

Helsinki Graduate Program in Biotechnology and Molecular Biology (GPBM)/  
Integrative Life Science (ILS) Doctoral Program

***MED12:***  
**A NOVEL PLAYER**  
**IN UTERINE LEIOMYOMAS**

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**Academic dissertation**

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## LIST OF ORIGINAL PUBLICATIONS

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

- I**            **Mäkinen N\***, Mehine M\*, Tolvanen J, Kaasinen E, Li Y, Lehtonen HJ, Gentile M, Yan J, Enge M, Taipale M, Aavikko M, Katainen R, Virolainen E, Böhling T, Koski TA, Launonen V, Sjöberg J, Taipale J, Vahteristo P, Aaltonen LA. *MED12*, the *mediator complex subunit 12 gene*, is mutated at high frequency in uterine leiomyomas. *Science* 2011, 334(6053):252-255.
- II**            **Mäkinen N**, Heinonen HR, Moore S, Tomlinson IP, van der Spuy ZM, Aaltonen LA. *MED12* exon 2 mutations are common in uterine leiomyomas from South African patients. *Oncotarget* 2011, 2(12):966-969.
- III**           **Mäkinen N**, Vahteristo P, Kämpjärvi K, Arola J, Bützow R, Aaltonen LA. *MED12* exon 2 mutations in histopathological uterine leiomyoma variants. *European Journal of Human Genetics* 2013, 21(11):1300-1303.
- IV**            **Mäkinen N**, Vahteristo P, Bützow R, Sjöberg J, Aaltonen LA. Exomic landscape of *MED12* mutation-negative and -positive uterine leiomyomas. *International Journal of Cancer* 2013, 134(4):1008-1012.

\*Equal contribution

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## ABBREVIATIONS

<b>bFGF</b>	basic fibroblast growth factor	<b>KAT6B</b>	K(lysine) acetyltransferase 6B
<b>BHD</b>	Birt-Hogg-Dubé syndrome	<b>let-7</b>	lethal-7
<b>bp</b>	base pair	<b>LOH</b>	loss of heterozygosity
<b>BRAF</b>	v-raf murine sarcoma viral oncogene homolog B	<b>LS</b>	leucine-serine-rich
<b>C</b>	cytosine	<b>MED12</b>	mediator complex subunit 12
<b>CCNC</b>	cyclin C	<b>MED12L</b>	mediator complex subunit 12-like
<b>cdNA</b>	complementary deoxyribonucleic acid	<b>MED13</b>	mediator complex subunit 13
<b>CDK19</b>	cyclin-dependent kinase 19	<b>MED13L</b>	mediator complex subunit 13-like
<b>CDK8</b>	cyclin-dependent kinase 8	<b>MIM</b>	Mendelian Inheritance in Man
<b>COL4A5</b>	collagen, type IV, alpha-5	<b>miRNA</b>	micro-ribonucleic acid
<b>COL4A6</b>	collagen, type IV, alpha-6	<b>mRNA</b>	messenger ribonucleic acid
<b>COMT</b>	catechol-O-methyltransferase	<b>MRI</b>	magnetic resonance imaging
<b>CUX1</b>	cut-like homeobox 1	<b>NGS</b>	next-generation sequencing
<b>D</b>	aspartic acid	<b>p</b>	short arm of a chromosome
<b>del</b>	deletion	<b>PCR</b>	polymerase chain reaction
<b>DNA</b>	deoxyribonucleic acid	<b>Pol II</b>	RNA polymerase II
<b>E</b>	glutamic acid	<b>PR</b>	progesterone receptor
<b>ECM</b>	extracellular matrix	<b>q</b>	long arm of a chromosome
<b>ER</b>	estrogen receptor	<b>RAD51B</b>	RAD51 paralogue B
<b>FASN</b>	fatty acid synthase	<b>RAS</b>	rat sarcoma
<b>FFPE</b>	formalin-fixed paraffin-embedded	<b>RB1</b>	retinoblastoma 1
<b>FH</b>	fumarate hydratase	<b>REST</b>	RE1-silencing transcription factor
<b>G</b>	guanine/glycine	<b>RNA</b>	ribonucleic acid
<b>gDNA</b>	genomic deoxyribonucleic acid	<b>SNP</b>	single-nucleotide polymorphism
<b>HE</b>	hematoxylin-eosin	<b>t</b>	translocation
<b>HGP</b>	the Human Genome Project	<b>TCAC</b>	tricarboxylic acid cycle
<b>HIF1</b>	hypoxia-inducible factor 1	<b>TGF-<math>\beta</math></b>	transforming growth factor beta
<b>HLRCC</b>	hereditary leiomyomatosis and renal cell cancer	<b>TP53</b>	tumor protein 53
<b>HMGA</b>	high mobility group A	<b>TSC</b>	tuberous sclerosis
<b>HMGA1</b>	high mobility group AT-hook 1	<b>TSC2</b>	tuberous sclerosis 2
<b>HMGA2</b>	high mobility group AT-hook 2	<b>V</b>	valine
<b>IGF</b>	insulin-like growth factor	<b>VEGF</b>	vascular endothelial growth factor
<b>ins</b>	insertion	<b>WHO</b>	the World Health Organization
		<b>WNT4</b>	wingless-type MMTV integration site family, member 4

Gene names and symbols are italicized in the text.

## ABSTRACT

Uterine leiomyomas, or fibroids, are benign tumors arising from the smooth muscle lining of the uterus, the myometrium. They represent one of the most common neoplasms in women with an estimated prevalence ranging from 20% to even as high as 77% during the reproductive years. Considering their high prevalence, the molecular mechanisms underlying leiomyomagenesis have remained relatively unknown. Decades of work with cytogenetics have shown that 40-50% of leiomyomas harbor somatic non-random chromosomal aberrations, including translocations between chromosome bands 12q15 and 14q24, interstitial deletions in 7q, and trisomy of chromosome 12. Rarely, uterine leiomyomas can be associated with a familial hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome. HLRCC is caused by heterozygous germline mutations in *fumarate hydratase (FH)*, which encodes the enzyme fumarase of the tricarboxylic acid cycle. The known somatic chromosomal aberrations and inherited genetic factors do not, however, explain the majority of uterine leiomyomas. Recent advances in next-generation sequencing technology have enabled comprehensive genome-wide characterization of somatic mutations in human tumors. The aim of this thesis was to elucidate the molecular genetic background of uterine leiomyomas with next-generation sequencing.

Exome sequencing of 18 uterine leiomyomas and the respective normal myometrial tissue from 17 Finnish (Caucasian) patients led to the identification of recurrent somatic mutations in *mediator complex subunit 12 (MED12)*. Verification of the mutations and subsequent screening of 207 additional tumors by Sanger sequencing revealed a remarkable 70% (159/225) of the tumors harbor *MED12* mutations. *MED12* encodes a component of the Mediator multiprotein complex, which participates in the regulation of global as well as gene-specific transcription. All the observed mutations resided in exon 2 or the intron 1-exon 2 junction, an evolutionarily conserved region of the gene, suggesting that malfunction of the region contributes to tumorigenesis. The mutation spectrum included missense, in-frame insertion-deletion, and intronic mutations, none of which resulted in a truncated protein product. Moreover, complementary deoxyribonucleic acid (cDNA) sequencing of 16 *MED12* mutation-positive leiomyomas confirmed predominant expression of the mutant alleles. Mutation-positive leiomyomas tended to be smaller in size compared to mutation-negative lesions ( $P=0.015$ ). Unsupervised hierarchical clustering of gene expression data from ten uterine leiomyomas (eight *MED12* mutation-positive and two *MED12* mutation-negative) and the corresponding normal myometrial tissue indicated that the mutation-positive tumors form a separate group from all the other samples. In addition, pathway enrichment analysis of the eight *MED12* mutation-positive tumors and their matched myometrial tissue identified three significantly altered pathways: focal adhesion, extracellular matrix receptor interaction, and Wnt signaling. High mutation frequency and clustering of the mutations into an evolutionarily conserved region of the protein indicate that *MED12* is likely to be a driver gene. Furthermore, the mutation pattern proposes a role for *MED12* as a putative oncogene. This is the first time *MED12* has been implicated in human tumorigenesis.



Uterine leiomyomas do not affect all ethnicities equally. To validate the finding in another ethnic group and to verify the role of *MED12* mutations in leiomyomagenesis, a series of 28 leiomyomas from 18 Black African or Coloured South African women went through Sanger sequencing for *MED12* exon 2 mutations. Altogether 50% (14/28) of the tumors displayed a mutation. Although South African women tended to carry fewer *MED12* mutations than Finnish women, the small number of cases makes it difficult to draw definitive conclusions from the mutation frequency and clinical correlations in this sample series. All in all, *MED12* mutations occurred recurrently in uterine leiomyomas of South African women, which confirms their role in the growth and development of these lesions regardless of ethnicity.

Original identification of *MED12* mutations took place in a series of histopathologically conventional uterine leiomyomas. To assess the relevance of *MED12* mutations for possible molecular classification of uterine leiomyomas, 137 tumors representing various relatively rare clinical uterine leiomyoma subtypes underwent Sanger sequencing for *MED12* exon 2 mutations. The sample series included 103 histopathological uterine leiomyoma variants (59 cellular leiomyomas, 26 mitotically active leiomyomas, and 18 leiomyomas with bizarre nuclei) and 34 uterine leiomyomas from 14 HLRCC-patients. Both the histopathological leiomyoma variants and leiomyomas from HLRCC-patients harbored *MED12* mutations significantly less frequently than conventional leiomyomas ( $P < 0.001$ ). Mitotically active leiomyomas represented the only group of tumors with no significant difference in the *MED12* mutation frequency from conventional leiomyomas. Overall, the results indicate that *MED12* mutation positivity is a key characteristic of conventional leiomyomas. Furthermore, the majority of uterine leiomyomas from HLRCC-patients displayed biallelic *FH* inactivation, however, none of the *MED12* mutation-positive tumors belonged to this group, suggesting that *MED12* mutations and biallelic *FH* inactivation may be mutually exclusive.

Thorough analysis of somatic variation in the coding regions of uterine leiomyoma genomes included exome sequencing of 27 uterine leiomyomas (12 *MED12* mutation-negative and 15 *MED12* mutation-positive) and their corresponding normal myometrial tissue. The study aimed at searching for novel driver mutations in *MED12* mutation-negative leiomyomas and additional contributing mutations in *MED12* mutation-positive leiomyomas. Focus was on recurrently mutated genes. No such genes, however, were observed in either tumor group. The complete lack of other recurrent somatic mutations in the examined sample series emphasizes the significance of *MED12* mutations for the tumorigenesis of uterine leiomyomas. Other types of changes, such as structural rearrangements, intronic variants, and epigenetic events undetectable by exome sequencing, probably have an impact to the development of *MED12* mutation-negative lesions.

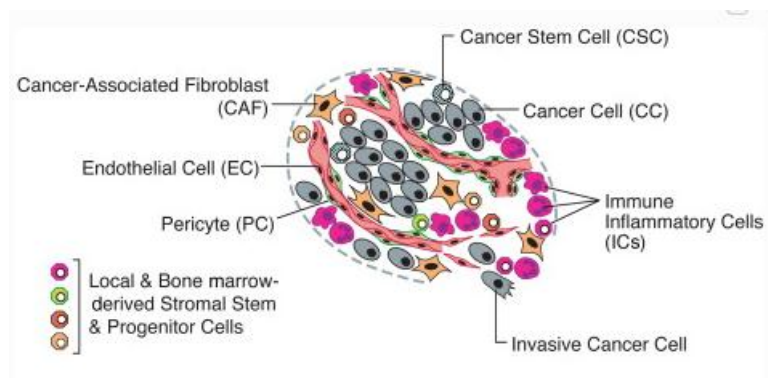
# REVIEW OF THE LITERATURE

## 1. Human tumor pathogenesis

Human tumor development and progression is a multistep process, which begins when a cell or a small group of cells break away from the normal constraints of cell division and start to proliferate uncontrollably. After multiple series of divisions and reproduction of abnormal cells, a tumor may form. Tumors can be benign or malignant; they can remain within the tissue of origin or have the ability to invade nearby tissues and establish new tumors (metastases) throughout the body.

As normal cells gradually transform into cancer cells, they acquire physiological capabilities, which enable cancer cells to survive, proliferate, and ultimately disseminate. These hallmark capabilities include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg, 2000); the last distinguishing a malignant tumor from a benign tumor. Two additional hallmark capabilities are emerging: reprogramming of cellular energy metabolism and avoiding immune destruction (Hanahan and Weinberg, 2011). The first involves major deregulation of cellular metabolism to support neoplastic proliferation and the second enables cancer cells to evade elimination by immune cells. In distinct tumor types, acquisition of hallmark capabilities can occur via various mechanisms and in different chronological order during tumor pathogenesis.

Tumors are not solely masses of cancer cells, but rather complicated tissues consisting of multiple distinct cell types (Figure 1). The majority of the cellular heterogeneity arises from various stromal cells, which create the tumor microenvironment that promotes neoplastic transformation, supports tumor growth, and protects the tumor from host immunity (Egeblad *et al.*, 2010; Hanahan and Coussens, 2012). Heterotypic signaling within the microenvironment changes progressively during the course of tumorigenesis (Quail and Joyce, 2013).



**Figure 1. Various cell types of the tumor microenvironment.** Adapted from Cell, 144, Hanahan and Weinberg, Hallmarks of Cancer: Next Generation, p.662, Copyright Elsevier (2011), with permission from Elsevier.

## 1.1 Genomic instability

Genomic instability in neoplastic cells generates the genetic and phenotypic diversity that drives tumorigenesis and is essential for acquisition of the hallmark capabilities (Hanahan and Weinberg, 2011). Rarely, certain genetic or phenotypic changes arise, which give selective advantage to a subclone of cells, allowing them to outgrow nearby cells. Multistep tumor progression is characterized by a series of clonal cell expansions, each of which is triggered by an alteration in the genome of a neoplastic cell (Nowell, 1976; Fearon and Vogelstein, 1990). Somatic alterations that confer selective growth advantage to a neoplastic cell in which they occur are called ‘driver’ mutations. The majority of somatic changes in neoplastic cells are, conversely, ‘passenger’ mutations, biologically neutral alterations, which are not subject to selection (Greenman *et al.*, 2007). As tumorigenesis proceeds, cells at geographically distinct parts of the tumor may start to display different genomic contents, a phenomenon called intratumoral heterogeneity (Yachida *et al.*, 2010; Vogelstein *et al.*, 2013).

The spontaneous mutation rate in normal human cells is remarkably low, approximately  $10^{-7}$  per gene during each cell cycle (Araten *et al.*, 2005; Bielas *et al.*, 2006). Several layers of highly evolved defense mechanisms protect cell genomes against both exogenous (e.g. tobacco, chemicals, and sunlight) and endogenous (hormones or the metabolism of nutrients within cells) mutagens, as well as deoxyribonucleic acid (DNA) replication errors. For a tumor to develop, neoplastic cells need to overcome these defenses to increase their mutability (Negrini *et al.*, 2010). The increased mutability improves the chances of cells to gain favourable somatic alterations, which confer them with a selective advantage through activation of oncogenes, inactivation of tumor suppressor or stability genes, or disruption of epigenetic regulation.

### 1.1.1 Oncogenes

Proto-oncogenes – non-mutated versions of oncogenes – are normal cellular genes that function under tight control in the cell signaling circuitry that promotes cell growth and differentiation. The products of proto-oncogenes include, for instance, transcription factors, growth factor receptors, apoptosis regulators, and signal transducers. Mutations in proto-oncogenes render the genes continuously active or active in a situation when they are not supposed to be leading to an uncontrolled flow of growth-promoting signals in a cell (Bishop, 1991). Activation of an oncogene typically confers a growth advantage or increased survival for a cell and enables the cell to become independent of growth signaling.

Oncogenes can be activated by three different mechanisms that affect either protein expression or structure: gene amplification, point mutation, or chromosomal rearrangement (Croce, 2008). A known proto-oncogene *ERBB2* (*HER2/neu*) is frequently amplified and overexpressed in breast carcinomas and is correlated with poor prognosis (Slamon, 1987; Press *et al.*, 1997). Elevated gene expression levels can be achieved also through a

translocation, which juxtaposes a proto-oncogene under the influence of a strong enhancer, as seen in the case of *MYC* in Burkitt lymphoma (Meyer and Penn, 2008). Translocations can, at the same time, create novel chimeric oncogenes with abnormal transforming properties. The Philadelphia chromosome, a product of a fusion gene *BCR-ABL1*, is present in 90% of chronic myeloid leukemia patients (Nowell and Hungerford, 1960; Rowley, 1973). Both *RAS* and *BRAF* oncogenes are frequently activated by point mutations in various different cancers (Garnett and Marais, 2004; Pylayeva-Gupta *et al.*, 2011). Notably, point mutations in proto-oncogenes occur recurrently at the same amino acid positions (Vogelstein *et al.*, 2013). For example, the majority of *BRAF* mutations change a valine (V) residue to glutamic acid (E) at codon 600 (previously p.V599E) (Davies *et al.*, 2002). This amino acid substitution permanently activates the kinase domain of BRAF, which then phosphorylates its downstream targets, resulting in aberrant cell growth (Wan *et al.*, 2004). Mutations in proto-oncogenes are dominant in nature; a single somatic mutation in one allele of the gene is sufficient to change the behavior of a cell.

### 1.1.2 Tumor suppressor genes

In order to prosper, neoplastic cells need to also circumvent programs that negatively regulate cell proliferation. Tumor suppressor genes encode proteins that are involved in many such programs. They prevent inappropriate cell cycle progression and ensure that anomalous cells are sentenced to death by apoptosis. Protein products of mutated tumor suppressor genes display a loss or reduction in their function, which allows cells to get pass the cell cycle checkpoints or to avoid apoptosis resulting in unrestricted replication. A few tumor suppressor genes encode proteins that modulate the microenvironment in which the cells grow (Kinzler and Vogelstein, 1998). The loss of their function generates an abnormal stromal environment that contributes to the neoplastic transformation of cells.

In general, both alleles of a tumor suppressor gene have to be inactivated before a cell gains a selective advantage, a phenomenon known as the ‘Knudson’s two-hit hypothesis’ (Knudson, 1971; Cavenee *et al.*, 1983). Inactivation of the first allele of a tumor suppressor gene can occur by deletions or insertions of various sizes, by point mutations, or by epigenetic silencing via promoter methylation (Vogelstein and Kinzler, 2004). Elimination of the remaining wild-type allele frequently involves a loss of heterozygosity (LOH) event at the gene locus, rather than a second independent mutational or promoter methylation event. Occasionally, only one inactivated allele of a tumor suppressor gene provides a growth advantage to a cell; a condition called haploinsufficiency, where single allele is insufficient for producing a normal phenotype (Santarosa and Ashworth, 2004). Haploinsufficiency can also result from a situation where one mutated allele can disrupt the function of a wild-type allele in a dominant-negative manner.

*RBI* and *TP53* are the two most frequently mutated tumor suppressor genes in neoplastic cells. Protein products of the genes function as key regulators of two interconnected signaling pathways that are essential for cell cycle progression or induction of senescence

and programmed cell death (Vogelstein *et al.*, 2000; Classon and Harlow, 2002). Both pathways are defected in a variety of human tumors leading to increased cell proliferation and decreased rate of apoptosis. Inactivation of *RBI* typically results from deletions or nonsense mutations, which disrupt the structure of the protein creating truncated protein products that undergo rapid degradation in cells. The loss of *RBI* function can also occur, at times, through promoter hypermethylation (Sakai *et al.*, 1991; Ohtani-Fujita *et al.*, 1997). The majority of mutated *TP53* alleles, on the other hand, carry missense mutations (Olivier *et al.*, 2010), which only slightly alter the protein product. The mutant protein may, in some instances, exert dominant-negative effects over the remaining wild-type allele, but in most cases both copies of the tumor suppressor gene are mutated (Olivier *et al.*, 2010; Muller and Vousden, 2013).

### *1.1.3 Stability genes*

Stability genes encode proteins that maintain and monitor the integrity of the cell genome. In contrast to oncogenes and tumor suppressor genes, stability genes do not directly regulate cell proliferation. Mutations in these genes lead to various genomic instabilities, such as microsatellites and chromosomal changes, which increase the cell's overall mutation rate and expedite the accumulation of genetic alterations in other genes, such as proto-oncogenes and tumor suppressor genes (Friedberg, 2003). As with tumor suppressor genes, both copies of stability genes must be typically inactivated through mutations or epigenetic silencing before their function is lost.

A large number of stability genes are DNA-repair genes, whose protein products correct mistakes made during normal DNA replication or as a result of exposure to mutagens, hence keeping DNA-level instability at minimum. For example, mismatch repair genes *MLH1* and *MSH2* are part of an evolutionary conserved pathway, which recognizes and repairs erroneous insertions, deletions, and misincorporated bases in the DNA sequence. Defects in these genes cause a 100- to 1,000-fold increase in mutation production (Ionov *et al.*, 1993; Bhattacharyya *et al.*, 1994). Other stability genes participate in processes, such as mitotic recombination, chromosomal segregation, and telomere maintenance, all of which involve large amounts of chromosomes. Inactivation of these genes causes chromosomal instability, the most common form of genetic instability in human tumors (Michor *et al.*, 2005).

### *1.1.4 Epigenetic modifications*

In addition to genetic alterations, epigenetic aberrations play a role in human tumor pathogenesis (Baylin and Jones, 2011; Sandoval and Esteller, 2012). Epigenetics is the study of changes in gene expression or phenotype that are heritable during cell division, but do not alter the DNA sequence. Mechanisms underlying these changes include, for instance, DNA methylation and histone modifications. DNA methylation, which refers to the addition of a methyl group to a cytosine (C) or guanine (G) residue, is associated with a number of key

biological processes, such as embryonic development, X-chromosome inactivation, and cell differentiation. In mammals, DNA methylation occurs mostly at CpG dinucleotides, which cluster at the promoter region of genes (CpG islands). Aberrant DNA methylation is a characteristic observed in various human malignancies. For example, hypermethylation of CpG islands has been implicated in the silencing of numerous tumor suppressor genes (Jones and Baylin, 2007; Irizarry *et al.*, 2009). Global hypomethylation frequently accompanies hypermethylation of promoter regions in tumors leading to chromosomal instability, possibly due to activation of transposable elements (Esteller, 2008). The methylation state of a cell is plastic, varying with the cell type, developmental stage, and signals from the microenvironment (Pelizzola and Ecker, 2011). Therefore, it is difficult to know which epigenetic aberrations are the ones that give selective advantage to the cell.

### 1.1.5 MicroRNAs

Micro-ribonucleic acid (miRNA) genes encode small non-coding RNAs of approximately 21-23 nucleotides in length that function as regulators of gene expression. Acting at the post-transcriptional level, miRNAs typically bind to a targeted messenger RNA (mRNA) and prevent protein production by suppressing translation and/or initiating mRNA degradation. A large number of miRNA genes localize at chromosomal regions that undergo rearrangements, deletions, and amplifications in tumors, supporting the notion that dysfunction of miRNAs play a frequent part in human malignancies (Calin *et al.*, 2004). MiRNA genes can be upregulated or downregulated in neoplastic cells: oncogenic or tumor-suppressive, respectively (Volinia *et al.*, 2006). Increased expression of oncogenic miRNAs represses target tumor suppressor genes, while the loss of tumor-suppressive miRNAs promotes the expression of target oncogenes (Kasinski and Slack, 2011). Upregulation can be due to amplifications, deregulation of a transcription factor, or demethylation of CpG islands in the promoter region of the miRNA gene. Downregulation, on the other hand, can be a result of deletions, epigenetic silencing, or loss of expression of one or more transcription factors. For example, members of the *let-7* miRNA family directly regulate expression of the *RAS* proto-oncogene in human cells. In lung cancer, deletions or underexpression of *let-7* results in overexpression of *RAS* (Johnson *et al.*, 2005). Although DNA methylation and histone modifications control the expression of certain miRNAs, conversely, miRNAs may also affect the methylation machinery and expression of proteins involved in histone modifications (Saito and Jones, 2006; Guil and Esteller, 2009).

## 1.2 Inherited tumor susceptibility

The vast majority of human tumors arise sporadically over the course of a lifetime and are strongly attributed to environmental and lifestyle factors. Inherited tumor susceptibility accounts only for approximately 5-10% of all human malignancies (Nagy *et al.*, 2004). Hereditary mutations that are passed on from parent to child through the germline increase the child's risk to develop a tumor, but do not directly cause it. Additional somatic mutations

are needed for a clinically detectable lesion to emerge. Patients with genetic predisposition to tumorigenesis often have multiple primary tumors that appear at an earlier age compared to the general population (Knudson, 2002).

Mutations in oncogenes, tumor suppressor and stability genes, as well as miRNAs can occur in the germline and result in hereditary tumor predisposition. Mutations are typically subtle in nature (point mutations or small insertions and deletions) and inherited in an autosomal dominant manner. Although oncogene-activating germline mutations are, in general, embryonically lethal, some exceptions exist, such as multiple endocrine neoplasia type 2 (MEN2) syndrome, which is caused by inherited mutations in the *RET* proto-oncogene, a receptor tyrosine kinase (Donis-Keller *et al.*, 1993; Mulligan *et al.*, 1993). Tumor suppressor genes account for the majority of dominantly inherited tumor susceptibility syndromes. Many of the genes function in developmental pathways, which frequently lead to phenotypic abnormalities when they are not functioning properly (Hodgson, 2008). For instance, germline mutations in the *APC* tumor suppressor gene predispose to familial adenomatous polyposis (FAP) (Kinzler *et al.*, 1991). In addition to tumor suppressor genes, inherited defects in stability genes are also common, including both autosomal dominant and recessive conditions. Hereditary breast and ovarian cancer syndrome is an example of dominant inheritance that arises as a result of a germline mutation in either *BRCA1* or *BRCA2* (Miki *et al.*, 1994; Wooster *et al.*, 1995). Ataxia telangiectasia, at the same time, is an autosomal recessive syndrome caused by inherited mutations in *ATM* (Savitsky *et al.*, 1995). Few germline mutations in miRNA genes have been observed so far, including one in *miR-125a*, which seems to associate with breast cancer (Li *et al.*, 2009).

Hereditary tumor susceptibility explains only a small fraction of the familial clustering of human malignancies. On various occasions, families have more cases of neoplasia than by chance, which occur at the average age of onset without a clear pattern of inheritance. A lot of this inherited tumor predisposition may be due to a combination of shared lifestyle factors, environmental exposures, common low-penetrance, and more rare moderate-penetrance gene mutations (Cazier and Tomlinson, 2010).

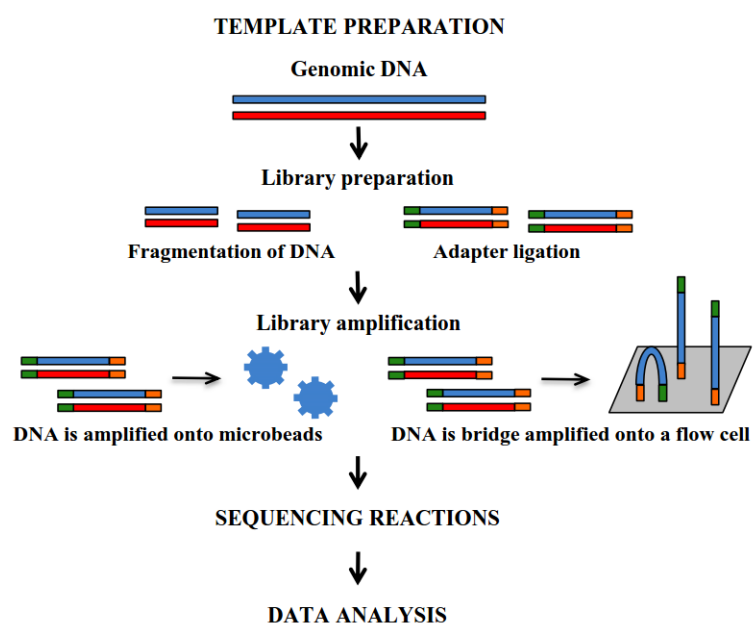
## 2. Next-generation DNA sequencing

Sanger sequencing was the gold standard for nucleic acid sequencing for approximately 25 years (Sanger *et al.*, 1977). The Human Genome Project (HGP), which established the first human baseline reference genome, leaned almost completely on this method (International Human Genome Sequencing Consortium, 2004). That project and numerous subsequent studies have had a huge impact on the current knowledge of the structure and function of the human genome. Since the completion of the HGP, Sanger sequencing has gradually been replaced by next-generation sequencing (NGS) technologies, which have significantly reduced the cost and increased the output and sensitivity of DNA sequencing. Over a very short time period, NGS efforts have discovered novel genes and mechanisms contributing to human tumor pathogenesis and considerably improved our understanding of cancer biology.

### 2.1 Applications of next-generation sequencing

Commercially available NGS technologies vary in several aspects, such as sequencing chemistries and signal detection (MacConaill, 2013), but they all follow a similar base methodology (Figure 2). Template preparation involves construction of a DNA sequencing library; a fragmented genomic DNA population with synthetic adapters ligated to the ends of the fragments. The library is subsequently amplified onto a solid surface (a bead or a glass slide) to separate the fragments spatially. This enables concurrent sequencing of millions of fragments in unison. Sequencing reactions rely on a sequencing-by-synthesis strategy. Detection of nucleotide sequencing information can take place through, for instance, pH changes or fluorescence.

**Figure 2. Schematics for NGS methodology.** Adapted by permission from MacMillan Publishers Ltd: Journal of Investigative Dermatology (Grada and Weinbrecht, 2013), copyright 2013.





Raw sequencing data is a subject to several preprocessing steps, such as the removal of adapter sequences and low quality reads, the alignment to the human reference genome, or less frequently *de novo* assembly. The National Center for Biotechnology Information (NCBI) maintains and updates the latest version of the human genome, which is available in public databases (<http://www.ncbi.nlm.nih.gov/genome/guide/human/>). To identify somatic mutations in the genomes of neoplastic cells, NGS data from both the individual's germline DNA and the tumor DNA is required. Furthermore, various public databases provide a growing number of NGS data that assist substantially with the removal of germline variation. Instead of whole-genome sequencing, a variety of NGS applications concentrate on targeted regions of the genome, ranging from hundreds of genes to the exome. The capture of the target DNA fragments from the sequencing library typically involves a hybridization reaction between the genomic DNA and a highly specific oligonucleotide probe set (Gnirke *et al.*, 2009).

### 2.1.1 Whole-exome sequencing

The exome consists of all protein-coding regions of the genes (exons). Although the exome accounts for only approximately 1% of the human genome, 85% of disease-causing mutations are thought to be located in this region (Majewski *et al.*, 2011). During the last few years, whole-exome sequencing efforts have made progress in the field of gene discovery. For example, identification of frequent *BAP1* mutations in metastasizing uveal melanomas and *ARID1A* mutations in ovarian carcinomas have linked these genes, for the first time, to these diseases (Harbour *et al.*, 2010; Wiegand *et al.*, 2010). Whole-exome sequencing enables robust detection of point mutations, and small insertions and deletions with an average of 100- to 150-fold coverage (Garraway and Lander, 2013). Deep coverage is important due to the cellular and molecular heterogeneity of most tumors. Advances of NGS in research have facilitated the entry of whole-exome sequencing, together with targeted gene panels, in clinical practise. In 2011, Ambry Genetics became the first CLIA-certified (Clinical Laboratory Improvement Amendments) laboratory to offer whole-exome sequencing along with medical interpretation for clinical diagnostic purposes.

### 2.1.2 Whole-genome sequencing

Whole-genome sequencing provides the most comprehensive picture of the genetic variation in the genome, including non-coding changes, copy-number alterations, and structural rearrangements, with an average of 30- to 60-fold coverage (Garraway and Lander, 2013). Since the publication of the first whole cancer genome effort – acute myeloid leukemia – in 2008 (Ley *et al.*, 2008), the number of whole-genome sequencing studies has continuously increased. Sequencing of tumor genomes has provided new insights into various mutational mechanisms contributing to tumor development and progression. For example, whole-genome sequencing of chronic lymphocytic leukemia resulted in the discovery of chromothripsis, a phenomenon where a single chromosome or a chromosome arm shatters

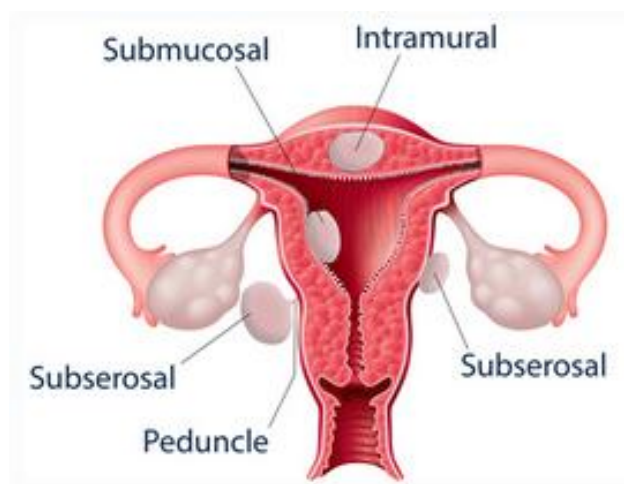
and reassembles back together in a random order in one single event (Stephens *et al.*, 2011). Subsequent studies have indicated that chromothripsis is present in approximately 2-3% of all human cancers (Forment *et al.*, 2012; Maher and Wilson, 2012). Advances in NGS technologies have launched several large-scale cancer genome projects, such as the International Cancer Genome Consortium (ICGC), which aims at generating a thorough catalogue of genomic alterations present in 50 clinically and societally important tumor types and/or subtypes (International Cancer Genome Consortium *et al.*, 2010). Although the use of whole-genome sequencing has started to increase in the clinical setting, several challenges have emerged, such as the incomplete depth of coverage for clinical mutation detection in the case of 10-19% of inherited disease genes (Dewey *et al.*, 2014).

## 2.2 Current limitations and challenges

NGS technologies, while revolutionizing the field of tumor biology, have technical limitations. A few of the biggest challenges are due to repetitive DNA, which accounts for nearly 50% of the human genome (Alkan *et al.*, 2011; Treangen and Salzberg, 2011). Regions of repetitive DNA might cause inaccurate sequencing or ambiguities in alignment or *de novo* assembly of short sequence reads. Another major limitation relates to data management and storage. Processing and analysing vast amounts of sequencing data can be time-consuming and expensive, posing notable bioinformatical challenges. Continuously increasing data volumes also seem to outrun the current archival storage capacity despite the development of methods that can facilitate electronic data storage, such as reference-based compression (Hsi-Yang Fritz *et al.*, 2011). In addition to technical limitations, interpretation of NGS data causes its own problems. At the moment, one of the greatest challenges is to distinguish driver mutations from passenger mutations in the genome. Most adult tumor genomes harbor an average of 1,000-10,000 somatic substitutions, the majority being passenger mutations (Stratton, 2011). Various approaches have emerged to identify genes with driver mutations (Vogelstein *et al.*, 2013). They rely on characteristics that serve as indicators of positive selection, such as mutation frequency, conservation status of the mutated residue, and mutation pattern. In clinical practise, ethical issues of privacy, confidentiality, and reporting of the NGS results to patients create challenges (Lolkema *et al.*, 2013). It is important to decide the extent to which patients are informed of incidental genetic findings, such as mutations predisposing to medically actionable conditions not related to the disease under study, or variants with uncertain pathogenic and functional significance. Unexpected discoveries and data security concern also the field of cancer research (Jarvik *et al.*, 2014).

### 3. Uterine leiomyomas

Uterine leiomyomas (fibroids or myomas) are benign tumors that arise from the cells of the myometrium. They are among the most common neoplasms in women with an estimated prevalence of 20-40% during the reproductive years, but also percentages as high as 77% have been presented (Cramer and Patel, 1990; Wallach and Vlahos, 2004). Leiomyomas are classified as subserous, intramural, or submucous depending on their location in relation to the layers of the uterus (Figure 3). Their size varies from microscopically small to over 20 cm in diameter. Leiomyomas can be either solitary or multiple, and their macroscopic appearance is typically spherical, circumscribed, and firm (Cramer and Patel, 1990).



**Figure 3. Location of uterine leiomyomas.** The figure was retrieved September 20, 2014, from NICHD Web Site (<http://www.nichd.nih.gov/health/topics/uterine/conditioninfo/Pages/default.aspx>).

Although the majority of leiomyomas are asymptomatic, approximately 25% of women with leiomyomas have clinically relevant lesions, which cause significant morbidity and require treatment (Baird *et al.*, 2003). The symptoms, which include prolonged or excessive menstrual bleeding, pelvic discomfort, and reproductive dysfunction, depend largely on the location, size, and number of leiomyomas. Submucous leiomyomas that grow partially or entirely in the uterine cavity are likely to cause abnormal bleeding, infertility, and increased risk of miscarriage (Garcia and Tureck, 1984; Stewart, 2001; Bukulmez and Doody, 2006; Klatsky *et al.*, 2008; Pritts *et al.*, 2009). They can, for instance, prevent the implantation, or possibly disrupt fertilization. Pelvic discomfort, on the other hand, may result from a massive uterus with multiple or subserosal leiomyomas, which can put pressure on the surrounding organs (Gupta *et al.*, 2008). Intramural leiomyomas, which grow within the uterine wall, represent the most common leiomyomas. Occasionally, they may expand considerably causing abnormal menstrual bleeding or pelvic pressure. Still today, symptomatic uterine leiomyomas are the primary indication for hysterectomy throughout the world. Approximately 200,000 hysterectomies are performed for leiomyomas in the United States per year (Farquhar and Steiner, 2002; Wu *et al.*, 2007). The estimated annual health care costs (surgery, hospital admissions, outpatient visits, and medications) are \$4.1-9.4 billion (Cardozo *et al.*, 2012).

### 3.1 Histopathology

Microscopically, leiomyoma consists of intersecting bundles of smooth muscle cells separated by abundant extracellular matrix (ECM) containing collagen, fibronectin, and proteoglycan. Tumor cells are uniform and elongated in shape with scarce mitotic figures. Frequently, leiomyoma may undergo degenerative changes, such as oedema or hyaline fibrosis. Conventional uterine leiomyomas account for approximately 90% of all leiomyomas (Oliva *et al.*, 2014). The remaining tumors consist of leiomyoma variants, which represent a group of heterogeneous lesions that mimic malignancy in one or more aspects (Table 1). Although associated with malignant features, leiomyoma variants are principally clinically benign and managed in the same way as conventional leiomyomas.

**Table 1. Examples of histopathological leiomyoma variants and growth patterns according to the WHO classification (Oliva *et al.*, 2014).**

<b>Leiomyoma variant</b>	<b>Features</b>
<b>Cellular leiomyoma</b>	Significantly increased cellularity compared to the surrounding myometrium Lack of nuclear atypia Infrequent mitotic figures Usually irregular borders mimicking invasion
<b>Leiomyoma with bizarre nuclei (formerly atypical leiomyoma)</b>	Mainly focal occurrence of highly atypical cells in an otherwise conventional leiomyoma Infrequent mitotic figures
<b>Mitotically active leiomyoma</b>	>10 mitotic figures/10 high power fields Usually lack of cytological atypia Often submucosal Sometimes associated with hormonal therapy
<b>Lipoleiomyoma</b>	Conspicuous number of adipocytes in an otherwise conventional leiomyoma
<b>Myxoid leiomyoma</b>	Smooth muscle cells separated by myxoid material Lack of cytological atypia Infrequent mitotic figures
<b>Epithelioid leiomyoma</b>	Epithelial-like appearance of tumor cells
<b>Intravenous leiomyomatosis</b>	Presence of benign smooth muscle within vascular spaces outside a leiomyoma Infrequent mitotic figures
<b>Diffuse leiomyomatosis</b>	Numerous small smooth muscle nodules merged together with myometrial smooth muscle Lack of cytological atypia
<b>Metastasizing leiomyoma</b>	Conventional leiomyoma found in the lungs of women with a history of uterine leiomyomas

### 3.2 Epidemiology and risk factors

The current literature on potential risk factors for the development of uterine leiomyomas has limitations and requires cautious interpretation. The paucity of epidemiological studies, reliability of self-reported diagnoses, and heterogeneity in study designs may complicate the characterization of these risk factors (Schwartz *et al.*, 2000). A large proportion of uterine leiomyomas are asymptomatic and may not come to medical attention. This may occasionally lead to a biased situation where study participants mainly represent symptomatic women.

The epidemiology of uterine leiomyomas seems to parallel the changes in estrogen and progesterone levels throughout the woman's reproductive life cycle. Leiomyomas rarely

occur, for instance, before puberty. Clinically relevant lesions are most prevalent within the reproductive years reaching the peak during perimenopause, and regress after menopause, when ovarian hormone concentrations and menstrual cyclicity fade (Marshall *et al.*, 1997; Flake *et al.*, 2003). Both early menarche (<10 years old) and nulliparity appear to increase the risk to develop leiomyomas, while late menarche (>16 years old), increased parity, and longer duration of breastfeeding, decrease the risk, respectively (Ross *et al.*, 1986; Parazzini *et al.*, 1996; Marshall *et al.*, 1998; Baird *et al.*, 2003; Terry *et al.*, 2010). One hypothesis suggests that lactation reduces the time of exposure to ovarian hormones by decreasing the number of menstrual cycles in woman's reproductive life. Alternatively, early onset of menstrual cycles and nulliparity may increase the number of cell divisions that the myometrium experiences during the reproductive lifespan, improving the chances of mutations to accumulate in myometrial cells (Marshall *et al.*, 1998). The potential effects of oral contraceptives or hormone replacement therapy on the growth of uterine leiomyomas have presented inconsistent results (Flake *et al.*, 2003). Therefore, no definitive relationship between exogenous ovarian hormones and leiomyomas currently exists.

Racial and ethnic differences in the incidence and severity of uterine leiomyomas are particularly apparent. For instance, African-American women tend to have a higher incidence rate and approximately three times greater risk of developing leiomyomas than Caucasian women (Marshall *et al.*, 1997; Baird *et al.*, 2003). The estimated cumulative incidence rate of leiomyomas by age 50 is >80% for African-American women, when the rate for Caucasian women is approximately 70%. African-Americans have also a younger age at diagnosis and at hysterectomy presentation, and at the same time, their leiomyomas are larger (heavier uterine weight), more numerous, and more symptomatic than in Caucasians (Kjerulff *et al.*, 1996; Huyck *et al.*, 2008). The basis for ethnic disparity in uterine leiomyomas remains unknown. One possible reason for the variation in the tumor incidence may come from the differences in estrogen metabolism between African-Americans and Caucasians (Taioli *et al.*, 1996).

Family history and various lifestyle factors may also contribute to the risk of uterine leiomyomas. For example, first-degree relatives of women with leiomyomas have an approximately 2.5 times higher risk of developing leiomyomas (Vikhlyaeva *et al.*, 1995; Schwartz *et al.*, 2000). Obesity, alcohol intake, diabetes, and hypertension seem to also increase the leiomyoma risk, while smoking reduces the incidence of leiomyomas in a dose-dependent manner (Summers *et al.*, 1971; Ross *et al.*, 1986; Shikora *et al.*, 1991; Parazzini *et al.*, 1996; Faerstein *et al.*, 2001; Wise *et al.*, 2004; Wise *et al.*, 2005). The influence of diet on the presence or growth of leiomyomas is still unclear due to the limited and discrepant information available.

### 3.3 Tumorigenesis

The majority of uterine leiomyomas are monoclonal in origin; in other words, they originate from a single myometrial cell (Linder and Gartler, 1965; Townsend *et al.*, 1970; Mashal *et*

*al.*, 1994; Hashimoto *et al.*, 1995). Rarely, polyclonality may also play a part in tumor formation (Ozisik *et al.*, 1993). The development of a uterine leiomyoma consists of two stages. At first, a normal myometrial cell transforms into a neoplastic myometrial cell, which then starts to proliferate in an uncontrolled manner, leading ultimately to a clinically apparent tumor. The precise mechanisms underlying leiomyoma pathogenesis are still largely unknown.

### 3.3.1 *Initiation*

The initiators of uterine leiomyomas remain unidentified, but several hypotheses exist. Transformation from a normal to an abnormal myometrial cell likely results from a genetic or epigenetic alteration induced by a preceding condition or injury in the uterus. For example, elevated levels of estrogen and progesterone in the myometrium may contribute to tumor formation by increasing the overall mitotic rate and expediting the accumulation of somatic mutations (Rein, 2000). Alternatively, significantly elevated estrogen receptor levels in the myometrium of leiomyomatous uteri compared to normal uteri suggest that women with leiomyomas may have an inborn anomaly in their myometrium (Richards and Tiltman, 1996). Another hypothesis proposes that different types of injury, such as ischemic injury associated with increased vasoconstrictive substances that render the myometrium hypoxic at the time of menstruation, may play a role in the genesis of leiomyomas (Stewart and Nowak, 1998).

In 2007, isolation and characterization of a stem/progenitor cell population from the human myometrium provided an entirely new angle to study the cellular origins of uterine leiomyomas, suggesting that the tumors may arise, instead of normal smooth muscle cells, from myometrial stem/progenitor cells (Ono *et al.*, 2007). These cells have the ability to proliferate and differentiate into multiple cell types *in vitro*, such as osteocytes, adipocytes, or mature smooth muscle cells. The spontaneous differentiation of stem/progenitor cells into smooth muscle cells *in vitro* occurs only under hypoxic conditions. Therefore, menstruation-induced hypoxia may potentially function as a driving force, which transforms myometrial stem/progenitor cells into more differentiated leiomyoma cells (Zhou *et al.*, 2011). At the same time, uterine leiomyomas seem to contain a population of stem/progenitor cells of their own, but at a lower percentage than normal myometrium (Chang *et al.*, 2010; Mas *et al.*, 2012; Ono *et al.*, 2012). One hypothesis suggests that these cells may arise from myometrial stem/progenitor cells as a result of a genetic alteration (Ono *et al.*, 2012).

### 3.3.2 *Growth and development*

After the initial transformation, several factors seem to participate in regulating the growth and development of uterine leiomyomas, including ovarian hormones and growth factors. As mentioned previously, leiomyomas are estrogen and progesterone dependent tumors. They exhibit higher mitotic activity and higher concentrations of estrogen and progesterone receptors (ERs and PRs) than the myometrium, as well as alterations in their estrogen

metabolism (Flake *et al.*, 2003). For example, reduced activity of the enzyme 17 $\beta$ -hydroxysteroid dehydrogenase, which converts estradiol to estrone, and increased levels of aromatase, an enzyme that catalyzes the conversion of androgens into estrogens, are particularly characteristic for leiomyomas (Pollow *et al.*, 1978; Eiletz *et al.*, 1980; Shozu *et al.*, 2004). Low levels of 17 $\beta$ -hydroxysteroid dehydrogenase may contribute to the accumulation of estradiol in the tumor cells, which in turn upregulates both ERs and PRs, leading to leiomyoma growth (Otubu *et al.*, 1982; Folkerd *et al.*, 1984).

The growth-promoting effects of estrogen and progesterone may occur through growth factors, which stimulate leiomyoma growth in autocrine and/or paracrine manner (Hyder and Stancel, 1999). Transforming growth factor- $\beta$  (TGF- $\beta$ ), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) are all examples of growth factors associated with uterine leiomyomas; the majority are present in leiomyomas with higher concentrations than in normal myometrium (Flake *et al.*, 2003). Elevated levels of TGF- $\beta$  signaling in leiomyomas may both promote mitogenesis and stimulate the production of ECM (Lyons and Moses, 1990). An increase in the size of a leiomyoma might originate from an increase in the number of tumor cells or from an expansion in the size of ECM. Basic FGF promotes angiogenesis and increases the proliferation of smooth muscle cells (Stewart and Nowak, 1996). Large amounts of this protein are present in the ECM of uterine leiomyomas, potentially contributing to tumor growth (Mangrulkar *et al.*, 1995).

Characteristics such as increased synthesis of ECM components, higher expression of hormone receptors, and the presence of myometrial contraction-associated proteins in uterine leiomyomas have led to a hypothesis that leiomyoma cells resemble myometrial smooth muscle cells of pregnancy (Andersen *et al.*, 1993; Andersen and Barbieri, 1995; Lee *et al.*, 1998; Cesen-Cummings *et al.*, 2000). On the other hand, transplantation studies in mice have indicated that multipotent leiomyoma stem/progenitor cells together with myometrial smooth muscle cells are capable of generating leiomyoma-like tissue (Mas *et al.*, 2012; Ono *et al.*, 2012). Because the stem/progenitor cells express extremely low levels of ERs and PRs, the growth of these cells may depend on the presence of ER- and PR-positive myometrial smooth muscle cells, which could mediate the ovarian hormone signals in a paracrine fashion.

### 3.4 Diagnosis and treatment

An enlarged or irregularly shaped uterus may indicate the presence of uterine leiomyomas during a routine pelvic examination. Various imaging techniques are available for confirming the diagnosis, including ultrasonography, saline-infusion sonography, and magnetic resonance imaging (MRI) (Parker, 2007). Ultrasonography, or ultrasound, serves as a routine imaging tool to differentiate uterine leiomyomas from other pelvic conditions, but it may be insufficient for assessing the exact number and position of the lesions (Dueholm *et al.*, 2002; Khan *et al.*, 2014). Saline-infusion sonography uses saline to distend

the uterine cavity and to obtain more accurate information on the inner lining of the uterus and its relation to potential submucous leiomyomas. While more costly, MRI is currently the most sensitive technique for the detection and localization of leiomyomas (Dueholm *et al.*, 2001; Dueholm *et al.*, 2002). MRI can differentiate submucous, intramural, and subserosal leiomyomas from each other, as well as identify lesions as small as 5 mm in diameter. Primarily, MRI is in use to evaluate suspected malignancy or before fertility preserving surgery. In addition to these imaging techniques, endometrial biopsy, sampling of the uterine mucosal lining for excluding hyperplasia and/or cancer from the diagnosis, takes place regularly in uterine leiomyoma diagnostics in Finland. Accurate assessment of the size, number, and location of leiomyomas is essential when selecting an optimal treatment for the patient. The age and reproductive desires of the individual, the presenting symptoms, and the skills of the surgeon also affect the management decisions.

The majority of diagnosed uterine leiomyomas are asymptomatic and managed expectantly. That said, various treatment options exist for women with symptomatic lesions, such as medical therapy, noninvasive or minimally invasive procedures, and surgery. Medical therapy is currently a short-term treatment option serving as a temporary relief of symptoms or as a pre-operative measure due to the notable risks with long-term therapy or lack of evidence regarding newer medical agents and their risks of long-term use (Khan *et al.*, 2014). Recommended medical treatments include nonsteroidal anti-inflammatory drugs (NSAIDs), tranexamic acid, combined oral contraceptive hormones, the levonorgestrel-releasing intrauterine system, gonadotrophin-releasing hormone analogs (GnRHa), selective estrogen and progesterone receptor modulators, and combination drug therapy. One example of progesterone receptor modulators recently subject to clinical trials is ulipristal acetate (Esmya), which serves at present as a pre-operative treatment for moderate to severe symptoms of uterine leiomyomas in Finland (Donnez *et al.*, 2012a; Donnez *et al.*, 2012b).

Surgical therapy is, even today, the standard method for treating uterine leiomyomas. Hysterectomy is the only definitive solution, which eliminates both the symptoms and chances of recurrence, while myomectomy (either open or minimally invasive surgery) is an option for women who wish to retain the uterus and thereby fertility. The introduction of several non- or minimally invasive procedures during the recent years has provided new options which not only preserve the uterus, but also reduce the recovery time and morbidity in comparison to open surgery (Khan *et al.*, 2014). For example, uterine-artery embolisation is an image-guided technique that blocks the arterial blood flow to leiomyomas and causes them to shrink (Banu *et al.*, 2007). Currently, embolisation is not recommended if the patient wishes to get pregnant early after the procedure (Mara *et al.*, 2008). In the future, better understanding of leiomyomagenesis may help to develop preventative treatment strategies that will reduce the incidence and morbidity of tumors.



## 4. Genetics of uterine leiomyomas

Although the causes and mechanisms of uterine leiomyoma tumorigenesis remain mostly unknown, numerous chromosomal and molecular analyses have provided data that support the role of genetic factors in the etiology of these tumors. For example, cytogenetic karyotyping has revealed that approximately 40-50% of leiomyomas have non-random, tumor-specific chromosome aberrations, including aneuploidy and various structural changes, such as translocations, deletions, and inversions (Nibert and Heim, 1990; Rein *et al.*, 1991). Furthermore, twin studies, familial clustering, and hereditary syndromes have provided strong support for genetic liability for uterine leiomyomas. The genetic background of leiomyomas appears to be heterogeneous, suggesting that diverse mechanisms may contribute to the growth and development of tumors. The known somatic chromosomal abnormalities and inherited genetic factors do not, however, explain the majority of uterine leiomyomas. Table 2 provides a summary of the key genetic changes that have been associated with leiomyomagenesis prior to this thesis work.

**Table 2. Key genetic events likely to contribute to the leiomyoma tumorigenesis reported prior to this thesis work.** The genetic changes are presented in the order in which they occur in the text.

	Chromosome band	Alteration type	Candidate gene	Function
<b>Somatic alterations</b>	12q15	Translocation	<i>HMG A2</i>	Transcription regulation
	14q23-24	Translocation	<i>RAD51B</i>	DNA repair
	7q22	Deletion	<i>CUX1</i>	Transcription regulation
	6p21	Translocation	<i>HMG A1</i>	Transcription regulation
	10q22	Translocation	<i>KAT6B</i>	Histone modification
<b>Predisposing factors</b>	1q42	Point mutation	<i>FH</i>	Enzyme (tricarboxylic acid cycle)

### 4.1 Chromosomal abnormalities

The presence of chromosomal abnormalities in uterine leiomyomas tends to correlate with increased tumor size and the anatomic location of the tumor (Brosens *et al.*, 1998; Rein *et al.*, 1998; Hennig *et al.*, 1999). For example, leiomyomas with 12q14-15 rearrangements have been shown to be larger in size than leiomyomas with either an apparently normal karyotype or with interstitial 7q22 deletions. At the same time, submucous leiomyomas display fewer abnormalities than intramural and subserous leiomyomas. In addition to conventional leiomyomas, both cellular leiomyomas with a greater mitotic index and leiomyomas with bizarre nuclei are more likely to have chromosomal abnormalities (Pandis *et al.*, 1991; Meloni *et al.*, 1992).

#### 4.1.1 Rearrangements of 12q14-15

The most common chromosomal aberrations in uterine leiomyomas are rearrangements of 12q14-15, present in approximately 20% of karyotypically abnormal tumors (Meloni *et al.*, 1992). The majority of these rearrangements consist of a single translocation, t(12;14)(q15;q23-24), although other rearrangements involving 12q14-15 do exist (Kazmierczak *et al.*, 1995; Wanschura *et al.*, 1997; Kurose *et al.*, 2000; Mine *et al.*, 2001; Quade *et al.*, 2003; Velagaleti *et al.*, 2010). In addition to uterine leiomyomas, rearrangements of 12q14-15 also occur commonly in a variety of other benign mesenchymal tumors, such as angiomyxomas, breast fibroadenomas, endometrial polyps, hemangiopericytomas, lipomas, pulmonary chondroid hamartomas, and salivary gland adenomas, supporting the importance of this region in tumorigenesis (Sandberg, 2005). Mapping of the chromosome 12q breakpoint region in both uterine leiomyomas and other benign mesenchymal tumors led to the identification of *high mobility group AT-hook 2* (*HMGA2*), an evolutionary conserved gene encoding a member of the non-histone chromosomal high-mobility group A (HMGA) protein family (Ashar *et al.*, 1995; Schoenmakers *et al.*, 1995). HMGA proteins regulate transcription indirectly by inducing conformational changes in DNA and thus influencing the access of other DNA-binding proteins to the target genes (Fusco and Fedele, 2007). They play a role in diverse cellular processes, such as proliferation, differentiation, growth, and apoptosis (Grosschedl *et al.*, 1994; Reeves, 2001). *HMGA2* is generally widely expressed in human tissues during early development, whereas the expression reduces entirely or to an almost undetectable level in adult tissues, apart from lung and kidney (Rogalla *et al.*, 1996; Gattas *et al.*, 1999; Gross *et al.*, 2003).

A significant proportion of the chromosome 12 breakpoints in uterine leiomyomas carrying t(12;14)(q15;q23-24) map outside the coding regions of *HMGA2*, the majority in the 5' region and the minority in the 3' region of the gene, suggesting dysregulation of *HMGA2* expression (Schoenberg Fejzo *et al.*, 1996; Quade *et al.*, 2003). Indeed, uterine leiomyomas with rearrangements of 12q14-15 commonly display *HMGA2* overexpression (Gattas *et al.*, 1999; Klemke *et al.*, 2009). Two mechanisms, the disruption of regulatory elements or the placement of an effective promoter/enhancer near the gene, may potentially account for the aberrant expression. *HMGA2* is also one of the major targets of *let-7* miRNAs, which can directly inhibit *HMGA2* expression by binding to its 3'UTR. Removal of the *let-7* binding site may provide an additional mechanism to upregulate *HMGA2* (Mayr *et al.*, 2007; Peng *et al.*, 2008; Klemke *et al.*, 2010; Mehine *et al.*, 2013). The presence of t(12;14)(q15;q23-24), high *HMGA2* expression levels, and low *let-7* expression levels all associate with large leiomyoma size (Rein *et al.*, 1998; Wang *et al.*, 2007; Peng *et al.*, 2008). Multiple lines of evidence show also a relationship between elevated *HMGA2* expression levels and cellular transformation, which supports the role for *HMGA2* in the regulation of cellular proliferation (Sandberg, 2005).

Involvement of the region 14q23-24 in rearrangements with 12q14-15 is relatively specific for uterine leiomyomas (Heim *et al.*, 1988; Turc-Carel *et al.*, 1988; Meloni *et al.*, 1992). *RAD51* *paralog B* (*RAD51B*), the target gene of the 14q breakpoint region, represents a candidate translocation partner for *HMGA2* (Ingraham *et al.*, 1999; Schoenmakers *et al.*, 1999). *RAD51B* is a member of an evolutionarily conserved protein family which plays an essential role in the homologous recombination of double-strand DNA breaks (Thacker, 2005). Although fusion transcripts involving *HMGA2* and *RAD51B* seem to occur in a small percentage of uterine leiomyomas, the key pathological event in leiomyomas with t(12;14)(q15;q23-24) remains unknown (Schoenmakers *et al.*, 1999; Takahashi *et al.*, 2001). One hypothesis suggests that aberrant expression of transcripts containing the intact *HMGA2* could be the primary molecular mechanism underlying these tumors, instead of fusion transcripts (Quade *et al.*, 2003). Of note, *HMGA2* overexpression is present also in uterine leiomyomas without the 12q14-15 rearrangements, indicating a more general role for *HMGA2* in leiomyomagenesis (Klemke *et al.*, 2009).

#### 4.1.2 Deletions of 7q

The second most common chromosomal aberration in uterine leiomyomas is an interstitial deletion of chromosome 7, del(7)(q22-32), accounting for approximately 17% of karyotypically abnormal leiomyomas (Sandberg, 2005). Although apparent in other benign mesenchymal tumors, this deletion occurs more frequently in leiomyomas than in any other solid tumor. Currently, the minimal deletion region on the long arm of chromosome 7 has been narrowed down to an area within 7q22 and the relatively high frequency of LOH in this region suggests the existence of a tumor suppressor gene (Zeng *et al.*, 1997; Sell *et al.*, 1998; van der Heijden *et al.*, 1998; Saito *et al.*, 2005; Vanharanta *et al.*, 2005). The gene-rich nature of the 7q22 region, however, has caused difficulties in defining the precise target sequence of the deletion that could promote tumorigenesis in uterine leiomyomas. In spite of the identification of numerous positional candidate genes on 7q22, such as *CUX1*, *ORC5L*, *PCOLCE*, and *ZNHIT1*, none have been proven to have a consistent causative role in uterine leiomyomas (Ishiai *et al.*, 1997; Zeng *et al.*, 1997; Ligon *et al.*, 2002; Mehine *et al.*, 2013; Schoenmakers *et al.*, 2013). One of the candidate tumor suppressor genes, *cut-like homeobox 1* (*CUX1*), encodes a member of the homeodomain family of DNA binding proteins which functions as a transcription factor in multiple cellular processes, including cell proliferation, cell motility/invasiveness, and apoptosis (Hulea and Nepveu, 2012; Vadnais *et al.*, 2012). The gene was recently identified as a frequent target of chromosome 7 deletions in myeloid neoplasms (McNerney *et al.*, 2013; Wong *et al.*, 2014).

The del(7)(q22-32) is present as the sole cytogenetic anomaly in a subset of uterine leiomyomas, but occurs also simultaneously with t(12;14)(q15;q23-24) in the tumors (Sait *et al.*, 1989). Leiomyomas with the sole deletion are smaller in size than leiomyomas with t(12;14) or leiomyomas with both the deletion and t(12;14) (Rein *et al.*, 1998; Hennig *et al.*, 1999). At the same time, leiomyoma cells with del(7)(q22-32) are frequently lost in tissue cultures, while they continue to persist in cultures when t(12;14)(q15;q23-24) is present

(Xing *et al.*, 1997). These observations suggest that 7q deletions may represent secondary changes in the development of uterine leiomyomas.

#### 4.1.3 Rearrangements of 6p21

Rearrangements of 6p21 are frequent events in various benign mesenchymal tumors, such as lipomas, pulmonary chondroid hamartomas, and endometrial polyps (Sandberg, 2005). They occur also in uterine leiomyomas, although with a much lower frequency, accounting for <5% of karyotypically abnormal tumors. The rearrangements in leiomyomas consist mainly of various translocations, occasionally involving 14q23-24, and inversions (Nilbert *et al.*, 1989; Kiechle-Schwarz *et al.*, 1991; Ozisik *et al.*, 1995; Sornberger *et al.*, 1999). *High mobility group AT-hook 1 (HMGA1)*, the gene encoding another member of the HMGA protein family, maps to 6p21 and represents the primary target region for these rearrangements (Kazmierczak *et al.*, 1996; Dal Cin *et al.*, 1997; Williams *et al.*, 1997; Xiao *et al.*, 1997). Breakpoints affecting the *HMGA1* locus have been demonstrated, in addition to uterine leiomyomas, in pulmonary chondroid hamartomas and hamartomas of the breast. Despite the sequence and structural similarity between *HMGA1* and *HMGA2*, the expression patterns of these genes seem to differ substantially from each other (Sandberg, 2005). For example, overexpression of *HMGA1* correlates, in particular, with a malignant phenotype in a number of epithelial tumors and leukemia (Cleynen and Van de Ven, 2008). In the case of uterine leiomyomas, 6p21 rearrangements appear to also upregulate *HMGA1* expression (Sornberger *et al.*, 1999; Tallini *et al.*, 2000). The level of upregulation, however, is much less strong than that of *HMGA2* in leiomyomas with 12q14-15 rearrangements in comparison to the myometrium (Nezhad *et al.*, 2010).

#### 4.1.4 Other chromosomal abnormalities

Trisomy 12, another non-random chromosomal aberration affecting chromosome 12, is present in approximately 12% of karyotypically abnormal leiomyomas (Nilbert *et al.*, 1990a; Vanni *et al.*, 1992). This anomaly may potentially result in the upregulation of *HMGA2* expression, as t(12;14)(q15;q23-24), but this time by increasing the gene dosage. A series of other chromosomal aberrations of lower frequency than those above exist in uterine leiomyomas, including monosomy 10 and various different rearrangements of chromosomes 1, 3, 10, 13, and X (Sandberg, 2005). For example, mapping of the 10q breakpoint region has identified *K(lysine) acetyltransferase 6B (KAT6B)*, a gene encoding a histoneacetyltransferase, as a possible candidate target gene (Moore *et al.*, 2004). Many of these aberrations occur simultaneously with other chromosomal changes in leiomyomas, suggesting their secondary nature in the tumorigenesis. On the other hand, chromosomal abnormalities at several loci in individual tumors are consistent with the multistep process of tumor development and progression.

#### 4.1.5 Clonality

Cytogenetic analyses of multiple leiomyomas from a single uterus have shown that the tumors can harbor different chromosomal abnormalities, suggesting that each tumor may develop independently (Ligon and Morton, 2000). Supporting evidence has come from X-chromosome inactivation studies, which utilize Lyonization as their basis to evaluate the clonal origin of tumors. Lyonization is a process in which one of the two X chromosomes in all female somatic cells is randomly inactivated early in embryogenesis (Morey and Avner, 2011). The inactivated X chromosome remains inactive throughout the lifetime of a cell and passes on to its descendants. A monoclonal tissue represents an identical pattern of X-chromosome inactivation, while a polyclonal tissue exhibits a mixture of cells expressing two different phenotypes. Analyses of X-chromosome inactivation of the glucose-6-phosphate dehydrogenase isoenzyme or androgen receptor CAG-repeat polymorphisms have indicated that most uterine leiomyomas arise independently from a single myometrial cell; individual leiomyomas within the same uterus express exclusively one or the other allele (Townsend *et al.*, 1970; Mashal *et al.*, 1994; Hashimoto *et al.*, 1995; Zhang *et al.*, 2006). Occasionally, however, observations of a common clonal origin for a subset of physically distinct leiomyomas in the uterus have emerged, suggesting that multiple tumors may develop from a single primary tumor (Nilbert *et al.*, 1990a; Nilbert *et al.*, 1990b; Canevari *et al.*, 2005; Mehine *et al.*, 2013).

#### 4.2 Copy-number alterations and gene expression profiling

After decades of work using standard karyotyping to characterize non-random chromosomal aberrations in uterine leiomyomas, a number of studies have introduced methods of increasingly higher resolution (microsatellite marker analysis, array comparative genomic hybridization (CGH), and single nucleotide polymorphism (SNP) array) to identify novel and previously undetectable submicroscopic losses and gains of chromosomal material in the tumors. Although various genomic regions with copy-number variation have emerged, including gains in chromosomes 9q and 19 and losses in chromosome 22q, these alterations seem to be infrequent events in the genomes of uterine leiomyomas (Packenham *et al.*, 1997; Levy *et al.*, 2000; Bowden *et al.*, 2009; Meadows *et al.*, 2011). The assessment of LOH, another method to detect genomic regions that may harbor critical genes involved in leiomyomagenesis, has also revealed little evidence of these gross chromosomal events in leiomyomas, with the exception of the regions 7q15-31 and 15q25-26 (van der Heijden *et al.*, 1998; Mao *et al.*, 1999; Canevari *et al.*, 2005; Vanharanta *et al.*, 2005; Meadows *et al.*, 2011). Alternatively, a series of studies have applied gene expression microarrays to identify differentially expressed genes and pathways between uterine leiomyomas and normal myometrium, which could potentially affect tumorigenesis. The gene expression profiles that have been generated have, however, differed substantially from each other. This may result from differences in microarray techniques, data analysis methods, and ethnicities of the patients, or not taking into account that the leiomyomas under study have distinct genetic statuses. Despite the variation in the expression profiles, dozens of genes, such as *ADHI*,

*EGR1*, *C-FOS*, *IGF2*, and *TGFBR2*, involved in retinoid metabolism, growth and proliferation, differentiation, and ECM formation have come up in several studies, suggesting that these factors may play an important role in the development of leiomyomas (Arslan *et al.*, 2005; Vanharanta *et al.*, 2006; Ishikawa *et al.*, 2007; Raimundo *et al.*, 2009; Csatlos *et al.*, 2013a; Csatlos *et al.*, 2013b). Future research efforts are vitally important to better understand the causes and mechanisms of leiomyomagenesis, which could in turn help to develop new strategies for prevention and treatment of the disease.

### 4.3 Predisposing genetic characteristics

Familial aggregation, research on various ethnic groups, twin studies, and association with hereditary syndromes have all provided supportive evidence for genetic predisposition to uterine leiomyomas. Recently, a genome-wide association study revealed three chromosomal loci (10q24.33, 22q13.1, and 11p15.5) associated with increased susceptibility to uterine leiomyomas in Japanese women (Cha *et al.*, 2011). This finding was afterwards examined in several ethnicities with contradictory results (Eggert *et al.*, 2012; Wise *et al.*, 2012; Edwards *et al.*, 2013). Another recent genome-wide linkage and association study in a cohort of women with European ancestry suggested *fatty acid synthase (FASN)* on chromosome 17q25.3 to be a novel leiomyoma risk allele in white women (Eggert *et al.*, 2012). This gene encodes an enzyme FASN, whose levels were three times higher in leiomyomas compared to the corresponding myometrium; the same phenomenon was previously associated with various neoplasms and linked to tumor cell survival (Pizer *et al.*, 1996; Pizer *et al.*, 1998; Liu *et al.*, 2010). Despite these results, so far the only well-known genetic factors that predispose to uterine leiomyomas are germline mutations in *fumarate hydratase (FH)* causing an inherited tumor susceptibility syndrome (Tomlinson *et al.*, 2002).

#### 4.3.1 Hereditary leiomyomatosis and renal cell cancer (HLRCC)

Hereditary leiomyomatosis and renal cell cancer (HLRCC; MIM 150800), also known as multiple cutaneous and uterine leiomyomatosis (MCUL), is a rare dominantly inherited tumor predisposition syndrome characterized by multiple cutaneous and uterine leiomyomas and a high risk of developing renal cell carcinomas (Launonen *et al.*, 2001). Cutaneous leiomyomas, the most distinctive feature of HLRCC, originate from arrector pili muscles attached to hair follicles. They tend to be numerous (ranging from one to hundreds), localize on the trunk and limbs, and cause pain in response to touch or temperature changes (Lehtonen, 2011). Occasionally, cutaneous leiomyomas can be overlooked or go unreported as a result of their subtle or tolerable nature at the time (Alam *et al.*, 2005; Tolvanen *et al.*, 2012). Uterine leiomyomas in HLRCC occur as large multiple lesions representing more severe symptoms and an earlier age of onset than their sporadic counterparts. In contrast to cutaneous and uterine leiomyomas, renal cell carcinomas (mostly papillary type II) are present only in a fifth of HLRCC-families worldwide (Launonen *et al.*, 2001; Tomlinson *et al.*, 2002; Toro *et al.*, 2003; Vahteristo *et al.*, 2010). They are, however, exceptionally

aggressive tumors, which can metastasize in the very early stages, creating a challenge for the diagnosis and treatment of these lesions.

Heterozygous germline mutations in *FH* at chromosome 1q42 underlie HLRCC (Tomlinson *et al.*, 2002). The gene encodes the enzyme fumarase, a component of the mitochondrial tricarboxylic acid cycle (TCAC) which catalyzes the hydration of fumarate to malate. The majority of *FH* germline mutations are missense (~58%), nonsense (~11%), or frameshift mutations (~18%) scattered throughout the gene (Bayley *et al.*, 2008; Lehtonen, 2011). Furthermore, HLRCC-associated tumors frequently display biallelic inactivation of *FH* indicating that the gene functions as a tumor suppressor. Loss of the wild-type allele occurs mainly through LOH, but occasionally point mutations can serve as second hits (Kiuru *et al.*, 2001; Launonen *et al.*, 2001). Biallelic inactivation of *FH* results in elevated levels of fumarate and succinate, an intermediate in the TCAC prior to fumarate (Pollard *et al.*, 2005a). TCAC is part of the aerobic respiration process in the cell's energy metabolism. The most extensively studied hypothesis on the molecular mechanisms of HLRCC tumorigenesis is "pseudohypoxia". The excessive amount of fumarate or succinate in a cell seems to aberrantly stabilize HIF1, a key-signaling molecule in the hypoxia pathway, despite the presence of oxygen (Isaacs *et al.*, 2005; Pollard *et al.*, 2005b). Activation of the pathway, in turn, leads to upregulation of several HIF1 target genes involved in vascularization, glycolysis, and glucose transport, processes that can promote tumor growth. Of note, biallelic inactivation of *FH* has been reported in only 1.3% of sporadic leiomyomas (Kiuru *et al.*, 2002; Lehtonen *et al.*, 2004; Vaidya *et al.*, 2012).

#### 4.3.2 Other hereditary syndromes associated with uterine leiomyomas

Uterine leiomyomas occur, in addition to HLRCC, in the context of other hereditary syndromes, such as tuberous sclerosis (TSC), Birt-Hogg-Dubé (BHD), and Cowden syndrome. TSC is a rare, multi-system genetic disease resulting from heterozygous germline mutations in the tumor suppressor genes *TSC1* and *TSC2* (European Chromosome 16 Tuberous Sclerosis Consortium, 1993; van Slegtenhorst *et al.*, 1997). One of the several animal models for TSC, the Eker rat with a germline mutation in one allele of *Tsc2*, has been shown to develop uterine leiomyomas spontaneously at a high frequency (~65%) (Everitt *et al.*, 1995; Walker *et al.*, 2003). These tumors are histologically similar to conventional human leiomyomas and display loss or reduction of tuberin, the gene product of *Tsc2*, in comparison to normal myometrium (Hunter *et al.*, 2002). As in their human counterparts, hormonal factors play an important role in the development of these leiomyomas (Walker, 2002). The Eker rat acts as an established animal model for uterine leiomyomas. Furthermore, spontaneous uterine leiomyomas arise also in German shepherd dogs, a canine model for BHD, carrying a germline *Bhd* mutation (Moe and Lium, 1997; Lingaas *et al.*, 2003). Of note, the co-occurrence of renal cell carcinomas and uterine leiomyomas in HLRCC and both animal models above suggests a possible genetic link between these carcinomas and uterine leiomyomas. Cowden syndrome, also known as multiple hamartoma syndrome, is caused by germline mutations in the tumor suppressor gene *PTEN* (Liaw *et al.*,

1997). Approximately 50% of women with the syndrome develop uterine leiomyomas, and hence the tumors are part of the minor criteria for the syndrome (Hobert and Eng, 2009).

#### 4.3.3 Genetic polymorphisms and ethnic disparity

Since the ethnic disparity in the incidence and biological behavior of uterine leiomyomas may potentially result from racial differences in estrogen metabolism, several studies have examined the role of gene polymorphisms in estrogen-metabolizing enzymes as prospective susceptibility factors for uterine leiomyomas. One of these enzymes is catechol-O-methyltransferase (COMT), which catalyzes methyl conjugation of the hydroxyl groups of catechol estrogens (Zhu and Conney, 1998). The regulation of COMT activity may indirectly modulate the biological effects of estrogen. The most studied polymorphism in the gene, a valine-to-methionine (M) substitution at codon 158, influences the enzymatic activity of the protein; the V/V genotype associates with the highest, and the M/M genotype with the lowest, activity (Lachman *et al.*, 1996). African-American women tend to have a higher frequency of the V/V genotype and a lower frequency of the M/M genotype, while the opposite applies for white women (Al-Hendy and Salama, 2006). The relationship between the V158M polymorphism and uterine leiomyoma susceptibility is still, however, unclear; results exist both for and against its involvement (Al-Hendy and Salama, 2006; Denschlag *et al.*, 2006; Gooden *et al.*, 2007; de Oliveira *et al.*, 2008; Morikawa *et al.*, 2008; Ates *et al.*, 2013; Shen *et al.*, 2014). In addition to polymorphisms in estrogen-metabolizing enzymes, the role of various polymorphisms in other candidate genes, such as ER $\alpha$ , ER $\beta$ , PR, androgen receptor, and VEGF, for leiomyoma susceptibility have been studied, but thus far with contradictory results.



## AIMS OF THE STUDY

The aim of this thesis work was to elucidate the molecular genetic characteristics of uterine leiomyomas using next-generation sequencing technology. The specific aim was:

1. To identify recurrent mutations in uterine leiomyomas from Finnish patients by exome sequencing

The discovery of highly frequent somatic *mediator complex subunit 12 (MED12)* exon 2 mutations clarified the subsequent aims of the study, which were:

2. To validate the finding and determine the frequency of *MED12* exon 2 mutations in uterine leiomyomas from South African patients
3. To determine the frequency of *MED12* exon 2 mutations in the most common histopathological uterine leiomyoma variants and uterine leiomyomas of HLRCC-patients
4. To discover driver mutations in *MED12* mutation-negative and additional contributing mutations in *MED12* mutation-positive uterine leiomyomas by exome sequencing

# MATERIALS AND METHODS

## 1. Samples

### 1.1 Finnish uterine leiomyoma patient samples (I, III, IV)

#### 1.1.1 Fresh frozen tissue samples (I, IV)

Uterine leiomyoma and the respective normal myometrial tissue samples were collected as fresh frozen tissue, in collaboration with Dr. Jari Sjöberg, from a cohort of hysterectomy patients in Helsinki University Central Hospital (HUCH), Helsinki, Finland in 2002-2004. Clinical information was unavailable to the researchers at the time of surgery. Samples comprised two categories: specimens from anonymous patients (“M” patients) and specimens from patients who signed an informed consent (“MY” patients). Subsequent retrieval of clinical data from pathology reports (only “MY” patients) included patient’s age at hysterectomy and number of tumors (solitary/multiple).

In Study I, the exome sequencing sample set consisted of 18 unselected uterine leiomyomas and their corresponding normal myometrial tissue, including two tumors from the same patient: one tumor (M32m1) with a somatic *FH* mutation, c.715G>A, p.A239T, and LOH as a second hit (Vanharanta *et al.*, 2006), and one *FH* mutation-negative tumor (M32m8). Additional validation set comprised 207 unselected leiomyoma specimens resulting in altogether 225 uterine leiomyomas from 80 patients; of these, tumor diameter was obtainable for 185 lesions (82%) (Table 3). Gene expression data of ten uterine leiomyoma-myometrium pairs were also available, nine of which were already part of the exome sequencing sample set.

Study IV included previously generated exome sequencing data of 17 (two *MED12* mutation-negative and 15 *MED12* mutation-positive) uterine leiomyoma-myometrium pairs from Study I, analyzed only in the context of *MED12*, and newly generated data of ten *MED12* mutation-negative uterine leiomyomas with matched normal myometrial tissue samples (Table 3). None of the *MED12* mutation-negative uterine leiomyomas displayed a mutation in *FH*.

**Table 3. Uterine leiomyoma patients and tumors from HUCH in Studies I and IV.**

	Exome sequencing					Validation set						
	M-series		MY-series		Total	M-series		MY-series		Total		
<b>Study I</b>	Patients	10	Patients	7	Patients	17	Patients	34	Patients	29	Patients	63
	Tumors	11	Tumors	7	Tumors	18	Tumors	88	Tumors	119	Tumors	207
<b>Study IV</b>	Patients	13	Patients	14	Patients	27						
	Tumors	13	Tumors	14	Tumors	27						
	<i>MED12</i> +	10	<i>MED12</i> +	5	<i>MED12</i> +	15						
	<i>MED12</i> -	3	<i>MED12</i> -	9	<i>MED12</i> -	12						

### 1.1.2 Formalin-fixed paraffin-embedded tissue samples (III)

Through collaboration with Dr. Ralf Bützow, a set of 103 anonymous formalin-fixed paraffin-embedded (FFPE) histopathological uterine leiomyoma variant samples was available from the Department of Pathology, HUCH, Helsinki, Finland. The set included 59 cellular leiomyomas (36 highly cellular and 23 cellular), 18 leiomyomas with bizarre nuclei, and 26 mitotically active leiomyomas. Additional 69 conventional FFPE leiomyomas served as controls.

### 1.2 South African uterine leiomyoma patient samples (II)

Prof. Zephne van der Spuy and Prof. Ian Tomlinson provided genomic DNA (gDNA) of 28 fresh frozen uterine leiomyoma and 14 respective normal myometrial tissue samples representing 18 patients with African ancestry from the Department of Obstetrics and Gynaecology, Faculty of Health Sciences, University of Cape Town/Groote Schuur Hospital, Cape Town, South Africa. Clinical information included patient's age at diagnosis, total number of tumors, and tumor size.

### 1.3 Finnish HLRCC-patient samples (III)

Altogether 34 fresh frozen or FFPE uterine leiomyoma samples and their corresponding normal myometrial tissue from 14 Finnish HLRCC-patients were available. The patients represented six different HLRCC-families with a known germline *FH* mutation status (Table 4).

**Table 4. Finnish HLRCC-families in Study III.**

Family name	No. of family members in Study III	Germline <i>FH</i> mutation		Reference
		Nucleotide change	Amino acid change	
FAM-1 (M)	7	c.671_672delAG	p.E224fs	(Launonen <i>et al.</i> , 2001)
FAM-2 (B)	2	c.671_672delAG	p.E224fs	(Launonen <i>et al.</i> , 2001)
FAM-3 (C)	2	c.1027C>T	p.R343X	(Kiuru <i>et al.</i> , 2001)
FAM-4 (D)	1	c.587A>G	p.H196R	(Lehtonen <i>et al.</i> , 2006)
FAM-5 (E)	1	c.587A>G	p.H196R	(Lehtonen <i>et al.</i> , 2006)
FAM-9 (N)	1	c.1027C>T	p.R343X	-

## 2. DNA and RNA extraction (I, III, IV)

Extraction of gDNA from fresh frozen tissue followed either a standard non-enzymatic method (Lahiri and Nurnberger, 1991) or FastDNA<sup>®</sup> Kit protocol (MP Biomedicals LLC, Solon, OH, USA). Genomic DNA from FFPE tissue was extracted with the NucleoSpin FFPE DNA Kit or the NucleoSpin FFPE RNA/DNA Kit (Macherey-Nagel, Düren, Germany).

Extraction and purification of total RNA were in accordance with manufacturers' instructions of TRIzol<sup>®</sup> Reagent (Invitrogen, Carlsbad, CA, USA) and RNeasy<sup>®</sup> MinElute<sup>™</sup> Clean Up Kit (Qiagen GmbH, Hilden, Germany). Conversion of RNA to cDNA complied with a standard protocol.

### **3. Histopathological evaluation (I, III, IV)**

Hematoxylin-eosin (HE)-staining of 5 µm FFPE or frozen uterine leiomyoma tissue sections complied with standard procedures. Classification of tumors was based on histopathological characteristics following WHO criteria (Hendrickson *et al.*, 2003). *MED12* mutation status was unknown during each histopathological assessment.

In Study I, pathologist Dr. Elina Virolainen reviewed HE-stained frozen tissue sections of all the tumors in the exome sequencing and on gene expression arrays to ensure high tumor percentage. Another pathologist, Dr. Tom Böbling, evaluated morphology and proliferation activity of a set of 93 randomly chosen uterine leiomyomas (67 *MED12* mutation-positive and 26 *MED12* mutation-negative). All the cases represented histopathologically conventional leiomyomas.

In Studies III and IV, pathologist Dr. Ralf Bützow reviewed HE-stained FFPE or frozen tissue sections from each uterine leiomyoma. Tumors were divided into conventional leiomyomas, cellular leiomyomas, mitotically active leiomyomas, or leiomyomas with bizarre nuclei (i.e. atypical). The number of mitoses per 10 high-power fields (HPF), the degree of cellularity (normal, cellular, and highly cellular), and severity of nuclear atypia (0–3) were recorded for each tumor.

### **4. Exome sequencing and data analysis (I, IV)**

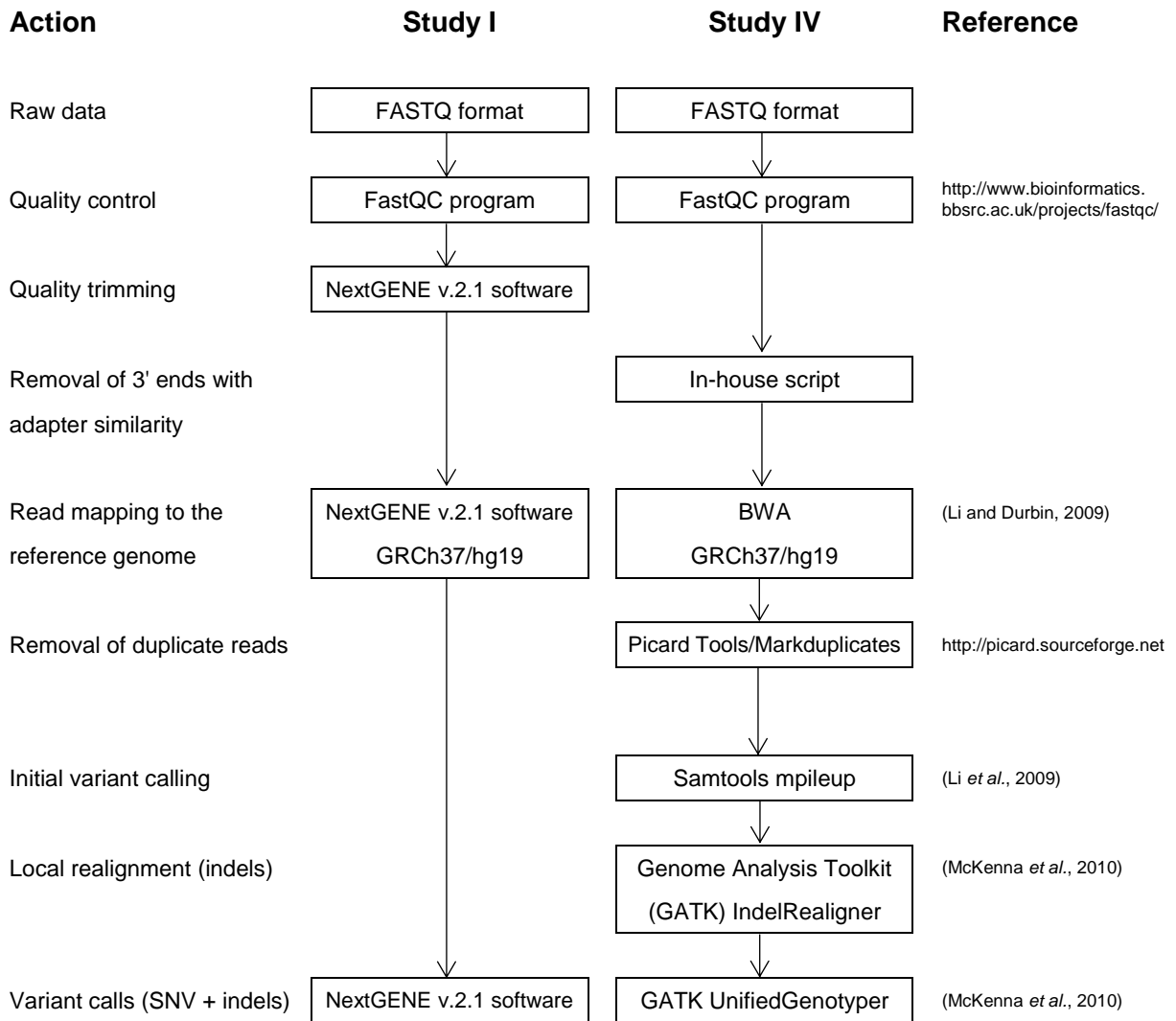
#### **4.1 Exome capture and sequencing**

Preparation of gDNA libraries of uterine leiomyoma and the respective normal myometrial tissue samples followed manufacturer's protocol of the NEBNext<sup>®</sup> DNA Sample Prep Reagent Set 1 Kit (New England Biolabs Ltd., Hitchin, United Kingdom). Exonic regions of the genome were enriched with the Agilent SureSelect Human All Exon Kit (Agilent, Santa Clara, CA, USA). Paired-end sequencing of 80-82 base pair (bp) reads was performed with Genome Analyzer II (Illumina Inc., San Diego, CA, USA) at the Institute of Molecular Medicine Finland (FIMM) Technology Center, Helsinki, Finland.

In Study I, the exome capture kit targeted approximately 38 Mb of coding DNA, whereas in Study IV the targeted region was about 50 Mb.

## 4.2 Read mapping and variant calling

Studies I and IV applied different pipelines for raw exome sequencing data (Figure 4).



BWA: Burrows-Wheeler Aligner; indel: insertion-deletion; SNV: single nucleotide variation

**Figure 4. Processing of raw exome sequencing data in Studies I and IV.**

## 4.3 Analysis of exome sequencing data

Exome sequencing data analysis and visualization were conducted with NextGENE v2.1 software (Softgenetics, State College, PA, USA) or Rikurator v1.3 Alpha (Katainen, 2013) (Table 5). In Study I, the average coverage for each base was 50 reads and 94% of the exonic regions covered displayed  $\geq 4$  reads. The corresponding numbers in Study IV were 41 reads and 90% ( $\geq 6$  reads).

**Table 5. Detailed information of exome sequencing data analysis in Studies I and IV.**

	<b>Study I</b>	<b>Study IV</b>
<b>Samples</b>	18 uterine leiomyoma-myometrium pairs	27 uterine leiomyoma-myometrium pairs
<b>Analysis software</b>	NextGENE v2.1	Rikurator v1.3 Alpha
<b>Analyzed regions</b>	Exons, $\pm 3$ bp of exon-intron boundaries	Exons, $\pm 2$ bp of exon-intron boundaries
<b>Quality filters</b>		
Minimum coverage		
1) Variation call	4 reads	6 reads
2) Mutated allele	20% of the reads	20% of the reads
Quality score	-	$\geq 40$ (GATK quality calculations)
<b>Additional filtering</b>	1. respective normal tissue samples	1. respective normal tissue samples
	2. rs-coded SNPs (Ensembl59, dbSNP132)	2. rs-coded SNPs (Ensembl59, dbSNP132)
	3. 156 in-house control exomes	3. 70 in-house control exomes
	4. mutation score $\geq 6$	4. 93 Finnish control genomes (1000 Genomes Project; phase 1 release v2)
	5. synonymous coding variants	5. synonymous coding variants
		6. variants in genes encoding uncharacterized proteins
		7. variants within 1-10 bp at the end of the reads and present only in the same direction reads

GATK: Genome Analysis Toolkit

After exome sequencing data filtering, only tumor-specific alterations remained in the analyses. The outcome in Study I was a list of the most frequently mutated genes in the examined uterine leiomyomas and in Study IV two lists for both *MED12* mutation-negative and *MED12* mutation-positive leiomyomas: 1) exactly the same variant in at least two tumors (common variants) and 2) different variants in the same gene in at least two tumors (unique variants).

## 5. Sanger sequencing

Oligonucleotide primers were designed with Primer3 (Untergasser *et al.*, 2012) with GRCh37/hg19 serving as a reference. Polymerase chain reaction (PCR) complied with a standard protocol. Purification of PCR products followed the ExoSAP-IT PCR Purification Kit (USB Corporation, Cleveland, OH, USA) and sequencing was carried out with the Big Dye Terminator v.3.1 Kit (Applied Biosystems, Foster City, CA, USA) on an ABI3730 Automatic DNA Sequencer (FIMM Technology Centre and DNA sequencing and Genomics laboratory, Institute of Biotechnology, Helsinki, Finland) or ABI3100 Capillary Sequence Analyzer (Sequencing Core Facility, Haartman institute, Helsinki, Finland) according to the manufacturer's instructions. Sequence graphs were analyzed both with Mutation Surveyor-software (Softgenetics) and manually.

### 5.1 Verification of exome sequencing variants (I, IV)

Sanger sequencing assessed the presence and somatic status of candidate exome sequencing variants. In Study I, this included all variants observed in *MED12*, the most frequently mutated gene among exome-sequenced uterine leiomyomas, and in Study IV, all common and unique candidate variants on the generated lists, as well as an unexpected *FH* mutation finding. Somatic status of the variants was verified from the corresponding normal myometrial tissue.

### 5.2 Mutation screening (I-IV)

Validation of *MED12* exon 2 mutations in Study I included direct sequencing of 207 additional fresh frozen uterine leiomyomas (Table 3). To confirm the expression of the mutant alleles in the tumors as well as to examine the effect of an intronic variant localized 8 bp upstream of the intron 1-exon 2 boundary, cDNA sequencing was performed for 19 *MED12* mutation-positive leiomyomas. Furthermore, a series of 30 uterine leiomyomas (20 *MED12* mutation-negative and 10 *MED12* mutation-positive) underwent whole gene sequencing of *MED12* for the identification of possible additional mutations.

In Studies II and III, the frequency of *MED12* exon 2 mutations was examined by Sanger sequencing. In Study III, two different amounts of DNA (15 and 25 ng) were used to ensure accuracy. In Study IV, one tumor with a germline *FH* mutation (MY31m4) went through whole gene sequencing of *FH* as a part of the assessment of biallelic *FH* inactivation.

### 5.3 Loss of heterozygosity analysis (III, IV)

Tumor DNA from patients with a germline *FH* mutation was sequenced for LOH in Studies III and IV. The assessment of LOH on tumor-derived DNA was based on visual observation of the sequence chromatograms by comparing the peak heights between the wild-type and mutant allele. Scoring of LOH required a recurring and significant reduction in the height of the wild-type allele peak when compared to that of the mutant allele. Study III included five parallel PCR reactions per FFPE sample to ensure accuracy. In addition, previous LOH data were available for 14 out of 34 uterine leiomyomas (Vanharanta *et al.*, 2006; unpublished data).

## 6. Gene expression profiling (I)

Previously generated transcriptome-wide gene expression data on GeneChip<sup>®</sup> Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) arrays of 10 uterine leiomyoma-myometrium pairs were available for Study I, including eight *MED12* mutation-positive and two mutation-negative leiomyomas. The gene expression data of the mutation-positive lesions were compared with the data of the mutation-negative lesions as well as respective normal myometrial tissue samples in an unsupervised hierarchical clustering using a paired t-test. Differentially expressed genes were defined by a fold change difference of >1.5. Furthermore, the differentially expressed genes from *MED12* mutation-positive uterine leiomyomas and their respective normal myometrium underwent pathway enrichment analysis. Pathways reached statistical significance with a false discovery rate of <0.05.

## 7. Statistical and computational analyses

### 7.1 Significance testing (I-III)

Statistical analyses were performed using R software, version 2.14.0 ([www.r-project.org](http://www.r-project.org)). Two-sided Wilcoxon rank sum test with continuity correction was utilized in Study I to assess the potential correlation between *MED12* mutation status and patient's age at hysterectomy or tumor size. In Study II, Pearson's chi-squared test was applied to evaluate the statistical significance of the difference between the amount of *MED12* mutation-positive leiomyomas in Finnish and South African patients. Fisher's exact test revised the same difference, when only leiomyomas with a diameter  $\geq 5.5$  cm were included in the analysis. The chosen size limit represented the average tumor size, when leiomyomas from both Finnish and South African patients were taken into account. Study III used solely Fisher's exact test to calculate the statistical significance of differences between *MED12* exon 2 mutation frequencies in various clinical uterine leiomyoma variants and conventional leiomyomas. Eight mitotically active leiomyomas with increased cellularity were included in both mitotically active and cellular leiomyoma groups. The *P*-value threshold for statistical significance was <0.05.

### 7.2 *In silico* predictions (I, IV)

Study I utilized several *in silico*-software applications to examine the possible consequences of intronic variants on splicing and to scrutinize the conservation status of the mutation hotspot region near the beginning of *MED12* exon 2 (Table 6). In Study IV, multiple types of software evaluated the potential functional effects of the identified exome sequencing variants (non-synonymous) (Table 6).



**Table 6. *In silico*-prediction programs in Studies I and IV.**

	<b>Software</b>	<b>Reference</b>
<b>Study I</b>		
<b>Splicing prediction</b>	ASSP	<a href="http://www.es.embnet.org/mwang/assp.html">http://www.es.embnet.org/mwang/assp.html</a>
	NetGene2	<a href="http://www.cbs.dtu.dk/services/NetGene2/">http://www.cbs.dtu.dk/services/NetGene2/</a>
<b>Peptide sequence alignment</b>	Muscle	(Edgar, 2004)
<b>Intronic sequence alignment</b>	Multiz alignments (UCSC)	<a href="http://genome.ucsc.edu/">http://genome.ucsc.edu/</a>
<b>Secondary structure prediction</b>	PSIpred	(Bryson <i>et al.</i> , 2005)
<b>Study IV</b>		
<b>Non-synonymous variant prediction</b>	Ensembl Variant Effect Predictor	(McLaren <i>et al.</i> , 2010)
	SIFT	<a href="http://sift.jcvi.org/">http://sift.jcvi.org/</a>
	Polyphen2	<a href="http://genetics.bwh.harvard.edu/pph2/">http://genetics.bwh.harvard.edu/pph2/</a>

## **8. Ethical issues (I-IV)**

Studies I, III, and IV were approved by the Ministry of Social Affairs and Health and the Ethics Committee for gynaecology and obstetrics, pediatrics and psychiatry of the Hospital District of Helsinki and Uusimaa, Finland. Patient samples were obtained either with an appropriate informed consent or, in the case of specimens from anonymous patients, with authorization from the director of the health care unit. Study II was approved by the local Human Research Ethics Committee, Cape Town, South Africa.

## RESULTS

### 1. Highly frequent *mediator complex subunit 12 (MED12)* mutations in uterine leiomyomas (I, II)

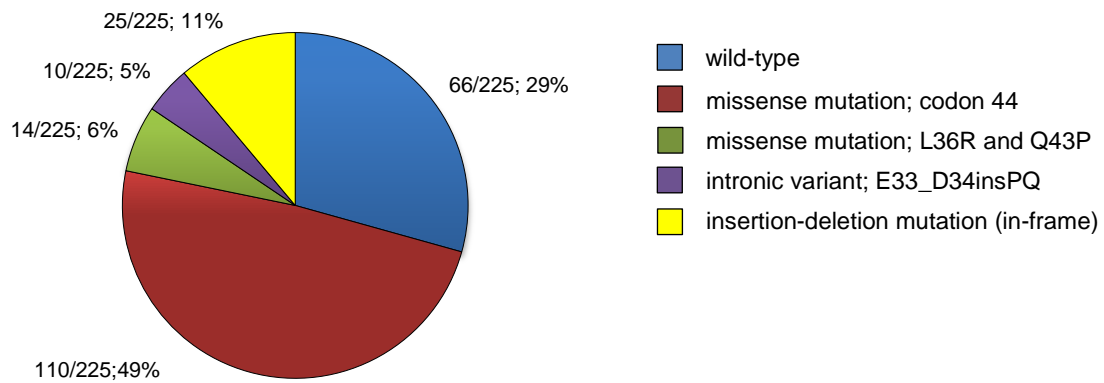
#### 1.1 Identification of *MED12* exon 2 mutations by exome sequencing (I)

Exome sequencing of 18 uterine leiomyomas from 17 Finnish patients revealed *MED12* as the most frequently mutated gene among the tumors. Tumor-specific *MED12* mutations were present in ten out of 18 specimens all locating in exon 2, the majority affecting specifically codon 44 (Table 7). None of the other 44 *MED12* exons displayed additional variation.

**Table 7. Observed *MED12* exon 2 mutations in 18 uterine leiomyomas by exome sequencing.** The nucleotide numbering and amino acid locations follow the transcript sequence: ENST00000374080.

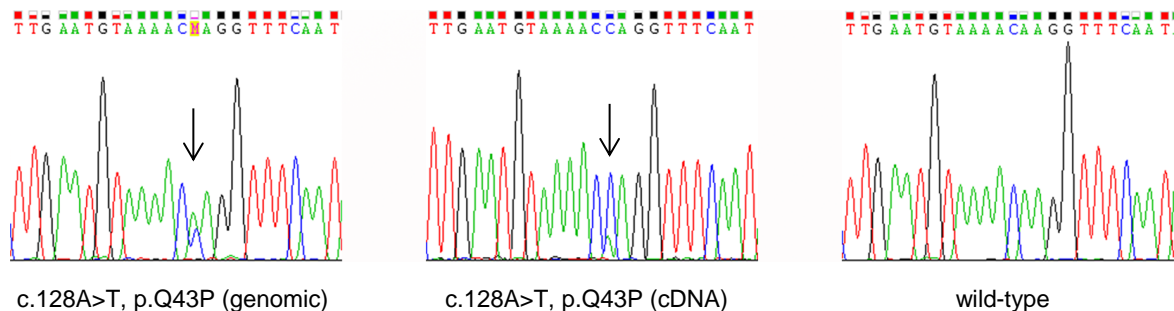
Mutation type	Position	Nucleotide change	Amino acid change	No. of mutations
Missense	X:70339251	c.128A>C	p.Q43P	1
	X:70339253	c.130G>A	p.G44S	2
	X:70339254	c.131G>C	p.G44A	2
	X:70339254	c.131G>A	p.G44D	2
	X:70339254	c.131G>T	p.G44V	2
Deletion	X:70339229	c.106_108del3	p.L36del	1

Verification of *MED12* exon 2 mutations came from Sanger sequencing of the original 18 tumors together with 207 additional uterine leiomyomas. Mutation screening discovered a remarkable frequency of somatic *MED12* mutations in the tumors. Altogether 159 out of 225 (70.7%) uterine leiomyomas harbored *MED12* exon 2 mutations. A notable amount of the mutations were located in codon 44 (69.2%; 110/159) including all six possible base pair substitutions leading to an amino acid change (Figure 5); a glycine (G)-to-aspartic acid (D) change being the most common. Other mutation hotspots consisted of codon 36 (6.9%; 11/159), codon 43 (1.9%; 3/159), and an intronic site 8 bp upstream of exon 2 (6.3%; 10/159). To validate the predicted effect of the intronic variant on the transcript, three available leiomyomas underwent cDNA sequencing confirming that the variant added the last six bases of intron 1 to the transcript. Various insertion-deletion type alterations, ranging from 3 bp to 43 bp in length, were observable in 15.7% (25/159) of *MED12* mutation-positive leiomyomas, all of which were predicted to result in an in-frame transcript. Of note, all *MED12* mutations were heterozygous, each tumor displaying not more than one mutation. Furthermore, multiple tumors from the same patient tended to have distinct *MED12* mutation statuses. Whole gene sequencing of 20 *MED12* mutation-negative and 10 *MED12* mutation-positive uterine leiomyomas did not reveal additional mutations. Overall, a remarkable 60 out of 80 patients (75%) had one or more *MED12* mutation-positive leiomyomas.



**Figure 5. *MED12* exon 2 mutation spectrum in Study I.**

*MED12* locates on chromosome Xq13.1 and hence it is subject to random X-chromosome inactivation. Analysis of the possible mutant allele expression in the tumors included cDNA sequencing of 16 *MED12* mutation-positive leiomyomas: 14 tumors with codon 44 mutations, one with codon 43, and one with codon 36 mutations. In each case, the mutant allele was predominantly present in the cDNA sequence (Figure 6).



**Figure 6. cDNA sequencing result of a *MED12* mutation-positive leiomyoma showing predominance of the mutant allele.**

### 1.2 *MED12* mutation status correlates with tumor size (I)

The correlation between *MED12* exon 2 mutation status and available clinical data was examined. *MED12* mutation-negative tumors (n=56; median 4 cm) tended to be larger than *MED12* mutation-positive tumors (n=129; median 3 cm) ( $P=0.015$ ). No correlation existed between the mutation status and patient's age at hysterectomy ( $P=0.69$ ). *MED12* mutation-negative tumors (n=66) did not distribute randomly among patients, but rather clustered among them ( $P<10^{-6}$ ). Five out of 42 patients (12%) with multiple tumors in the study harbored only *MED12* mutation-negative tumors, whereas 16 patients (38%) displayed only *MED12* mutation-positive tumors. The remaining 21 patients (50%) had both *MED12* mutation-positive and -negative tumors, the majority representing mutation-positive lesions.

### 1.3 Gene expression profiling (I)

Unsupervised hierarchical clustering of eight *MED12* mutation-positive leiomyoma, two *MED12* mutation-negative leiomyoma, and their respective normal myometrial tissue samples indicated that *MED12* mutation-positive lesions constitute a separate cluster from the other samples. Four of the mutation-positive tumors harbored different codon 44 mutations and four tumors displayed the intron 1 mutation. Pathway enrichment analysis of the eight *MED12* mutation-positive leiomyomas and the matched normal myometrium revealed three significantly changed pathways: focal adhesion, extracellular matrix receptor interaction, and the Wnt signaling pathway.

### 1.4 *MED12* mutations in uterine leiomyomas from South African patients (II)

To validate the finding of *MED12* exon 2 mutations in other ethnic groups, the mutation frequency was examined in 28 uterine leiomyomas from 18 South African patients representing both Black African and Coloured. Half of the tumors displayed *MED12* exon 2 mutations (50%; 14/28), the majority affecting codon 44 (57%; 8/14) (Table 8). Somatic status of the mutations was confirmed in cases in which normal myometrium was available (71%; 10/14).

**Table 8. Identified *MED12* exon 2 mutations in uterine leiomyomas from South African women.** The nucleotide numbering and amino acid locations follow the transcript sequence: ENST00000374080.

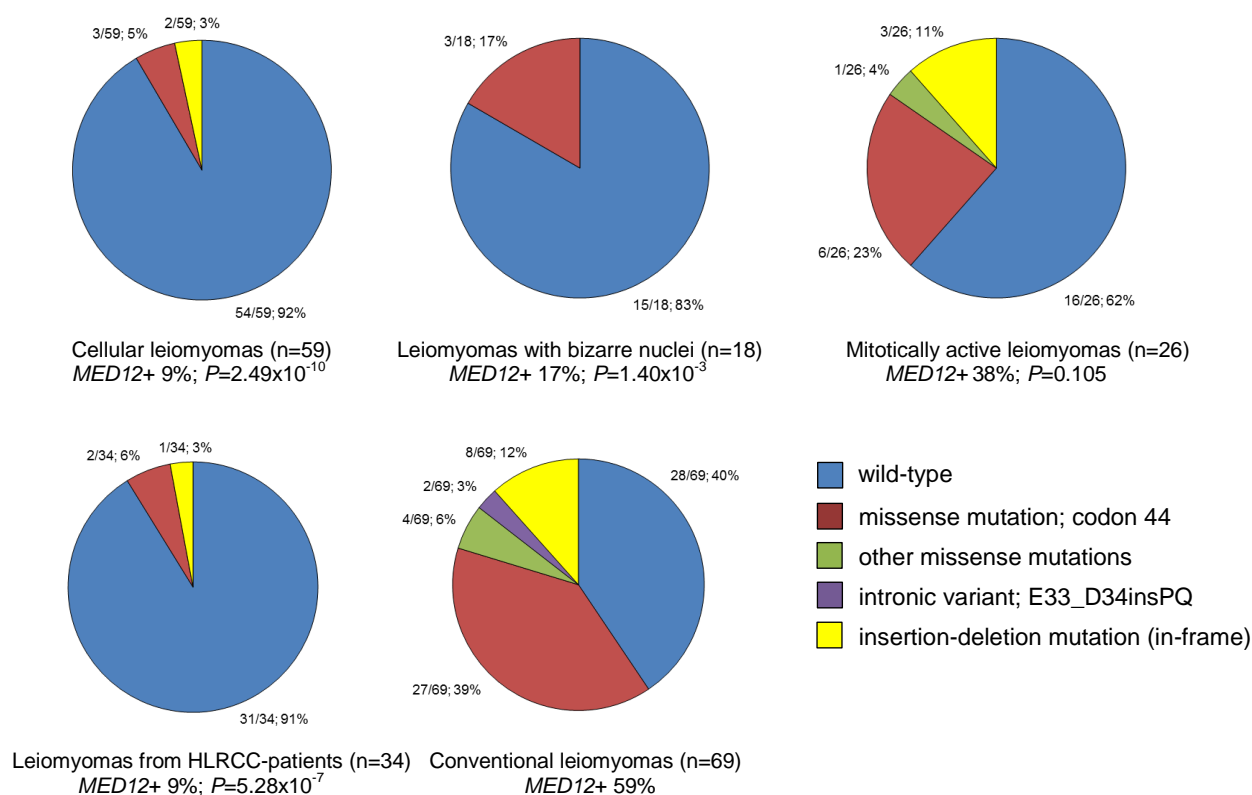
Mutation type	Position	Nucleotide change	Amino acid change	No. of mutations out of 28 (%)
<b>Missense</b>	X:70339230	c.107T>G	p.L36R	2 (7.1)
	X:70339251	c.128A>C	p.Q43P	1 (3.6)
	X:70339253	c.130G>C	p.G44R	1 (3.6)
	X:70339253	c.130G>A	p.G44S	1 (3.6)
	X:70339253	c.130G>T	p.G44C	1 (3.6)
	X:70339254	c.131G>C	p.G44A	1 (3.6)
	X:70339254	c.131G>A	p.G44D	4 (14.3)
<b>Splice site</b>	X:70339215	c.100-8T>A	p.E33_D34insPQ	1 (3.6)
<b>Deletion</b>	X:70339245	c.122_148del27	p.V41_P49	1 (3.6)
	X:70339272	c.149_163del15	p.A50_D54del	1 (3.6)

The difference between the frequencies of *MED12* mutation-positive leiomyomas in Finnish and South African women had borderline significance ( $P=0.045$ ). Because women with African ancestry tend to have larger tumors than Caucasian women, the difference was re-calculated taking into account only uterine leiomyomas with a diameter  $\geq 5.5$  cm (Finnish  $n=38$ ; South African  $n=7$ ). This time, the difference was no longer significant ( $P=0.69$ ). Of note, 45% (17/38) of the larger leiomyomas in Finnish and 57% (4/7) in South African women harbored *MED12* exon 2 mutations. On the other hand, the difference between the frequencies of *MED12* mutation-positive leiomyomas in these ethnic groups was statistically significant ( $P=0.011$ ) when tumors with a diameter  $< 5.5$  cm (Finnish  $n=147$ ; South African  $n=7$ ) were considered. Altogether 77% (113/147) and 29% (2/7) of the smaller leiomyomas in

Finnish and South African women were *MED12* mutation-positive. No statistically significant correlation occurred, however, between the *MED12* mutation status and tumor size (*MED12*+ n=6, median 9 cm; *MED12*- n=8; median 5 cm) in South African patients ( $P=0.40$ ).

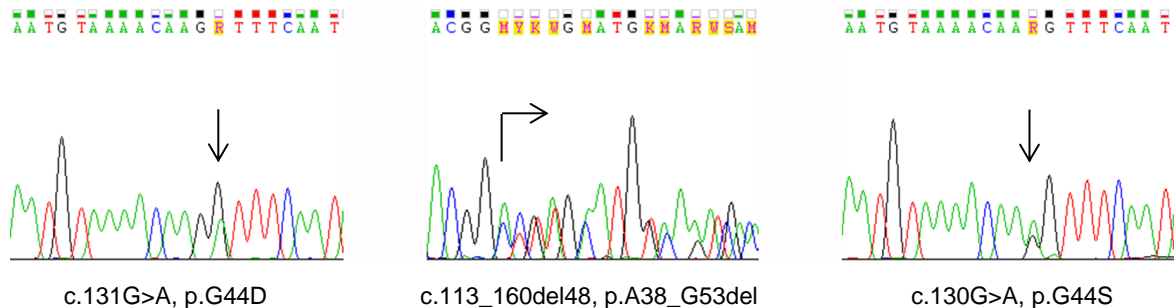
## 2. *MED12* mutations in clinical uterine leiomyoma subtypes (III)

*MED12* exon 2 mutations were originally identified in a series of histopathologically conventional uterine leiomyomas. To examine the frequency of the mutations in rarer clinical uterine leiomyoma subtypes, 103 histopathological uterine leiomyoma variants, 34 leiomyomas from HLRCC-patients, and a control set of 69 conventional leiomyomas went through Sanger sequencing. Both the histopathological leiomyoma variants (17%; 18/103;  $P=2.93 \times 10^{-8}$ ) and leiomyomas from HLRCC-patients (9%; 3/34;  $P=5.28 \times 10^{-7}$ ) harbored significantly fewer *MED12* mutations than the conventional leiomyomas (59%; 41/69) (Figure 7). Mitotically active leiomyomas were the only variants with no significant difference in the *MED12* mutation frequency from conventional leiomyomas (38%; 10/26;  $P=0.11$ ). Highly cellular leiomyomas, on the other hand, displayed the lowest frequency of *MED12* mutations (5%; 2/42;  $P=1.51 \times 10^{-9}$ ). The majority of the observed *MED12* exon 2 mutations located in codon 44 regardless of the clinical leiomyoma subtype in question (Figure 7). Novel mutation findings included all the observed 14 insertion-deletion mutations and one missense mutation; c.122T>A, p.V41E.



**Figure 7. *MED12* exon 2 mutation spectrum in clinical uterine leiomyoma variants in Study III.** Eight mitotically active leiomyomas with elevated levels of cellularity were count in both mitotically active and cellular leiomyomas. In terms of mutation spectrum the eight tumors were only part of the mitotically active leiomyomas.

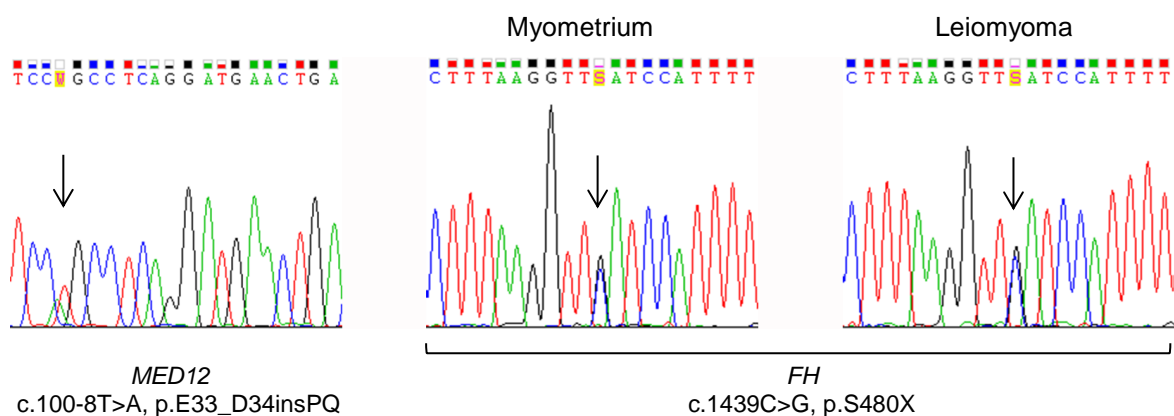
Most leiomyomas from HLRCC-patients (62%; 21/34) showed clear biallelic *FH* inactivation, as a marker of *FH* driven tumorigenesis, when assessed for LOH. Of note, none of the three *MED12* mutation-positive leiomyomas in the series (Figure 8) exhibited LOH at the site of the *FH* mutation.



**Figure 8.** Sequence chromatograms of three *MED12* exon 2 mutation-positive uterine leiomyomas from HLRCC-patients.

### 3. Exomic landscape of uterine leiomyomas (IV)

Exome sequencing data of 15 *MED12* mutation-positive and 12 *MED12* mutation-negative leiomyomas were thoroughly examined to scrutinize somatic variation in uterine leiomyomas; the focus was on genes harboring mutations in at least two tumors. No additional contributing mutations in *MED12* mutation-positive leiomyomas or novel driver mutations in *MED12* mutation-negative leiomyomas were observable. Altogether two unique variants, one in *MED12* mutation-positive and one in *MED12* mutation-negative leiomyoma, were verified: *NPR2* c.1329G>T, p.L443F and *GRIN2B* c.1658C>G, p.P553R (Table 9). One *MED12* mutation-positive leiomyoma (MY31m4) unexpectedly displayed a heterozygous germline *FH* mutation, c.1439C>G, p.S480X (Figure 9). Assessment of LOH and other somatic point mutations did not show biallelic *FH* inactivation.



**Figure 9.** One patient with a *MED12* mutation-positive leiomyoma (MY31m4) and a germline *FH* mutation, but no LOH present at the *FH* mutation site in the tumor.

**Table 9. Candidate exome sequencing variants in *MED12* mutation-positive and *MED12* mutation-negative uterine leiomyomas in Study IV.**

Gene	Variant	<i>In silico</i> prediction	Validation status
<b><i>MED12</i> mutation-positive uterine leiomyomas</b>			
<b>Common variation</b>			
<i>TCHHL1</i>	c.1114insC, p.L372fs		False
<i>WDR6</i>	c.626_627delCT, p.A209fs		False
<b>Unique variation</b>			
<i>SCNN1D</i>	c.1934C>A, p.S645X		False
	c.2024T>A, p.L675H	deleterious	False
<i>PBXIP1</i>	c.542A>T, p.Q181L	neutral	False
	c.1294C>A, p.L432M	deleterious	False
<i>CA9</i>	c.527C>A, p.A176D	neutral	False
	c.636G>T, p.M212I	neutral	False
<i>NPR2</i>	c.1329C>G, p.L443F	neutral	False
	<b>c.1578_1588del11, p.S526fs</b>		<b>True</b>
<b><i>MED12</i> mutation-negative uterine leiomyomas</b>			
<b>Unique variation</b>			
<i>TTLL4</i>	c.1372C>A, p.Q458K	deleterious	False
	c.2048G>T, p.S683I	neutral	False
<i>MUC4</i>	c.4145_4192del48, p.L1382fs		ND
	c.11921_11969del48, p.R3974fs		ND
	c.12496_12542del48, p.A4166fs		ND
<i>PRRC2B</i>	c.2905G>T, p.E969X		False
	c.5755C>A, p.P1919T	deleterious	False
<i>C1RL</i>	c.451C>A, p.H151N	ND	False
	c.1141G>A, p.E381K	ND	False
<i>GRIN2B</i>	<b>c.1658C&gt;G, p.P553R</b>	<b>deleterious</b>	<b>True</b>
	c.3448G>T, p.D1150Y	deleterious	False

ND; not determined

## DISCUSSION

Uterine leiomyomas are one of the most frequent clinically relevant human tumors. That said, the molecular genetic background of their tumorigenesis has remained obscure. For several decades, classical cytogenetic karyotyping was the primary method to search for tumor-specific chromosomal aberrations in leiomyomas. Although various different cytogenetic changes emerged, including rearrangements of 12q14-15 and deletions on chromosome 7q22, the majority of the tumors remained karyotypically normal (Sandberg, 2005). Subsequent molecular genetic analyses have concentrated on identifying novel and previously undetectable submicroscopical chromosomal events, such as LOH and copy-number alterations, but suggest that they are relatively rare events in uterine leiomyomas. Furthermore, a large number of studies on differential gene expression between uterine leiomyomas and normal myometrium have so far led to inconsistent results. Over the past decade, NGS technologies have revolutionized the field of tumor genomics by enabling comprehensive genome-wide characterization of somatic alterations in a large number of tumor specimens. For us, the emergence of these technologies provided an opportunity to explicitly detect small tumor-specific alterations (point mutations or insertion-deletions) not previously studied in the genomes of uterine leiomyomas, potentially leading to the discovery of new candidate driver genes and improving our knowledge of the molecular genetic characteristics of these tumors.

### 1. *MED12* in tumorigenesis

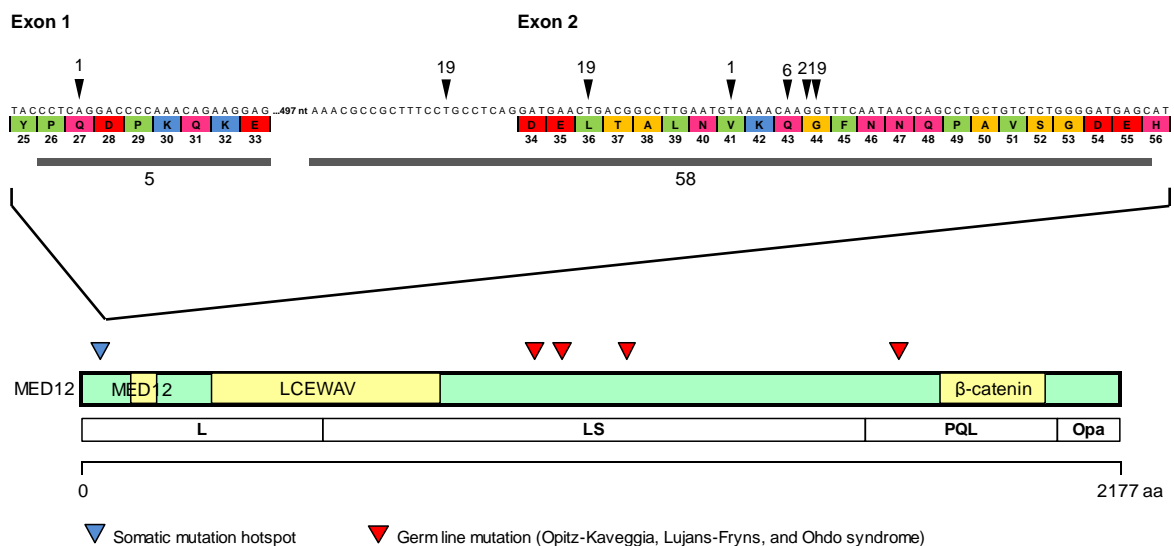
#### 1.1 *MED12* is the only frequently mutated gene in uterine leiomyomas

Identification of recurrent somatic *MED12* mutations by exome sequencing of 18 uterine leiomyomas from unselected Finnish (Caucasian) patients and further validation in a series of 207 additional leiomyomas revealed that a striking 70% of the tumors display *MED12* mutations. *MED12* is a component of the Mediator complex, a large multiprotein assembly involved in the gene transcription by RNA polymerase II (Pol II) (Taatjes, 2010). All the observed mutations were located in either exon 2 or near the intron 1-exon 2 junction, with the majority affecting one single codon: amino acid 44. The mutation spectrum consisted of missense, in-frame insertion-deletion, and intronic mutations, none of which truncated the protein product (Figure 10). In each case, no more than one mutation was present in the tumor. All the mutations were heterozygous, which is consistent with the fact that *MED12* is an X-chromosomal gene subject to lyonization. Since only one *MED12* allele is expressed in each tumor cell due to random X-chromosome inactivation, the expression of *MED12* mutant alleles was confirmed by cDNA sequencing in a set of 16 mutation-positive leiomyomas. The results indicated that all of the *MED12* transcripts derived predominantly from the mutant alleles. A minor normal allele peak was apparent in each cDNA sequence chromatogram suggesting potential normal tissue contamination. Alternatively, cellular



heterogeneity in the tumors may have led to the same outcome. A mostly monoclonal nature and a high tumor percentage of uterine leiomyoma samples, however, argue against the latter. Whole gene sequencing of 30 leiomyoma samples, both *MED12* mutation-positive and -negative, did not reveal additional mutations in any other exon of the gene emphasizing the specificity of *MED12* mutations in these tumors.

Overall, the molecular genetic evidence strongly suggests that *MED12* mutations contribute to the tumorigenesis of uterine leiomyomas. Furthermore, all the mutations affect an evolutionary conserved region of the protein; glycine at codon 44 represents the most conserved amino acid among the observed mutation hotspots. This region and especially codon 44 seem to be crucial for the normal *MED12* function in uterine myometrial cells, aberrant function potentially leading to the development and progression of a leiomyoma. Currently, one of the major challenges concerning next-generation sequencing data interpretation has been the classification of somatic mutations into drivers and passengers. In this case, the high frequency of *MED12* mutations in uterine leiomyomas and clustering of the mutations into a specific, evolutionarily conserved region of the protein indicate that the mutations are likely to confer a selective advantage to the cell, consequently establishing *MED12* as a candidate driver gene. Moreover, the mutation pattern in *MED12* suggests that the gene functions as an oncogene (Vogelstein *et al.*, 2013). As with oncogenes, the majority of *MED12* mutations is located recurrently at the same amino acid positions and represents missense mutations. This is the first time *MED12* has ever been associated with human tumor development.



**Figure 10. Schematic view of *MED12* and the distribution of *MED12* hotspot mutations.** (Top) The number of all the observed *MED12* exon 1 and 2 mutations in uterine leiomyomas, including the clinical leiomyoma subtypes, from the following studies: Mäkinen *et al.*, 2011a, Mäkinen *et al.*, 2013, Heinonen *et al.*, 2014, and Kämpjärvi *et al.*, 2014. Missense and splice-site mutations are above and insertion-deletion mutations below the amino acid sequence. Grey underlines represent the regions where the insertion-deletion mutations are distributed. (Bottom) Overview of *MED12* with high-confidence Pfam protein domains in yellow, underneath which the protein is divided into domains based on structural and sequence identity (L, leucine-rich; LS, leucine-serine-rich; PQL, proline-glutamine-leucine-rich). The leiomyoma-linked mutation hotspot and hereditary syndrome-linked germline mutations are marked above the protein. aa; amino acid

Uterine leiomyomas do not have an equal impact in all ethnicities. Ethnic differences in the incidence and severity of uterine leiomyomas between Caucasian women and women of African ancestry are particularly evident. Women of African descent are three times more likely to develop leiomyomas, have an earlier age at diagnosis and hysterectomy, as well as harbor larger, more numerous, and more symptomatic leiomyomas than Caucasians (Kjerulff *et al.*, 1996; Marshall *et al.*, 1997; Baird *et al.*, 2003; Huyck *et al.*, 2008). The reasons behind ethnic disparity have so far remained unclear. To validate our finding and to verify the role of *MED12* mutations in tumorigenesis of uterine leiomyomas in an ethnicity other than Caucasian, a series of 28 leiomyomas from 18 South African women underwent Sanger sequencing for *MED12* exon 2 mutations. All in all, 50% of the tumors harbored somatic *MED12* mutations, codon 44 persisting as the most frequent mutation hotspot. In comparison to the Finnish patient series, South African women had a tendency to carry fewer *MED12* mutations. It is noteworthy that *MED12* mutation positivity correlated with a smaller tumor size in uterine leiomyomas of Finnish origin. At the same time, uterine leiomyomas of South African origin tended to be larger in size than their Finnish counterparts. When the frequency of *MED12* mutation-positive leiomyomas was examined both in tumors with a diameter  $\geq 5.5$  cm and  $< 5.5$  cm in South African women, larger tumors appeared to harbor more *MED12* mutations than the smaller ones, as opposed to the Finnish sample series.

The uterine leiomyoma sample series from South African patients was relatively small (n=28) compared to the Finnish sample series, simultaneously representing two distinct subpopulations: Black African and Coloured. The Black Africans account for the majority of South Africa's total population, while the Coloured form a minority group with diverse ethnic backgrounds, such as Black, White, Indian, and Chinese. The small number of the tumors makes it difficult to draw definitive conclusions, especially regarding the correlation between *MED12* mutation status and tumor size, and hence more samples are needed for further analyses. Despite the scarcity of uterine leiomyomas, our results indicate that women with African ancestry also harbor recurrent *MED12* mutations in their leiomyomas confirming the role of *MED12* mutations in tumorigenesis of these lesions regardless of ethnicity. Furthermore, *MED12* does not seem to explain the ethnic differences between Caucasian women and women with African ancestry.

Since the identification of highly frequent *MED12* exon 2 mutations in uterine leiomyomas of both Finnish and South African origin, several studies have validated and replicated the finding in various ethnic groups around the world (Table 10). In each case, the frequent occurrence of *MED12* mutations in uterine leiomyomas has successfully been confirmed, with the reported mutation frequencies varying generally between 50% and 80%. Of note, the number of patients and leiomyomas has substantially differed between the studies, which may partially explain the variation in the frequencies. Also the differences in tumor characteristics, such as size and multiplicity, may have had an impact. For example, our recent study indicated that by collecting all feasibly obtainable tumors, even the smallest ones, from unselected uterine leiomyoma patients at hysterectomy the *MED12* mutation frequency could increase even further (Heinonen *et al.*, 2014). Thus, the inclusion of only large tumors in these studies may not represent leiomyomas in general. The correlation

between *MED12* mutation positivity and a smaller tumor size has been confirmed by some (Markowski *et al.*, 2012; de Graaff *et al.*, 2013; Heinonen *et al.*, 2014), but not all, subsequent studies (Je *et al.*, 2012; Bertsch *et al.*, 2014). Despite a few outliers, *MED12* mutations seem to distribute evenly among the different ethnicities (Table 10). Moreover, the majority of uterine leiomyomas in each ethnic group harbor *MED12* mutations.

Both the *MED12* mutation pattern and spectrum have proved to be similar in uterine leiomyomas across all the studies: the clear majority of *MED12* mutations consist of missense mutations at codon 44, especially the amino acid change G44D. So far, all the observed exon 2 mutations have been heterozygous and no more than one mutation has occurred per tumor with the exception of one case: a leiomyoma expressing simultaneously two codon 44 mutations (c.130G>A, p.G44S and c.131G>A, p.G44D) (Markowski *et al.*, 2012).

**Table 10. Summary of all the studies presenting *MED12* exon 2 mutation frequencies for conventional uterine leiomyomas.** The studies are presented in chronological order.

<b>Study</b>	<b>Ethnicity/nationality (no. of patients)</b>	<b><i>MED12</i> exon 2 mutation frequency</b>
Mäkinen <i>et al.</i> , 2011a	Caucasian/Finnish (80)	159/225 (71%)
Mäkinen <i>et al.</i> , 2011b	African/South African (18)	14/28 (50%)
Markowski <i>et al.</i> , 2012	Caucasian/German (50)	47/80 (59%)
McGuire <i>et al.</i> , 2012	Mixed/American (148)	100/148 (68%)
	White American (120)	79/120 (66%)
	Black American (23)	18/23 (78%)
Je <i>et al.</i> , 2012	Asian/Korean (67)	35/67 (52%)
Perot <i>et al.</i> , 2012	Caucasian/French (9)	6/9 (67%)
Ravegnini <i>et al.</i> , 2013	Mixed/American (-)	9/13 (69%)
Markowski <i>et al.</i> , 2013	Caucasian/German (-)	10/21 (48%)
Matsubara <i>et al.</i> , 2013	Asian/Japanese (-)	36/45 (80%)
Mäkinen <i>et al.</i> , 2013	Caucasian/Finnish (-)	41/69 (59%)
de Graaff <i>et al.</i> , 2013	Caucasian/Dutch (16)	11/19 (58%)
Rieker <i>et al.</i> , 2013	Caucasian/German (2)	11/12 (92%)
Schwetye <i>et al.</i> , 2014	Mixed/American (28)	15/28 (54%)
Bertsch <i>et al.</i> , 2014	Mixed/American (133)	133/178 (75%)
	White American (52)	53/74 (72%)
	Black American (64)	64/81 (79%)
	Hispanic (13)	13/16 (81%)
	Asian (4)	4/6 (67%)
Heinonen <i>et al.</i> , 2014	Caucasian/Finnish (28)	138/164 (84%)
Halder <i>et al.</i> , 2014	Mixed/American (135)	92/143 (64%)
	Black American (68)	45/68 (66%)
	White American (43)	26/43 (60%)
	Hispanic (24)	13/24 (54%)

- ; not clear from the study

The original identification of *MED12* exon 2 mutations took place in a series of histopathologically conventional uterine leiomyomas, which in general account for approximately 90% of all leiomyomas (Oliva *et al.*, 2014). In order to evaluate the pertinence of *MED12* mutations for possible molecular classification of uterine leiomyomas, an exceptionally large tumor collection (n=137) of various relatively rare clinical uterine leiomyoma subtypes was screened for the mutations. These subtypes included three different histopathological uterine leiomyoma variants (cellular leiomyomas, mitotically active leiomyomas, and leiomyomas with bizarre nuclei; n=103) as well as uterine leiomyomas from HLRCC-patients (n=34). The latter can exhibit the same histopathological characteristics as the variants, such as increased cellularity and atypia, but other distinct features may also exist, including, for example, orangeophilic nucleoli surrounded by a perinuclear halo (Lehtonen, 2011; Sanz-Ortega *et al.*, 2013). All the clinical uterine leiomyoma subtypes, except mitotically active leiomyomas, harbored significantly fewer *MED12* mutations than conventional leiomyomas. The majority of the observed *MED12* mutations represented missense changes at codon 44 (67%; 14/21). Interestingly, while most of the studied uterine leiomyomas from HLRCC-patients showed biallelic *FH* inactivation, a well-known attribute of tumors in HLRCC, none of the *MED12* mutation-positive tumors displayed loss of the remaining *FH* allele.

Our results suggest that *MED12* mutation positivity is a key feature of conventional leiomyomas. Cellular leiomyomas and leiomyomas with bizarre nuclei in particular may develop primarily through distinct molecular mechanisms. Similar kinds of results regarding the rare occurrence of *MED12* mutations in these two histopathological leiomyoma variants have emerged, with the mutation frequencies ranging between 0-33% (Perot *et al.*, 2012; Matsubara *et al.*, 2013; Ravegnini *et al.*, 2013). The number of examined tumors in these studies, however, has been extremely small (from 1 to 6). The *MED12* mutation frequency in mitotically active leiomyomas did not significantly differ from that of conventional leiomyomas. This result agrees with the fact that mitotically active leiomyomas actually resemble conventional leiomyomas histopathologically, with the exception of increased mitotic activity. The elevated number of mitoses may result from hormonal factors, because these tumors frequently occur in pregnancy, during the secretory phase of the menstrual cycle, and in patients on hormonal therapy (Prayson and Hart, 1992). For example, patients using progestin preparations have presented significantly higher mitotic activity in their leiomyomas compared to control patients (Tiltman, 1985). None of the uterine leiomyomas from HLRCC-patients simultaneously harbored a *MED12* mutation and biallelic *FH* inactivation, proposing two different pathogenic pathways. *MED12* mutation-positive leiomyomas may represent incidental tumors not associated with germline *FH* mutation.

Recently, somatic *MED12* exon 1 mutations were identified at a low frequency (2.5%) in uterine leiomyomas (Kämpjärvi *et al.*, 2014). All of the observed mutations represented in-frame insertion-deletion mutations, which were located at the end of exon 1, affecting the same evolutionarily conserved region of the protein as the previously identified exon 2 mutations (Figure 10). Of note, the *MED12* exon 2 mutation-negative uterine leiomyomas analyzed in this study came from our three previously described studies: the original exome

sequencing study and two subsequent *MED12* mutation screening studies in respect to the leiomyomas of South African origin as well as clinical uterine leiomyoma subtypes (Mäkinen *et al.*, 2011a; Mäkinen *et al.*, 2011b; Mäkinen *et al.*, 2013). Four conventional uterine leiomyomas of Finnish origin and one of South African origin, but none of the clinical leiomyoma subtypes, harbored a *MED12* exon 1 mutation. The low occurrence of exon 1 mutations in uterine leiomyomas may explain why these mutations went initially unnoticed in the whole *MED12* gene sequencing of 20 *MED12* mutation-negative leiomyomas (Mäkinen *et al.*, 2011a). Interestingly, the study discovered two new cases concurrently displaying two different *MED12* mutations. Both cases harbored a deletion accompanied by a nearby point mutation in exon 1, suggesting that the mutations could have resulted from the same mutational event. All in all, the identification of *MED12* exon 1 mutations slightly increases the amount of *MED12* mutation-positive leiomyomas and emphasizes the significance of these hotspot mutations in the development of uterine leiomyomas.

To examine the somatic variation in uterine leiomyoma exomes in more detail, we analyzed exome sequencing data of 12 *MED12* mutation-negative and 15 *MED12* mutation-positive uterine leiomyomas. The aim was to search for novel driver mutations in the remaining minority of uterine leiomyomas lacking *MED12* mutations and additional contributing mutations in *MED12* mutation-positive leiomyomas, with the main focus on genes mutated in at least two different tumors. Of note, each *MED12* mutation-negative leiomyoma lacked both exon 1 and exon 2 mutations. No recurrently mutated genes were detected in either tumor group. The absence of other somatic recurrent point mutations or small insertion-deletions in *MED12* mutation-positive leiomyomas highlights the significance of these mutations for the genesis of uterine leiomyomas even further. In the case of *MED12* mutation-negative leiomyomas, the existence of rare driver mutations cannot be excluded due to the fairly small number of tumors. Moreover, several different driver genes may underlie this tumor group making them difficult to identify from a sample set of this size. Also other types of genomic changes, such as structural rearrangements and intronic variants, as well as epigenetic events undetectable by exome sequencing, could potentially contribute to the development of these lesions. For example, chromosomal rearrangements of 12q14-15 giving rise to *HMGA2* overexpression have been associated, in particular, with *MED12* mutation-negative leiomyomas (Markowski *et al.*, 2012; Mehine *et al.*, 2013).

Surprisingly, one sporadic *MED12* mutation-positive leiomyoma displayed a truncating heterozygous *FH* mutation, which turned out to be of germline origin. The patient harbored five additional tumors each displaying a different *MED12* mutation (unpublished data). None of the six tumors showed biallelic *FH* inactivation through LOH or somatic point mutations as a second hit in the tumor, favouring the notion that *MED12* mutations and biallelic *FH* inactivation are mutually exclusive. Additional support for the hypothesis of mutual exclusivity comes from a recent study reporting an HLRCC-patient with 15 uterine leiomyomas all lacking *MED12* mutations (Heinonen *et al.*, 2014).

## 1.2 Characteristics of MED12

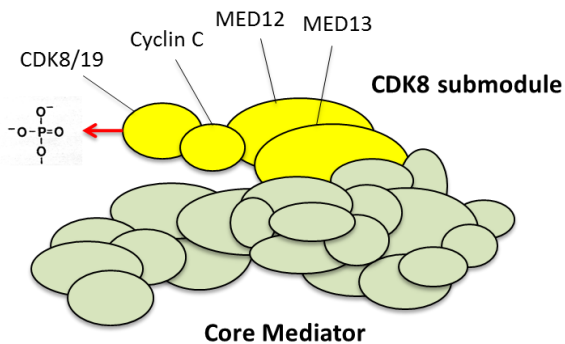
*MED12* consists of 45 exons, which span approximately 25 kb of genomic DNA on chromosome Xq13 (Philibert *et al.*, 1999; Risheg *et al.*, 2007). The gene encodes a 2177 amino acid protein divided into four distinct domains on the basis of structural and sequence identity: a leucine-rich (L), a leucine-serine-rich (LS), a proline-glutamine-leucine-rich (PQL), and an Opa domain (Figure 10). The amino acid sequence is mostly conserved across eukaryotic species, specifically in mammals (Kitano *et al.*, 2003). *MED12* is ubiquitously expressed in multiple human tissues, showing increased expression during fetal development (Philibert *et al.*, 1998; Philibert *et al.*, 1999).

*MED12* is a component of an evolutionarily conserved multiprotein complex called Mediator, which participates in the regulation of global gene transcription in eukaryotic cells (Borggreffe and Yue, 2011; Conaway and Conaway, 2011). The complex integrates and conveys regulatory information directly to Pol II by acting as a bridge between transcription factors and the enzyme, important for both initiation and elongation steps of the transcription cycle (Donner *et al.*, 2010; Taatjes, 2010; Takahashi *et al.*, 2011). Mediator consists of four structurally distinct modules: three core modules (head, middle, and tail) and a kinase module (CDK8 module), which variably associates with the core Mediator (Figure 11). As a result, the complex exists in the cell in two different configurations. The core Mediator alone stimulates basal transcription (Mittler *et al.*, 2001; Baek *et al.*, 2002), whereas binding of the CDK8 module to the core results in decreased gene transcription caused by a structural change within the core that prevents interactions with Pol II (Elmlund *et al.*, 2006; Knuesel *et al.*, 2009a; Tsai *et al.*, 2013). In addition to its role as a major regulator of global gene transcription, Mediator also participates in gene-specific transcription (Borggreffe and Yue, 2011). Various subunits of the complex serve as targets for different co-regulatory factors at different genes, leading to gene-specific physiological effects. Despite the constantly increasing amount of studies, the molecular mechanisms by which Mediator regulates gene expression are not yet fully understood.

The CDK8 kinase module, a four-subunit complex containing *MED12*, *MED13*, CDK8, and Cyclin C, can act as a co-repressor or co-activator of gene transcription (Borggreffe *et al.*, 2002; Samuelsen *et al.*, 2003). Besides binding to the Mediator and sterically blocking its interactions with Pol II, the CDK8 module can inhibit transcription by phosphorylating the C-terminal domain of the Pol II subunit before the formation of the preinitiation complex, hence disrupting Mediator-Pol II interaction (Hengartner *et al.*, 1998). Furthermore, the module seems to be necessary for transcriptional activation of various networks, including for example p53 (Donner *et al.*, 2007), serum response (Donner *et al.*, 2010), and HIF1A networks (Galbraith *et al.*, 2013). On the other hand, the CDK8 module may also function independently of Mediator (Knuesel *et al.*, 2009b). *MED12* plays a crucial part in the regulation of the CDK8 module since the protein is essential for CDK8 kinase activity (Knuesel *et al.*, 2009b). *MED12* interacts with several different transcription factors related to a variety of developmental pathways, such as the nuclear hormone receptor, Wnt, and

Sonic hedgehog signaling pathways (Belakavadi and Fondell, 2006; Kim *et al.*, 2006; Zhou *et al.*, 2006; Zhou *et al.*, 2012). MED12 binds, for example, directly to  $\beta$ -catenin, a key nuclear effector of the canonical Wnt pathway, and activates the transcription of target genes. MED12 can also modulate Sonic hedgehog signaling through a direct interaction with Gli3. Furthermore, MED12 contributes to epigenetic silencing of neuronal gene expression in non-neuronal cells by linking RE1-silencing transcription factor (REST) with G9a histone methyltransferase (Ding *et al.*, 2008). MED12 also interacts directly with several members of Sox family, such as Sox9 and Sox10, representing DNA-binding transcription factors important for controlling embryonic development (Zhou *et al.*, 2002; Rau *et al.*, 2006; Vogl *et al.*, 2013).

In vertebrates, three of the four CDK8 kinase module subunits, MED12, MED13, and CDK8, have undergone gene duplications to produce paralogs designated as MED12L, MED13L, and CDK19, respectively (Sato *et al.*, 2004; Bourbon, 2008). The role of these paralogs on the function of the CDK8 module or Mediator remains unclear, particularly in the case of MED12L and MED13L. A recent study indicated that all these paralogs are mutually exclusive of each other, albeit not exclusive of the other kinase module members, suggesting that CDK8 module may exist in several configurations in the cell (Daniels *et al.*, 2013). The increased amount of components in the module probably refers to the extended number of functions in vertebrates, which could possibly contribute to the gene-specific and context-dependent transcription regulation by Mediator.



**Figure 11. Schematic view of CDK8 module associated with Mediator complex.** Adapted from Fertility and Sterility, 102, Mehine *et al.*, Genomics of uterine leiomyomas: insights from high-throughput sequencing, p.623, Copyright Elsevier (2014), with permission from Elsevier.

### 1.3 *MED12* hotspot mutations in leiomyomagenesis

Although the cellular origin of uterine leiomyomas remains obscure, identification of *MED12* hotspot mutations has provided a few insights to the matter. Firstly, the occurrence of different *MED12* mutations in multiple leiomyomas from the same patient supports the notion that the majority of these tumors arise independently (Mäkinen *et al.*, 2011a; Markowski *et al.*, 2012; de Graaff *et al.*, 2013). A recent whole-genome sequencing study of uterine leiomyomas indicated that a small subset of physically distinct leiomyomas might, however, have a common clonal origin (Mehine *et al.*, 2013). The results are consistent with

some previously published karyotypical findings (Nibert and Heim, 1990; Nilbert *et al.*, 1990a). Of note, all of the clonally related tumors in the study were *MED12* mutation-negative, which could partially explain the observed clustering of tumors lacking *MED12* mutations in a subset of patients in our original exome sequencing study. Secondly, stem/progenitor cells from uterine leiomyoma tissue appear to carry *MED12* mutations, while no mutations are present in the adjacent myometrium or myometrial stem/progenitor cells (Ono *et al.*, 2012). These data suggest that a *MED12* mutation may lead to tumorigenic transformation in a cell already part of a leiomyoma cell lineage. On the other hand, another study did identify *MED12* mutations in the adjacent histologically unremarkable myometrium of a uterine leiomyoma, proposing that these mutations occur early in pathogenesis (Schwetye *et al.*, 2014). Further work is necessary to elucidate the role of *MED12* as a potential initiator of leiomyomagenesis.

Based on our pathway enrichment analysis of eight *MED12* mutation-positive uterine leiomyomas and their respective normal myometrium tissue samples, three significantly altered pathways emerged, including the Wnt signaling pathway. Since then, a few studies have examined the potential role of this pathway in the development of leiomyomas. *MED12* mutation-positive leiomyomas appear to show clear upregulation of *WNT4*, a member of the Wnt signaling pathway, which regulates the formation of the Müllerian ducts, the structures that give rise to the uterus, fallopian tubes, cervix, and the upper part of the vagina (Markowski *et al.*, 2012). Because estrogen stimulates *Wnt4* expression in several cell types (Hou *et al.*, 2004; Miyakoshi *et al.*, 2009), it was hypothesized that *MED12* and estrogen could cooperate in leiomyomagenesis. At the same time, immunohistochemical stainings of  $\beta$ -catenin in uterine leiomyomas have demonstrated that the Wnt signaling pathway is not constitutively active in tumors with *MED12* mutations (Perot *et al.*, 2012; de Graaff *et al.*, 2013). Nuclear staining of  $\beta$ -catenin, a strong indicator of the activated canonical Wnt pathway, was either not present at all or present in approximately half of the mutated tumors, depending on the study. Although these results suggest that *MED12* mutations probably do not generally affect tumorigenesis through the Wnt signaling pathway, activation of the pathway may occasionally play a part in a subset of *MED12* mutation-positive tumors. It is noteworthy that the original pathway enrichment analysis included only eight uterine leiomyoma-myometrium pairs, thus the results should be verified with a larger sample series in the future.

Recently, functional analyses have indicated that *MED12* exon 2 mutations uncouple Cyclin C-CDK8/19 from the core Mediator, resulting in diminished CDK8 kinase activity (Turunen *et al.*, 2014). At first, affinity-purification mass spectrometry revealed a specific decrease in the association of Cyclin C, CDK8, and CDK19 with mutant *MED12* (G44D) versus wild-type *MED12*. Immunoprecipitation-western blot confirmed the finding and extended the results to apply to other *MED12* exon 2 mutations, including L36R, Q43P, and G44S. Mapping of the Cyclin C-binding domain on *MED12* to the first 100 amino acids of the protein, a region largely encoded by exons 1 and 2, suggested that *MED12* mutations disrupt the binding interface between *MED12* and Cyclin C. Through this direct binding, *MED12* seems to activate the cyclin-dependent CDK8 kinase activity. Subsequently, uterine



leiomyoma-linked *MED12* exon 1 mutations have been shown to share a similar molecular basis for their tumorigenic role as exon 2 mutations (Kämpjärvi *et al.*, 2014). Overall, these findings have implicated aberrant CDK8/19 activity to the genesis of uterine leiomyomas and elucidated molecular interactions underlying Mediator kinase activity. Further analyses are necessary, however, to assess the next downstream targets of *MED12* in leiomyomagenesis.

Defects in other components of the CDK8 module, including CDK8/19, Cyclin C, *MED13*, and *MED13L*, have been implicated in various human tumor types. Especially, the dysregulation of *CDK8* and *CCNC* is frequently associated with numerous cancers (Schiano *et al.*, 2014). Mutation screening of *CDK8/19*, *CCNC*, and *MED13* in 70 *MED12* mutation-negative uterine leiomyomas did not identify somatic coding region mutations in these genes, suggesting that mutations in these components of CDK8 module do not principally contribute to the development of uterine leiomyomas (Mäkinen *et al.*, 2014). These results highlight the specificity of *MED12* hotspot mutations and their functional effects.

#### 1.4 *MED12* mutations in other human tumor types

##### 1.4.1 *MED12* hotspot mutations

Ever since the identification of *MED12* exon 2 mutations in uterine leiomyomas, numerous studies have examined the role of these mutations in other human tumor types, both benign and malignant. Uterine leiomyosarcomas, the malignant counterparts of uterine leiomyomas, for example, have been of particular interest. Uterine leiomyosarcomas represent extremely rare, clinically aggressive tumors with a poor 5-year survival and high recurrence rate (Major *et al.*, 1993; Mayerhofer *et al.*, 1999; Giuntoli *et al.*, 2003; Dinh *et al.*, 2004). These tumors are traditionally thought to arise *de novo*; however, according to the literature, some leiomyosarcomas may originate from a leiomyoma precursor (Lee *et al.*, 1994; Mittal and Joutovsky, 2007; Mittal *et al.*, 2009; Yanai *et al.*, 2010). The discovery of recurrent *MED12* mutations in uterine leiomyosarcomas indicated that occasionally leiomyosarcomas could arise from *MED12*-mutated leiomyomas (Je *et al.*, 2012; Kämpjärvi *et al.*, 2012; Markowski *et al.*, 2012; Perot *et al.*, 2012; de Graaff *et al.*, 2013; Matsubara *et al.*, 2013; Ravegnini *et al.*, 2013; Bertsch *et al.*, 2014). Furthermore, two *MED12* mutation-positive uterine leiomyosarcomas displayed identical *MED12* mutations with their associated benign components (Matsubara *et al.*, 2013). On the other hand, *MED12* mutations may give leiomyosarcomas a growth advantage. The overall mutation frequencies have proven to be, however, much lower than in uterine leiomyomas, only reaching up to 30%, suggesting that the majority of leiomyosarcomas and leiomyomas probably develop through different genetic pathways. Of note, smooth muscle tumors with uncertain malignant potential (STUMP), a rare heterogeneous group of tumors histopathologically impossible to diagnose as benign or malignant, also harbor *MED12* mutations at a low frequency (11%) (Perot *et al.*, 2012). All in all, leiomyoma-linked *MED12* exon 2 mutations are not restricted to benign tumors.

Rarely, *MED12* exon 2 mutations occur also in leiomyomas outside the uterus, but whether these tumors actually have a uterine origin is unclear (de Graaff *et al.*, 2013; Markowski *et al.*, 2013; Ravegnini *et al.*, 2013). Only one single extrauterine leiomyosarcoma has displayed a *MED12* mutation: a soft tissue mass in the thigh with femoral involvement (Schwetye *et al.*, 2014). In addition to smooth muscle tumors, *MED12* exon 2 mutations have been reported at very low frequencies in colon carcinomas (0.3-0.5%) (Je *et al.*, 2012; Kämpjärvi *et al.*, 2012) and in single cases of endometrial polyps (Markowski *et al.*, 2012), basal triple-negative breast carcinomas (Shah *et al.*, 2012), and chronic lymphatic leukemias (CLL) (Schuh *et al.*, 2012). To date, no *MED12* exon 1 mutations have been reported in other human tumors (Kämpjärvi *et al.*, 2014).

Until lately, leiomyoma-linked *MED12* hotspot mutations have appeared to be uterus-specific and rare in other human tumors. Recent identification of *MED12* exon 2 mutations in 59% of breast fibroadenomas with the observed mutation spectrum nearly identical to that of uterine leiomyomas strongly suggests that the tumors share a common genetic basis (Lim *et al.*, 2014). Breast fibroadenomas represent common benign tumors with a different anatomical location and tissue type compared to uterine leiomyomas. Both tumor types, however, are female-specific and hormone-dependent, localizing in key target tissues of estrogen (Zhu and Conney, 1998). This proposes that *MED12* exon 2 mutations may relate to hormonal expression. Depletion of *MED12* has been previously shown to impair ER $\alpha$ -regulated transcription (Prenzel *et al.*, 2012). In the future, it would be interesting to see if the loss of CDK8 kinase activity influences the transcription of genes involved in estrogen metabolism. Moreover, since the N-terminal *MED12* mutations are present only in a few tissue types, their tumorigenic effect likely has something to do with gene-specific, rather than global, transcription.

#### 1.4.2 Other *MED12* mutations

In addition to leiomyoma-linked *MED12* hotspot mutations, mutations in other parts of *MED12* have also been associated with human diseases. Exome sequencing analyses of two hormone-related cancers, prostate and adrenocortical carcinomas, have identified somatic *MED12* mutations at low frequency in the tumors (Barbieri *et al.*, 2012; Assie *et al.*, 2014). Despite the low frequency, *MED12* is at the moment one of the most recurrently mutated genes in prostate carcinomas. Nevertheless, the location and type of the mutations suggest that the molecular mechanisms underlying these tumors most likely differ from that of uterine leiomyomas and breast fibroadenomas. For example, single mutations observed in adrenocortical carcinomas affect the  $\beta$ -catenin binding site at the C-terminal region of *MED12*.

#### 1.5 *MED12* germline mutations

Various *MED12* germline mutations affecting the LS domain of the protein (Figure 10) are responsible for three different forms of X-linked mental retardation syndromes: Opitz-

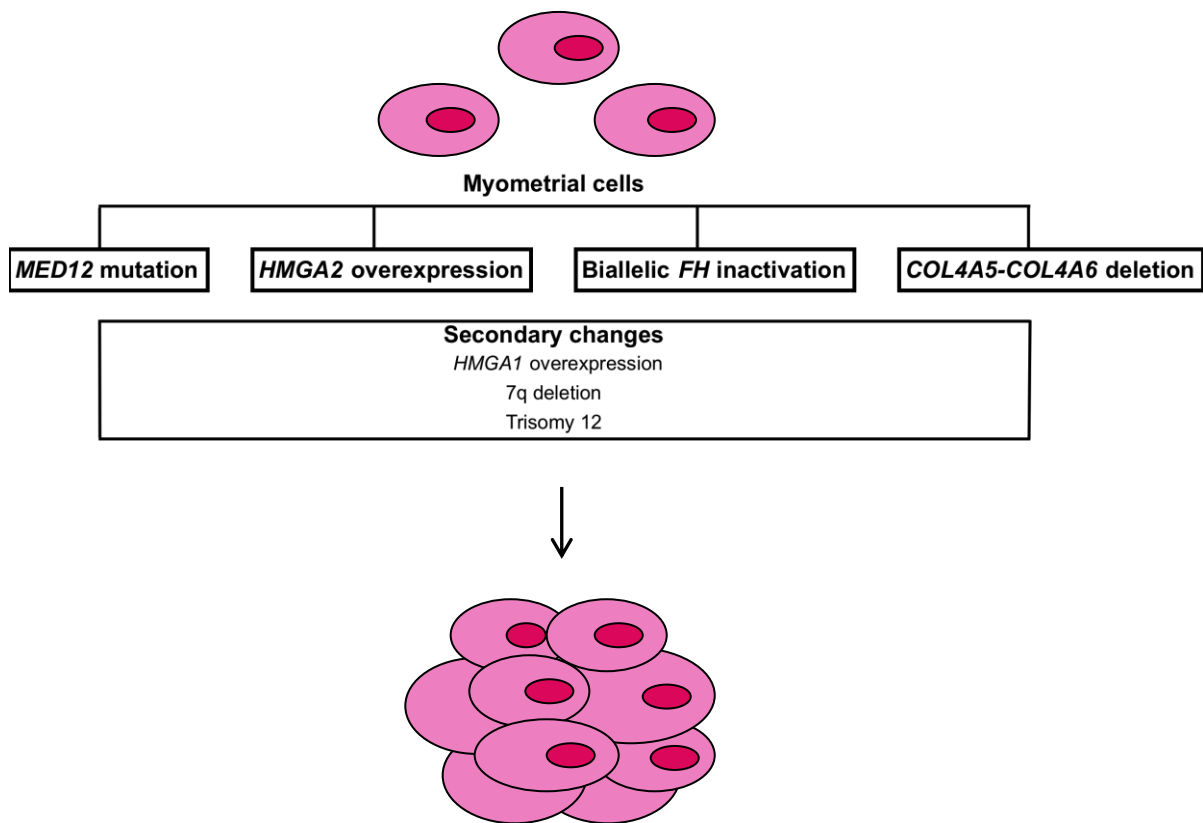
Kaveggia syndrome (MIM 305450) (Risheg *et al.*, 2007; Rump *et al.*, 2011), Lujan-Fryns syndrome (MIM 309520) (Schwartz *et al.*, 2007), and Ohdo syndrome (Maat-Klevit-Brunner type) (Vulto-van Silfhout *et al.*, 2013). None of these syndromes are associated with tumor predisposition. Once again, the molecular mechanisms underlying the syndromes are likely to be different. The current hypothesis suggests that *MED12* germline mutations may impair the recruitment of Mediator to RE1-silencing elements disrupting REST-imposed epigenetic restrictions on neuronal gene expression (Ding *et al.*, 2008; Vulto-van Silfhout *et al.*, 2013).

## 2. Molecular classification of uterine leiomyomas

Traditionally karyotypic chromosome abnormalities, such as deletions of 7q, trisomy 12, or rearrangements of 12q14-15 or 6p21, have enabled classification of uterine leiomyomas into subgroups. Since the identification of *MED12* exon 2 mutations, the coincidence of the mutations and chromosomal abnormalities has been under study (Markowski *et al.*, 2012; McGuire *et al.*, 2012; Bertsch *et al.*, 2014). The clear majority of *MED12* mutations are present in leiomyomas with an apparently normal karyotype, whereas rearrangements of 12q14-15 occur exclusively in leiomyomas lacking *MED12* mutations. These findings strongly suggest that *MED12* mutations and *HMGA2* overexpression due to chromosomal rearrangements are independent genetic events, which represent two alternative pathways in the tumorigenesis of uterine leiomyomas (Figure 12). Further evidence for mutual exclusivity comes from gene expression profiling analyses. While our preliminary gene expression analysis of ten uterine leiomyoma-myometrium pairs showed separate clustering of *MED12* mutation-positive leiomyomas from *MED12* mutation-negative tumors and the respective myometrial tissue samples, the small number of specimens caused speculation that the observed correlation could result from changes other than these mutations. Since then, a hierarchical clustering analysis of 38 uterine leiomyomas has confirmed the unique gene expression profile of *MED12* mutation-positive leiomyomas, which evidently differs from that of *HMGA2*-overexpressed tumors (Hodge *et al.*, 2012; Mehine *et al.*, 2013). Taken together, *MED12* and *HMGA2* pathways might account for as much as 80-90% of uterine leiomyomas. Of note, *RAD51B*, the candidate translocation partner for *HMGA2*, has appeared as the most significantly upregulated gene among *MED12* exon 1 and 2 mutation-positive leiomyomas, further suggesting a role for the gene in leiomyomagenesis (Mehine *et al.*, 2013; Kämpjärvi *et al.*, 2014).

In contrast to *HMGA2*, rearrangements of 6p21 affecting *HMGA1* can co-exist with *MED12* mutations in the same tumor, providing novel information on their possible role as secondary events in respect to *MED12* in uterine leiomyomas (Markowski *et al.*, 2012). At the same time, chromosome 7q deletions not only co-occur with *MED12* mutations, but also with *HMGA2* rearrangements, confirming earlier observations suggesting that these chromosomal changes represent secondary alterations in the development of leiomyomas (Mashal *et al.*, 1994; Xing *et al.*, 1997). Moreover, one leiomyoma with trisomy 12 has also been shown to display a *MED12* mutation (Markowski *et al.*, 2012). Recent whole-genome sequencing analysis of uterine leiomyomas discovered that a subset of tumors harbor interconnected

complex chromosomal rearrangements resembling chromotripsis; a phenomenon in which one or a few chromosomes shatter into pieces and randomly assemble back together in a single event (Stephens *et al.*, 2011; Mehine *et al.*, 2013). Chromotripsis has previously been associated with malignant neoplasms, poor prognosis, and *TP53* mutations (Forment *et al.*, 2012). Thus, this finding suggests that the phenomenon is not only limited to malignant tumors, but can also exist in benign lesions lacking *TP53* mutations. The majority of the leiomyomas harboring complex chromosomal rearrangements were *MED12* mutation-negative showing either *HMGAI* or *HMGAI2* overexpression. These data together with exome sequencing data indicate that *MED12* mutation-positive leiomyomas are stable at the nucleotide level and carry relatively few chromosomal aberrations. This suggests that the mutations alone may be vital for leiomyomagenesis. Curiously, a similar pattern has been seen previously in melanocytic nevi, which harbor specific missense mutations in *BRAF*; critical in the initiation of melanocytic neoplasia, but alone insufficient for malignant progression (Pollock *et al.*, 2003).



**Figure 12. Schematics of the various potential independent pathways in the tumorigenesis of uterine leiomyomas.** Secondary changes are likely to confer additional growth advantage to a neoplastic cell during tumor development.

*FH*-deficient uterine leiomyomas, in other words tumors showing biallelic *FH* inactivation, harbor a distinct gene expression profile from *MED12* mutation-positive and *HMGAI2* overexpressed tumors, providing further evidence for the mutual exclusivity of biallelic *FH* inactivation and *MED12* mutations (Vanharanta *et al.*, 2006; Mehine *et al.*, 2013).

Furthermore, another distinct gene expression pattern consisting of leiomyomas with *COL4A5-COL4A6* alterations has emerged (Mehine *et al.*, 2013). So far, these alterations have not co-existed with *MED12* mutations, *HMGA2* rearrangements, or biallelic *FH* inactivation, but data are insufficient to draw conclusions. Alterations in *COL4A5-COL4A6* have previously been observed in the germline of patients with Alport syndrome and diffuse leiomyomatosis (Garcia-Torres *et al.*, 2000; Thielen *et al.*, 2003). Both *FH*-deficient uterine leiomyomas and leiomyomas with *COL4A5-COL4A6* alterations may represent two rare independent subgroups of uterine leiomyomas, leaving only a small portion of the tumors genetically unexplained (Figure 12). Overall, these data are in line with previous notions that diverse mechanisms may contribute to the growth and development of uterine leiomyomas. Further work is necessary to unravel the precise molecular mechanisms underlying each tumor subgroup. In conclusion, NGS technologies have provided several new insights into the tumorigenesis of uterine leiomyomas and substantially advanced the molecular classification of the tumors.

### 3. Clinical relevance

Most studies on uterine leiomyomas rely on tissue samples removed at surgery from women with symptomatic lesions, who have not benefited from previous non-surgical treatments. The samples may also be biased towards larger tumor size, which can be easier to collect for research purposes. Thus, the possibility exists that the research samples do not completely represent leiomyomas in general. At the same time, they do reflect those leiomyomas which have proven to be clinically relevant.

In addition to tumor size, the correlation between *MED12* mutation status and several other clinical variables has been assessed. Patients with *MED12* mutation-positive tumors seem to have more lesions in their uteri than patients with tumors lacking *MED12* mutations (McGuire *et al.*, 2012; Heinonen *et al.*, 2014). The smaller size of *MED12* mutation-positive tumors may potentially result from the multiplicity of the tumors, or alternatively the tumors may possess a different growth pattern. Although leiomyomas lacking *MED12* mutations, such as those harboring *HMGA2* rearrangements, can on behalf of their larger size cause notable symptoms, the smaller *MED12* mutation-positive lesions are likely to result in a similar outcome due to their numbers. Higher body mass index is a clinical variable that may slightly increase the chances to develop *MED12* mutation-positive leiomyomas (Heinonen *et al.*, 2014). Obesity is a known risk factor for uterine leiomyomas, increasing the amount of biologically available estrogen in the tissue, which potentially can affect tumor development and growth. Other clinical factors related to hormone metabolism, such as parity and menarche, however, exhibited no correlation with *MED12* mutation status. Several studies have reported no correlation either between the mutation status and patient's age at hysterectomy (Je *et al.*, 2012; McGuire *et al.*, 2012; de Graaff *et al.*, 2013; Bertsch *et al.*, 2014; Heinonen *et al.*, 2014). In the future, a larger sample series with comprehensive clinical data is essential to have a better understanding of the correlations between distinct uterine leiomyoma subgroups and clinical factors.

Identification of extremely common somatic *MED12* hotspot mutations has improved our knowledge regarding the pathogenesis of uterine leiomyomas, but at the same time, provided an attractive as well as specific target for drug development. It is still, however, early days to design targeted therapies and further analyses are necessary to clarify the molecular biology underlying *MED12*-related leiomyomagenesis. Instead of *MED12* itself, the *MED12*/Cyclin C interface or other potential downstream components of the pathway could also serve as compelling drug targets. It is worth noticing that uterine leiomyomas do not represent a uniform, but rather heterogeneous, group of tumors not responding only to one kind of therapy. The majority of leiomyomas display *MED12* mutations or *HMGGA2* overexpression making these two independent tumorigenic pathways currently the most promising targets for therapy. An accurate molecular classification of the tumors is a requirement for improved management and personalized medical treatments in the future.

In addition to uterine leiomyomas, identification of *MED12* mutations has provided new perspectives on the biology of uterine leiomyosarcomas, representing one of the few recurrent genetic alterations observed in both benign and malignant counterparts. In most cases, the clinical behavior of uterine smooth muscle tumors can be reliably predicted based on their gross and microscopical appearances. Diagnostic challenges emerge still in daily pathological practice in distinguishing uterine leiomyosarcomas from benign leiomyoma variants, which mimic malignancy. Any new knowledge of the mechanisms underlying the potential progression to malignancy is essential, because the diagnosis of uterine leiomyosarcomas is often unexpected and postoperative. The low frequency of *MED12* mutations in both leiomyosarcomas and histopathological leiomyoma variants suggests that most of these tumors develop likely through distinct genetic mechanisms. In the future, it would be interesting to further study the possibility that some conventional leiomyomas or histopathological leiomyoma variants could act as precursors for leiomyosarcomas.

NGS technologies have considerably improved our understanding of the somatic genomic landscapes of different human tumor types over a very short period of time, providing information on, for example, novel mutational processes and new disease genes, as in uterine leiomyomas. At the same time, high-throughput sequencing of tumors has led to unexpected germline findings, unrelated to the research in question. One of the continuing bioethical debates in genomics research is how much information on these incidental findings should be passed to the patient. Occasionally, these unexpected findings may offer the patient clinically relevant health-related information. In those cases, if the patient has wished to receive the research results based on signed informed consent, he can be informed and guided to appropriate counseling and formal genetic testing. It is important, however, that the patient has been appropriately instructed prior to the next-generation sequencing effort that unexpected genetic information, which has nothing to do with the ongoing research, can be identified. It is also good to bear in mind that patients represent the general population, varying in education and genetic expertise.

## CONCLUDING REMARKS AND FUTURE PROSPECTS

This thesis work describes a prominent new finding in the genetics of uterine leiomyomas: the identification of recurrent somatic *MED12* exon 2 mutations in up to 70% of the tumors. Concurrently, the thesis work implicates *MED12* in human tumorigenesis for the first time. The finding has since been validated in a variety of ethnic groups worldwide indicating a major role for these mutations in leiomyomagenesis. All in all, the high *MED12* mutation frequency, clustering of the mutations into a definite evolutionarily conserved region of the protein, and mutation pattern establish *MED12* as a novel candidate driver gene, which is likely to function as an oncogene.

*MED12* is part of a multiprotein Mediator complex, which is necessary for regulating the transcription of Pol II-dependent genes. Functional studies of *MED12* mutations have so far associated aberrant CDK8/19 kinase activity with the genesis of uterine leiomyomas. The disruption of the *MED12*-Cyclin C binding interface, required for the activation of Cyclin C-dependent CDK8/19 kinase activity, detaches the Cyclin C-CDK8/19 from the core Mediator. Further analyses are essential to assess whether uncoupling of Cyclin C-CDK8/19, for example, enables these proteins to carry out their possible independent functions or activates/represses the function of the core Mediator. Pathway enrichment analysis with a large sample series could potentially produce a more accurate view of signaling and metabolic pathways, or molecular networks involved in leiomyomagenesis and help to predict their downstream effects. Interestingly, the existence of *MED12* exon 2 mutations in the majority of breast fibroadenomas suggests that the mutations may play a part in hormonal expression. One hypothesis could be that the loss of CDK8/19 kinase activity affects the transcription of genes involved in estrogen metabolism. Other benign hormone-dependent tumors in females naturally represent prospective research subjects to further examine the plausible link between *MED12* mutations and hormones. It is worth noting that the discovery of *MED12* exon 2 mutations has not only provided new insights into leiomyomagenesis, but also elucidated molecular interactions important for Mediator kinase activity. Leiomyoma-linked mutations have also given new tools for researchers to study the function of *MED12* further. For example, future functional analyses on the N-terminal region of the protein could clarify molecular mechanisms underlying CDK8-driven cancers.

During the last few years the use of NGS technologies has substantially improved our knowledge of the molecular genetic background of uterine leiomyomas. NGS has led to several major findings in these tumors, such as the identification of novel driver genes and complex chromosomal rearrangements resembling chromotripsis. These discoveries together with previous cytogenetic data have considerably advanced the molecular classification of leiomyomas. Although most driver genes in the tumors may have been recognized, other less frequent genomic changes affecting tumor initiation and progression are likely to exist. In forthcoming studies, it would be important to examine if certain clinical variables are associated with certain molecular genetic characteristics in leiomyomas. Consequently, the

stratification of uterine leiomyomas into clinically relevant subgroups enables improved management and can lead to more personalized medical treatments.

In contrast to conventional leiomyomas, histopathological leiomyoma variants, uterine leiomyomas from HLRCC-patients, and malignant uterine leiomyosarcomas harbor significantly fewer *MED12* mutations, suggesting that they may develop primarily through distinct molecular mechanisms. In the case of tumors from HLRCC-patients, biallelic *FH* inactivation, present in the majority of tumors, seems to be mutually exclusive with *MED12* mutations, explaining the result. Further genetic analyses on histopathological leiomyoma variants and uterine leiomyosarcomas, however, could possibly reveal additional factors involved in the tumorigenesis of these lesions. In the future, it would also be interesting to study the possible malignant transformation of conventional leiomyomas or histopathological leiomyoma variants. New knowledge of the mechanisms underlying the potential progression into malignancy would provide valuable data to the current diagnosis of uterine leiomyosarcomas, which is often unexpected and postoperative.



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## Websites and public databases:

1000Genomes	<a href="http://www.1000genomes.org/">http://www.1000genomes.org/</a>
ASSP	<a href="http://www.es.embnet.org/mwang/assp.html">http://www.es.embnet.org/mwang/assp.html</a>
dbSNP	<a href="http://www.ncbi.nlm.nih.gov/SNP/">http://www.ncbi.nlm.nih.gov/SNP/</a>
Ensembl	<a href="http://www.ensembl.org">http://www.ensembl.org</a>
FastQC	<a href="http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc">http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc</a>
Multiz alignments	<a href="http://genome.ucsc.edu/">http://genome.ucsc.edu/</a>
NCBI	<a href="http://www.ncbi.nlm.nih.gov/genome/guide/human/">http://www.ncbi.nlm.nih.gov/genome/guide/human/</a>
NetGene2	<a href="http://www.cbs.dtu.dk/services/NetGene2/">http://www.cbs.dtu.dk/services/NetGene2/</a>
OMIM	<a href="http://www.omim.org">http://www.omim.org</a>
Picard Tools	<a href="http://picard.sourceforge.net">http://picard.sourceforge.net</a>
Polyphen2	<a href="http://genetics.bwh.harvard.edu/pph2/">http://genetics.bwh.harvard.edu/pph2/</a>
Primer3	<a href="http://frodo.wi.mit.edu/primer3/">http://frodo.wi.mit.edu/primer3/</a>
R	<a href="http://www.r-project.org/">http://www.r-project.org/</a>
SIFT	<a href="http://sift.jcvi.org/">http://sift.jcvi.org/</a>