Paper V and VI). The targets were labelled and barcoded to tolerate massive pooling of multiple samples. The samples and the pool of amplicons were analyzed on an Illumina MiSeq instrument (Paper II) or an Illumina HiSeq instrument (Papers V and VI), and the obtained sequences were subsequently aligned to the reference genome. In short, the targets are bound to primary adapters that in turn are able to bind to other, secondary adapters that are attached to the surface of an Illumina flow cell, like sprucely organized trees in a forest that only one single unique squirrel can attach (to use a metaphor). The secondary adapters are used as primers in an amplification reaction within the flow cell and a reaction referred to as sequencing by synthesis is initiated when labeled nucleotides are added to the flow cell, one by one. An image is captured of the flow cell after each added nucleotide where an emitted light from each adapter (tree) shows whether the nucleotide could be incorporated. The series of captured images can be read by the instrument to construct a sequence.

Whole-exome sequencing

As the exons (including the coding regions of the DNA) only constitute approximately 1% of the human genome, sequencing of those regions has been shown to be efficient for identification of novel functional mutations (255). Also, since PCR amplification of each coding sequence is expensive and cumbersome, this new method is today playing a major role in disease gene discovery and also in the clinical setting at some university hospitals. In Paper V and VI, we used the NimbleGen whole-exome protocol for labeling of DNA, followed by sequencing on an Illumina HiSeq platform (32,204) as described above and previously in a published article (255). Both tumor- and matched normal blood/tissue DNA were included in the WES approach. The sequences were subsequently aligned to the human reference genome using standardized protocols (ELAND, Illumina). Differences between reference and non-reference read proportions between tumors and their corresponding normal sample were called variants or indels, and the significance of the aberrations were statistically

calculated (Fisher's exact test). Constitutional variants that were annotated in the 1000 Genomes database[22] were excluded.

siRNA transfection, stable over-expression and cell motility

In Paper V, functional studies regarding *KMT2D* were performed in which PC12 cells (rat pheochromocytoma) were used for both siRNA transfection (knock-down) and stable transfection (over-expression) of *KMT2D* in a mock medium (lipofectamine-2000, Life technologies). For the siRNA transfection, siRNA targeting *KMT2D* was used and for the constitutive transfection, Neo- and MLL2- plasmid vectors were used (Origene). The transfection efficiencies were verified using qRT-PCR for both *KMT2D* silencing and over-expression. After siRNA transfection, the migratory potential of the cells were measured after 4, 12, 16 and 24 hours using modified Boyden Chambers (BD Biosciences), and after the over-expression the cell motility was measured through counting of migrated cells after 2, 4, 8 and 12 hours. Additionally, protein was extracted from the cells and used for Western Blot analysis of the KMT2D protein. RNA isolated from *KMT2D* over-expressing cells and mock-transfected[23] PC12 cells was used for gene expression profiling as described below.

Gene expression microarray

While qRT-PCR was used to analyze the expression of individual genes, an Affymetrix-based RNA microarray approach was used in Paper IV and V for genome-wide analysis of ~29k annotated genes (32). Microarray analysis uses the same basic principle with a flow cell as described for targeted sequencing above, but with the difference that oligonucleotides are arranged at a surface that are able to recognize and attach to cDNA. In Paper V, the overall gene expression profiles between *KMT2D* over-expressing and mock-transfected PC12 cells were studied. For both Papers IV and V, the same microarray platform was used, but with different Gene Chips to analyze the human and rat genome respectively. For the Affymetrix microarray, a median of 26 probe sets covered each gene from which a mean was calculated as a measurement for the specific gene expressions. Benjamini-Hochberg calculations were used to correct for multiple testing and t-tests were performed for testing of differentially expressed genes between the sample groups. In Paper IV, after normalization, hierarchical clustering of the microarray expression data was performed using a gene set of 454 genes as defined by Burnichon *et al.* (227)

[22] According to the 1000 Genomes homepage (www.1000genomes.org), the project is the first effort to sequence the genomes of a large number of people, with the goal to find the most genetic variants that have frequencies of at least 1% in the population to provide a complete resource on human genetic variations.

[23] Mock-transfected cells are treated with the transfection reagent only and no addition of siRNA.

PCR product cloning

In Paper II, two *EPAS1* mutations were found in the same tumor. In order to investigate if the mutations occurred on the same or on different alleles, a technique to clone the PCR product into a vector was used (31). The technique involves transformation of the vector into *E.coli* bacteria cells and is thoroughly described in a related publication where the *EPAS1* gene was analyzed in detail (137).

Protein analysis

Immunohistochemistry (IHC)

The IHC method used to detect proteins in tissue sections, was invented in 1950 (256) and is still in use as a diagnostic tool in the clinical practice as well as in research. The method was used in this thesis to detect protein expression of NF1 (Paper III), and of KMT2D, H3K4me3, H3K27me3 and SDHB (Papers V and VI). By application of a primary antibody that is directed to a specified target amino acid sequence (epitope), the target protein can be identified and visualized in the tissue section. The method can provide information about presence and subcellular localization, or absence of the protein investigated. The avidin biotin complex (ABC) method that amplifies the signal intensity was used for protein detection in all papers included in this thesis where IHC has been performed. If the amplification obtained by the ABC kit is not preferred, a HRP-conjugated secondary antibody can be used instead.

IHC is performed on 4 µm thin paraffin embedded slides, and prior to the antibody incubation, xylene is used to remove the paraffin in order to get access to the proteins in the tissue. Heated citrate solution was used for antigen retrieval for all cases, and since our developing method (with 3,3'-Diaminobenzidine, DAB chromogen) is based on a peroxidase reaction, endogenous peroxidases in the tissues need to be inhibited with a hydrogen peroxide (H_2O_2) solution. As the ABC method uses the proteins avidin and biotin to visualize the target, endogenous avidin and biotin is blocked with biotin and avidin respectively. The biotinylated secondary antibody has a high affinity to the primary antibody and the avidin used in the ABC solution binds to the biotin attached to the secondary antibody. In order to avoid unspecific binding of the secondary antibody, the slides need to be blocked with a protein solution (such as bovine serum albumin). When DAB and H_2O_2 is added, a brown color appears when H_2O_2 is converted to O_2 and H_2O by the peroxidase that is attached to the avidin (which is attached to the biotin of the secondary antibody).

Primary antibody incubations were performed in wet chambers over night at different concentrations for the different antibodies used in Papers III, V and VI. After counterstaining in HTX, the slides were evaluated in a graded scale as presented in the papers (32,159,204).

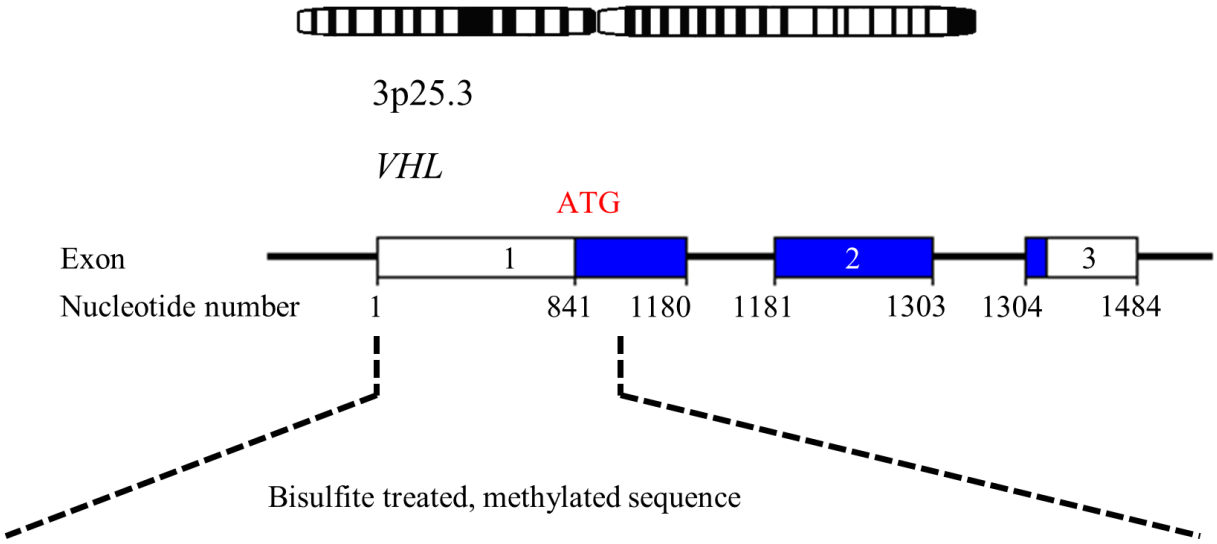
RESULTS AND DISCUSSION

I - EPIGENETIC INACTIVATION OF THE *VHL* GENE

In Paper I (51), we sought to investigate the susceptibility gene promoter methylation status in a large cohort of PPGL. This was performed by bisulfite Pyrosequencing of the promoter regions of the following 11 genes: *EGLN1, MAX, MEN1, NF1, RET, SDHAF2, SDHB, SDHC, SDHD, TMEM127* and *VHL* in 96 tumors and 34 normal adrenal samples. For all genes except *VHL* and *RET*, both PPGLs and normal adrenal tissue samples exhibited low levels of promoter methylation. Regarding the *RET* gene, promoter hypermethylation was observed in two single PGLs. The main finding of the study was a frequently observed increase of *VHL* promoter methylation levels. The *VHL* methylation levels were increased in PGL compared with PCC, in PPGL compared with normal adrenal samples and in malignant PPGL compared with benign tumors. To expand on this, we studied the *VHL* gene expression with qRT-PCR and found an inverse correlation between *VHL* methylation status and *VHL* mRNA expression. Previous efforts for assessing the methylation levels of the PPGL susceptibility genes have failed to find hypermethylation of the *VHL* promoter (53,118,126,244). The discrepancy is probably stemming from the fact that the CpG sites analyzed in those studies are not same as the ones assessed herein (Figure 12). Specifically, Welander *et al.* (118), Dammann *et al.* (244) and Cascón *et al.* (53) used primers as indicated in blue in Figure 12, originally designed by Herman *et al.* (257) while Letouzé *et al.* (126) used the Illumina HM27 (27K) methylation assay covering 27578 CpG sites with the closest dinucleotide located around 800 bases downstream of the sequence in this study.

Interestingly, we found that the methylation density of the four individual CpG sites analyzed in our assay differed with higher levels of methylation at the early positions. Since the sequences of all other attempts of assessing the *VHL* promoter methylation levels are located downstream of our assay as indicated in Figure 12, it might be a true phenomenon that the methylation levels are decreasing downstream of the promoter region.

In summary, our results indicate that the *VHL* gene is inactivated through promoter methylation and that the hypermethylation could play a role in the PPGL development. Since the publication of the study in 2013, it has been found that several Ras-association domain family members (RASSF) also are hypermethylated in their promoter regions and could play a role in the tumor development (258).



TGAGTGTTTATGTTTGTAGTTTTAATTGTTTTGAAGTAAATATTTGATTTTTTAATTTTTATTAGAGTGTTTTGTAT
ATAGTAGGTTTAATTATTTTTTTTTTTTTATTAATTATTTATGTTTTGTAAGAATTTAGTTAGTTGATTTTTTGTATT
TTATAAG**CG**TGATGATTGGGTGTTTT**CG**TGTGAGATG**CG**TTATTTT**CG**AATTTTGTTA**CG**A**CG**T**CG**GTATATTG**C**
GCGTTTGATATGAAGAAAAAAAAAATTTAGTTAGTTTATTAGGTATAGTGGTTAAGGTTTGTAATTTTTGTATTT
TGAGAGGTTAAGGTAGGAGGATTATTTGAATTTAGGAGTT**CG**AGATTAGTTTAGGTAATATAG**CG**AGATTT**CG**T
TTTAAATAATAAATAAAAATAATTAGT**CG**GGTATGGTGGTG**CGCG**TTTATAGTATTAATTATT**CG**GGAGGTTGA
GG**CG**AGA**CG**AT**CG**TTTGAGTTAGGGAGGTTAAGGTTGTAGTGAGTTAAGTTCGCGTTATTGTATTTTAGTT**CG**G
GCGATAGAGTGAGATTTTGTTTTAAAAAAAAAAAAAATATTAAATTTTAGAGGGG**CG**AAAAAAAATTTTATAGT
GGAAATATAGTAA**CG**AGTTGGTTTAGTTT**CG**TTTT**CG**TTATAA**CG**GTTTA**CG**GTGTTGGAGGATTTTTTTG**CG**TA
CGCGTATAGTTTT**CG**GT**CG**GTTATTTT**CGCG**AG**CGCG**TTTTATTTTTTAT**CG**AG**CGCGCGCG**AAGATTA**CG**GAG
GT**CG**ATT**CG**GGAG**CGCG**TA**CG**TAGTTT**CG**TTT**CGCG**TT**CG**ATT**CGCG**GATTTCGCGGCGTTCGGTTCGGGTGGT
TTGGAT**CGCG**GAGGGAATGTTT**CG**GAGGG**CG**GAGAATTGGGA**CG**AGGT**CG**AGGTAGG**CGCG**GAGGAGGTAG
G**CG**T**CG**AAGAGTA**CG**GTTTTGAAGAAGA**CG**G**CG**GGGAGGAGT**CG**GG**CG**T**CG**AGGAGTT**CG**GTT**CG**GAAGAG
TT**CG**GTT**CG**GAGGAATTGGG**CG**T**CG**AGGAGGAGATGGAGGT**CG**GG**CG**GT**CGCG**GTT**CG**T --> 200 bases

TGA = Start of the *VHL* gene, transcript 1.
GAA = *VHL* gene promoter, Genbank accession No AF010238
TCG = Sequence analyzed in this study
CG = CpG site
GTA = Primer sites previously analyzed by Welander *et al*, Dammann *et al* and Cascón *et al*.
ATG = Starting of protein translation.
--> = Position of first CpG site covered by the Illumina K27 array

Figure 12.

Overview of the start of the *VHL* gene and its promoter region.

The *VHL* gene is located on chromosome 3, at 3p25.3 and transcript 1 consists of three exons as shown in blue and white. The bisulfite treated, methylated sequence of the gene differs from the human reference since unmethylated cytosine (C) residues are converted to thymidine (T). The sequences analyzed here and in other publications are indicated in different colors and by arrow. The figure is modified from Paper I, figure 4 (51).

II - IDENTIFICATION OF RARE GERM-LINE MUTATIONS

In the second study (31), we identified rare germ-line mutations and characterized somatic mutations by targeted sequencing of the PPGL susceptibility genes in 86 PPGL. The targeted sequencing approach (Illumina MiSeq) was designed to cover all exons of the genes that (at the time of the study design) were known to be associated with PPGL: *EGLN1, EPAS1, KIF1Bβ, MAX, MEN1, NF1, RET, SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127* and *VHL*. All suspected mutations/variants found with the MiSeq sequencing were verified with Sanger sequencing in the tumors and also investigated in corresponding constitutional DNA when available. The sequencing approach was found to be significantly faster and economical compared with Sanger sequencing. A possible drawback of the technique is that since the publication of the study, the susceptibility gene list has grown rapidly and frequent updates of the gene coverage design is therefore needed. Additionally, there is always a risk of incidental findings of mutations/variants when screening many genes. This might lead to ethical issues both for the patient and physicians due to the difficulties of evaluating the pathogenicity of novel variants.

As the title indicates, rare germ-line mutations were found including one *KIF1Bβ* mutation in the constitutional tissue of a patient with a PCC. Also, a somatic *KIF1Bβ* mutation was found in a PCC already known to be endowed with a *NF1* gene mutation. Patients endowed with germ-line mutations were generally younger at their age of diagnosis compared to the patients with somatic mutations. Moreover, the most frequently mutated gene was *NF1*, followed by *EPAS1* and *RET*. One PGL harbored two *EPAS1* mutations that after PCR product cloning was found to occur on the same allele. No mutations were found in *MEN1, SDHC, SDHD* or *SDHAF2*. For the other genes, mutations were found in frequencies as previously published.

In summary, we found that a large subset of the PPGL investigated was endowed with mutations in PPGL susceptibility genes. We also proved the usefulness of targeted susceptibility gene sequencing for genetic screening that might be considered in the clinical setting.

III - NF1 IHC DOES NOT PREDICT *NF1* GENE MUTATIONS

In Paper II, it was found that the *NF1* gene is the most frequently mutated gene in PPGL patients, both in tumors and in the germ-line DNA of the patients. To follow up on this, In Paper III (159), we sought to investigate if the *NF1* mutations reflect an aberrant NF1 protein expression and also whether NF1 IHC could be used as a screening tool to find patients for further (germ-line) genetic testing of the *NF1* gene.

We included a total of 67 PCCs (with known *NF1* gene status: 49 *NF1* wild-type and 18 *NF1* mutated) and assessed NF1 expression using IHC. Although the majority of the *NF1* mutated PCCs displayed absent NF1 immunoreactivity, the NF1 expression was only retained in a minority of *NF1* wild-type PCCs. Our data therefore indicate that NF1 IHC exhibits a robust sensitivity but poor specificity for use as a screening tool for *NF1* mutations, and hence cannot be recommended as a clinical adjunct for screening purposes. Therefore, in the clinical context, family history of NF1 phenotypes, co-occurrence of other clinical NF1 characteristics and genetic screening therefore remains the most efficient and reliable tool to pinpoint these patients. However, the finding of absent NF1 expression in the majority of *NF1* wild-type PCCs suggests important alternative modes of *NF1* inactivation apart from mutations.

Interestingly, the NF1 immunoreactivity was specifically prominent in those tumor cells close to the tumor capsule and around blood vessels (Figure 13). Although this phenomenon stands without a credible explanation; we hypothesized that regional differences in NF1 degradation and/or subclonal expansions of NF1 could underlie the obtained staining patterns.

In summary, we found that NF1 IHC alone is not a sufficient tool for *NF1* mutation screening.

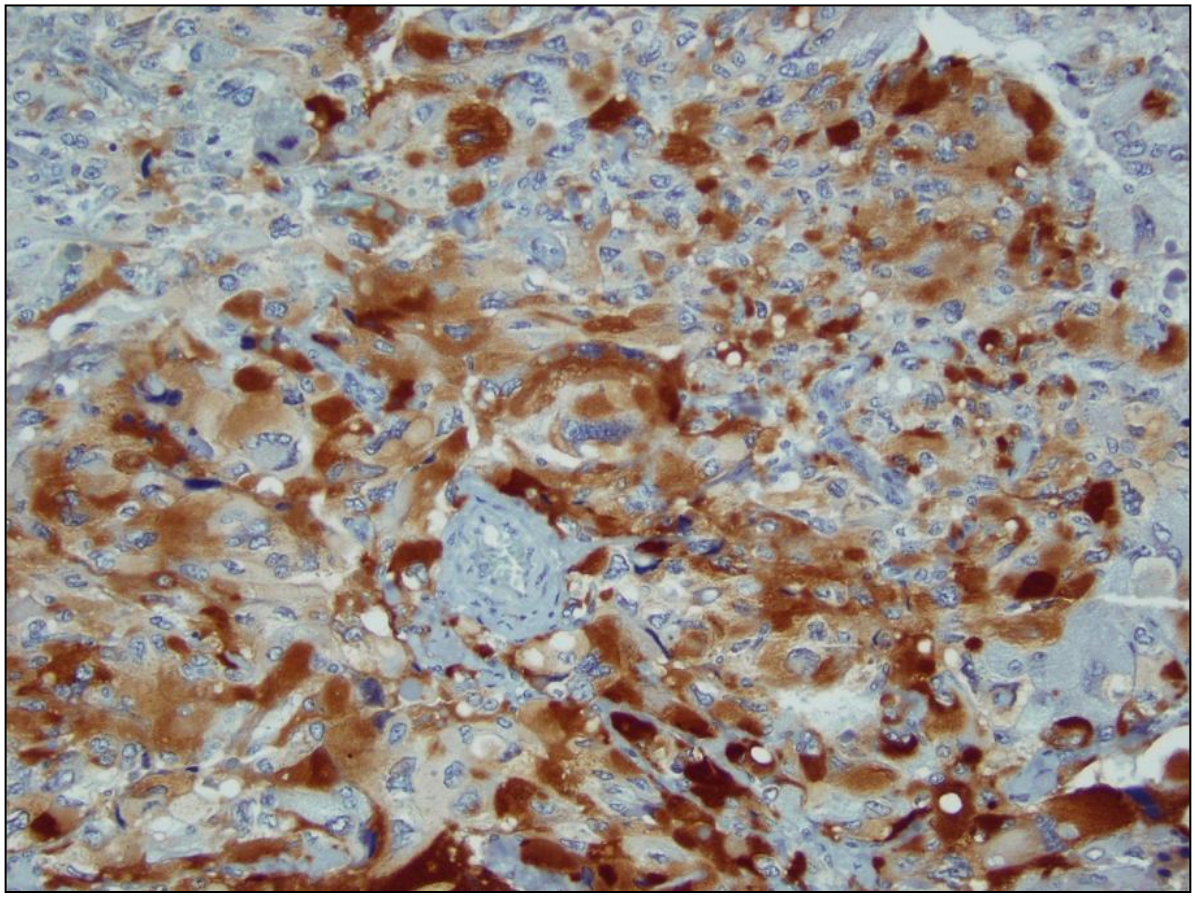


Figure 13.

NF1 immunoreactivity around blood vessel.

The image shows a previously unpublished NF1 staining of a PCC with strong, prominent NF1 immunoreactivity (brown) around a blood vessel (blue), a phenomenon without an obvious explanation. Cell nuclei are stained blue.

IV – ASSESSMENT OF *HRAS* MUTATION PREVALENCE AND ASSOCIATED EXPRESSION PROFILE

In Paper IV, we characterized the *HRAS* mutation prevalence in a large cohort of tumors and investigated the expressional profiling of *HRAS* mutated tumors. Since the publication of Paper II, new PPGL susceptibility genes have been verified with *HRAS* as one of them. To further study the PPGL etiology we collected 156 PPGL (142 PCCs and 14 PGLs) and screened them for mutations in the *HRAS* gene. We found activating *HRAS* mutations in 11/156 (7.1%) cases and identified a novel *HRAS* mutation (Q61L) in a PGL (Figure 14). Additionally, we compared the *HRAS* mutation status with microarray-based gene expression profiles for a previously defined set of 454 genes (227) for 93 of the cases.

All tumors with *HRAS* mutations included in the mRNA expression profiling were found to cluster together with the *NF1*- and *RET*-mutated tumors (Figure 9). The gene expression profiles of these tumors have been shown to be associated with kinase pathway activation (Figure 10). We also found that somatic *HRAS* mutations did not occur in patients with any other known PPGL susceptibility gene driver event which is in line with preceding findings (120,198,199,215) and none of the *HRAS*-mutated tumors in our cohort were malignant.

Taken together with the previously published frequencies of *HRAS* mutations in PPGL, the overall *HRAS* mutation frequency was calculated to 5.2% among all PPGL and 8.8% among PPGLs without known susceptibility gene mutation (Table 2).

In summary, our data suggest a role of *HRAS* mutations as a somatic driver event in benign PPGL and that *HRAS* mutated pheochromocytomas cluster together with *NF1*- and *RET*-mutated tumors associated with activation of kinase pathways.

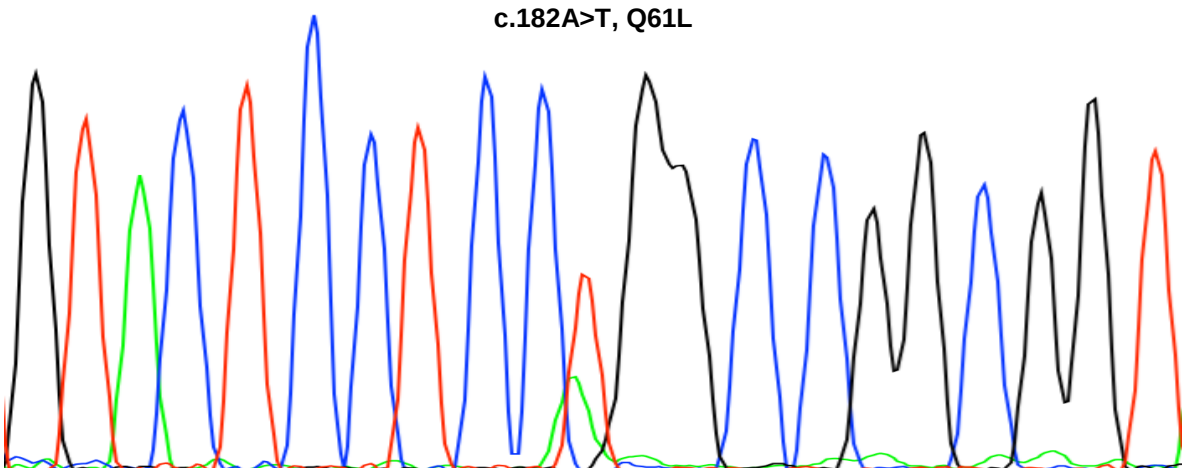


Figure 14.

Chromatogram of a PGL with the *HRAS* Q61L mutation.

The figure shows the Q61L mutation in a PGL (c.182A>T, COSM498), in a reversed complementary sequence. The heterozygous variant is shown in the middle of the chromatogram.

V - IDENTIFICATION OF *KMT2D* AS A RECURRENTLY MUTATED GENE IN PCC

In the fifth study (32), we characterized a discovery cohort of 15 PCCs devoid of mutations in established PCC susceptibility genes using WES of matched PCC and normal tissue samples with the aim of detecting novel driver genes. Besides *KMT2D* as the title indicates, several interesting variants were identified including mutations in known cancer genes such as *ZAN, MITF, WDTC1, CAMTA1* and *CDKN2C*. A low rate of 6.1 non-silent single nucleotide variants (SNVs) per sample was detected. Among the 15 PCCs, one somatic *HRAS* mutation and one somatic *EPAS1* mutation were observed as the only PPGL related genes.

In the discovery cohort, two somatic mutations and one constitutional mutation were found in the *KMT2D* gene in one sample each. Further focused screening of 83 additional PCCs led to the detection of *KMT2D* mutations/variants in 11 additional cases, of which two were recurrent in two samples each (N5223S and G2735S) (Figure 8). Altogether, heterozygous missense *KMT2D* mutations were found in 14 (11 somatic, two constitutional, one undetermined) out of 99 PCCs, resulting in an overall *KMT2D* mutational frequency of 14%. Five of the 14 *KMT2D* mutations (36%) were found in the functional FYR or SET domains of the gene (Figure 8). Further expressional analysis using qRT-PCR showed that *KMT2D* expression was significantly up-regulated in PCCs as compared to normal adrenal tissue samples. Copy number analysis showed that the vast majority of PCCs (91%) carried two copies of *KMT2D* while 2% of the cases exhibited one copy, 6% three copies and 1 case (1%) had four copies of the gene. IHC showed a positive correlation between *KMT2D* mutations and KMT2D and H3K4me3 nuclear staining, a finding that might suggest that the mutations affect histone methyltransferase activity.

As the size of the *KMT2D* gene substantially obstructed a site-directed mutagenesis approach to test the pathogenicity of the mutation(s), we instead chose to study the effects of *KMT2D* over-expression. This showed that *KMT2D* over-expression positively affected cell migration in a PCC cell line. Furthermore, the expressional profile of PC12 cells with and without *KMT2D* over-expression was determined using an Affymetrix microarray technique. A significant difference in expressional patterns between mock-transfected and transfected PC12 cells was found for a total of 594 transcripts and gene ontology analysis suggested an enrichment of genes within the TGF-beta signaling network and extracellular matrix-receptor interaction pathways. This analysis may explain why *KMT2D* transfected PC12 cells exhibited an increased migratory potential, since the TGF-beta pathway as well as the triad extracellular matrix, focal adhesion and regulation of actin cytoskeleton are important players in mediating migratory potential and invasive properties of various tumor types.

In summary, we found that somatic *KMT2D* mutations are frequent in PCCs. Our findings highlight a possible novel gene implicated in PCC tumorigenesis, and suggest that methyltransferase dysregulation could constitute a novel pathogenic mechanism for subsets of PCC.

VI – LACK OF *KMT2D* MUTATIONS IN PGL

In study VI (204), we report the screening of PGLs for mutations in the *KMT2D* gene. The study is a continuation of Paper V where somatic *KMT2D* mutations were reported in 14% of PCCs and methyltransferase dysregulation was proposed as a novel disease mechanism in this tumor entity. Here we sought to assess whether PGLs display any *KMT2D* gene mutations as well as investigate *KMT2D* gene expression, copy number alterations and lysine-specific histone methylation levels. Thirteen PGLs were included and all exhibited *KMT2D* wild-type sequences only. *KMT2D* gene expression was found to be significantly higher in PGLs than in normal adrenal samples and positive nuclear KMT2D immunoreactivity was found in the majority of cases. We could also see a significant association between KMT2D protein and positive H3K4me3 staining. Also, subsets of PGLs displayed copy number gain of the KMT2D locus. In summary, our data suggest that *KMT2D* mutations in PGLs are rare or absent. The finding of deregulated *KMT2D* expression compared to normal adrenals might also suggest an abnormal methyltransferase regulation in PGLs, which expands our current notion regarding PGL tumorigenesis and its known coupling to histone methylation aberrancies.

When performing gene expression analysis, an ideal comparison to PCCs would be normal adrenal medulla rather than whole adrenal glands as used in Papers I, V and VI since adrenocortical tissue is more predominant in the adrenal gland compared to the medullary cells. However, due to the miniscule nature of a normal adrenal medulla and the rarity of normal adrenal tissues at a whole, these tissue specimens are very hard to obtain. As an effort to comply with this problem, we have done two (non-published) serious attempts. In the first, we employed a microscopy-guided dissection technique using formalin-fixed paraffin-embedded (FFPE) material, in which sections of 12 normal adrenal tissues were collected from the Karolinska University Hospital biobank, and subsequently dissected to remove cortex from medulla. RNA was extracted using an RNA extraction kit specialized for FFPE samples, but unfortunately all samples failed the amplification step with qRT-PCR, probably due to fragmented material. The second attempt included a macroscopic dissection technique in which multiple 1x0.5 mm biopsies were taken directly postoperatively from a normal adrenal gland where one part of the biopsy was snap frozen in liquid nitrogen and the other part was directly put in formalin and processed as a paraffin block. Representativity slides from each block were stained for heamatoxylin-eosin and investigated by an experienced endocrine pathologist using light microscopy. Using this technique, a single biopsy proved to contain >80% adrenomedullary cells, supported by strong chromogranin A and synaptophysin immunostaining, which are neuroendocrine cell markers known to be expressed in adrenal medulla. This biopsy was then assessed for *KMT2D* gene expression by qRT-PCR. The results as here presented in Figure 15, show that the normal adrenomedullary biopsy exhibited *KMT2D* expressional levels similar to the normal adrenals.

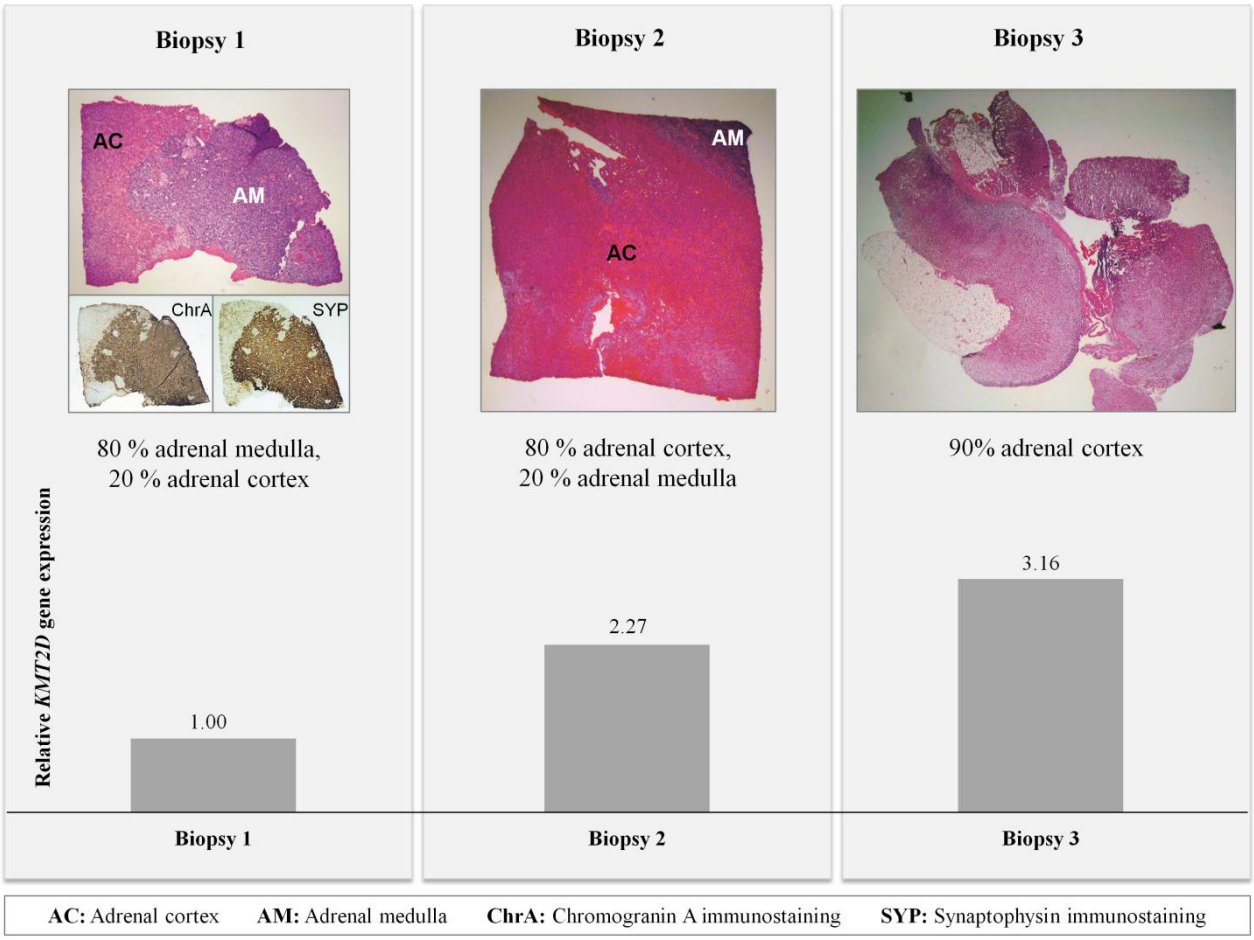


Figure 15.

KMT2D expression in biopsies from normal adrenal gland.

The figure shows H&E-stained representativity slides from three biopsies (1, 2 and 3), with ChrA and SYP immunohistochemical stainings for biopsy 1 with their relative *KMT2D* gene expression (normalized to biopsy 1 = 1) below.

CONCLUDING REMARKS

All six studies included in the thesis show that the genetic and molecular background of PPGL is remarkably diverse with a very high degree of heritability and up to date, 25 genes (and counting) that are associated with PPGL tumorigenesis to some extent. The work as presented in this thesis aimed to further characterize the genetic and molecular background of these tumors, and may have contributed to a piece of the PPGL puzzle that together with other studies can contribute to better diagnosis, prognosis and treatment for the patients. The specific conclusions for the papers are outlined below.

In Paper I, our results suggest that the *VHL* gene is inactivated through promoter methylation which could trigger PPGL development, a finding that in the future may have implications for better prognosis and/or treatment for the PPGL patients.

In Paper II, a comprehensive NGS mutation screening analysis showed that a large subset of the PPGL investigated had mutations in the susceptibility genes. This knowledge is of certain importance in order to detect patients that might be in risk as well as facilitate early detection of tumors and knowledge of how to treat the different diseases. We were also able to prove the usefulness of a fast and cost-efficient sequencing method.

In Paper III, we concluded that NF1 immunohistochemistry is not a satisfactory screening method alone for *NF1* mutations and that genetic screening together with clinical workup remains the most reliable tool to pinpoint NF1 patients.

In Paper IV, we found that *HRAS* mutated PCC cluster together with *NF1*- and *RET*-mutated tumors and that these mutations are considered to constitute a somatic driver event in benign PPGLs without other known susceptibility gene mutations.

In Paper V, a whole exome sequencing approach was used in order to screen for novel susceptibility genes. The *KMT2D* gene was frequently mutated in PCCs which highlights a possible novel gene implicated in the tumor development. As of this, we also suggest that methyltransferase dysregulation could represent a novel pathogenic mechanism in the tumors.

In Paper VI, we screened PGLs for mutations in the *KMT2D* gene due to the findings in Paper V and the similitude of PGLs and PCCs. All PGLs showed wild-type sequences of the *KMT2D* gene and we therefore concluded that mutations in this gene are rare or absent in PGL.

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