Genetic and molecular alterations in aldosterone producing adenomas

Ravi Kumar Dutta

FACULTY OF MEDICINE AND HEALTH SCIENCES
Linköping University Medical Dissertation No. 1710, 2020
Department of Clinical and Experimental Medicine
Linköping University
SE-581 83 Linköping, Sweden
www.liu.se
Genetic and molecular alterations in aldosterone producing adenomas

Ravi Kumar Dutta

Division of Cell Biology
Department of Clinical and Experimental Medicine
Faculty of Medicine and Health Sciences
Linköping University
SE-58185 Linköping
Supervisors
Professor Peter Söderkvist
Linköping University

Professor Oliver Gimm
Linköping University

Ravi Kumar Dutta 2020

Articles have been reprinted with permission of the respective copyright owners.

ISBN: 978-91-7929-976-7
ISSN: 0345-0082

Printed by LiU-Tryck, Linköping, Sweden, 2020
To Aprajita and Ved
ABSTRACT

Aldosterone producing tumors (APA, also known as Conn tumors) are adrenal tumors that overproduce aldosterone, a hormone that regulates the sodium levels in blood and contributes to blood pressure (BP) regulation. Excessive production of aldosterone causes hypertension and approximately 5-15% of hypertensive patients have hyperaldosteronism, known as primary aldosteronism (PA). Major causes of PA are bilateral adrenal hyperplasia (BAH) or aldosterone producing adenoma (APA) and about 30% of PA patients have APAs. In most cases, the disease is unilateral, in rare cases bilateral. Patients with APA are often detected when they have elevated blood pressure (BP>160/100mmHg) or when BP cannot be controlled with drugs. Surgery dramatically normalizes or lowers BP in patients with APA.

In this thesis, we first explored the mutation frequency in susceptibility genes in sporadic APAs. About 60% of APAs displayed complementary mutations in the KCNJ5, ATP1A1, ATP2B3, CTNNB1, CACNA1D and CLCN2 genes (Paper I, II & III). Copy number variation analysis of 35 APAs identified amplification of chromosome 10q24.31 in two tumors, where CALHM1-3- genes encoding for potential calcium ion channels are located. Only CALHM2 is expressed in adrenals and sequencing of CALHM2 revealed three different heterozygous sequence variants; c.341_42delCT (CALHM2P114Rfs*12), c.286G>A (CALHM2A96T) and c.580G>A (CALHM2V194M) in 5 APAs. CALHM2 is expressed in the mitochondrial membranes and Ca$^{2+}$ imaging revealed that CALHM2 has selection of another ion rather than Ca$^{2+}$. The genetic variant CALHM2V194M converts CALHM2 into a non-selective channel and results in higher Ca$^{2+}$ conductance in mitochondria. We further found that loss of CALHM2 function upregulates REELIN/LRP8 signaling activating β-catenin dependent transcription of target genes (Paper II). In Paper IV, we investigated Scandinavian APA cases (n=35) and Swedish controls (n=60) for GWAS and discovered a susceptibility locus on chromosome Xq13.3 in a 4 Mb region to be significantly associated with APAs. Significance level was still same after genotyping the sentinel SNP rs2224095 in a replication cohort of APAs (n=52) and controls (n=740). Sequencing of an adjacent gene of the sentinel SNP, MAGEE1, identified a rare variant in one APA, which is complementary to other mutations in our primary cohort.

In Summary, our studies have increased the knowledge of molecular genetic events in APAs. The results may contribute to find future non-surgical treatments for APAs.
Aldosteronproducerande adenom (APA, kallas också Conn tumörer) är binjuretumörer som överproducerar aldosteron, ett hormon som reglerar blodets natrium/kalium nivåer och medverkar i blodtrycksregleringen. Patienter med APA upptäcks nästan alltid när hen har förhöjt blodtryck (BT >160/100mmHg) eller när BT inte kan kontrolleras med läkemedel. Kirurgi normaliserar eller sänker dramatiskt BT hos patienter med APA. Orsaken till APA beror på medfödda eller under livet förevärvade förändringar i binjurebarkcellernas arvsmassa som resulterar i en ökad tillväxt och medföljande ökad hormonproduktion. Genetiska variationer eller förändringar hos en del jonkanalproteiner (t ex KCNJ5, ATP1A1, ATP2B3, CACNAID) resulterar i förhöjda intracellulära kalciumnivåer, som i sin tur stimulerar aldosteronproduktionen och bidrar till högt BT. De hittills identifierade genetiska förändringarna förklarar ca 50% av APA.

Och området för APA-statistikerna är ett hårt eller ett mjukt majestät, är det förstås ett mått att bestämma om och hur det förbättrar behandlingen av patienter med APA. Projektet började med sekvensering av kända mottagliga gener i vår kohort av skandinaviska patienter och hos cirka 60% fann vi somatiska, icke ärftliga, mutationer i genen KCNJ5. Hos de patienter där inga mutationer i kända orsaksgener kunde visas analyserades människans alla arvsanlag (s k exom) och variation av kopietal för att identifiera ytterligare förändringar i APA. På kromosom 10q23 identifierar vi en kopietalsökning och sekvensering av gener i detta kromosomala område finner vi både ärftliga och förvärvade mutationer i CALHM2-genen. CALHM2-genen var förändrad hos 12% av APA patienterna. Proteinet är en kalciumjonkanal och finns i cellens mitokondrier och är en viktig jontransportör i cellens mitokondrier. Årftliga mutationer är viktiga att identifiera, dels för att kunna ge rätt behandling och för tidig upptäckt av APA, dels för att kunna identifiera släktingar som löper en ökad risk för att drabbas av högt BT. Hos muterade celler ökar kalciumkonzentrationen i cellen och det resulterar i en ökad bildning av tillväxtsignaler, REELIN, LRP8 och så småningom aktiveras transkriptionsfaktorn β-catenin som stimulerar celltillväxt. Samtidigt stimulerar bildningen av enzymer som ökar cellens aldosteronproduktion, en aktivierungssignal som återfinns hos mer än 70% av APA. I nästa projekt utrede vi huruvida en kloridjonkanal, CLCN2, som orsakar familjär hyperaldosteronism också kan vara involverad i sporadisk APA. Vi fann att 1% av sporadiska APA patienter hade samma mutation som de familjära fallen. Mutationen var komplementär till de andra APA mutationerna som tidigare analyserats, vilket antyder att den utgör ytterligare en orsaksgen. En analys av 41 nya fall från Norge resulterade inte i några ytterligare mutationer. I en analys av nukleotidvariationer hos APA patienter kunde vi identifiera ytterligare en kromosomal region, på X-kromosomen, som kunde kopplas till APA och en undersökning av arvsanlagen i detta kromosomala område avslöjade andra förändringar i MAGEE1-genen.

Sammanfattningsvis har dessa studier resulterat i en ökad kunskap om vilka genetiska förändringar som bidrar till uppkomsten av sporadiska APA. Både årftliga och sporadiska mutationer i CALHM2 och CLCN2 generna representerar nya arvsanlag som kan bidra till uppkomst av APA. En bättre förståelse av mekanismerna för de ganska vanliga aldosteronproducerande tumörerna kan leda till framtagande av mer specifika läkemedel och förbättra dagens ganska ospecifika medicinska behandling som ett alternativ till kirurgisk behandling.
Table of Contents
LIST OF PAPERS .......................................................................................................................... 2
ABBREVIATIONS .......................................................................................................................... 3
BACKGROUND ............................................................................................................................. 5
Cancer ........................................................................................................................................... 5
Aldosterone and blood pressure ................................................................................................. 6
Primary aldosteronism (PA) ......................................................................................................... 9
Diagnosis and treatment of PA .................................................................................................... 9
Genetics of sporadic aldosterone producing adenomas ............................................................. 10
Genetics of Familial hyperaldosteronism (FH) .......................................................................... 15
AIMS ............................................................................................................................................ 17
MATERIALS AND METHODS ...................................................................................................... 19
Samples and nucleic acid isolation ............................................................................................. 19
Sanger Sequencing ....................................................................................................................... 19
Quantitative real-time PCR ....................................................................................................... 19
DNA microarrays ......................................................................................................................... 20
Luciferase reporter assays ........................................................................................................... 21
Calcium imaging assays ............................................................................................................. 21
Statistics ...................................................................................................................................... 22
RESULTS AND DISCUSSION ..................................................................................................... 23
Paper I: Complementary somatic mutations of KCNJ5, ATP1A1 and ATP2B3 in sporadic aldosterone producing adrenal adenomas (Endocr Relat Cancer 2014; 21:L1-4) ...................... 23
Paper II: Germline sequence variants of CALHM2 encoding for a mitochondrial channel are associated with aldosterone-producing adenomas (Submitted) ........................................... 24
Paper IV: Genome-wide association study identifies a susceptibility locus on the X-chromosome in patients with aldosterone producing adenomas (submitted) ........................................... 29
Conclusions ................................................................................................................................. 30
Acknowledgements .................................................................................................................. 31
References .................................................................................................................................... 33
APPENDIX (PAPERS I-IV) ........................................................................................................... 41
LIST OF PAPERS
The thesis is based on the following papers.


II. Dutta RK, Vesa Loitto, Thomas Arnesen, Martin Walz, Piero Alesina, Urban Karlsson, Luca Conti, Oliver Gimm, Peter Söderkvist. Germline sequence variants of CALHM2 encoding for a mitochondrial channel are associated with aldosterone-producing adenomas. *Manuscript*


ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensinogen converting enzyme</td>
</tr>
<tr>
<td>APA</td>
<td>Aldosterone producing adenomas</td>
</tr>
<tr>
<td>ARR</td>
<td>Aldosterone to renin ratio</td>
</tr>
<tr>
<td>ATP1A1</td>
<td>ATPase Na+/K+ transporting subunit alpha 1</td>
</tr>
<tr>
<td>ATP2B3</td>
<td>ATPase plasma membrane Ca2+ transporting 3</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AVS</td>
<td>Adrenal venous sampling</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>BAH</td>
<td>Bilateral adrenal hyperplasia</td>
</tr>
<tr>
<td>CACNA1D</td>
<td>Calcium voltage-gated channel subunit alpha 1 D</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>Catenin beta 1</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response elements</td>
</tr>
<tr>
<td>CYP11B1</td>
<td>Cytochrome P450 family 11 subfamily B member 1</td>
</tr>
<tr>
<td>CYP11B2</td>
<td>Cytochrome P450 family 11 subfamily B member 2 (Aldosterone synthase)</td>
</tr>
<tr>
<td>CYP21A2</td>
<td>Cytochrome P450 family 21 subfamily A member 2</td>
</tr>
<tr>
<td>DOC</td>
<td>Deoxycorticosterone</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>eQTL</td>
<td>Expression quantitative trait loci</td>
</tr>
<tr>
<td>FH</td>
<td>Familial Hyperaldosteronism</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
</tr>
<tr>
<td>HSD3B2</td>
<td>3β-Hydroxysteroid dehydrogenase-2</td>
</tr>
<tr>
<td>KCNJ5</td>
<td>Potassium inwardly-rectifying channel subfamily J member 5</td>
</tr>
<tr>
<td>NR4A1</td>
<td>Nuclear receptor subfamily 4 group A member 1</td>
</tr>
<tr>
<td>NR4A2</td>
<td>Nuclear receptor subfamily 4 group A member 2</td>
</tr>
<tr>
<td>PA</td>
<td>Primary aldosteronism</td>
</tr>
<tr>
<td>StAR</td>
<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>ZG</td>
<td>Zona glomerulosa</td>
</tr>
</tbody>
</table>
BACKGROUND

Cancer

According to World Health Organization, 18.1 million of new cases of cancer are diagnosed and 9.6 million deaths are predicted every year. One in 5 men and one in 6 women worldwide develop cancer during their lifetime. In western countries, increased lifespan with lifestyle, such as smoking, alcohol consumption, obesity and also reproductive and hormonal factors are considered as major causes of cancer.

Cancer is a genomic disease where changes in genome or mutations transform the normal cell to a cancer cell which acquire the ability of uncontrolled proliferation. There are key features called ‘Hallmark of Cancer’ that differentiate normal cell and cancer cells. Cancer cells should have ability to sustain proliferative signaling, evade growth suppressors, resist cell death, enable unlimited replication and induce angiogenesis. Each normal cell in the body has a specific role and it divides, creating new cells to maintain homeostasis. Cell divisions are tightly regulated and errors at the single nucleotide level activate apoptosis of cells. If the mutant cell continues through the cell cycle and replicate, the cell acquires the first step in carcinogenesis.

The mutation occurs randomly at any location in the genome. However, it often targets proto-oncogene and tumor suppressor genes. These genes are highly conserved through evolution and regulates the cell cycle, cell division and differentiation. Proto-oncogenes promotes the cell proliferation; and mutation in it causes over activation of protein which leads to uncontrolled cell growth. Therefore, mutation in one allele is often enough for dominant effect on cell. Tumor suppressor have repressive effect on the proliferation of the cell cycle. Both alleles of a tumor suppressor needs to be affected to show loss of the normal function.

In this thesis, we have studied the genetic and molecular alteration in aldosterone producing adenomas (APAs). APAs are benign tumor producing enormous aldosterone. We have focused research on genes and proteins affecting APAs.
**Aldosterone and blood pressure**

The zona glomerulosa (ZG) cell layers of the adrenal cortex produces aldosterone, a mineralocorticoid, which under physiological conditions is regulated by the renin-angiotensin system. Aldosterone is an important hormone that maintain the blood pressure and blood volume\(^7\). Low blood pressure and depletion in blood volume leads to secretion of a proteolytic enzyme, renin, from the kidneys\(^8\)\(^9\). Renin converts angiotensinogen, produced by the liver, to angiotensin I, which is subsequently cleaved by angiotensinogen converting enzyme (ACE) to angiotensin II (Figure 1). Angiotensin II and serum potassium levels regulate the secretion of aldosterone\(^10\). To a lesser extent, the adrenocorticotropic hormone (ACTH), estrogen and urotensin II also stimulates the secretion of aldosterone from the adrenals\(^11\).

---

**Figure 1.** Aldosterone production. Under physiological condition, decrease in blood pressure and volume activate the kidney granular cells to produce renin which cleaves angiotensinogen into angiotensin I. Angiotensin I is subsequently cleaved into angiotensin II by angiotensinogen (ACE). Angiotensin II binds to receptors (AT1A, AT1B and AT2) on zona glomerulosa cells of the adrenal cortex. This triggers the production of aldosterone. This figure is modified from previously published figure (Dutta et al. 2016)
Angiotensin II binds to angiotensin II type 1 (AT1) receptors on the surface of ZG cells which activates the secondary messenger inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (Figure 2) \cite{12,13}. IP$_3$ trigger the export of Ca$^{2+}$ from the endoplasmic reticulum through IP$_3$ receptors, increasing cytosolic Ca$^{2+}$ that activates the Ca$^{2+}$/calmodulin-dependent protein kinases (CaMKs). Diacylglycerol regulates the StAR (Steroidogenic acute regulatory protein) levels via cAMP response elements binding (CREB) sites in target gene promotors \cite{12,14-16}. StAR is involved in the transportation of cholesterol, a substrate for aldosterone production. The CaMK complex mediates the transcription of the nuclear receptors (NR4A1 and NR4A2) which initiate the transcription of aldosterone synthase (CYP11B2)\cite{12}. Angiotensin II type 1 (AT1) receptors also inhibits the leakage in K$^+$ channels which results in depolarization of the cell membrane and activation of voltage gated Ca$^{2+}$ channels. It triggers the influx of calcium and increases aldosterone synthesis. K$^+$ concentration across the membrane sets the membrane potential\cite{10}. Change in extracellular K$^+$ depolarizes the cell membrane and activates the voltage gated Ca$^{2+}$ channels.

![Diagram of Angiotensin II receptor signaling](image)

**Figure 2.** Angiotensin II receptor signaling. Binding of angiotensin II to angiotensin type II receptor generates the secondary messenger inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ trigger the export of Ca$^{2+}$ from the endoplasmic reticulum through IP$_3$ receptor. Simultaneously, it blocks the potassium channel and depolarize the membrane which leads to opening of voltage gated channels. It triggers the influx of calcium and increases aldosterone synthesis. Calcium is the main signal for aldosterone production.
Production of aldosterone takes place in mitochondria and aldosterone is then exported out of the cells. The enzymes involved in aldosterone production are either located in the mitochondrial or the endoplasmic reticulum (ER) membranes. Aldosterone production begins with the conversion of cholesterol to pregnelolone by CYP11A1 enzyme in mitochondria. Pregnelolone is then transported to the ER where it is converted into deoxycorticosterone (DOC) by 3β-hydroxysteroid dehydrogenase-2 (HSD3β2) and 21-hydroxylase (CYP21A2). DOC is then transported back to mitochondria and converted into aldosterone by aldosterone synthase (CYP11B2). In the zona fasciculata of the adrenal cortex, steroid 11β-hydroxylase (CYP11B1) converts DOC to cortisol. CYP11B1 and CYP11B2 are homologous enzymes, 95% similarity on protein, and their expression is limited to the zona fasciculata and zona glomerulosa, respectively.

Figure 3. Aldosterone production. Cholesterol is converted into aldosterone by series of enzymetic conversion. Red and yellow color enzymes are found in mitochondria and endoplasmic reticulum.

Aldosterone play a key role in Na+ homeostasis. It stimulates Na+ reabsorption by binding to a cytoplasmic/nuclear mineralocorticoid receptor (MR) in epithelial cells of distal tubule in kidney, colon and sweat glands. MR acts as a transcription factor and controls the expression of Na+.
channels, Na⁺/K⁺ ATPase and NaCl co-transporters. The aldosterone driven reabsorption of Na⁺ regulates the extracellular fluid osmolarity and volume, and hence blood pressure.

Primary aldosteronism (PA)

Autonomous production of aldosterone is a pathological condition called primary aldosteronism (PA) and can be found in 5–20% of hypertensive patients. The two most common causes of primary aldosteronism are an aldosterone-producing adenoma (APA) and bilateral idiopathic adrenal hyperplasia (BAH), both are characterized by a constitutive over-production of aldosterone, independent of angiotensin II.

Diagnosis and treatment of PA

Patients with PA have resistant hypertension. The blood pressure of these patients is uncontrolled, defined to be >140/90 mmHg with 3 conventional antihypertensive drugs or controlled, <140/90 mmHg on four drugs. The diagnosis of PA requires demonstration of over-production of aldosterone, independent of angiotensin II. The Endocrine Society guidelines recommend the measurement of plasma aldosterone and renin concentration. If the aldosterone to renin ratio (ARR) is >200, confirmative tests such as the captopril-challenge test, the upright furosemide-loading test, or the saline-loading test should be performed. Then, imaging of the adrenal glands is performed to discriminate between APAs and BAH, also called idiopathic hyperaldosteronism (IHA). However, very small APAs or adrenal hyperplasia cannot be visualized with either CT or MRI. The distinction between APAs and BAH is important to identify appropriate treatment. Therefore, adrenal venous sampling (AVS) is recommended to determine which adrenal is secreting more aldosterone. Furthermore, the guidelines suggest that familial history of early onset of hypertension must be taken into consideration for younger PA patient (<20 age). These patients should undergo genetic testing for Familial hyperaldosteronism I (FH-I) and for germline mutations of the KCNJ5-gene causing Familial hyperaldosteronism III (FH-III). APA patients undergo adrenalectomy which often normalizes the blood pressure. Patients with BAH are treated with antagonists of mineralocorticoid receptor lifelong.
Genetics of sporadic aldosterone producing adenomas

All the genes found to be mutated in sporadic APAs are either ion channels or ion exchangers localized on the cell membrane except for β-catenin that acts as a transcriptional co-activator. Ion channels regulate the membrane potential and mutations in the genes lead to proteins that depolarize the membrane resulting in influx of Ca\(^{2+}\). Ca\(^{2+}\) seems to be a stimulator for aldosterone production. The effect of these mutations on cell proliferation is unclear except for APAs with mutations in CTNNB1, which is a well-known oncogene that encodes for β-catenin. The susceptibility genes are briefly described below.

**KCNJ5**

*KCNJ5* encodes the potassium ion channel Kir3.4 which is expressed in many cell types. Kir3.4 forms the ion channel as homo- or heterotetramers with Kir3.1 (*KCNJ3*). In human adrenal cortex, it is highly expressed in the zona glomerulosa. Potassium channels have a highly conserved stretch of amino acid, a K\(^+\) channel signature sequence or GYG motif which forms the selectivity filter region for K\(^+\) ions. The Kir3.4 channel is an inward-rectifier channel that is very selective for monovalent ions and remarkably discriminates between K\(^+\) and Na\(^+\) ions. Loss-of-function germline mutations in *KCNJ5* have been reported in congenital long QT syndrome. Germline and somatic mutations were identified in *KCNJ5* genes in patients with familial PA and sporadic forms of APA. Two hot spot somatic mutations were identified at p.Gly151Arg and p.L168R of the Kir3.4 channel. The amino acids G151 and L168 are situated on the highly conserved glycine-tyrosine-glycine (GYG) motif of the selective filter and the second transmembrane (TM) domain of *KCNJ5*, respectively. The mutation p.G151R is the most frequently identified mutation (>60%). It affects the first glycine of the GYG motif which is present in the extracellular loop of all four subunits of channels and forms the narrowest part of the pore. The mutant channels lose the selectivity filter which leads to higher Na\(^+\) conductance and persistent depolarization of the cell. Further, this depolarization results in the opening of the voltage gated Ca\(^{2+}\) channels, which results in an influx of Ca\(^{2+}\) and increased aldosterone production.

Since the discovery of first *KCNJ5* mutations in APAs, several other *KCNJ5* mutations have been identified (p.R52H, L157S, p.E246K, p.G247R, p.E282Q etc). Among the *KCNJ5* mutations,
G151R and L168R are found in approximately 90-99% of all cases, i.e., other mutations are rare. Studies on a multinodular adrenal case identified two different \textit{KCNJ5} mutation in two different nodules\textsuperscript{45}. It showed the importance of the \textit{KCNJ5} channel for the zona glomerulosa cells. On the expression level, no obvious gene expression clustering pattern could be identified that differentiates APAs with and without \textit{KCNJ5} mutations\textsuperscript{46}. However, the expression of \textit{CYP17A1} (a marker of the zona fasciculata) was higher in \textit{KCNJ5} mutant tumors\textsuperscript{46}. However, it is not yet known whether it is a consequence of the mutation that leads to increased expression of \textit{CYP17A1}\textsuperscript{37}.

\textit{KCNJ5} mutations are present in approximately 30-65\% of APA patients, with a higher prevalence in the Asian population. In clinical presentation, patients harboring \textit{KCNJ5} mutations were younger, had larger tumors and higher aldosterone levels compared to wild type \textit{KCNJ5} patients and more prevalent in females.

\textit{ATP1A1}

\textit{ATP1A1} encodes for the $\alpha$ subunit of the Na$^+$/K$^+$ ATPase which is a membrane-bound ion transporter of P-type ATPase. Na$^+$/K$^+$ ATPases consist of $\alpha$- and $\beta$-subunits, where the $\alpha$-subunit contains Na$^+$/K$^+$ and ATP binding sites whereas the $\beta$-subunits are responsible for directing the $\alpha$-subunit to the plasma membrane\textsuperscript{47}. The $\alpha$ subunit is the most abundant and the major form found in the kidney and epithelial cells\textsuperscript{47}. Na$^+$/K$^+$ ATPases are expressed throughout the adrenal cortex; the highest expression is found in the zona glomerulosa cell layer\textsuperscript{29}. At the expense of ATP, three Na$^+$ transported out of the cells for two K$^+$ ions by Na$^+$/K$^+$ ATPase\textsuperscript{48}. The exchange of ions generates an electrochemical gradient across the membrane that facilitates the cellular uptake of ions. The $\alpha$ subunit is a 110 kDa protein with more than 1000 amino acid residues arranged in 10 transmembrane domains with large cytoplasmic domains. Residues of TM4, TM5 and TM6 forms the K$^+$ ion binding site \textsuperscript{47}. Amino acids from TM5 (Y778), TM6 (G813 & T814) and TM9 (E960 &E961) are involved in a Na$^+$-specific binding site. L104 and V332 interact with E334 and cooperate in K$^+$ binding and gating of the K$^+$ ions \textsuperscript{48}.

Somatic mutations in APAs were identified at or around amino acid L104, E334 and E961 which lies in TM1, TM4 and TM9, respectively\textsuperscript{27,29,37,49}. The reported mutations are at conserved amino
acid residues present in all ATPases. Expression of mutant L104R and E334G results in membrane depolarization, leakage of H+ and acidification of adrenal cells. An increase of extracellular K+ results in an increase of pH in the mutant-expressing cells. Acidification of adrenal cells have been shown to increase the expression of CYP11B2 mRNA and aldosterone levels. The four amino acid deletion at the Na+ binding site (p.A963SdelGGT) still transport K+ ions under normal physiological conditions. ATP1A1 mutations are found in 5-8% of patients with APAs. Patients harboring ATP1A1 mutations have high aldosterone levels and the mutations are more prevalent in males than in females.

**ATP2B3**

*ATP2B3* encodes a plasma membrane calcium transporter, ATPase 3 (PMCA3), which belongs to the superfamily of P-type transporters. It is highly expressed in the adrenal cortex. The Ca2+ ATPases are conserved and have a single polypeptide chain organized into 10 transmembrane (TM) domains and four cytoplasmic domains. There are two calcium ion binding sites (I and II). Site I is located in the space between TM4 and TM5 with some contribution of TM8. Site II is formed on TM4. Ca2+ ATPases stay inactive under physiological cytoplasmic Ca2+ concentration. Binding of calmodulin to the calmodulin binding site on Ca2+ ATPase’s, activate the transportation of Ca2+. Ca2+ ATPase transport one Ca2+ ion with exchange of one H+ ion at the expense of one ATP. Germline mutations in *ATP2B3* have previously been reported in X-linked congenital cerebellar ataxia and Laminin syndromes.

Mutations in *ATP2B3* are located in the TM4 domain. At least two amino acids deletion were identified between amino acids L425 and L433. The amino acids L425, V426, and V427 interact with glutamic acid at 462 and are crucial for calcium binding. The mutations potentially lead to the distortion of Ca2+ binding region which reduced the capacity of exporting Ca2+ from cells. Mutations also depolarize the membrane which leads to opening of voltage gated calcium channels and influx of calcium.

*ATP2B3* mutations have a low frequency (~1.5%) in APAs, are more prevalent in females, and are associated with a more severe form of PA.
**CACNA1D**

*CACNA1D* encodes the α1 subunit of L-type voltage gated calcium channel, Cav1.3. The α1 subunit is the pore forming region of L-type channels. Depolarization of the membrane activates the Cav1.3 channel. CACNA1D is arranged in four homologous repeats (I-IV); each repeat contains six transmembrane segments (S1-S6). The S5 and S6 segments and the membrane-associated pore loop between them form the pore that lines the channel. The narrow external end of the pore is lined by the pore loop which contains a pair of glutamate (E) residues in each domain. These glutamate residues are required for Ca²⁺ selectivity that is unique to a Ca²⁺ channel. Mutations in *CACNA1D* have been reported in sinoatrial node dysfunction and deafness syndrome, patients with sporadic autism and intellectual disability.

Somatic mutations have been identified in the *CACNA1D* gene in sporadic cases of APAs all over the gene. Mutations have been mainly found at S4, S5 and S6 segments of all four repeats. All mutations were at conserved sites in orthologues that range from invertebrates to humans. Expression of mutant channels found early opening of channel (lower potential) and have a sustained activation which increases the intracellular Ca²⁺ levels.

Prevalence of *CACNA1D* mutations varies in different ethnic groups. Sequencing of DNA from aldosterone producing cell cluster found the higher percentage of *CACNA1D* mutation (>40%) in black population. Unlike *KCNJ5* mutants, *CACNA1D* mutants have been reported to be more prevalent in males, older, and smaller APAs. Patients harboring *CACNA1D* mutations have high aldosterone levels compared to patients with APAs harboring other mutations and are associated with a more severe form of PA.

**CTNNB1 (β-catenin) and Wnt signaling pathways**

Wnt-signaling is one of the evolutionary conserved fundamental pathways which controls the developmental process. The best understood Wnt signaling is the canonical Wnt signaling (Figure 3). In the absence of Wnt signaling, β-catenin becomes phosphorylated by a degradation complex, which consists of Axin, adenomatous polyposis coli (APC), GSK3β, casein kinase-1β protein proteins and targets β-catenin for ubiquitination and proteasome dependent degradation.
Activation of Wnt/β-catenin pathway by Wnt ligands inhibits phosphorylation of β-catenin and proteasomal degradation, translocating it to the nucleus. Upon translocation, β-catenin binds with DNA-binding proteins of the Tcf/Lef family in target gene promoters. Tcf/Lef proteins acts as repressors in the absence of β-catenin and Wnt signaling and binding of β-catenin converts Tcf/Lef factors into transcriptional activators. Gain of function mutations in CTNNB1 have been identified in several different forms of cancer including benign and malignant adrenocortical tumors.

Figure 4. Wnt signaling. Upon binding of wnt ligand to its receptors, Frizzled (Fz) and Lrp5/6, it inhibit the degradation complex to phosphorylate β-catenin (CTNNB1). Stable β-catenin, then, translocate to nucleus and binds with DNA-binding proteins of Tcf/Lef family which eventually leads to expression of target genes.

β-catenin signaling is essential for the development of the adrenal cortex, especially for adrenal glomerulosa. Mutations in the Wnt/β-catenin pathway results in a defective zone of adrenal cortex and development of PA in mice models. In sporadic cases of APAs, very low frequency mutations are found on critical phosphorylation sites of CTNNB1. The mutations result in the loss of phosphorylation sites which prevents ubiquitination by the E3 ligase, TrCP1, and further proteasomal degradation of β-catenin. However, active β-catenin is found in the majority of APAs, where it regulates the transcription of the aldosterone rate-limiting enzyme, CYP11B2.
Genetics of Familial hyperaldosteronism (FH)

Less than 5% of PA cases have a germline mutation in patients. Currently, familial hyperaldosteronism (FH) is divided into four forms (I-IV).

FH I

FH I is one familial form of PA which is also known as glucocorticoid-remediable-aldosteronism. The molecular etiology of FH I is unequal crossing over of homologous genes CYP11B1 and CYP11B2 during meiosis. The fusion gene includes the promotor region of CYP11B1 and most of the coding sequence of CYP11B2. Consequently, patients with FH I produces aldosterone from all three layers of adrenal cortex and is regulated by ACTH instead of angiotensin II. Each individual of FH I has a different pattern of crossing over break points of CYP11B1 and CYP11B2 which suggests that mutations arise independently.

FH II

FH II is the most common form of inherited PA and is clinically indistinguishable from the sporadic form of PA. Clinical and biochemical features as well as the severity of the disease vary among FH II patients. Criteria for the diagnosis of FH II are at least two first-degree members of the same family have confirmed PA and FH-I and familial hyperaldosteronism type-III (FH-III) have been excluded. Six different mutations have been identified in CLCN2, encoding for a chloride channel, at conserved regions of the channel (p.M22K, p.G24D, p.Y26N, p.R172Q, p.delK362 and p.S865R). Mutation leads to open the channel and abolish the voltage dependency of the channel. This may lead to depolarization of the adrenal cell membrane and activation the voltage gated calcium channel to Ca^{2+} influx.
**FH III**

FH-III is a very rare form of inherited hypertension\(^7^9\). The patients have a very severe clinical phenotype\(^2^6\). Resistant hypertension has been found among family members at an early age with bilateral adrenal hyperplasia and hypokalemia. Bilateral adrenalectomies normalizes the patients’ blood pressure and aldosterone levels. The genetic etiology behind FH-III are mutations in the \( KCNJ5 \) gene which encodes a potassium channel\(^2^6\). Mutation was identified at p.T158A which affects the selectivity filter region of the channel protein Kir3.4.

**FH IV**

Germline mutations in the \( CACNA1H \) gene in familial cases are considered as FH IV\(^8^0\). FH IV is characterized by the early onset of hypertension with autonomous adrenal aldosterone production and no evidence of adrenal hyperplasia or tumor at the time of presentation.
AIMS
The overall aim of thesis was to elucidate the genetic background of sporadic aldosterone producing adenomas. By using various methods, we expected to identify new genes involved in the development of these tumors and in addition, we planned to characterize those genes functionally.

The specific aims for this thesis were:
1. To identify mutations in susceptibility genes in apparently sporadic APAs. (Paper I & III)
2. To identify a candidate gene by DNA microarray and their association with aldosterone producing adenomas. (Paper II)
3. To identify a genomic locus associated with aldosterone producing adenomas. (Paper IV)
MATERIALS AND METHODS

Samples and nucleic acid isolation

The studies included the tumor tissues and corresponding adjacent normal tissue and/or blood samples from aldosterone producing adenoma patients diagnosed and treated in Norway, Sweden, and Germany. All patients had drug-resistant hypertension and pathologic aldosterone to renin ratio (ARR is > 200) at the time of diagnosis. Following removal of the affected adrenal gland, the tumors were snap frozen and stored at -80°C until analysis. A specialized pathologist confirmed the diagnosis of an adenoma of the adrenal cortex. DNA and RNA were isolated from tumor tissue and blood using Maxwell 16 tissue and blood purification kits (Promega) and RNeasy minikit (Qiagen). Written informed consent was received from patients prior to inclusion in the study. All studies were conducted with the approval of an ethical committee of Linkoping University, Sweden.

Sanger Sequencing

In last two decades, the most widely used sequencing method is capillary based Sanger sequencing. It was developed by Frederick Sanger in 1977. In this method, genomic region of interest is amplified by the polymerase chain reaction (PCR). A same PCR product is further amplified using mixture of deoxynucleosidetriphosphates (dNTPs) and modified di-deoxynucleosidetriphosphates (ddNTPs). ddNTPs are modified dNTPs where 3'-hydroxyl group is replaced by a hydrogen molecule. The 3'-hydroxyl is essential for chain elongation. ddNTPs are also labeled with different fluorescent dyes. Therefore, the result of the reaction is a mixture of DNA fragments of different sizes, which can be separated by size using capillary gel electrophoresis. The identity of the terminating nucleotides is detected by the fluorescent emission that follows laser excitation of the fluorescent dye. In all paper, sequencing is performed by capillary Sanger sequencing.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) is method to detect and quantify a nucleic acid in real time. Fluorescent dyes like SYBR® Green or sequence specific DNA binding probes labeled with a fluorescent reporter are two commonly used method to detect nucleic acid in qPCR. Fluorescent dye like SYBR binds with double stranded DNA, regardless of unspecific product or primer dimer. A second approach commonly known as TaqMan assays is more specific. It contains two primers
and reporter probe which binds with complementary sequence of PCR product. The reporter probe consists of fluorophore and quencher and as long as reporter stay unbounded, quencher suppresses the fluorophore. Once PCR amplified the target region, reporter probe binds with target and DNA polymerase cleaves the probe through its 5’-3’ exonuclease activity. It results in a detectable fluorescent signal. We have used this method for gene expression and SNP genotyping. In paper I-II, we have performed expression analysis with both SYBR® Green and predesign TaqMan assays. For this method, RNA was reversely transcribed into cDNA by reverse transcription. cDNA was further used for qPCR. The relative expression was measured with reference to housekeeping gene HPRT1 which has stable expression in different tissue form\textsuperscript{81}. The comparative CT method (2\textsuperscript{-ΔΔCT}) was used to calculate to relative expression\textsuperscript{82, 83}.

In paper III & IV, we used qPCR method for SNP genotyping. In this approach, we used the predesign TaqMan probe from thermoFisher scientific, which contain two probes, each specific for one allele, is labeled for detection with the reporter dyes FAM or VIC. Upon PCR amplification, each probe hybridized with polymorphic site.

**DNA microarrays**

The microarray technique provides a powerful method to obtain genetic information on a genome wide scale from a sample. DNA microarrays can be used to study single nucleotide polymorphism (SNPs) as well as copy number variations (CNVs) in DNA. A microarray consists of oligonucleotides that are attached to surface in a specific pattern, and can be used to detect complementary oligonucleotides in a sample. In this method, a genomic DNA is fragmented enzymatically and ligated with adaptor\textsuperscript{84}. Fragmented DNA is amplified with generic primers which recognize the adaptor sequences and then labeled with fluorescence probe. Thereafter Labelled DNA hybridized to the oligonucleotide on a microarray. After hybridization, Arrays are scanned for fluorescence intensities of each spot. In paper II, we used a microarray of from affymetrix (Genome-Wide Human SNP Array 6.0) covering more than 906,600 single nucleotide polymorphisms (SNPs) and more than 946,000 probes for the detection of copy number variation. CNVs were assessed using Genotype Console (Affymetrix) software and GeneSpring GX (Agilent)\textsuperscript{85 86}. Reference data was generated using local Swedish healthy person’s DNA. In paper IV, we used the SNPs data to investigate the genome wide association study.
siRNA

Small interfering RNA (siRNA) is a double-stranded (non-coding) RNA of 20-25 base pairs which interferes with the expression of specific genes with complementary nucleotide sequences by degrading mRNA after transcription, preventing translation. In papers II, siRNA against CALHM2 of was used to downregulate it in H295R adrenal cancer cell line at a concentration of 30nM. Expression of CALHM2 was confirmed by qPCR on mRNA level and at protein level using Western blot.

Luciferase reporter assays

Luciferase is a generic term in which product of the enzymatic conversion of substrate emits the light. Luciferase is widely used in research field to assess transcriptional activity in the cells. A vector containing the luciferase gene under the control of a promoter construct of interest is transfected in cells to evaluate the transcriptional activity. In paper II, we measured the activity of β-catenin after silencing of CALHM2. Upon activation of Wnt signaling, β-catenin binds with the promoter of TCF4. A luciferase reporter vector containing TCF4 promoter (TOP flash) and mutant TCF4 promoter (FOP flash) were transfected in CALHM2-silenced H295R adrenal cancer cells. Mutant TCF4 promoter (FOP flash) were used to for removing noise from data. Pierce™ Firefly Luc One-Step Glow Assay Kit was added to each well to detect luciferase activity. Cells without any treatment were used for normalization of data.

Calcium imaging assays

Calcium imaging is used to measure the intracellular calcium level in isolated cell or tissue. Two common calcium indicators used to detect calcium are chemical indicators and genetically encoded calcium indicators which responds to the binding of Ca$^{2+}$ ions by changing their fluorescence properties. In Paper II, we performed single cells calcium imaging of cytoplasm (Fluo-4) and mitochondria (Rhod-2) using chemical indicator. Cells were incubated with either fluo-4 or Rhod-2 for 30-60min at 37°C. Then, cells were treated with 100 μM histamine perfused with Krebs–Ringer Glucose (KRG) buffer containing 1mM of Ca$^{2+}$. Histamine treatment releases Ca$^{2+}$ from
endoplasmic reticulum (ER) stores. Images were taken at intervals of 1s using 40× water immersion objective (Carl Zeiss) with appropriate filter-sets for fluorescent labels.

**Statistics.**

Statistical analysis was performed using GraphPad Prism. A two-tail student t-test was performed between two groups and two-way ANOVA for more than two groups. Genotype frequencies were compared using the Chi-square test or Fisher’s exact test. Odds ratios (OR), relative risks (RRs), and 95% CIs were calculated. P-values of less than or equal to 0.05 were considered statistically significant.
RESULTS AND DISCUSSION

Paper I: Complementary somatic mutations of KCNJ5, ATP1A1 and ATP2B3 in sporadic aldosterone producing adrenal adenomas (Endocr Relat Cancer 2014; 21:L1-4)

The aim of this study was to determine the frequency of mutations in the APA susceptibility genes KCNJ5, ATP1A1, and ATP2B3 in apparently sporadic APAs. When this study was performed, mutations in KCNJ5 were identified in about 30–45% of patients. KCNJ5 encodes an inwardly rectifying K⁺ channel, Kir3.4, which exists either as a homo tetrameric channel or as a hetero tetrameric channel with KCNJ3. Hetero tetrameric channels are more active than the homo tetrameric channels. ATP1A1 encodes a sub-unit of a Na⁺/K⁺ exchangers. ATP2B3 encodes a plasma membrane calcium pump. Mutations in ATP1A1 and ATP2B3 have been reported in about 6% and 2% of the tumors, respectively.

We sequenced the entire coding region of KCNJ5, KCNJ3, ATP1A1 and ATP2B3 in 35 sporadic cases of APAs. We identified frequent somatic mutations in KCNJ5, ATP1A1 and ATP2B3. Mutations in KCNJ5 were identified at two hot spot sites, p.G151R and p.L168R. The frequency of mutations (31%) is similar to other reports. Mutations in ATP1A1 were identified at p.L104R in 2(6%) APAs. The wild type amino acid L104 interacts with E334 by van der Waals interaction which is crucial for K⁺ binding and gating in ATP1A1. The mutation p.Leu104Arg disrupts the binding of K⁺. Deletions of 425A_426V and 428A_429V were identified in ATP2B3 in 3 (9%) APAs. The mutations remove one of the calcium binding sites of ATP2B3. Mutations in KCNJ5 were more prevalent among female patients and younger patients. However, there was no gender deference in ATP1A1 and ATP2B3 mutations. Patients harboring mutations in genes encoding for ATPase’s had higher aldosterone levels and smaller APAs compared to patients with KCNJ5 mutations. All mutations were complementary to each other.

With this paper, we showed the presence of KCNJ5, ATP1A1 and ATP2B3 mutation in our Scandinavian cohort. We acknowledge that we had a fairly a small cohort of sporadic cases of APAs and frequency of ATPase’s mutation differs from previous reports. However, mutation in KCNJ5 was in agreement with previous reports.
Under physiological condition, Binding of Angiotensin II to Angiotensin II type I receptor results in closure of TASK K⁺ channels. K⁺ channels maintains the cell membrane potential and open at hyperpolarized state (negative membrane potential). Closure of K⁺ channels depolarizes the membrane which results to opening of voltage gated Ca²⁺ channels. Hyperaldosteronism and hypertension have been identified in female KCNK3–/– mice (KCNK3 encodes TASK-1). The mutation p.G151R lies in the GYG conserved motif of KCNJ5 protein which is outer line of the pore region of Kir3.4 channel. The mutation p.L168R lies in the 2nd transmembrane domain which forms the covalent bond with GYG-motif of extracellular loop. Both mutations destroy the selectivity filter of channel Kir3.4 which converts it into a non-selective channel. Furthermore, mutant channels results in higher conductance of Na⁺ ion. Loss of inward K⁺ current results in chronic depolarization of the cell membrane and opening of voltage-gated calcium channels, rise of intracellular Ca²⁺, which subsequently leads to increased aldosterone biosynthesis.

In case of ATP1A1 and ATP2B3, mutations remove the K⁺ and Ca²⁺ binding sites. Previous study have shown that angiotensin II inhibits the Na⁺/K⁺ pump (ATP1A1) activity in glomerulosa cells which results in depolarization of cell membrane and opening voltage gated ion channels. ATP2B3 transports out the Ca²⁺. Mutation in ATP2B3 results in accumulation of calcium inside cell. ATPase mutations also result in depolarization of the cell membrane and opening of voltage-gated calcium channels.

**Paper II: Germline sequence variants of CALHM2 encoding for a mitochondrial channel are associated with aldosterone-producing adenomas (Submitted)**

The aim of the second study was to identify new genes involved in the development of APAs. In addition, we planned to characterize those genes functionally. To accomplish our hypothesis, we used high density DNA microarray which contains both probes for copy number variations and SNPs. We detected several chromosomal alterations. Among them, amplification of chromosome 10q24.31 was identified in two APAs. Chromosome 10q24.31 contains the genes CALHM1-3 that encode for potential calcium channels. Sequencing of CALHM1-3 revealed three different heterozygous sequence variants: c.341_42delCT (CALHM2P114Rfs*12, rs200457105), c.286G>A (CALHM2A96T) and c.580G>A (CALHM2V194M, rs2232662) in 5 APAs. All mutations were
germline and have been reported in the ExAC database (http://exac.broadinstitute.org/) as well as in the Swedish 1000 genome database (SweGen, https://swegen-exac.nbis.se/) as rare variants. The impact of SNPs on the risk of developing APA was evaluated in a case-control study design (35 APA cases and 752 regional population controls). The frequency of the CT deletion (rs200457105) allele was higher in patients with APAs (12%) than in controls (4.5%), with an odds ratio (OR) of 2.8 (CI 1.2–11.2, p = 0.03). SNP rs2232662 is the second rare SNP found in the APA cohort, and this SNP was neither detected in a subset of the healthy regional control cohort (n = 376) nor in the SweGen database (n=1000). Mutations in CALHM2 were complementary to mutations in other APA susceptibility genes, except for one APA with two sequence variants (CALHM2 and ATP2B3). Patients with CALHM2 mutations were all males, older, had smaller APAs and yet high levels of plasma aldosterone.

We also sequenced CTNNB1 and CACNA1D. Three somatic and one germline mutations were identified in CACNA1D (~10% of APAs). One somatic mutation was identified in CTNNB1. Patients with CACNA1D mutations were all males, older, had smaller APAs and yet very high levels of plasma aldosterone.

To further investigate the effect of the identified CALHM2 mutations, we performed mRNA microarray analysis of tissue from APAs with CALHM2 mutation and normal adrenal cortex derived from patients operated on for pheochromocytoma. First, we studied the expression of CALHM1-3. Among CALHM1-3, only CALHM2 was expressed in normal and tumor tissues, and there was no difference regarding the expression levels of CALHM2 between wild type CALHM2 and mutant CALHM2 APAs. Next, we performed genome wide tests for differential expression and identified more than 200 differentially expressed genes. Among these, REELIN and LRP8 were two rational genes upregulated among mutant APAs. REELIN is a glycoprotein and acts as a ligand for LRP8 (ApoER2). To examine the role of REELIN/LRP8 in mutant APAs, we further performed gene set enrichment analysis by preparing the list of genes in REELIN/LRP8 pathway identified by Telese et al.97. We observed a significant (P=0.0001, FDR= 0.02) enrichment of the same set of genes in the REELIN/LRP8 signaling. REELIN/LRP8 signaling has been identified to inhibit GSK3β dependent phosphorylation of β-catenin, allowing non-phosphorylated β-catenin translocation to the nucleus and activation of gene transcription. Further, staining of the tumor
tissues against β-catenin showed the presence of β-catenin in the nucleus in CALHM2<sup>P114Rfs*12</sup> adenomas and cytoplasmic in CALHM2<sup>V194M</sup> adenomas. The average fold change (FC) of LRP8 in CALHM2<sup>P114Rfs*12</sup> is 11 times higher than the expression of LRP8 in CALHM2<sup>V194M</sup>, indicating the activation of the REELIN/LRP8 canonical pathway in CALHM2<sup>P114Rfs*12</sup> mutant APAs.

We next compared the transcription profiles of APAs with the CALHM2 germline variant CALHM2<sup>P114Rfs*12</sup> and APAs with the somatic KCNJ5 mutation p.G151R found in the APAs of four males to understand if they differ regarding their expression patterns. The transcriptions profiles of APAs with KCNJ5 and CALHM2 mutations clustered differently in unsupervised hierarchical clustering, except for the one APA with double mutations which clustered together with APAs having KCNJ5 sequence variants. Among those genes that were upregulated in APAs with CALHM2 mutations were steroid synthesizing enzymes (CYP11B2 and CYP11A1), transcription factors (NR4A1 and NR4A2), and receptors (MC2R and NR5A1), all involved in aldosterone synthesis.

In the beginning of this study, CALHM2 was not characterized functionally. Therefore, we began with determining the localization of CALHM2 and identified it to be expressed on the mitochondria membrane in adrenal cells. It formed a hexameric channel with its own monomeric unit. By single cell calcium imaging, we found that calcium may not be the main target ion of the CALHM2 channel. The genetic variant p.V194M made the CALHM2 protein non-selective for ions and lead to an increase of the mitochondrial calcium level.

Next, we silenced CALHM2 by siRNA in the H295R cell line and measured the mRNA of genes involved in steroidogenesis together with genes identified in the tumor expression array experiment. We found a higher expression of CYP11B2, REELIN, LRP8 and TCF4 indicating the activation of β-catenin. We therefore used luciferase assay containing TCF4 promoter and β-catenin inhibitor, FH535, to see the effect on CALHM2 silenced cells. Silencing of CALHM2 increased the luciferases activity. Furthermore, we inhibited CALHM2 silenced cells with FH535 which antagonizes β-Catenin/TCF mediated transcription. Expression of CYP11B2 decreased together with LRP8. In summary, loss of CALHM2 acts through the REELIN/LRP8 pathway and activates β-catenin.
Figure 5. Proposed model of CALHM2’s function in APAs. (A) At resting condition, binding of angiotensin II (Ang II) to angiotensin receptor I (AT1R) closes the voltage gated K⁺ channels and depolarizes the cell membrane. Subsequently, opening of voltage gated Ca²⁺ channel increases the cytoplasmic Ca²⁺ and activates the transcription of aldosterone synthase (CYP11B2) and aldosterone production in mitochondria. (B) In CALHM2 variant cells, REELIN and LRP8 is upregulated by unknown mechanisms. REELIN/LRP8 inhibits GSK3β dependent phosphorylation of β-catenin and β-catenin translocate to the nucleus which in turn increases the expression of steroidogenic genes and subsequent aldosterone production. Inactivation of CALHM2 also increases the Ca²⁺ in mitochondrial matrix which enhances the activity of aldosterone synthase and aldosterone production.
In conclusion, we reported CALHM2 as a new susceptibility gene for sporadic APAs. CALHM2 is a functional mitochondrial ion channel and mutational inactivation of its function in adrenocortical cells is associated with the development of APAs.


In the third study, we sequenced the gene CLCN2 which was identified to be mutated in familial hyperaldosteronism II\(^{77, 78}\). CLCN2 encodes for a plasma membrane homo-dimeric chloride channel (CIC-2)\(^{98, 99}\). CIC-2 channels are slowly activated at hyperpolarized conditions\(^{96}\). They are sensitive to extracellular pH, cell swelling and intracellular Cl\(^-\) concentrations. The entire coding region of CLCN2 was sequenced from tumor DNA of 39 apparently sporadic APAs. One somatic (p.G24D) and one germline (p.R73H) mutations were identified in CLCN2 in two patients. We further sequenced the entire coding region of the CLCN2 gene in additional 41 sporadic APAs from Norway without finding any additional mutations. The wild type amino acid G24 is in the region (16-60 amino acids) which is sensitive towards voltage, cell-swelling and extracellular pH\(^{100}\). The mutation p.G24D leads to opening of the channel at resting potential. The strong increase in Cl\(^-\) current may depolarize the adrenal cell membrane and activate voltage gated calcium channels.

The germline mutation p.R73H (rs144412275) is a rare variant with a minor allele frequency of 0.002 in ExAC and SweGen databases. Bioinformatics analysis predicted the single amino acid substitution p.R73H as a benign alteration. Several vertebrate species have histidine at position 73. A previous study reported that deletion of amino acids 66 to 76 had no effect on the channel activity\(^{100}\). No familial history of hyperaldosteronism or hypertension is reported in the patient’s family and the patient has not developed any recurrence in 8 years. With these consideration, p.Arg73His seems to be a rare benign or neutral variant.

In summary, a CLCN2 mutation was identified in about 1% of sporadic APAs\(^{101}\). This mutation was complementary to mutations in other susceptibility genes for sporadic APAs and may thus be a driving mutation in APA formation.
Paper IV: Genome-wide association study identifies a susceptibility locus on the X-chromosome in patients with aldosterone producing adenomas (submitted)

The genome-wide association is revolutionary approach which allows to examine the whole genome to identify chromosomal regions that may be associated to the disease. In this study, we used SNP data from our second study. We discovered a susceptibility locus on chromosome Xq13.3 to be significantly associated with APAs (rs2224095, OR= 7.9, 95%CI= 2.8-22.4, P=1 x10^{-7}). Direct genotyping of the sentinel SNP rs2224095 in a replication cohort of APAs (n=91) and population controls (n=740) identified a strong association for this region in APAs (OR=6.1, 95% CI=3.5-10.6, P<0.0005). We sequenced an adjacent gene of the sentinel SNP, MAGEE1, and identified a rare variant p.G327E in one APA. This mutation is complementary to other mutations identified in our primary cohort.

One of the susceptibility genes of APAs, ATP2B3, lies on the X-chromosome and mutations in ATP2B3 have been found in ~2% of cases. ATP2B3 is located on the 77.12 Mb downstream from the GWAS locus. Expression of ATP2B3 was significantly higher in the presence of the risk allele indicating a potential impact of the SNP on ATP2B3 expression. However, there is no evidence that shows a direct connection of genes on chromosome Xq13.3 and the regulation of ATP2B3.

To gain further insight into the functional basis of SNP rs2224095, we performed an expression quantitative trait loci (eQTL) analysis using expression data of 34 APAs and identified 24 trans-eQTL. Expression of TMEM47 was strongly associated with the SNP rs2224095 genotype, and the risk allele (C) was associated with higher expression. This association remained significant after adjusting for multiple testing (P=2.7 x 10^{-5}, FDR= 0.05). Other relevant genes were KCNE5 (P<0.05) and CASK (P<0.05) which also displayed strong association with the risk allele (C). TMEM47 and CASK revealed higher expression with the risk allele while KCNE5 showed significantly lower levels of mRNA expression. We further sequenced the genes TMEM47, KCNE5 and CASK in a subset of 14 tumors of the GWAS discovery cohort. There was no somatic or germline mutation detected in these tumors.

In conclusion, our study provides further insights into the genetic background of APAs. Some of the identified genes by trans-eQTL point towards a novel mechanistic explanation for the association of the SNPs with APAs. Further studies will be required to examine the relevance of the chromosomal region Xq13.3 and eQTL on APAs.
Conclusions

In the last decade, the genetic knowledge of APAs has broadened. Today, we know the genetic causes of ~70% of APAs and it is increasing. Genetic knowledge is important to facilitate early detection or for finding potential drugs. In sporadic APAs, there is still lack of knowledge regarding tumor progression. Active β-catenin is found in the majority of APAs but \textit{CTNNB1} is seldom mutated. However, activation of β-catenin is poorly understood. Accumulating evidence suggests that ion channels or ion exchangers play a major role and are affected by somatic mutations in sporadic APAs. This thesis has aim to further characterize the genetic and molecular alterations in sporadic APAs.

- About 60% of sporadic APAs have mutations in the known susceptibility genes \textit{KCNJ5}, \textit{ATP1A1}, \textit{ATP2B3} and \textit{CACNA1D}.

- Germline mutations were identified in \textit{CALHM2} in 14% of patients with sporadic APAs. \textit{CALHM2} is mitochondrial channel and mutations abolish the selectivity of the channel. Loss of \textit{CALHM2} activates β-catenin through the \textit{LRP8/REELIN} pathway.

- Somatic mutations in \textit{CLCN2} were found in approximately 1% of sporadic cases of APAs which is the main cause for FH II.

- Using a genome wide association study, we identified a locus on X-chromosome to be associated with APAs.
Acknowledgements

I would like to express my sincere gratitude to all the people who have contributed to this thesis.

Peter Söderkvist & Oliver Gimm, my supervisors: I would like to thank you for believing in me, giving me an opportunity to work on this project and to develop as a researcher with your supervision. Thank you for all inspiring discussion, creating friendly environment and open atmosphere at the lab. I would also like to thank Peter for all those enlightenment hours in my office.

Malin Larsson, my bioinformatics supervisor: I would like to express my gratitude for your guidance and support during my studies. It was really a great opportunity to learn about Bioinformatics.

Vesa Loitto. Though I cannot call you my co-supervisor, you have been like to me. Thank you for all the help, support and technical discussions.

Gizeh: Thank you very much for your support and guidance during my studies. You were always there to listen and help me whenever I needed.

Mouna & Gosia: Well!! What to say about you both!! Thank you for the useful scientific discussion (haha..). Scientific discussion? well! Astrology and palmistry are still science©. I will miss you guys, Gizeh, Mouna & Gosia. Sharing an office with you guys made this time much more memorable.

I would like to thank all my collaborators. Fredrik Elender, Urban Karlsson and Luca conti: Thank you for the useful discussion and your valuable suggestions for my project. And thank you for allowing to use your laboratory. Thomas Arnesen: Thank you for providing human material and your valuable suggestions on all manuscripts.

I would like to thank all my colleagues in the Peter Söderkvist lab for creating a friendly environment to work. Annette Molbæk and Åsa Schippert: the most valuable person in the lab. Thank you for all the technical support and laboratory help. Without your magical hand, nothing would have been possible here. Deepti Verma: thank you for kindness and advice. Thanks to Jenny Welander, Mohmed Ali Mosrati, Kerstin Willander, Lena Thunnel, Naomi Yamada-Fowler, Jonas Ungerbäck.

To all the students who worked with me: Marcus, Clara, Helena, Hugo. It was nice to work and share knowledge with you all.

I take this opportunity to express my gratitude to all my current and previous colleagues for their inspiring discussion, creating friendly environment and friendly atmosphere at floor 9: Mihaela-Maria Martis, Chuanwen Fan, Vian Osman, Maria Turkina, Cecilia Bivik, Charlotta Enerbäck, Annelie Abrahamsson, Gabriela Vazquez, Josefine Sandström, Carolin Jönsson, and Elin Karlsson.

I would like to specially thank to Liv Gröntoft and Bo Hägqvist for helping and guiding me for immunohistochemistry experiments. Amaya Jauregi Miguel: thank you for advice for β-catenin experiment.
To all my friends: Sumit, Mehdi, Mayur, Sudeep, Jaya Prakash, Zaheer, Pratheek, Raghu. I really had wonderful time with you all. Thanks for being there for me every time.

Uncle, Govind lal Das. Thank you Govind mama for your inspiration and endless help throughout my life.

My Brothers, Lav kumar Dutta, Chandan Karn and Navin Karn; and sister Sonal: Thank you for your endless support and love. Thank you lav dai, this would not have been possible without your support.

My Parents: Even the word ‘Thank you’ cannot be enough to show the gratitude but still thank you for making me realize the role of education, in life.

Thank you my beautiful wife, Aprajita for holding my hands while mine hands were busy in holding the Ph.D workload. I know it’s hard to grow child alone. And my little prince Ved (the little dynamite) who made my life wonderful. You both have added colors to my life.
References


Reincke M. Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. Nat Genet 2013 45 440-444, 444e441-442.


Murthy M, Xu SX, Massimo G, Wolley M, Gordon RD, Stowasser M & O'Shaughnessy KM. Role for Germline Mutations and a Rare Coding Single Nucleotide Polymorphism Within the KCNJ5 Potassium Channel in a Large Cohort of Sporadic Cases of Primary Aldosteronism. Hypertension 2014 63 783-789.


89. Baldwin TO. Firefly luciferase: The structure is known, but the mystery remains. *Structure* 1996 4 223-228.
Papers

The papers associated with this thesis have been removed for copyright reasons. For more details about these see:

http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-162592
Genetic and molecular alterations in aldosterone producing adenomas

Ravi Kumar Dutta