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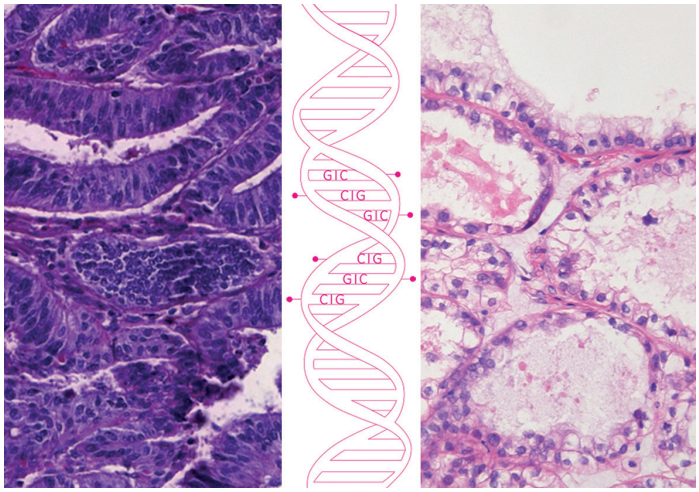
Molecular Alterations of Endometrial and Ovarian Tumorigenesis in Lynch Syndrome Mutation Carriers and the General Population



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Molecular alterations of endometrial and ovarian tumorigenesis in Lynch syndrome mutation carriers and the general population

Anni Niskakoski

Department of Medical and Clinical Genetics
Medicum
University of Helsinki

Integrative Life Science Doctoral Program
University of Helsinki
Helsinki, Finland

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Supervisors	Professor Päivi Peltomäki, M.D., Ph.D. Department of Medical and Clinical Genetics Medicum, University of Helsinki Finland Docent Sippy Kaur, Ph.D. Department of Oral and Maxillofacial Diseases University of Helsinki and Helsinki University Hospital Finland
Thesis committee	Docent Miina Ollikainen, Ph.D. Institute for Molecular Medicine Finland University of Helsinki Finland Docent Auli Karhu, Ph.D. Research Program Unit University of Helsinki Finland
Reviewers	Professor Robert Winqvist, Ph.D. Laboratory of Cancer Genetics and Tumour Biology University of Oulu Finland Docent Minna Pöyhönen, M.D., Ph.D. University of Helsinki, Finland Hospital District of Helsinki and Uusimaa Finland
Opponent	Docent Annika Auranen, M.D., Ph.D. University of Turku, Finland Department of Obstetrics and Gynecology Tampere University Hospital, Tampere, Finland
Custos	Professor Päivi Peltomäki

Cover layout by Anita Tienhaara
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\- *Tommy Tabermann*

CONTENTS

ABBREVIATIONS

APC	Adenomatous polyposis coli
ARID1A	AT-rich interaction domain 1A
ATP	Adenosine triphosphate
bp	Base pair
BMPR1A	Bone morphogenetic protein receptor type 1A
BRAF	B-Raf proto-oncogene, serine/threonine kinase
BRCA1/2	Breast cancer 1/2, early onset
CABLES1	Cdk5 and Abl enzyme substrate 1
CAH	Complex hyperplasia with atypia
CDH13	Cadherin 13
CDKN2B	Cyclin-dependent kinase inhibitor 2B
CH	Complex hyperplasia without atypia
CH_3	Methyl group
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CMMRD	Constitutional MMR deficiency
CpG	Cytosine-guanine dinucleotide
CRISPR	Clustered regularly interspaced short palindromic repeats
CTNNB1	Catenin beta 1
CxCa	Cervical adenocarcinoma
DAPK	Death-associated protein kinase
Dm	Methylation dosage ratio
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMT1/3A/3B	DNA methyltransferase 1/3 isoform A/3 isoform B
EnCa	Endometrial cancer
EPCAM	Epithelial cell adhesion molecule
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin embedded
GTPase	Guanosine triphosphatase
HAT	Histone acetyltransferase
HBOC	Hereditary breast and ovarian cancer syndrome
HDAC	Histone deacetylase
HNPCC	Hereditary non-polyposis colorectal cancer syndrome
HOXA9/10	Homeobox A9/10
IHC	Immunohistochemistry
KRAS	Kirsten rat sarcoma viral oncogene homolog
let-7a-3	MicroRNA let-7a-3
LINE-1	Long interspersed nuclear element 1
LOI	Loss of imprinting
LS	Lynch syndrome
L1CAM	L1 cell adhesion molecule
miRNA	MicroRNA
MIR34B	MicroRNA 34b
MLH1/3	MutL homolog 1/3

MMR	DNA mismatch repair
mRNA	Messenger RNA
MSH2/6	MuS homolog 2/3
MSI	Microsatellite instability
MS-MLPA	Methylation-specific multiplex ligation-dependent probe amplification
MSS	Microsatellite stable
MYC	MYC proto-oncogene, bHLH transcription factor
NGS	Next-generation sequencing
NE	Normal endometrium
OMIM	Online Mendelian inheritance in man
OPCML	Opioid binding protein/cell adhesion molecule like
OvB	Borderline ovarian tumor
OvCa	Ovarian cancer
OvCC	Clear cell ovarian cancer
OvE	Endometrioid ovarian cancer
PCR	Polymerase chain reaction
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PMS2	Postmeiotic segregation increased 2
PROM1	Prominin 1
PTEN	Phosphatase and tensin homolog
p53	Protein encoded by TP53 gene
RASSF1	Ras association domain family member 1
RNA	Ribonucleic acid
RSK4	Ribosomal protein S6 kinase A6
SAH	Simple atypical hyperplasia
SFRP2	Secreted frizzled related protein 2
SH	Simple hyperplasia
SMAD4	SMAD family member 4
SPARC	Secreted protein acidic and cysteine rich
SSCP	Single strand conformation polymorphism
STK11	Serine/threonine kinase 11
SWI/SNF	switch/sucrose non-fermentable
TET	10–11-translocation proteins
THBSO	Total hysterectomy and bilateral salpingo-oophorectomy
TMA	Tissue microarray
TP53	Gene encoding protein p53
TP73	Cellular tumor antigen p73
TSA	Trichostatin A
TSG	Tumor suppressor gene
VHL	Von Hippel-Lindau disease tumor suppressor
WHO	World Health Organization
WT1	Wilms tumor 1
WT1-AS	WT1 antisense RNA
5-aza-dC	5-aza-2'-deoxycytidine

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications which are referred to in the text by Roman numerals I-III.

I Niskakoski A, Kaur S, Renkonen-Sinisalo L, Lassus H, Järvinen HJ, Mecklin J-P, Bützow R, Peltomäki P (2013) Distinct molecular profiles in Lynch syndrome-associated and sporadic ovarian carcinomas. *International Journal of Cancer*. 133:2596–2608.

II Niskakoski A*, Kaur S*, Staff S, Renkonen-Sinisalo L, Lassus H, Järvinen HJ, Mecklin J-P, Bützow R, Peltomäki P (2014) Epigenetic analysis of sporadic and Lynch-associated ovarian cancers reveals histology- specific patterns of DNA methylation. *Epigenetics*. 12:1577-1587. *equal contribution.

III Niskakoski A, Pasanen A, Lassus H, Renkonen-Sinisalo L, Kaur S, Mecklin J-P, Bützow R, Peltomäki P. Molecular changes preceding endometrial and ovarian cancer: A study of consecutive endometrial specimens from Lynch syndrome surveillance. *Modern Pathology*. 2018 Mar 27. doi: 10.1038/s41379-018-0044-4.

In addition, this thesis includes some unpublished data.

ABSTRACT

Endometrial and ovarian cancers are among the most prevalent malignancies in females all around the world. Carcinomas belonging to the type I subset exhibit many similarities in their genetic and epigenetic profiles. Lynch syndrome (LS) is one of the most prevalent hereditary cancer susceptibility syndromes in the world. LS is a result of defective mismatch repair (MMR) caused by a germline mutation in MMR genes, which combined with other molecular alterations, is known to accelerate tumorigenesis. In addition to a high prevalence in colon cancer, type I endometrial and ovarian cancers predominate in women with LS. Apart from the MMR abnormalities, the molecular profile of LS-associated ovarian cancer remains unknown. Moreover, the developmental changes occurring in LS patients and in the general population prior to endometrial and ovarian cancer are poorly understood. Type I endometrial and ovarian non-serous carcinomas are believed to originate from the endometrial lining of the uterus, termed the endometrium. Women with LS have been offered regular gynecological surveillance in Finland since 1996. This surveillance program provides invaluable consecutive endometrial samples before cancer diagnosis and represents an excellent model with which to investigate the molecular changes resulting in the development of endometrial and ovarian tumors. The aims of this thesis were to identify and compare the molecular alterations in LS-associated and sporadic ovarian cancer, and to determine genetic, epigenetic and gene expression alterations in consecutive specimens prior to the appearance of endometrial and ovarian cancer.

In total, 213 endometrial and ovarian carcinomas, as well as endometrial biopsy specimens from 66 LS mutation carriers were compared to 197 sporadic specimens of the corresponding histological types and profiled with established genetic and partly novel epigenetic markers. Immunohistochemistry (IHC) was used to analyze the expression of MMR, *ARID1A*, and *L1CAM* genes, whereas epigenetic DNA methylation alterations of 37 tumor suppressor genes (TSGs) were evaluated using both commercial and custom-designed methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assays. Additionally, ovarian carcinomas were investigated by IHC for p53 protein,

hypomethylation of LINE-1 marker (a retrotransposon) was detected by MS-MLPA and we also conducted a mutational analysis of hotspot sites in *KRAS*, *BRAF*, and *PIK3CA* by PCR, followed by sequencing.

Novel molecular characteristics of LS-associated ovarian cancer were identified: An extremely high frequency of loss of ARID1A protein expression, MMR deficiency, no *BRAF* and *KRAS* mutations, normal p53 protein expression, a unique hypermethylation of selected TSGs, and an absence of LINE-1 hypomethylation in endometrioid and clear cell ovarian carcinomas, and frequent L1CAM overexpression specifically in clear cell ovarian cancer. Molecular analyses of LS surveillance specimens revealed closely related pathways in endometrial and ovarian type I tumorigenesis. For example, both MMR deficiency and TSG promoter methylation of specific genes appeared in histologically normal endometrial tissue preceding endometrial and ovarian cancer and there was ARID1A loss in complex hyperplasia with or without atypia prior to the appearance of the endometrial cancer. Additionally, we identified a high degree of similarity in the molecular alterations present in the hyperplastic lesions that occurred prior to or concurrently with the detection of endometrial or ovarian carcinoma collected from the same patient. This discovery suggests that endometrial hyperplasia may contribute to the development of ovarian tumors in addition to its well-established role in endometrial tumorigenesis.

Our findings provide novel and valuable information about the gynecological tumorigenesis of LS as well as the corresponding tumor with a sporadic origin. Further investigations are warranted with larger patient series. Our results may facilitate the prediction of the malignant potential of pre-neoplastic specimens, guide treatment decisions and identify those women who could benefit from prophylactic surgery.

INTRODUCTION

Cancer is an extensive global cause of death regardless of the endless time and money spent on attempts to learn how to control the disease or destroy metastatic cells. Cancer is a genetic and epigenetic disease — this means that genetic and epigenetic alterations in genes allow cells to function abnormally, especially to grow and divide without control, which can ultimately lead to tumor development. Genetics refers to information based on the structure of the DNA sequence, whereas epigenetics means the inherited information restored in gene expression patterns (1). In fact, genetic and epigenetic events together with lifestyle and other environmental influences are closely intertwined in cancer development and progression; epigenetic alterations can introduce mutations into genes, whereas mutations often occur in genes involved in epigenome modifications (2).

Changes that contribute to tumorigenesis can be inherited from the parents or they can be acquired during an individual´s life e.g. from endogenous sources (such as hormones and free radicals from cellular metabolism) or external mutagens (such as chemical carcinogens from cigarette smoke, physical risk from radiation, and pathogenic bacterial and viral infections) as well as errors in DNA replication. In normal cells, these errors are quickly repaired by several layers of effective DNA repair mechanisms. Therefore, a damaged DNA repair system, such as mismatch repair (MMR) mechanism, may promote tumorigenesis by the accumulation of mutations with a growth advantage in the cell´s genome (3).

Lynch syndrome (LS) is a hereditary cancer syndrome, associated with inherited autosomal dominant alterations in MMR genes and rare cases in *EPCAM* gene (4-6). In addition to its high prevalence of colorectal carcinomas, as many as 57% and females with LS develop endometrial carcinoma and this is also the case in 24% of ovarian carcinomas (7, 8). In addition to being common cancers as part of LS, in general, endometrial and ovarian carcinomas are some of the most prevalent cancers among women (4th and 8th place, respectively), in the United States (9, 10). Survival from ovarian cancer is poor, and

regardless of the fact that there are major histological and molecular differences within ovarian cancer, the different diseases are currently treated as a single entity. Endometrial cancer is the most common but ovarian cancer is the most lethal of gynecological cancers, but for both, the molecular changes that precede cancer development are currently unknown. Interestingly, epidemiological findings suggest that pathways leading to endometrial and ovarian tumorigenesis may intertwine in the early steps of tumor development, even before malignant progression.

The unsatisfactory management of most of the ovarian and part of the endometrial carcinomas reflects the poor knowledge of what molecular alterations actually lead to malignant development, starting from an uncertainty of the cell type of origin. An increased understanding of the molecular pathogenesis of ovarian and endometrial carcinoma will be required before we can expect improvements in the diagnosis and management of both hereditary and sporadic cases. MMR alterations are known to accelerate tumorigenesis in LS and sporadic cases (11, 12), but other mechanisms, such as mutations or epigenetic hypermethylation of specific genes may be important in the initiation of tumorigenesis but also in determining in which tissue and how fast tumor development progresses.

LS offers an excellent model with which to study epigenetic factors that facilitate tumorigenesis, since both the genotype and phenotype of a patient with LS often display a poor correlation. In addition, LS is an invaluable model for investigating the molecular changes preceding endometrial and ovarian cancer, as the basic tumorigenesis in LS significantly resembles that in corresponding sporadic cases but is accelerated. Furthermore, invaluable consecutive endometrial biopsy specimens from surveillance against gynecological cancer are available from these patients (13). Since the risk for gynecological carcinomas among LS mutation carriers increases after 40 years of age (7, 14), prophylactic surgery is recommended around that age, but its exact optimal timing remains an open question. Molecular findings from biopsy specimens could help to resolve this question.

REVIEW OF THE LITERATURE

1 Cancer – Overview

Cancer is a diverse disease of multiple organs as a result of genetic and epigenetic alterations in specific genes, which disrupt the cells' abilities to maintain normal growth and division. Although the start of cancer development is monoclonal, the high rate of new mutations and the different forms of genomic instability soon divide cancer cells into new populations (15). In 2000, Hanahan and Weinberg (16) proposed six hallmarks that all cancers have in common and which explain the properties that permit cancer cells to live, divide and spread. Every cancer is a unique combination of these hallmarks and aberrations that may occur in changing orders or simultaneously. The hallmarks include (1) the ability of cancer cells to stimulate their own proliferation, (2) the cancer cells are resistant to signals that inhibit their growth, (3) they have the capacity to avoid cell death, (4) they have an endless potential to replicate, (5) they possess a capability to grow and maintain blood vessels, and (6) cancer cells display a potential to move from the original site to invade distal organs (16). In 2011, the same scientists added two emerging hallmarks, the ability of cancer cells to modify cellular metabolism and to escape from immune destruction, as well as two facilitating hallmarks known as genome instability and inflammation which facilitate cancer cells to receive the main core and emerging hallmarks (17). In addition to these well-known hallmarks, another important hallmark exists; global alterations in the epigenetic landscape (18). Moreover, epigenetic mechanisms may be involved in each of the hallmarks proposed.

In the United States, it is predicted that around 1 735 350 new cancer cases will be diagnosed in 2018 (19). The most prevalent cancers in the Finnish population are shown in **Table 1**. On the positive side, the cancer death rate is declining (especially the death rates of the most common cancers of lung, colorectal, breast, and prostate), due to a reduction in smoking, early diagnosis and improvements in treatment.

Table 1. The five most common cancers in Finland and cancers included in the study (in 2015).

		Female		
Primary cancer	**Order of prevalance**	**New cases**	**Deaths**	**Incidence***
Breast	1.	5161	841	96.9
Colon	2.	1014	394	14.13
Skin, non-melanoma	3.	789	30	7.9
Lung and trachea	4.	936	779	13.27
Endometrium	5.	846	203	12.55
Ovarian	10.	436	349	7.12
		Male		
Prostate	1.	4855	921	78.77
Lung and trachea	2.	1690	1456	27.36
Bladder and urinary tract	3.	991	217	15.42
Colon	4.	981	378	16.09
Skin, non-melanoma	5.	896	32	12.68

*relative to world standard population by age adjusted, 1/100 000 people (20).

2 Tumorigenic pathways

Genetic and epigenetic alterations that contribute to cancer mainly affect two major types of genes: Tumor suppressor genes (TSGs) and proto-oncogenes, which are involved in the growth and division of normal cells. DNA repair genes are an important subclass of TSGs, tightly implicated in normal cellular functions (21). In cancer, proto-oncogenes become activated to become oncogenes that drive cell division or prevent cells from apoptosis (programmed cell death) whereas TSGs become silenced so that they cannot resist these oncogenic processes to happen (22). Moreover, DNA repair genes become faulty and lead to permanent DNA damage, thus causing the accumulation of mutations. Although thousands of alterations in different genes take place in a neoplastic cell, only approximately 140 of those are so-called driver genes which have the ability to promote tumorigenesis. Usually, 2 to 8 driver gene mutations are found in a tumor and all the rest (more than 99.9% of the alterations) are passenger mutations which do not enhance nor impair the tumor growth (21). The driver genes are involved in key processes of a cell, including specific cell fate, cell survival, and genome maintenance (21).

2.1 Altered tumor suppressor genes and proto-oncogenes in cancer

Mutations affecting proto-oncogenes are typically dominant and speed up tumorigenesis by a gain of function in gene expression, leading to enhanced cell division or prevention of cell death. *KRAS* is the most commonly altered proto-oncogene in cancer (23); it encodes a GTPase, a key component of the P13K/AKT pathway, and this proto-oncogene plays an important but stringently regulated role in normal cell signaling growth. However, mutations in *KRAS* can transform it into a constitutively active oncogene, causing over-production of its gene product further promoting uncontrolled growth (24, 25).

In contrast to proto-oncogenes, mutations and epigenetic alterations that occur in TSGs are frequently recessive, meaning that both of their parental alleles need to be inactivated (by Knudson´s "two- or multiple hit" hypothesis) to achieve complete expression of the modified phenotype (26, 27). TSGs can be silenced by different kinds of "hits", such as mutational inactivation, loss of heterozygosity (partial or complete loss of gene) or the gene can be turned off by epigenetic mechanisms (2). Occasionally, inactivation of only one allele of a TSG may predispose to a change in gene expression; in haplo-insufficiency, one allele alone is unable to produce a wild-type phenotype (28). This can also be achieved by a situation in which one mutated allele can disturb the function of the other allele by dominant-negative manner. *p53* is a good example of a crucial TSG often associated with different cancers; it is also known as "the guardian of the genome", which in normal cell becomes active in response to DNA damage (29, 30). Loss of expression of p53 increases cell proliferation (31) and interrupts p53-dependent cell death promoting tumorigenesis (32).

2.1.1 An epigenetic tumor suppressor, *ARID1A*

A recent intriguing finding in cancer research, discovered through whole genome sequencing, has been the realization that nearly all cancers harbor alterations in genes involved in creating the epigenetic machinery. These modified genes may therefore alter

the epigenome, resulting in changed gene expression and evoking genomic instability. Moreover, the frequency of many of these mutations is sufficiently high to suggest that they are "driver" mutations, meaning that a disturbance of the epigenome may be an early event in the initiation of cancer (2, 33). *ARID1A*, a gene involved in chromatin/nucleosome remodeling, is one of these genes often altered in several different types of cancer (34).

ARID1A (AT-rich interaction domain 1A) is a TSG which is often mutated in human cancers and believed to play a role both in tumor initiation and progression (35). *ARID1A* encodes a large protein, BAF250a, a key component of the ATP-dependent chromatin remodeling complex SWI/SNF (switch/sucrose non-fermentable). This complex regulates the transcription of specific genes by changing the accessibility of the chromatin around gene promoters; it is involved in several cellular processes such as DNA synthesis, DNA repair and genomic stability (36). As part of the chromatin remodeling complex, ARID1A is able to inhibit cell growth in normal cells (37). Disrupted *ARID1A* may cause distinct problems in SWI/SNF complexes, including a disturbance of nucleosome sliding, targeting to certain genomic sites and the assembly of coactivators or corepressors (38). Mutations in *ARID1A* are expected to lead to direct epigenetic modifications in cancer cells by changing the chromatin structure. Therefore, mutated *ARID1A* in cancer can partly explain why DNA methylation and chromatin differ between cancer and normal cells (39).

2.2 DNA repair

Despite the massive amount of errors that occur in a cell's genome, only a very low number (10^{-7}-10^{-11}bp/cell generation) of mutations remain in the genome due to the multilevel repair mechanisms that proofread DNA and correct most of the genomic alterations (40). Most mutations that are left in the genome are harmless to the cell, but on rare occasions the cell acquires a growth promoting mutation that will confer on the cell ability to achieve the hallmarks of cancer and eventually become neoplastic (16, 17, 41). If a defence mechanism such as MMR becomes defective, mutations start to build up high rate in a

cell´s genome, causing hypermutability and an increased risk for additional destructive mutations that often lead to tumor development.

There are a variety of DNA repair strategies, each repairing specific types of damage and restoring lost information. For instance, base excision (42), nucleotide excision (43) and mismatch repair (44) are needed to repair single-strand breaks on DNA, whereas double-strand breaks are often repaired by non-homologous end joining or by homologous recombination (45).

2.2.1 DNA mismatch repair (MMR)

The mismatch repair (MMR) machinery plays a critical role in the protection of genome stability by recognizing and stimulating repair of base pair mismatches and insertion/deletion loops in DNA caused by environmental factors and cellular processes as well as replication errors that escape DNA proofreading (46). **Figure 1** illustrates the MMR repair of single base pair mismatches. Defects in MMR cause an accumulation of small mono- and dinucleotide deletions and insertions, which lead to a variable number of these repeats causing microsatellite instability (MSI) (see Chapter 2.3.2 below). MSI can be used as a marker to detect MMR deficiencies (47). Defective MMR promotes tumorigenesis in two alternative ways: First, defective MMR causes increase in the number of replication errors leading to hypermutability, which may increase tumor heterogeneity and generate mutations, which are advantageous for neoplastic cells (3). Second, the absence of sensors for DNA damage, may lead to accelerated cell divisions and evading apoptosis (48).

Both genetic and epigenetic silencing of many genes have been implicated in the MMR pathway (4). A defective MMR system may be the starting point for tumorigenesis and inherited aberrations in the MMR machinery are well known to underlie a hereditary cancer syndrome called Lynch syndrome.

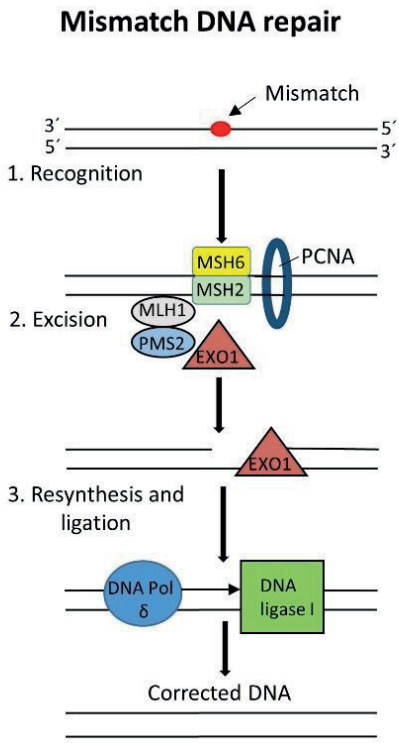


Figure 1. Simplified outline of the MMR pathway. Three main steps are involved in MMR repair: (1) Recognition of damaged DNA and initiation of repair, (2) excision of mismatched DNA and (3) resynthesis. (1) The DNA damage is recognized by the hMutSα complex, consisting of a MSH2 and MSH6 heterodimer, which binds to single base pair mismatches (as shown in the figure). The MSH2 and MSH3 heterodimer, called hMutSβ complex, detects longer insertions or deletions (not shown). Proliferating cell nuclear antigen (PCNA) is also involved in the recognition of mismatches. (2) After the recognition of DNA damage, the hMutLα complex, consisting of MLH1-PMS2 heterodimer, is recruited to the site and starts dissembling the mismatched DNA by exonuclease EXO1. (3) Finally, a new complementary strand of DNA is synthesized by DNA polymerase δ and further ligated to the old, undamaged strand by DNA ligase I.

2.3 Genomic Instability

Genomic instability in cancer is the driving force that leads to genetic heterogeneity inside a tumor, generating the genetic diversity for cancer cells to survive through natural selection, and providing extensive variety in patient phenotypes (49, 50). Genomic instability is a feature of nearly all human cancers, but the molecular basis and the time point when it arises in carcinogenesis remains still largely unknown. In hereditary cancer, this phenomenon has been linked to defective DNA repair mechanisms causing a high

spontaneous mutation rate (mutator hypothesis) (51) which is present already in precancerous lesions and confers on the neoplastic cell an ability to undergo favorable genetic changes and to achieve the hallmarks of cancer (17, 52). On the other hand, the molecular background of genomic instability in sporadic cancer is still not fully understood. It has been proposed that oncogene-induced errors in DNA replication as well as telomere erosion could be at least partly responsible for the genomic instability encountered in sporadic tumors (53, 54). Chromosomal instability (CIN) and MSI are two distinct forms of genomic instability (55, 56).

2.3.1 Chromosomal instability (CIN)

The amount of chromosomal alterations is highly increased in cancer, and it is the major form of genomic instability in cancer occurring in more than 90% of solid tumors (57). Alterations that contribute to CIN are large-scale rearrangements of the chromosome structure and number, involving aneuploidy (changes in numbers of chromosomes) as well as intrachromosomal inversions, deletions, translocations and amplifications (55, 58). Aneuploidy is a consequence of unequal division of chromosomes to daughter cells in mitosis and this feature of CIN is unique to neoplastic cells as the accurate arrangement and the number of chromosomes is strictly regulated in normal cells (55). CIN provides cancer cells with the possibility to obtain heterogeneity, allows them to rapidly collect mutations and modify tumor genomes, which further drives tumor progression (59).

2.3.2 Microsatellite instability (MSI)

Microsatellite DNA refers to short repeated sequences of DNA (typically dinucleotides) scattered throughout the human genome. The lengths of the repeats vary among the population, but are unique in an individual (60, 61). A defective MMR system can lead to an accumulation of base pair mismatches in microsatellites causing MSI; this can be observed as deletions or insertions of only a few nucleotides at repeat sequences (47, 62). MSI is a characteristic of almost all LS-associated tumors (over 90% of colorectal and

endometrial carcinomas (63, 64) but additionally, acquired aberrations in the MMR system are estimated of being the causal factor in 15 to 20% of colon and up to 30% of endometrial cancers of sporadic origin (63, 65-67). These sporadic carcinomas develop as a result of a defective MMR machinery caused by a somatic mutation or by DNA methylation (68). Tumors displaying MSI show a major acceleration of the mutations rate by between 100 to 1000 fold in comparison with normal cells (69).

3 Epigenetics and Cancer

The initiation and progression of cancer by silencing of TSGs, activation of oncogenes and the acquisition of genomic instability is achieved by genetic and epigenetic dysregulation. Epigenetics refers to all of the mechanisms involved in the regulation of gene expression not involving changes in the primary DNA sequence (70). Thus, in contrast to genetic mutations, epigenetic alterations (epimutations) do not alter the genetic code of the DNA itself but are able to regulate gene expression by other, potentially reversible, mechanisms. Furthermore, epigenetic regulation is affected by genetic factors and the environment. Epigenetic mechanisms consist of DNA methylation, histone modifications, non-coding RNAs (mainly miRNA expression) and modifications of chromatin remodeling systems (demonstrated in **Figure 2**). These epigenetic mechanisms which carefully regulate normal homeostasis of expressed genes in a cell, become completely disrupted in the neoplastic cell (1). Additionally, recent findings from next-generation sequencing (NGS) of whole cancer genomes have shown that epigenetic genes, for instance those encoding parts for chromatin remodeling machinery as well as enzymes that modify histones, are frequently mutated in cancer, transforming them to behave like TSGs or oncogenes (71, 72).

The reversible but heritable nature of epigenetic aberrations has led to the emergence of the promising field of epigenetic therapy, which is already making progress with the recent FDA approval of four epigenetic drugs for use in the cancer treatment of T-cell lymphomas and myeloma (73).

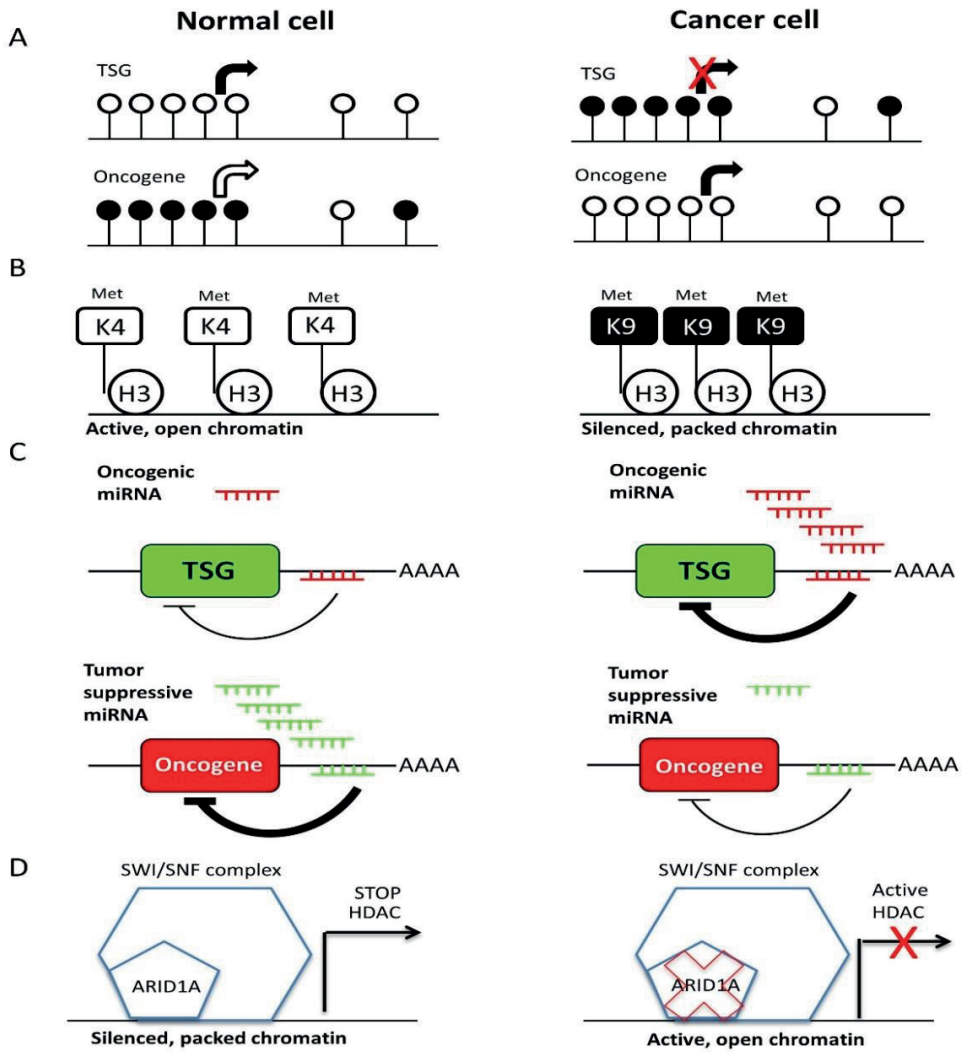


Figure 2. Epigenetic alterations in cancer, including DNA methylation (**A**), histone modifications (**B**), miRNAs (**C**) and chromatin modifiers (**D**). **A** (top), in cancer, TSG promoters become methylated (black balls) and silenced (bent arrow indicates the transcription starting point); **A** (bottom), oncogene promoters become hypomethylated and activated (open arrow indicates low and solid arrow high expression). **B**, The number of inactivating histone marks increases in cancer cells resulting in compact chromatin and silencing of TSGs. **C** (top), inactivation of a TSG by increased oncogenic miRNA expression; **C** (bottom), oncogene activation by decreased expression of tumor suppressive miRNA. **D**, chromatin remodeling complex SWI/SNF modifies chromatin accessibility. In normal cells (left), SWI/SNF-complex activates genes normally silenced by packed chromatin structure established by HDACs. SWI/SNF complex represses expression of HDACs leading to active chromatin and expression of genes regulated by HDACs. In cancer (right), the loss of ARID1A, expression leads to a dysfunction in the SWI/SNF-complex resulting in expression of HDACs, subsequent repressive chromatin state and inactivation of genes targeted by HDACs. Modified from Peltomäki et al. (74)

3.1 DNA hypermethylation and cancer

DNA methylation is an epigenetic change in DNA sequence that typically occurs at a 5´cytosine located next to guanine forming CpG dinucleotides (2). Patterns of DNA methylation are established in the early stages of development and maintained respectively stable throughout life. Furthermore, methylation patterns are frequently inherited to daughter cells in cell division (75) and even aberrant hypermethylation (epimutations) of specific TSGs has been shown to be inherited in the germline from parents to progeny (76, 77). Particularly stable form of DNA methylation is involved in genomic imprinting which is a way of silencing gene expression of one parental allele by DNA methylation (78). DNA methylation in humans is produced and maintained by DNA methyltransferase (DNMT) enzymes, which add a methyl group (CH_3) into the 5´ position of a cytosine adjacent to a guanine (by DNMT1) and maintain (by DNMT3A and DNMT3B) the methylation through cell divisions. On the contrary, 10–11-translocation proteins (TET) can remove methyl groups and further demethylate DNA (79).

In human genome, approximately 60% of gene promoters contain a high number of CpG dinucleotides, called CpG islands (80). The DNA methylation patterns of these islands are tissue specific (81). Hypermethylation of CpG islands around gene promoters is associated with inactivation of the gene. In normal cells, most of the CpG islands around gene promoters are unmethylated, as it allows open and active chromatin and expression of genes when equivalent transcription factors are accessible. In contrast to hypermethylation and consequent inactivation associated with promoters, hypermethylation that occurs in a gene body either enhances or has no effect on gene expression (2). In cancer, usually 5 to 10% of CpG sites located in gene promoters become heritably hypermethylated (33). This may cause transcriptional inactivation of the TSGs, which in normal conditions would suppress tumor formation (18, 33, 82). These alterations in methylation are believed to drive the cancer formation as they appear already in early stages of cancer development (83). Genes known to be hypermethylated in cancer are

involved in all of the key cellular pathways, such as the cell cycle (*CDKN2B*), DNA repair (*MLH1*), metabolic reprogramming (*VHL*) and cell death (*DAPK*) (1, 79).

Abnormal hypermethylation of gene promoters is a main mechanism associated with tumor suppressor gene inactivation in carcinogenesis (See **Figure 2A**) (84). In nearly all cancer types, hundreds of genes may be silenced by promoter hypermethylation. However, only a small fraction of these hypermethylated genes are "drivers" and hence affect cancer initiation and progression (2, 85). Three main routes involving DNA methylation are known to promote tumorigenesis. These changes often occur concurrently and include genome-wide hypomethylation, specific hypermethylation of TSG promoters, and direct mutational processing of sites containing methylated cytosines by ultraviolet radiation, deamination or by other carcinogenic mutagens (2, 86).

DNA methylation has been utilized as a biomarker in diagnosis, prognosis, and in response to treatment (79). At the present, clinical treatments with demethylating agents have been limited by their non-specific nature. This may change in the future, as the CRISPR-mediated system holds the promise of site-specific epigenetic editing of the genome (87).

3.2 Hypomethylation, LINE-1 and cancer

Hypomethylation, the genome-wide decrease in 5-methylcytosine, was the first epigenetic alterations identified in human tumors (88). Global hypomethylation is often a characteristic of tumor progression but sometimes it can be also observed in the early stages of tumor development (89, 90). Hypomethylation is a frequent feature of cancer cells and in contrast to hypermethylation of CpG sites in gene promoters, hypomethylation is often present in the remainder of the genome and can be detected in vast areas of the genome. Hypomethylation stimulates carcinogenesis in diverse ways such as activating oncogenes (91), generating CIN by disruption of genes via retrotransposition of long interspersed element 1 (LINE-1), and by loss of imprinting (LOI) (92, 93). LOI activates an

allele that has been imprinted and therefore silenced by DNA methylation and this activation will result in an overabundance of the gene product expressed now by both alleles (94).

Highly repeated DNA sequences, such as the interspersed Alu and LINEs comprise nearly half of the human genome, and are the typical place for hypomethylation to take place. LINE-1 retrotransposon, up to 6 kb in length and the only active and abundant LINE-element still in human, provides a useful marker to measure global hypomethylation of the cancer cell genome (95, 96). DNA hypomethylation activates LINE-1 transcription and enables the retrotransposition of these elements into new sites in the genome. In tumor development, these elements can disrupt gene function by insertion and can also act as surrogate splice sites or as alternative promoters (93). Alterations in DNMTs and TET2 have been detected in some cancers which may cause global hypomethylation (79).

3.3 Histone modifications

DNA in the human nucleus is tightly wrapped around histone proteins. Histones have an unfolded domain, called the histone tail, which is bound by different epigenetic marks (see **Figure 2B**). These epigenetic signatures on histone tails contribute to packing of the chromatin and influence the binding of proteins to chromatin. The histone tails can be modified with many different chemical bounds, such as methylation, acetylation, phosphorylation and ubiquitination (97, 98). The chemical modification together with its position of the histone tail, specify influence the chromatin. For example, trimethylation of lysine 4 in histone 3 (H3K4Me3) is an activating signal, whereas the same modification in lysine 9 in histone 3 (H3K9Me3) mediates a repressive function. Furthermore, the modification with a different chemical of the same position may produce an opposite event such as acetylation of lysine 9 in histone 3 (H3K9ac) may cause an activation of transcription (79). In cancer, these covalent histone marks around promoter regions often become altered together with DNA methylation changes (2, 82, 99).

3.4 MicroRNAs

MicroRNAs (miRNAs) are another set of crucial factors involved in epigenetic regulation. MiRNAs belong to small non-coding RNAs, which regulate the expression of selected genes at the post-transcriptional level (100). MiRNAs regulate the translational processing of genes by specific targeting of 3´-untranslated region of messenger RNA (mRNA) followed either by target mRNA degradation or by blocking mRNA translation into protein. MiRNAs may be important players in tumor development, because they target and consequently regulate specific proto-oncogenes (tumor suppressive miRNAs such as miRNA *let-7a* which inhibits *MYC* oncogene) (101) and TSGs (oncogenic miRNAs such as *MIR34B*) as shown in **Figure 2C**, and furthermore, miRNAs may mediate regulatory communication between oncogenes and TSGs in carcinogenesis (102). In addition, MiRNAs directly target components of the epigenetic machinery, such as DNMTs (103) and histone deacetylases (HDACs) (104), resulting in indirect modulation of genes regulated by epigenetic modifications (105).

On the contrary and making this network even more complex, miRNA themselves can be regulated at the transcriptional level by binding of specific proto-oncogenes and TSGs to miRNA host gene promoter which encode miRNA (106), as well as through genetic alterations of the host gene promoter (107). Moreover, recent breakthrough findings have shown that the genes encoding for miRNAs can also be epigenetically regulated by promoter methylation, acetylation and methylation of histones as well as chromatin modifications (105, 108). This ability of a cancer cell to epigenetically regulate specific miRNAS may help the cell to transform its transcriptome to an oncogenic phenotype.

3.5 Chromatin modifiers in cancer

Defective epigenetic machinery may often underlie the epigenetic alterations in a cancer cell´s genome as demonstrated in **Figure 2D** (2). During cancer progression, the maintenance of transcriptionally repressed and active chromatin states becomes altered.

Therefore, chromatin modifiers and their alterations may play a major part in carcinogenesis. Several candidates have been acknowledged: HDACs are proteins that remove acetylation from histones as well as from other proteins and establish a tightly packed and silenced chromatin structure (109). Other candidates are histone acetyltransferases (HATs), which function as transcriptional activators by adding acetyl-groups to histone tails, as well as the SWI/SNF protein complex, together with its subunit *ARID1A*, which actively modifies the chromatin around promoter regions as well as changing the localization of nucleosomes to promote gene expression (1, 38).

3.6 Technology to identify DNA methylation changes

There are many different techniques available for analyzing gene-specific or genome-wide DNA methylation patterns. The determination of the DNA methylation patterns and their distribution in the genome is essential in understanding their function in normal cellular functions as well as in disease, such as cancer. These techniques are primarily divided into three categories depending on the pre-treatment step applied: (1) Restriction enzyme based assays, (2) affinity enrichment based assays, and (3) sodium bisulfite based assays (110, 111). The optimal choice for DNA methylation assay depends on several aspects such as the scientific question, the amount and quality of the sample to be analyzed, the information available of the sequence under analysis, the required sensitivity of the assay, the bioinformatics knowledge available, as well as economic issues.

For many methods, the limiting factor is the high quality and/or large quantities of DNA required and therefore only a few assays are suitable for formalin-fixed paraffin embedded (FFPE) samples. Many assays, such as restriction landmark genomic scanning (RLGS) and the methylated-CpG island recovery assay (MIRA) require high quality DNA, but for instance, Illumina Infinium 450K assay can be used to analyze low quality, FFPE samples (110, 112, 113). As the whole-genome methylation assays have developed becoming more sensitive and cheaper, they are replacing the methods used to analyze gene-specific methylation. Still today, gene-specific methods are pivotal due to their low cost, their

ability to generate quantitative data, the possibility to detect methylation changes from low quality FFPE DNA (such as MS-MLPA), and in cases where the interest in detecting DNA methylation only requires a low number of specific genes, such as MLH1 methylation in colorectal and endometrial cancer (5). In addition, the plethora of data produced by whole-genome methylation assays, require time and bioinformatics knowledge to sort out the data, whereas analyzing gene-specific methylation is often fast and requires less bioinformatics skills.

New platforms using NGS and/or genome-wide hybridization to investigate genome-wide DNA methylation patterns have enabled the identification of large sets of genes methylated in cancer (67, 114). The widely used, Illumina Infinium 450K microarray platform detects around 450,000 candidate CpG sites throughout the genome giving a broad perspective of the methylation changes in the human genome. Although, the coverage of this platform is broad, it is not very specific in a given region of the genome and thus it is mainly used as a first screening method and subsequently followed by methods that carry a higher number of probes for a specific region (115). In addition, direct sequencing of all CpG sites after bisulfite modification of DNA (for instance, whole genome bisulfite sequencing, WGBS) (116) is currently available and allows extensive information of nearly all CpGs throughout the genome (117).

4 Hereditary cancer

Most cancers are caused by somatic mutations in driver genes, but around 10% of cases are a consequence of a germline mutation that may be inherited from parents to offspring. More than 110 genes are known to be associated with hereditary cancer syndromes (118). The most common inherited alterations involved in hereditary cancer are due to defects in DNA repair genes (119).

Most hereditary cancer syndromes are passed on to the children in an autosomal dominant manner and in most cases, a carrier is heterozygous for the inherited germline mutation.

This means that the carrier inherits one defective allele of a specific TSG (the so-called first hit in Knudson´s two hit hypothesis) (26, 27) which increases the risk for developing cancer, but only after the second somatic hit has been acquired in a cell of a target tissue, may tumor development be initiated. In sporadic cancer however, two hits affecting both alleles of a TSG need to occur somatically before tumor development can begin. Either one or both of these inactivating hits can occur genetically or epigenetically. Examples of hereditary autosomal dominant cancer syndromes include Lynch syndrome (described below), hereditary breast and ovarian cancer syndrome (described below), and Peutz-Jeghers syndrome (germline mutation in *STK11* gene) (120) which predispose to several cancers, whereas familial adenomatous polyposis (germline mutation in *APC* gene) mainly predisposes to colon cancer (121).

4.1 Lynch syndrome

Lynch syndrome (LS, OMIM #120435, #120436), earlier referred to as hereditary non-polyposis colorectal cancer syndrome (HNPCC), was first reported by Doctor Aldred Scott Warthin in 1913 (122). Patients with LS were defined according to clinical and family history alone until the year 1993, when Peltomäki et al. (123) identified the first susceptibility locus for this syndrome. LS is a severe hereditary cancer susceptibility syndrome caused by autosomal dominant mutation or epimutation in one of the genes belonging to the MMR system (5). LS mutation carriers have a high risk of developing early onset colon or gynecological cancer (endometrial and ovarian) and most tumors present with a MSI phenotype due to aberrant MMR (124). Most women with LS become affected with endometrial and/or ovarian cancer at some stage of life and the latest analysis of cancer risk in LS patients has shown that the risk of gynecological cancer in women with LS outweighs the risk for colon cancer (7). Other cancer types such as stomach, urinary tract, bladder, breast, brain (glioblastoma) and cancer of the kidney are less common in LS patients but nonetheless, the incidence exceeds that of average population (see **Figure 3**) (125, 126). MMR genes are expressed in all tissues, but the cancer risk varies according to

the tissue. This may be a consequence of the amount of MMR product produced in the tissue, the proliferation rate of cells, the power of immune defense, and the way in which the tissue has been exposed to endogenous or exogenous carcinogenic agents (127). It is typical that an LS mutation carrier may be diagnosed with different cancers in their life but fortunately, the survival is higher for most cancers compared to sporadic cancers (8). The incidence of an individual with Lynch syndrome developing any type of cancer before the age of 70 is 75% in women and 58% in men (8).

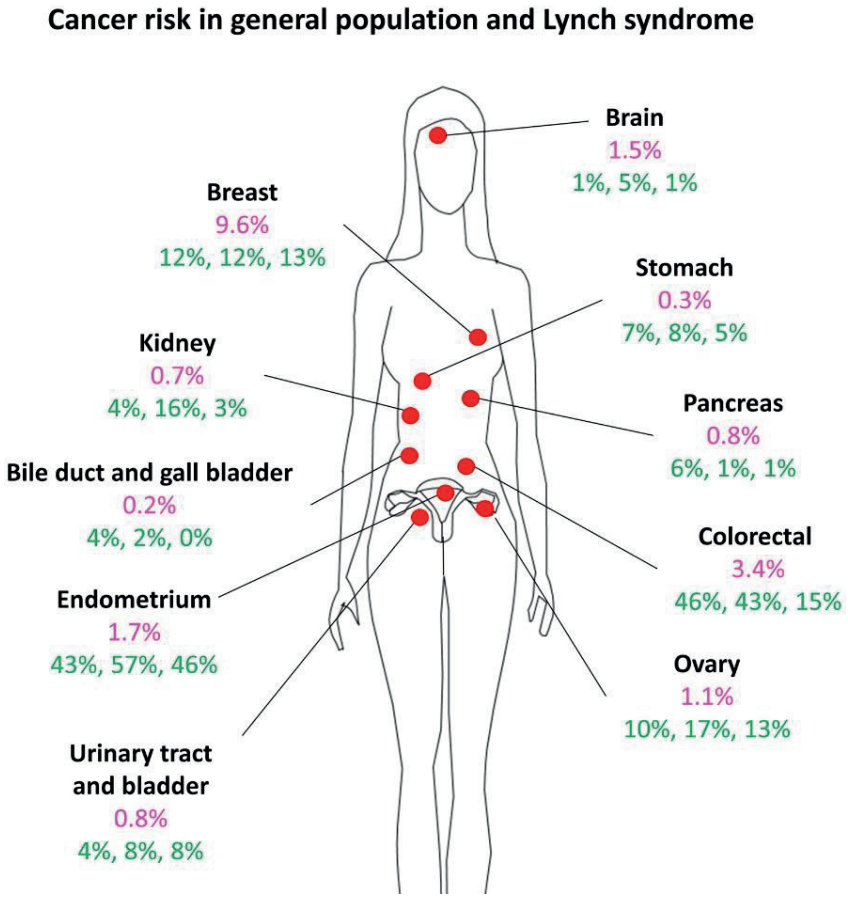


Figure 3. Cumulative cancer risk of LS-associated cancers from age 25 up to 75 years of age in general population and in LS germline mutation carriers. Cumulative risk values in general population are shown for females only (pink color). The cancer risk in Lynch syndrome for endometrial, ovarian, and breast cancer is shown in females only; for all other cancers, the risk values are shown for combined genders. In LS-associated cancers, the cumulative risk is shown separately in *MLH1*, *MSH2* and *MSH6* mutation carriers, respectively (green color). The data for general population is collected from the NORDCAN-database (128, 129) and for Lynch syndrome from Møller et al. (2017) (130).

The incidence of LS in the population can be as high as 1 in every 370 individuals and even this value may be an underestimation (124). It has been estimated that there are more than 10 000 LS mutation carriers in Finland and over a million in Europe, moreover, LS has been evaluated as being the causal factor in 3% of the newly diagnosed colorectal carcinomas (5). In Finland, there are currently approximately 280 known families with a verified mutation causing LS (131). Because of the dominant characteristic of the LS causing mutation, the risk for passing on a defective allele to a child is 50%. In a rare condition, constitutional MMR deficiency (CMMRD), the child inherits defective MMR alleles from both parents causing a severe form of cancer syndrome and in which cancers (mainly hematological malignancies, colorectal cancer, and brain tumors) occur already in childhood (132).

LS is a consequence of germline mutation in one of the MMR genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*), or by a large deletion in *EPCAM* gene. *EPCAM* germline mutation is a rare event, but it can also cause LS by epigenetic inactivation of its adjacent *MSH2* promoter by DNA hypermethylation followed by silencing of *MSH2* gene (6, 133). Almost 3500 unique LS-associated variants have been discovered in *MLH1*, *MSH2*, *MSH6*, *PMS2* and *EPCAM*, and the shares of the variants among MMR genes are approximately 38%, 33%, 19%, and 10% respectively (134). On rare occasions, Lynch syndrome may be inherited via a constitutional epimutation (135). This means that hypermethylation of a specific allele (non-imprinted), such as *MLH1* in LS, occurs in a germline and is therefore spread throughout the normal tissues in the body. This constitutive epimutation of *MLH1* is typically the first hit and LOI frequently the second hit that inactivates the gene and drives tumor formation in a tissue (76). Clinically, this resembles the *MLH1* mutation or methylation of *MLH1* in sporadic cases (136). On the contrary, an *MSH2* epimutation is secondary and caused by a deletion of the *EPCAM* gene (6, 77). In LS-associated cancers both two hits, hereditary and later acquired somatic, are frequently genetic (MMR or *EPCAM*) (137, 138).

4.1.1 Diagnostics and screening pathways

The identification of colorectal and gynecological carcinoma patients with LS is important in order to save lives by guiding these patients and their affected relatives to surveillance programs and to start prevention interventions. It has been estimated that colonoscopy surveillance for colorectal carcinoma in verified LS patients decreases the overall death incidence from colorectal carcinoma by 65% (139). The estimates with gynecological surveillance have not shown a similar effect (140). However, Auranen and Joutsiniemi (2011) performed a systematic review of gynecological surveillance in women with LS and revealed a 5% to 6.5% detection rate of pathological endometrial findings in surveillance visits that involved endometrial biopsies (141).

Different guidelines (Amsterdam Criteria and Bethesda Guidelines) have been developed for clinicians to identify colorectal and endometrial carcinoma patients that have a high risk of being LS mutation carriers and who should be guided to further analysis of MMR defects (142-145). The problem with these guidelines is that they are either not sensitive (Amsterdam Criteria) or specific (Bethesda Guidelines) enough and hence most Lynch mutation carriers remain undetected. The currently used guidelines Amsterdam criteria II (145) and Bethesda (revised in 1999) (143) for LS diagnosis are shown in **Table 2**.

Table 2. Amsterdam Criteria II and revised Bethesda guidelines for diagnosis of LS.

Amsterdam Criteria II
1. At least three relatives with LS-associated cancer[a], of whom one affected individual is a first-degree relative of the other two.
2. Affected individuals detected in two generations
3. At least one of the LS-associated cancers[a], diagnosed under 50 years of age
4. Familial adenomtous polyposis (FAP) excluded in CRC cases
Revised Bethesda guidelines
1. CRC diagnosed in an individual under 50 years of age
2. Synchronous or metachorous LS-associated tumors[a] detected regardless of age
3. CRC tumor with MSI-high histology[b] diagnosed in an individual under 60 years of age
4. Diagnosis of CRC in at least one first-degree relative with LS-associated tumor, with one of the tumors diagnosed under 50 years of age
5. Diagnosis of CRC in at least two first- or second-degree relatives with LS-associated tumors, at any age.

[a]LS-spectrum tumors include CRC, endometrial, ovarian, stomach, urinary tract, bladder, breast, brain (glioblastoma), skin, pancreas, and cancer of the small bowel. [b]MSI-high histology is determined as the presence of tumor-infiltrating lymphocytes, mucinous/signet-ring differentiation or medullary pattern of growth. Abbreviations: LS, Lynch syndrome; CRC, colorectal cancer.

If patient is suspected of being LS, at present immunohistochemical (IHC) analysis of MMR genes is recommended. This analysis is able to detect an absent MMR protein and thus identifies which MMR gene to test by mutational analysis in order to find out the exact mutation causing the syndrome (146). If the absence of MLH1 is detected by IHC, an additional methylation analysis of *MLH1* promoter is needed before mutational testing to exclude common sporadic cases caused by methylation of *MLH1* gene promoter (5).

The final identification of LS mutation carriers is based on the detection of MMR or *EPCAM* germline mutations which can be found by sequencing. At present, a tumor sample is needed in the characterization of a mutation, but a functional assay is being developed that would detect a MMR deficiency from a healthy relative by using fibroblasts (147). Until the present time, sequencing has been expensive and time-consuming and the analysis and interpretation of sequencing data of MMR genes have required substantial effort due to their large size and high number of variants known in these genes. Therefore, only patients showing convincing proof by fulfilling clinical criteria of LS and having a defective MMR by IHC are at present guided to undertake final genetic testing. All verified mutation carriers should be provided with genetic counseling and enrolled into surveillance programs to prevent cancer development (5). Families meeting the clinical criteria of LS but showing negative for tested predisposing mutations should be considered for further epigenetic testing to exclude constitutional epimutation that also predisposes to LS (76, 148).

Currently, immunohistochemistry (IHC) analysis of four MMR proteins is recommended for all colorectal cancer cases and it can also be considered for the detection of Lynch syndrome among endometrial carcinomas (at least when diagnosed at an age of less than 70 years) internationally (5, 149). Despite these recommendations, it is very clinic- and clinician-dependent, deciding which patients will be selected for genetic counseling and further IHC testing of the samples.

4.2 Hereditary Breast and Ovarian Cancer Syndrome

Tumor suppressor genes, *BRCA1* and *BRCA2*, involved in the repair of a damaged DNA are the genes most often involved in hereditary ovarian cancer causing Hereditary Breast and Ovarian Cancer Syndrome (HBOC, OMIM #604370, #612555) and account for approximately 14% of the epithelial ovarian carcinomas, and 65 to 85% of all hereditary ovarian carcinomas (150-152). Mutations in *BRCA1/2* predispose carriers to a high risk of breast and ovarian cancer but also other cancers such as prostate cancer in males. The cumulative risk for developing ovarian cancer before the age of 80 was recently estimated by Kuchenbaeker et al. (153) and found to be 44% in *BRCA1* mutation carriers and 17% in carriers of *BRCA2* germline mutation. Ovarian carcinomas diagnosed in these *BRCA1/2* mutation carriers are generally of the high-grade serous histological type, often an aggressive form of ovarian cancer which predominates in the general population as well (154).

5 Ovarian and endometrial cancer

5.1 Closely intertwined epidemiology of endometrial and ovarian carcinoma

The epidemiology of endometrial and ovarian carcinoma is tightly entangled, including highly comparable risk factors as well as age and geography which are correlated with the incidence of cancer rates. Many factors, such as a late onset of first menstruation, giving birth, breastfeeding and healthy weight reduce the risk for both cancers. On the contrary, obesity, late onset of menopause, nulliparity, and heredity increase the risk for endometrial and ovarian carcinomas. All these factors contribute to hormonal levels (especially the estrogen and progesterone involved in these cancers) and to number of ovulatory cycles (155).

5. 2 Type I and type II ovarian and endometrial cancer

Histopathological and epidemiological characteristics underlie the clinical outcome and divide both ovarian and endometrial cancer into two major types, designated as type I and type II. Tumors of both endometrial and ovarian origin belonging to the type I category as being highly similar to each other (156, 157). The differences in genetic, epigenetic and gene expression profiles between type I and type II highlight the distinct origin and molecular pathways involved and may provide new ways to improve the prognosis due to the development of subtype specific treatments (157). The hypotheses of the endometrial and ovarian tumorigenesis of type I and II are demonstrated in **Figure 4**.

Approximately 80 to 85% of all endometrial carcinomas belong to type I category, which consists of tumors with low-grade endometrioid histology, that are associated with an intense estrogen expression (158), often have a good prognosis, and generally develop through hyperplastic endometrial lesions (159). Type II tumors, on the other hand, mainly display a serous and clear cell histology, are likely to originate from atrophic endometrium and typically have a poor prognosis after their diagnosis (160).

Type I ovarian cancers mainly comprise low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas, whereas high-grade serous is the main histology of type II tumors. Type I ovarian tumors typically grow slowly and are thought to originate from endometriosis or borderline tumors (157, 161) whereas type II tumors are often aggressive and are likely to originate from precursor lesions in fallopian tubes or endosalpingiosis (162). In comparison to endometrial type I tumors, which account for the majority of all endometrial carcinomas, high-grade serous are the most common histological type and are responsible for approximately 70% of all epithelial ovarian tumors (163).

Endometrial and ovarian type I tumors often have mutations in *ARID1A*, *KRAS/BRAF*, *PIK3CA*, *PTEN*, and *CTNNB1* (67, 114, 164-166). MSI is a rare feature in normal endometrial tissue, but a common finding (13 to 30%) in endometrial carcinoma as well as in preceding

hyperplastic tissues, as well as in ovarian type I carcinomas (10%) of sporadic origin (67, 167-170). Type II endometrial and ovarian tumors frequently harbor mutations in *p53* and are chromosomally unstable (67, 114, 157, 166).

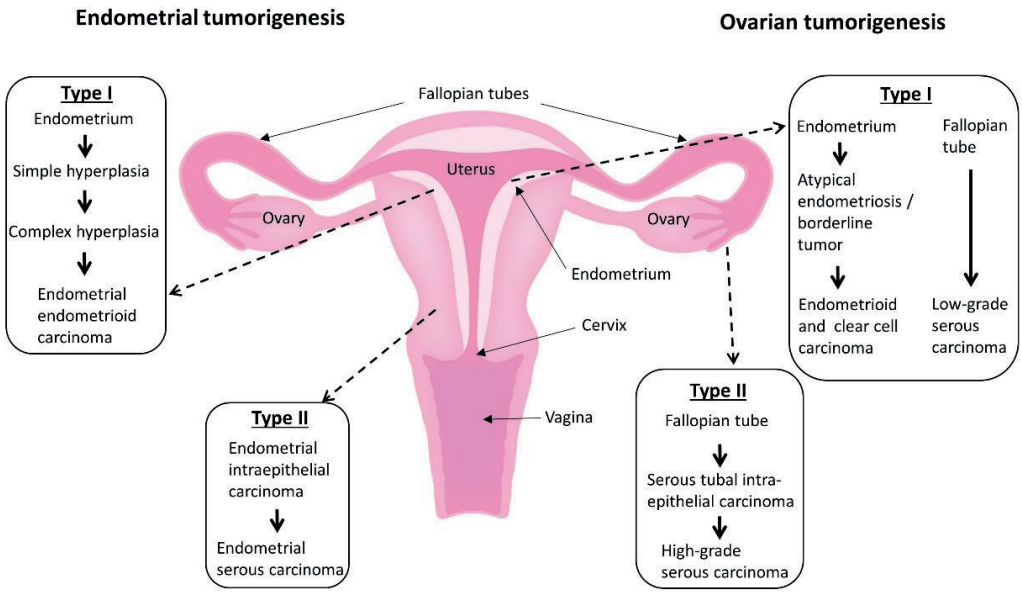


Figure 4. Hypothesis of endometrial and ovarian tumorigenesis.

5.3 Ovarian cancer

In the United States, there were 22 440 new ovarian carcinoma cases and 14 080 deaths expected in 2017 (171). The early signs of ovarian carcinoma are typically mild and at the time of diagnosis, already around 75% of ovarian carcinomas have spread out of the ovaries leading to the high fatality rate. Most of the women diagnosed with ovarian cancer die of this disease (163).

Ovarian cancer is a highly diverse disease comprising of variable tumors and histological cell types within the tumors, which complicates the treatment of ovarian cancer. More than 95% of ovarian tumors are epithelial, but there are also germ cell and sex cord stromal cell tumors. Epithelial ovarian carcinomas are principally classified by the cell type in the tumor

into serous (high-grade 70%, low-grade 5%), endometrioid (10%), clear cell (10%) and mucinous (3%). The main four histological types of ovarian cancer are shown in **Figure 5**. In addition, undifferentiated and non-malignant borderline ovarian tumors exist (163). It is acknowledged that the different histological subtypes are separate entities and should be considered as distinct diseases, since they have different clinical presentations, responses to treatment, and overall outcome. However, at present, epithelial ovarian cancer is treated as a single disease. This is partially attributable to the lack of knowledge of the driver molecular events behind each disease. Nonetheless, it is vital to learn more about the molecular mechanisms and detect drivers if we are to establish novel and individual treatments against all types of ovarian cancer. Other obstacles in the treatment of ovarian cancer are the absence of reliable markers for early detection and the acquisition of chemoresistance as treatment progresses (172).

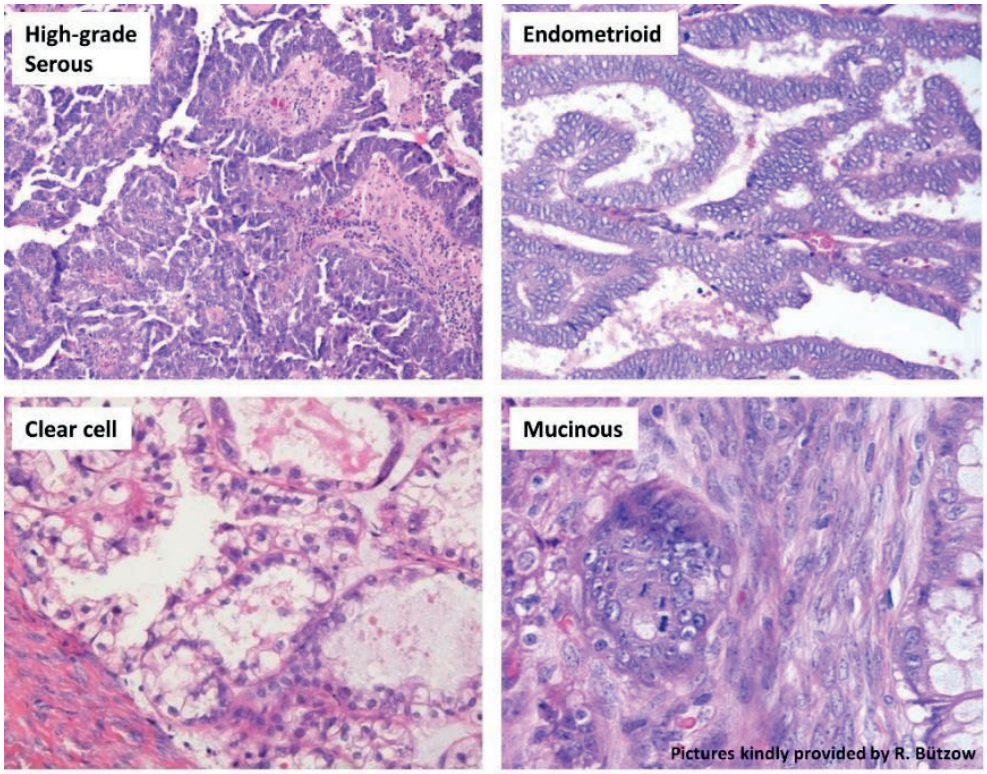


Figure 5. The main four histological types of epithelial ovarian cancer.

Histology and tumor grade can be used to divide ovarian carcinomas into two different categories, namely Type I and Type II (as described above in Chapter 5.2) (157). Additionally, ovarian carcinomas are classified into four stages according to the International Federation of Gynecology and Obstetrics (FIGO) staging system. The stage depends on the invasiveness of the cancer. Accordingly, in stage I, the tumor is confined to ovaries or fallopian tube(s), in stage II, the tumor involves one or both ovaries/fallopian tube with some pelvic peritoneal extension, in stage III, it has spread to the peritoneum outside the pelvis or metastasis to retro-peritoneal lymph nodes has occurred, and by stage IV, the metastasis is found in distal organs (173).

5.3.1 Origin of ovarian cancer

In 1872, Sir Spencer Wells claimed that epithelial ovarian cancer arises from ovarian surface epithelial cells and for more than a century, it was believed that the origin of ovarian cancer was in the ovary itself (174). But with the latest evidence, it has appeared that the origin and early steps of epithelial ovarian carcinoma development take place outside the ovaries. There is recent data suggesting that serous ovarian cancer originates from the fallopian tubes and endosalpingiosis whereas endometrioid and clear cell ovarian carcinomas originate from endometrioid epithelial cells through a process involving atypical endometriosis and/or borderline tumors (see **Figure 4**) (162, 175, 176). It still remains a mystery how endometriosis contributes to the development of endometrioid and clear cell types of ovarian cancer, but it has been speculated that repetitive damage and repair of endometriotic epithelial cells in their microenvironment abundant with free iron which induces the production of free radicals and leads to the abnormal growth of these cells (177, 178). Although, it has been acknowledged that endometrioid and clear cell ovarian carcinomas originate from atypical endometriosis, the importance of alterations in endometrial epithelia that contributes to endometriosis has been largely uninvestigated.

5. 3.2 Lynch syndrome associated ovarian cancer

Lynch syndrome is the second most common cause of hereditary ovarian cancer after *BRCA1/2* (causing HBOC syndrome) and is responsible for 8% to 13% of all hereditary ovarian cancers and up to 2 % of all ovarian carcinomas (179, 180). The lifetime risk of ovarian cancer in women with LS varies according to the mutation carried by the carrier and is 10% in *MLH1*, 17% in *MSH2* and 1% in *MSH6* carriers (181) and it occurs typically before menopause (7). LS-associated ovarian cancer differs from sporadic ovarian cancer in several ways both clinically and histologically. The mean age at diagnosis of ovarian cancer in female LS mutation carriers is 45 which is 15 to 20 years earlier than in patients with sporadic cases (182). The histology of ovarian carcinomas is mainly non-serous (typically endometrioid or clear cell) and presents as well-differentiated and early stage tumors at the time of diagnosis compared to sporadic cases which are typically of serous histological type and displayed in advanced tumor stages (182). In addition, more than 20% of women with LS have a synchronous endometrial cancer at the time of ovarian cancer diagnosis in comparison to sporadic ovarian carcinomas where the frequency of synchronous tumors is less than 10% (183, 184).

5.4 Endometrial cancer

In the United States, it has been estimated that 61 380 new endometrial carcinoma cases and 10 920 deaths would occur in 2017 (171). Endometrial cancer is a heterogeneous disease from a histopathological standpoint. The vast majority of endometrial carcinomas have their origin in the endometrial lining called the endometrium and these cancers are referred to as adenocarcinomas. Furthermore, sarcomas exist, which arise mainly from the smooth muscle tissue or stromal cells of the uterus (160).

5.4.1 Precursor lesions of type I endometrial cancer and its tumorigenesis

Tumors of (low-grade) endometrioid histology and categorized as type I are believed to develop through hyperplastic lesions likely as a consequence of excessive estrogen

stimulation combined with inadequate progesterone levels (185). Endometrial hyperplasia means thickening of the endometrium, the lining of the uterus, caused by cellular overgrowth. Molecular alterations in *KRAS*, *BRAF* and *PTEN*, a high frequency of MSI, and hypermethylation of specific TSGs have been detected in endometrial hyperplastic lesions and seem to be early developments in endometrioid endometrial carcinogenesis (186-191).

For 20 years, according to the World Health Organization 1994 (WHO94) and the revised WHO2003 schema, the differences in histological complexity of the glandular architecture (either simple or complex), the presence or absence of nuclear atypia (atypical and non-atypical), and the risk of precursor progression into cancer, have been used to divide hyperplasias into four categories, namely simple and complex non-atypical as well as simple and complex atypical hyperplasia (159, 192-194). Based on several publications, the risk of atypical endometrial hyperplasia to progress into carcinoma has been estimated as being up to 30%, and therefore these are currently seen as precursor lesions of endometrial carcinoma (190, 192). According to these results, in 2014 the WHO decided to classify tumors into two categories, namely non-atypical and atypical hyperplasia, and this classification is based only on nuclear atypia (WHO2014) (195). This WHO2014 schema is currently recommended to be used on categorizing of hyperplasias in Finland.

The categorization of endometrial hyperplasia is not universal and in addition to the WHO2014, another schema for endometrial atypical hyperplasia classification exists, referred to as endometrial intraepithelial neoplasia (EIN) (196). This schema, developed by the International Endometrial Collaborative group, takes into account the clonal origin of the lesions as well as all of the criteria and terminology that clearly divide atypical hyperplastic lesions into different categories that can be managed differently with a specific management protocol recommended for each category. This system classifies the precursor lesions into benign (non-atypical hyperplasias which are hormone-dependent and these changes are reversible), premalignant (atypical hyperplasias) and malignant

(carcinoma) according to the data emerging from histological, genetic, and clinical analyses (159, 185, 196, 197).

At present, total hysterectomy (removal of uterus and cervix) is the standard method of treating atypical hyperplasia whereas hyperplasia without atypia is treated with medication in the majority of the cases (185). Too little is still known about the efficacy of the nonsurgical methods, such as progestin-based therapy of atypical hyperplasia, which is a desirable alternative in patients who wish to retain fertility and when surgery is not an applicable option for the patient (185).

5.4.2 Lynch syndrome associated endometrial cancer

Around 3 to 5 % of endometrial carcinomas are likely caused by inherited predisposition with LS being responsible for most of these cases (198, 199). Moreover, around 10% of all early-onset (under age 40) endometrial carcinomas are diagnosed with a deleterious LS causing mutation (200). The cumulative incidence of endometrial carcinoma in female LS mutation carriers ranges from 43% to 57% depending on the type of mutation (181). Compared to the general population, LS-associated endometrial cancer occurs in younger women (mean 50 years vs. 68 years), typically before menopause, most (~90%) LS-associated endometrial carcinomas have an endometrioid histology belonging to type I carcinomas (201), and a lower uterine segment involvement is detected in up to 29% of the cases compared to less than 5 % in sporadic cases (202). In addition, over 90% of the LS-associated cases show MSI (203). There are conflicting results whether the Lynch-associated endometrial cancer has a worse prognosis compared to the respective sporadic cases (204).

The identification of defective MMR system among endometrial carcinoma patients is important, because it may enhance prognostication, it can help in guidance of targeted therapy and it improves the identification of LS patients (205). It is still a matter of debate, whether all endometrial carcinomas should be tested with IHC of MMR proteins.

Eventually, as the cost and feasibility of sequencing will become reasonable, all endometrial carcinoma cases will be sequenced to detect mutations in the MMR genes. Until that day, if resources permit, IHC will become the primary method to detect abnormal MMR protein expression in laboratories and hospitals (140).

AIMS OF THE STUDY

In addition to the MMR defects, little is known about the molecular background of LS-associated ovarian carcinoma. Moreover, molecular changes prior to endometrial and ovarian cancer and the sequence of events leading to their appearance remain unsolved. The aim of the thesis projects was to identify genetic and epigenetic alterations involved in LS-associated and sporadic ovarian and endometrial tumorigenesis.

The specific aims were:

1. To identify epigenetic, genetic and gene expression alterations in LS-associated and sporadic ovarian cancer (I-III)
2. To investigate epigenetic mechanisms in ovarian tumorigenesis and in particular, to discern differences and reveal similarities between LS-associated and sporadic ovarian cancer as well as between different histological types of ovarian cancer (II)
3. To determine the molecular changes that precede endometrial and ovarian cancer (III)

MATERIALS AND METHODS

1 Cell lines (II-III)

Commercial cell lines (endometrial and colorectal cancer cell lines purchased from American Type Culture Collection, ATCC, Rockville, USA and ovarian cancer cell lines provided by R. Bützow who was involved in all thesis projects) were used in optimization and validation of custom MS-MLPA test (specified in section 6.2) as well as in the epigenetic drug treatments to detect methylation consequences (specified in section 6.3.1). The DNA from cell lines was extracted using the method described by Lahiri and Nurnberg (1991) (206).

2 Patient samples (I-III)

For the studies I and II, all available LS-associated ovarian carcinomas and their respective normal samples were identified from the nationwide Hereditary Colorectal Cancer Registry of Finland, followed by collection of all available archival FFPE samples. In the third study, we took advantage of a surveillance program against gynecological carcinoma which has been offered to women with LS in Finland since 1996. Thus, additional newly diagnosed cases of LS-associated ovarian cancer as well as all patients diagnosed with endometrial carcinoma and/or endometrial hyperplasia and their consecutive aspiration biopsies from this surveillance program were identified from the registry and collected. Sporadic samples of ovarian (207) and endometrial carcinoma (208), endometrial hyperplasias (189)(and original Publication III), reference normal endometria (189, 209) and fallopian tubes (n=22) (used in thesis studies I and II) representing histological types common in LS carriers were collected from larger sporadic cohorts and studied for comparison. The number of specimens included in studies are given in **Table 3**. For more information of the tumor characteristics, please see original publications I-III. A four category system for the classification of hyperplasias (simple hyperplasia, SH; simple atypical hyperplasia, SAH; complex hyperplasia without atypia, CH; and complex hyperplasia with atypia, CAH)

according to WHO2003 (194) was applied for interpretation of hyperplasias because that same categorization was being used at the time of diagnosis.

Table 3. Number of specimens of Lynch and sporadic used in studies.

	Ovarian cancer	Endometrial cancer	Endometrial hyperplasias	Normal endometrium
Lynch				
I	20	–	–	49
II	19	–	–	7
III	23	35	56	99
Sporadic				
I	87	–	–	18
II	84	–	–	18
III	87	36	76	38

All patient material consisted of FFPE tissue archived in blocks, which had been cut into 4 µm sections with a microtome, stained with hematoxylin and eosin for visual inspection. Areas with over 60% tumor cell coverage were chosen and manually microdissected for DNA extraction which was performed according to the customized protocol devised by Isola et al. (210). All ovarian and endometrial tissue material was reviewed by a gynecological pathologist at the time of diagnosis, and the diagnosis was further re-evaluated by a collaborator (Bützow R.) when samples were collected.

The studies were approved by the Institutional Review Boards of the Departments of Surgery (466/E6/01) and the Obstetrics and Gynecology (040/95) of the Helsinki University Central Hospital (Helsinki, Finland) and the Jyväskylä Central Hospital (Jyväskylä, Finland) (Dnro 5/2007). The archival specimen collection was approved by the National Authority for Medicolegal Affairs (Dnro 1272/04/044/07, original publications I and II) and the National Supervisory Authority for Welfare and Health (Valvira/Dnro 10741/06.01.03.01/2015, original publication III).

A detailed description of patient materials and cell lines as well as methodology used in thesis, including information primers and probes, can be found in the original publications I-III. A summary of the methods included in the publications see **Table 4**.

Table 4. Summary of methods included in thesis.

Method	Publication
Processing of FFPE samples for DNA extraction and IHC	I-III
DNA extraction and quantification	I-III
RNA extraction and quantification	I
Primer design and DNA sequencing	I-III
Mutational analyses	I
Single-strand conformation poymorphism analysis (SSCP)	I
RNA profiling by microarray	I
Identification of patients with OvCa, EnCa and endometrial hyperplasia from hereditary CRC registry of Finland and collection of samples nation-wide	III
Microsatellite instability (MSI) analysis	I-III
Immunohistochemistry (IHC)	I, III
DNA bisulphite conversion	II-III
DNA bisulphite sequencing	II-III
Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)	I-III
Statistical analyses	I-III

3 Protein expression by immunohistochemistry (I, III)

Immunohistochemistry (IHC) was used to analyze the protein expression status of the specimen. At the beginning of IHC of each protein analyzed, the 4µm FFPE tissue sections were deparaffinized with xylene and dehydrated with graded alcohols. After antigen retrieval, tissue slides were counterstained with hematoxylin (Mayers HTX, Histolab), dehydrated, cleared in xylene, and mounted. IHC was carried out on individual whole-slide sections from LS-associated cases and on tissue microarray (TMA) slides containing sporadic ovarian and endometrial carcinomas. The slides were scored by two investigators and pathologists specialized in gynecology (Bützow R. and Pasanen A.).

3.1 p53 protein (I)

Expression of p53 protein was analyzed using ultraView Universal DAB Detection Kit with Cell Conditioning Solution (CC1, Ventana Medical Systems INC, Tucson, AZ). Anti-p53 (1:200; clone DO-7, Dako, Glostrup, Denmark) was used as the primary antibody. The expression of p53 was regarded as abnormal by two distinct expression profiles; (1) if over 50% of tumor nuclei were strongly stained indicating overexpression and (2) if expression was completely lost but stromal cells stained positive indicating silencing of p53.

3.2 ARID1A and L1CAM proteins (III)

PT-Module (Lab Vision, CA, USA) was performed for antigen retrieval at 98C°/20 minutes using Envision TM Flex Target Retrieval solution, pH 6.1 for ARID1A and pH 9 for L1CAM (Agilent technologies, USA). The following antibodies were used: Covance SIG-39110-200 produced in mouse for L1CAM (1:40 for 20 minutes, CD171, clone 1E11, Covance) and anti-ARID1A antibody produced in rabbit for ARID1A (1:200 for 20 minutes, HPA005456, polyclonal, Lot D104841, Sigma-Aldrich, USA). Autostainer 480 automated immunostainer (Lab Vision, CA, USA) was used for staining. Examples of expression and scoring are shown in **Figure 6**.

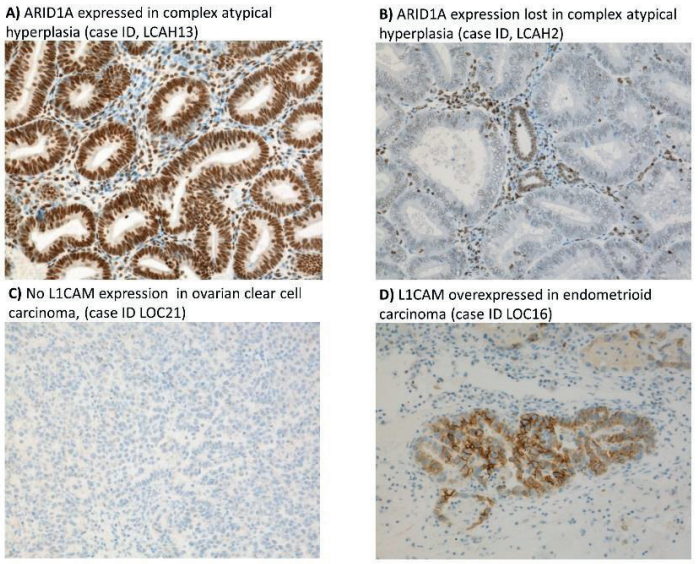


Figure 6. Examples of normal and abnormal IHC results of ARDI1A and L1CAM proteins. **A**, ARID1A is positive/normal in all nuclei and **B**, negative/abnormal when there is no nuclear staining of the tumor cells but stromal cells show positive expression functioning as an internal control. **C**, Membranous L1CAM staining of cells is scored as negative/normal when less than 10% of tumor cells express L1CAM and **D**, positive/abnormal when more than 10% of tumor cells express L1CAM.

3.3 MMR genes (I, III)

Whole-slide and TMA sections were immunohistochemically stained with mouse primary antibodies detecting MLH1 (anti-MLH1, 1:40, clone G168-15, BD Biosciences/Pharmingen, Erembodegem, Belgium), MSH2 (anti-MSH2, 1:60, clone FE11, Calbiochem/Oncogene

Research, Darmstadt, Germany), MSH6 (anti-MSH6, 1:60, clone 44/MSH6, BD Biosciences) and PMS2 (anti-PMS2, 1:400, clone A16-4, BD Biosciences). MMR expression was regarded as negative/abnormal when there was no nuclear staining of tumor cells and the internal control (typically stromal cells, tumor infiltrating lymphocytes or endothelium) displayed a positive expression. Negative immunostaining of the tumor tissue was interpreted to indicate the inactivation of the particular MMR gene.

4 Microsatellite instability (MSI) analysis (I, III)

MSI was analyzed by DNA fragment analysis using polymerase chain reaction (PCR) with mononucleotide repeat markers BAT25 and BAT26. These MSI-markers are sensitive and specific and have been shown to define the MSI-status with high accuracy (211, 212). Products labeled with fluorescent dyes were sequenced with ABI 3730 Automatic DNA Sequencer and GeneMapper 4.0 and 5.0 softwares (Applied Biosystems) were obtained for visual interpretation of results. Samples with stable repeat markers were interpreted as microsatellite stable (MSS), whereas those with at least one unstable repeat marker was considered as MSI.

5 Mutation analysis

5.1 *KRAS*, *BRAF* and *PIK3CA* (I, III)

Known hotspot mutations in *KRAS*, *BRAF* and *PIK3CA* were analyzed by exon-specific DNA sequencing. Before sequencing, all gene products were amplified by PCR. The primer sequences and PCR protocol are described in original Publication I. The PCR products were sequenced with ABI 3730 Automatic DNA Sequencer (Applied Biosystems) using BigDye Terminator v.3.1 chemistry.

5. 2 Single strand conformation polymorphism (SSCP) analysis (I)

SSCP analysis allows two sequences of identical length to be distinguished from each other on the basis of their distinct conformations in gel electrophoresis (213). Thus, all the

samples identified as positive for the *KRAS* mutation by sequencing were further investigated by SSCP analysis to verify the mutational status against positive reference samples carrying known *KRAS* mutations. Sample DNA amplified in PCR was separated on a polyacrylamide gel accompanied with 1 x MDE Gel Solution (Cambrex BioScience Rockland Inc., ME, USA) at 3W for 20 hours followed by silver staining of the gel for visual detection of DNA.

6 DNA methylation analysis (I-III)

6.1 Bisulfite modification, direct bisulfite sequencing, and sequencing after cloning (II, III)

Bisulfite modification and direct bisulfite sequencing was used as a method to select a representative region for the MS-MLPA probe design and to validate the methylation data obtained from a custom designed MS-MLPA test (original publications II and III). In brief, 600ng of DNA from 13 cancer cell lines, normal colon and endometrial DNA (purchased from AMS Biotechnology, UK) as well as blood from a so-called healthy donor were bisulfite modified by using EZ DNA methylation Direct™ Kit (Zymo research, CA, USA) following the manufacturer´s instructions (version 1.0.7). Bisulfite modification refers to treating of DNA with sodium bisulfite ($NaHSO_3$) which deaminates unmethylated cytosine nucleotides converting them into a uracil nucleotide, whereas methylation protects cytosine from conversion and leaves it intact (214). The MethPrimer-program (215) and manual designing when appropriate were used to build bisulfite primers for selected ovarian and endometrial cancer related gene promoters. The detailed characteristics of primers and PCR protocol can be found in original Publication II. The PCR products were sequenced with Applied Biosystems ABI3730 Automatic DNA Sequencer.

Bisulfite sequencing was additionally used after cloning of bisulfite-converted and PCR amplified fragment (*SFRP2* was used as an example) to prove the quantitative nature of MS-MLPA test (see original publication II). PCR amplification products of *SFRP2* gene from

five cancer cell lines and normal colon and endometrial samples representing distinct types of methylation statuses were cloned into *Escherichia coli* bacterial cells in pCR2.1 TOPO vector by utilizing the TOPO TA Cloning System (Invitrogen, USA). After cloning, all produced white bacterial colonies were collected, DNA was extracted and sequenced with bisulfite sequencing primers. The methylation status of the HhaI restriction site (GCGC) chosen to be included in MS-MLPA probe was analyzed for each clone and interpreted as either methylated or unmethylated to determine the proportion of methylated DNA. Methylation dosage ratio (Dm) values between clones and the MS-MLPA result of *SFRP2* were concordant.

6.2 MS-MLPA (I-III)

All methylation data produced from patient samples was conducted by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) test (**Figure 7**), first introduced by Nygren et al. in 2005 (216). MS-MLPA test is based on probes that contain a restriction site (GCGC) for the methylation-sensitive endonuclease HhaI (Promega, USA), which binds to the unmethylated CpG dinucleotide of a GCGC site and subsequently digests the site. If the GCGC is methylated, then the site stays undigested and will generate a signal peak in PCR. All MS-MLPA analyses involving TSGs were conducted according to the manufacturer´s protocol (217) using 100 to 250ng of DNA extracted from FFPE samples. The PCR products were separated by capillary electrophoresis performed with ABI 3730 Automatic DNA sequencer (Applied Biosystems, USA) and analyzed by GeneMapper 4.0 and 5.0 genotyping software (Applied Biosystems). For each sample analyzed, the MS-MLPA method produces a Dm-value which is calculated as described in Gylling et al. (218). The Dm-value varies between 0 and 1 corresponding to the frequency of methylated DNA in the specific GCGC site analyzed.

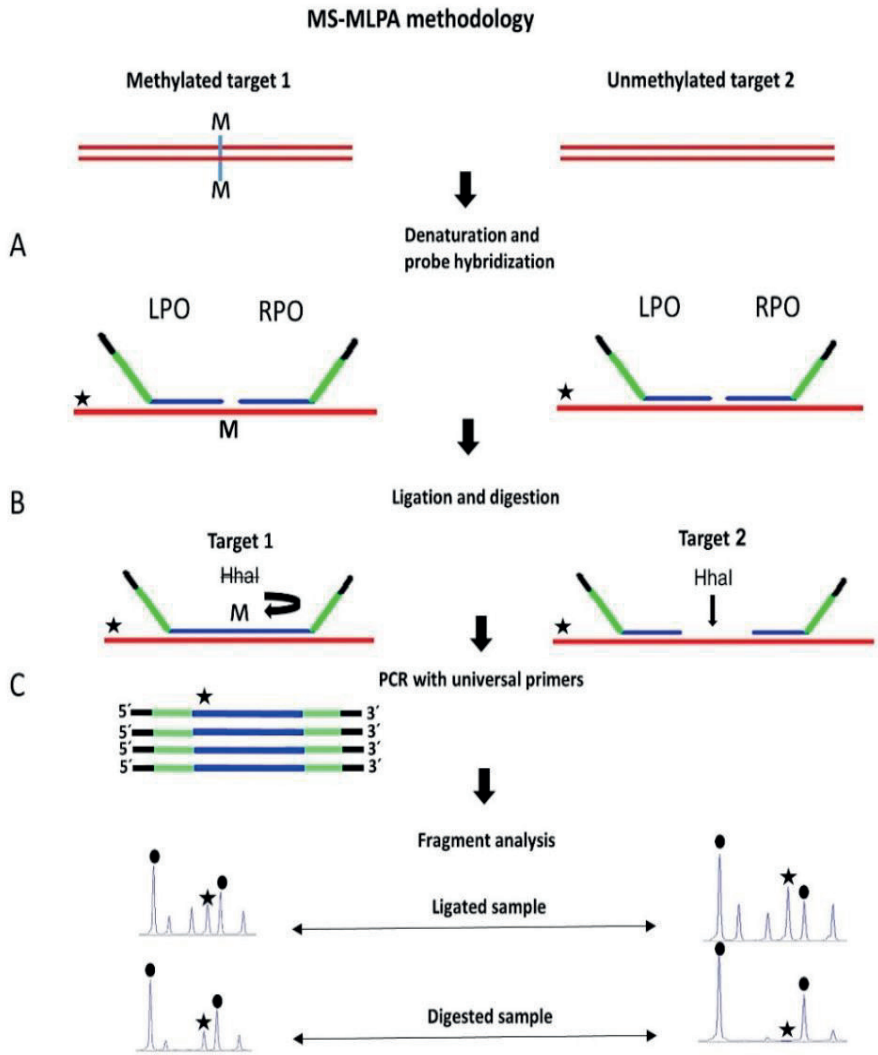


Figure 7. General outline of the MS-MLPA method. The probe targeting a specific gene promoter consists of two oligonucleotides (left and right oligo probes, LPO and RPO), each probe containing a universal primer sequence (black), a hybridizing sequence (blue), and a stuffer sequence (green) when needed. A, the method starts with denaturation of DNA and hybridization of the probes to their target sequences. B, ligation and digestion are performed in two tubes: The first tube both ligation and digestion take place whereas in the second tube only ligation reaction is carried out (not shown in the figure). In the first tube, only the probe pairs that target a methylated GCGC site will ligate, since the HhaI restriction enzyme does not recognize and digest the site. If the target site is unmethylated, HhaI enzyme will digest the site and the LPO and RPO will not be ligated. C, only ligated probes are exponentially amplified in subsequent PCR and show as peaks in the electropherogram. Black dots indicate the reference probes, that do not contain HhaI restriction site and will be amplified and generate a peak in ligated and digested reactions. A star indicates the target DNA sequence and shows a peak in the digested sample only when the target DNA is methylated.

Commercial MS-MLPA test (SALSA ME001-C2 Tumour suppressor 1, MRC Holland, The Netherlands) was applied to analyze methylation patterns of 24 general tumor suppressor genes (TSGs) (219) often known to be methylated in several cancer types. The threshold Dm-value of 0.15 or above was considered to represent methylation as previously described (218) for all commercial TSGs except for *CDKN2B* (Dm cut-off = 0.34), which was noticed by MRC-Holland to give higher values than expected.

A custom MS-MLPA test was designed to detect abnormal methylation of 11 TSGs and two miRNA genes (genes specified in original publication II) often methylated in endometrial and ovarian cancer. The methylation patterns of CpG islands in promoters of selected genes containing a GCGC site were first investigated by bisulfite sequencing of cancer cell line and normal sample DNA followed by designing of custom MS-MLPA probes that were optimized according to the results obtained from bisulfite sequencing to target representative HhaI restriction sequences (GCGC) and by bacterial cloning of *SFRP2* gene as described briefly above and more detailed in original publication II. The custom MS-MLPA probes were designed following the instructions from MRC Holland (217). CpG islands of selected genes were detected using EMBOSS CpG Plot software (220). Custom probes were combined with SALSA MLPA kit P-300-B1 human DNA reference-2 (221) to carry out MS-MLPA reactions. Since the baseline level for methylation that distinguishes tumor from normal depends on the normal tissue as well as probe analyzed, the thresholds for methylation were calculated individually for each endometrial and ovarian cancer related gene (see original Publications I and III). In brief, the thresholds for each gene were calculated separately for LS-associated and sporadic case according to average methylation levels in normal endometrium (for comparison of non-serous samples) and in fallopian tubes (for comparison of serous samples) plus 1 standard deviation.

6.2.1 LINE-1 hypomethylation analysis (I)

A custom-made MS-MLPA test designed by Pavicic et al. (222) was used to measure hypomethylation from LS-associated ovarian carcinomas and their respective normal

samples when available as well as from sporadic ovarian carcinomas by using 50 to 100 ng of FFPE DNA. This custom MS-MLPA test contains three LINE-1-specific probes with HhaI restriction site and is combined with the SALSA MLPA kit P300-A1 Human DNA Reference-2 (221). The test was carried out following the customized standard MS-MLPA protocol, defined by Pavicic et al. (222).

6.3 Expression based methods

6.3.1 Cell culturing and epigenetic drug treatments

Gene expression can be regulated by epigenetic modifications, such as DNA methylation and histone acetylation. Chemical agents, 5-aza-2'-deoxycytidine (5-aza-dC) and trichostatin A (TSA) can be used to modify these epigenetic alterations and change the regulative stage of a protein. 5-aAza-dC functions as a strong inhibitor of methyltransferase causing demethylation and reactivation of epigenetically inactivated genes (223). TSA, on the other hand, inhibits HDAC activity and leads to an opening of the chromatin structure (224, 225). 5-Aza-dC and TSA have been reported to exert a synergistic effect in the re-activation of expression of genes epigenetically silenced by methylation (226, 227). Therefore, both chemical agents were used in order to achieve the highest possible reactivation state of genes silenced by promoter methylation and further to confirm the methylation consequences of selected genes.

Different cell lines were chosen for treatments to serve as models for different types of carcinomas and MMR status. The cell lines were cultured as described in the supplier´s instructions (ATCC, Rockville, MD, USA) and then treated according to protocol by Derks et al. (228) with 5-aza-dC (1µM, Sigma, A3656) and TSA (300nM, Sigma, T1952) for 96h and 18h, respectively. All drug treatments were done in duplicate to verify the effect of the treatment on the cell line. After treatments, the cell line DNA was extracted using the standard protocol by Isola et al. and total RNA was isolated with miRNeasy mini kit (Qiagen, CA, USA). The performance of drug treatments was verified by comparing the results before

and after treatment by investigating the promoter methylation of selected TSGs using SALSA MS-MLPA ME001-C1 (219) test.

6.3.2 Genome-wide RNA expression profiling of cell lines (I)

Genome-wide mRNA gene expression analysis was accomplished using Affymetrix Human Genome U133 plus 2.0 GeneChip® microarrays (Affymetrix, Santa Clara, CA) whereas miRNA expression was analyzed using Agilent´s human miRNA microarrays (8 x 15 K, Agilent Technologies, G4470B). RNA isolated from treated and untreated cell lines as well as respective normal samples (Amsbio, Abingdon, UK or extracted from fresh-frozen tissues obtained from national hospitals) were amplified, labeled and hybridized as characterized in Nymark et al. (229). Array image and fluorescent signals were analyzed using GeneChip operating software (from Affymetrix) for mRNA data whereas fluorescent signal intensities from miRNA expression array were calculated according to the Feature Extraction software (version 10.7.3.1, Agilent).

Microarray data analysis was carried out by GeneSpring GX software, version 12 (Agilent Technologies, Santa Clara, CA) for both mRNA and miRNA expression. The following parameters were established to evaluate distinct mRNA and miRNA expression patterns between treated and untreated cell lines and normal samples: (1) mRNA expression was RMA normalized whereas quantile normalization was used for miRNA data, (2) the statistical significance of gene expression changes was identified by moderated t-test integrated with Benjamini and Hochberg correction for multiple testing and (3) filters based on p-value cut-off of 0.05 and fold change cut-off +/-1.5 were chosen to identify distinct expression.

7 Statistical analyses (I-III)

Statistical evaluations were performed using SPSS software, versions 20.0 and 22.0 (IBM® SPSS® Statistics, Inc. Chicago, IL, USA) as well as using Vassarstats programs (230). Fisher´s exact test was applied to calculate frequency data in pairwise comparisons of gene

expression status as well as MMR status (two-tailed *P*-values) and adjusted for multiple comparisons by Bonferroni correction when appropriate. Comparisons between two groups involving Dm-values of specific genes or numbers of methylated genes, Shapiro-Wilk test was implemented first to test if the data were normally distributed. Student´s T-test was applied for normally distributed samples and Mann-Whitney U test for samples not normally distributed. The non-parametric Kruskal-Wallis test with pairwise comparisons was applied when analyzing statistical significance of methylation changes between multiple categories of endometrial specimens. Kruskal-Wallis was chosen because either all groups did not reach the homogeneity of variances studied by Levene´s test or were not normally distributed. *P* values < 0.05 (2-tailed) were considered significant. Pearson product-moment correlation coefficient for normally distributed (tested by Shapiro-Wilkins test) data and Spearman rank correlation coefficient for data not normally distributed was applied to test statistical significance between methylation and expression correlation. The detailed description of statistical analyses used for gene expression data are depicted above in section 6.3.2.

RESULTS

1 Novel molecular profile of Lynch syndrome associated ovarian cancer (I-III)

At the beginning of this thesis project in 2011, rather little was known about the exact molecular background of LS-associated ovarian cancer and in general, the origin of ovarian cancer was just starting to be revealed. The clinical and histological differences between LS-associated and sporadic ovarian cancer had been acknowledged but the underlying genetic and epigenetic causes of these differences other than MMR defects remained a mystery. It was particularly interesting to examine whether there are molecular differences that can explain the better survival of ovarian cancer in LS-associated versus sporadic ovarian cancer (183). Prompted by this lack of knowledge, we decided to investigate established genetic and new epigenetic markers involved in ovarian cancer from LS-associated ovarian carcinomas and compare results to cases from sporadic cohorts with corresponding histological types. Among the genetic markers studied, there were the known hotspot mutation sites from *KRAS*, *BRAF*, and *PIK3CA* which were identified by exon specific sequencing, whereas immunohistochemistry (IHC) was used to detect aberrant protein expression of the MMR, *p53*, *ARID1A*, and *L1CAM* genes. Additionally, MSI analysis was carried out to verify the MMR status and to detect MMR deficient cases missed by MMR IHC. Epigenetic analysis was carried out by investigating promoter methylation of 37 TSGs shown to be involved in tumorigenesis.

All available cases with ovarian cancer were identified from the nationwide Hereditary Colorectal Cancer Registry of Finland and collected as part of the project. Overall, 14 cases of endometrioid and 9 cases of clear cell ovarian carcinomas were collected from 22 MMR mutation carriers and the results were compared to 39 and 28 cases of clear cell and endometrioid type of ovarian cancer, respectively, from sporadic cases. Additionally, LS-associated cases included two serous cases (one low- and one high-grade). For comparison of the results from carcinomas, 18 normal unrelated endometrial samples (the expected origin of endometrioid and clear cell ovarian carcinomas) were analyzed. The sporadic high-

grade serous category was included in the analysis in original Publications I and II. The next sections will concentrate on the findings from non-serous (endometrioid and clear cell) histological types of ovarian cancer, which are the prevalent histological types among LS mutation carriers.

1.2 Genetic profile of Lynch syndrome associated ovarian cancer (I, III)

1.2.1 Deficient MMR status is a key feature of ovarian carcinomas from Lynch mutation carriers (I-III)

Lynch patients inherit one inactive allele of a MMR gene (or *EPCAM* gene in rare cases). One inactive gene copy in all of the cells of an individual´s body predisposes the individual to a high risk of cancer. However, in order for tumor development to start, a second somatic alteration of the other parental allele needs to occur in the target tissue (140). Deficient MMR status of a sample marks inactivation of the wild type allele by somatic alteration. Accordingly, we regarded MMR status deficient if MSI was found by microsatellite analysis, or a loss of MMR protein expression detected by IHC, or both. Deficient MMR is a key feature of LS-associated ovarian carcinomas but also a common characteristic of type 1 ovarian cancer of sporadic origin (231). All (23/23, 100%) LS-associated type I ovarian carcinomas were MMR deficient (see **Figure 8** and **9**). In sporadic ovarian carcinomas, MMR-deficiency was detected in 14% (4/28) of endometrioid and 15% (6/39%) of clear cell ovarian carcinomas. The difference was statistically significant (P<0.001) between LS and sporadic cases of corresponding histological types.

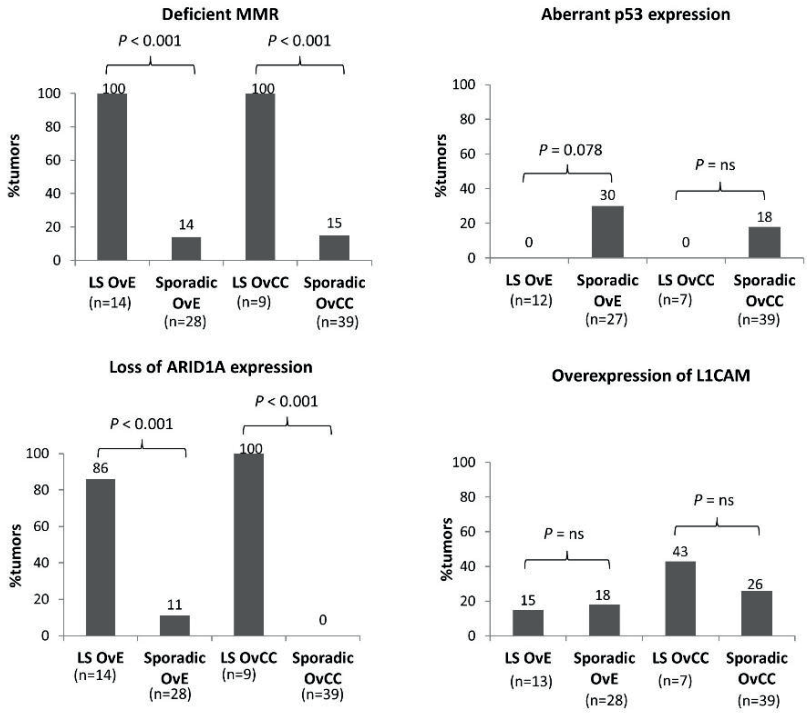


Figure 8. Frequencies of abnormal status of p53, MMR, ARID1A, and L1CAM expression in LS-associated versus sporadic type I ovarian cancer. Abbreviations: OvE, endometrioid ovarian cancer; OvCC, clear cell ovarian cancer.

1.2.2 p53, ARID1A and L1CAM expression profiles in LS-associated ovarian cancer (I, III)

A TSG p53 is often abnormally expressed in several cancer types and is a typical feature of type II (mainly high-grade serous) ovarian carcinomas (114). Normal expression of p53 was detected in all LS-associated endometrioid (12/12) and clear cell (7/7) ovarian carcinomas whereas abnormal expression was present in 30% (8/27) of endometrioid and 18% (7/39) of clear cell ovarian carcinomas of sporadic origin as demonstrated in **Figures 8** and **9**. When endometrioid and clear cell histological types were combined and the results were compared between LS (0%, 0/19) and sporadic cases (23%, 15/66), sporadic cases showed a significantly higher frequency of aberrant p53 expression (*P*=0.035) in non-serous tumors.

ARID1A, a tumor suppressor and a subunit of SWI/SNF chromatin remodeling complex, is often mutated in type 1 ovarian tumors, irrespective of MMR-status (114, 232). Surprisingly, LS-associated ovarian carcinomas revealed an exceptionally high frequency of aberrant ARID1A expression. All LS-associated clear cell (9/9) and 86% (12/14) of the endometrioid ovarian carcinomas had lost their ARID1A expression which was in striking contrast to sporadic cases where ARID1A expression was absent in none of the clear cell (0/39) and in only 11% (3/28) of the endometrioid ovarian carcinomas. The differences between different histological types of LS and sporadic ovarian tumors of corresponding histological types were significant (P<0.001) as shown in **Figure 8**.

Overexpression of L1CAM is connected to invasion and metastatic potential in cancer and aberrant expression of L1CAM has been detected in endometrial and ovarian cancer by several studies (233-237). Compared to the high prevalence (86% – 100%) of aberrant ARID1A expression and MMR defects in LS-associated ovarian carcinomas, L1CAM aberrations were less frequent. L1CAM overexpression showed the highest frequencies in ovarian clear cell carcinomas: 43% (3/7) in LS-associated and 26% (10/39) in sporadic clear cell ovarian carcinoma compared to 15% (2/13) and 18% (5/28) of endometrioid ovarian carcinomas in LS-associated and sporadic cases, respectively (A.N. et al. unpublished data). The differences between LS and sporadic cases were not statistically significant which in part may reflect the small sample series (**Figure 8**). Although, overexpression of L1CAM has been associated with a dismal prognosis in ovarian cancer (235) in a recent publication by Soovares et al. (207) the dismal prognosis was shown to be associated only with endometrioid but not in clear cell type of ovarian cancer. According to results from Soovares et al. (207) our finding of frequent L1CAM overexpression in LS-associated clear cell ovarian cancer is in agreement with the high survival among LS-associated ovarian carcinomas (8, 183). Moreover, the only two endometrioid ovarian carcinomas that were detected with aberrant L1CAM expression were characterized as grade 2 carcinomas, thus showing a higher grade (A.N. et al. unpublished data).

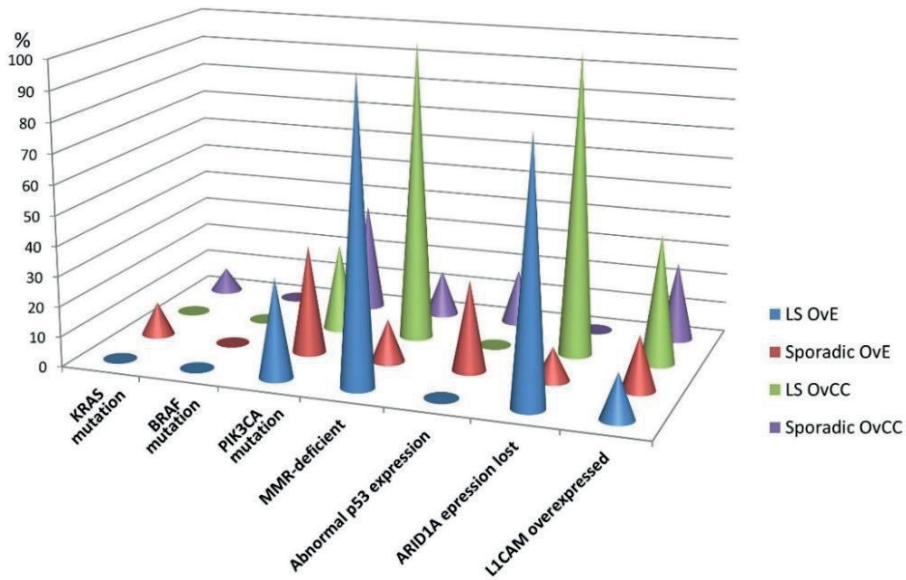


Figure 9. Genetic profile of LS-associated vs. sporadic non-serous ovarian carcinomas.

1.2.3 *KRAS*, *BRAF* and *PIK3CA* mutations in LS-associated ovarian carcinomas (I)

Type I ovarian carcinomas often show mutations in *KRAS*, *BRAF* and *PIK3CA* genes (157, 238). As a unique feature of LS-associated ovarian carcinomas, these completely lacked mutations in *KRAS* exon 2 and *BRAF* V600E. The BRAF mutations were also absent in sporadic endometrioid and clear cell ovarian carcinomas, instead *KRAS* mutations were detected in 11% (3/27) of endometrioid and 8% (3/37) of clear cell ovarian carcinomas as shown in **Figure 9**. The observed differences between LS and sporadic ovarian carcinomas did not reach statistical significance.

PIK3CA mutations are detected in 20 to 40% of sporadic endometrioid and clear cell (non-serous) but are rare in serous ovarian carcinomas according to the literature (239, 240). Our results were in agreement with previous findings from sporadic tumors: *PIK3CA* mutations were present in similar frequencies in LS-associated (6/19, 32%) and sporadic (24/67, 36%) non-serous ovarian cancer, but only in 5% (1/20) of sporadic serous ovarian carcinoma. Thus, hotspot mutations of *PIK3CA* seem to be a characteristic of non-serous histological type and common regardless of hereditary or sporadic background (**Figure 9**).

The lack of p53 expression aberrations and *KRAS* mutations but frequent detection of *PIK3CA* alterations agree with the good prognosis in LS ovarian carcinoma patients, since p53 abnormalities and *KRAS* mutations have been shown to associate with advanced stages and poor prognosis, whereas *PIK3CA* mutations which activate P13K/AKT/mTOR pathway are connected to a propitious prognosis in ovarian cancer (25, 239-241). In addition, our data resembled the genetic background of LS-associated colorectal carcinomas, which also have fewer p53 expression aberrations (242), *BRAF* mutations (243), better stage-specific survival compared to sporadic cases and also harbor *PIK3CA* mutations in approximately 20% of the cases (137).

1.3 Epigenetic profile of LS-associated ovarian cancer (I-III)

In addition to genetic alterations, DNA methylation analyses (by MS-MLPA) were motivated as epigenetic changes provide fingerprints of cancer cell origins (244), are histology-specific (67), and likely to promote tumorigenesis in MMR deficient cells. Hypermethylation of TSG promoters and genome-wide hypomethylation are likely to drive tumorigenesis, since they arise in early steps of tumor development (83). Therefore, it is important to look deeper into the epigenetic changes and differences between different backgrounds and histological subtypes of ovarian carcinoma in order to enhance diagnosis, treatments and survival of the patients.

1.3.1 Hypermethylation of specific gene promoters is a frequent event in LS ovarian tumorigenesis (I-III)

A panel of epigenetic markers to study hypermethylation (13 endometrial and ovarian cancer related and 24 general TSGs often methylated in cancer) was chosen for sample profiling. In addition to commercial MS-MLPA assay including 24 TSGs often methylated in several cancer, we wanted to design a custom test including genes that would be more specific for endometrial and ovarian cancer. Briefly, gene candidates that would be highly informative epigenetic markers specifically for endometrial and ovarian carcinomas were

identified from the literature and by expression profiling of cancer cell lines treated with demethylating chemicals. After evaluation for the most informative markers, a custom MS-MLPA test was designed to include 13 genes. The selected gene candidates fulfilled two key prerequisites: (1) Abnormal methylation at the gene promoter can be used as a marker of a malignant process based on literature and (2) methylation was shown to correlate with expression by expression profiling or by literature. MS-MLPA was chosen as a method to investigate promoter methylation, because it can be used to analyze low quality and fragmented DNA extracted from FFPE tissue blocks. Normal endometrial tissue specimens (18 non-related cases) were used as a reference. All promoters of genes included in the studies except the promoters of *WT1* and *CABLES1* (showing low levels of methylation in tumor and normal samples) as well as *let-7-3a* (oncogenic miRNA showing high methylation levels in tumor and normal samples) displayed a low degree of methylation in normal endometrial tissue and increased methylation levels in non-serous (endometrioid and clear cell) ovarian tumors.

The highest hypermethylation frequencies were detected in genes *RSK4*, *PROM1*, and *MIR34B* in LS-associated clear cell ovarian carcinomas among all histological types and LS and sporadic origin. Moreover, LS-associated endometrioid ovarian carcinomas resembled those found in sporadic cases. *RSK4*, *SPARC*, *PROM1*, *HOXA10*, *HOXA9*, *WT1-AS*, *SFRP2*, *OPCML,* and *MIR34B* were frequently hypermethylated in LS-associated and sporadic ovarian non-serous tumors compared to normal endometrial tissue.

LS-associated and sporadic endometrioid and clear cell ovarian carcinomas were combined for analysis of clinical correlations. One interesting finding was made according to grade analysis with endometrioid ovarian carcinomas, where Dm values of *RSK4*, *SPARC* and *HOXA9* were shown to decrease together with increasing grade of tumors, showing a shift towards the characteristics of high-grade serous tumors (see original publication II for more information). Therefore, lower methylation levels among these three genes may predict a

more aggressive (high-grade) phenotype within endometrioid carcinomas which generally have a favorable prognosis (245).

The same trend of high hypermethylation frequencies of selected genes in non-serous tumors of LS and sporadic background were observed when analyzing the methylation of 24 general TSGs using commercial MS-MLPA test. **Figure 10** demonstrates the methylation profiles of the TSGs most frequently methylated among Lynch-associated ovarian tumors compared to normal endometrium.

Promoter methylation of TSGs has previously been shown to be a common feature of LS-associated endometrial, colorectal, gastric, urinary tract, brain and breast tumors using the same commercial MS-MLPA assay (189, 218, 246-249). The former studies have highlighted that TSG promoter methylation levels and the average number of methylated genes are tissue specific, and additionally, hereditary background affects the methylation profile (246-248). Agreeing with results from other LS-associated tumors, methylation profiles of TSGs in ovarian carcinomas varied depending of the histological type of tumor and hereditary vs. sporadic origin. The most frequently methylated TSGs among 24 were *APC*, *RASSF1*, *TP73*, and *CDH13* and hypermethylation of these genes appears to be a common feature of LS-associated and sporadic non-serous ovarian carcinomas. All these genes, except *TP73* were also frequently methylated in LS-tumors of different types from other studies (189, 218, 246-249). Moreover, Strathdee et al. (250) detected hypermethylation of *TP73* in sporadic ovarian carcinomas, but the frequencies were rather low (13%). Although the frequencies of hypermethylated promoters of *TP73* and *CDH13* were high in tumors, the frequencies were high in normal endometrial tissue of Lynch and sporadic origin as well, and therefore no significant differences were detected between tumor and normal tissue (see **Figure 10**). Since *TP73* and *CDH13* were often methylated already in normal endometrium from LS-associated cases, it may indicate that the promoters of these genes become hypermethylated and silenced early in ovarian (and endometrial) tumor development.

An interesting observation among LS-associated ovarian tumors, was the finding that LS-associated ovarian carcinomas displayed the highest number of hypermethylated genes (4.2/24) and the lowest levels of LINE-1 hypomethylation (Dm=0.91) as compared to the results from all other LS-associated tumors collected from 8 different organs and analyzed using the same techniques (218, 222, 247, 248). These findings suggest that TSG hypermethylation is an important part of ovarian tumorigenesis, especially in LS.

BRCA1 and *BRCA2* are among the most investigated genes in ovarian cancer due to their important role in hereditary and sporadic types of the disease (150-152). Promoter methylation of *BRCA1* is thought to be the cause for the loss of BRCA1 expression in sporadic ovarian cancer (251). Complete or partial silencing of the *BRCA1* gene due to promoter methylation has been observed in 15% of ovarian tumors with a sporadic background and the methylation has been shown to be more frequent among advanced stages (II and III) and serous histological type of ovarian cancer than in stage I and non-serous tumors (250, 252, 253). In contrast to *BRCA1* methylation profile in ovarian cancer, *BRCA2* does not seem to be differentially methylated in ovarian cancer compared to normal tissue (254). *BRCA1* and *BRCA2* were included in the 24 TSG panel, but no methylation in either of the gene promoters were detected among LS-associated or sporadic ovarian carcinomas of different histological types. Our sporadic series included a serous set of ovarian carcinomas (n=20) but no signs of methylation were detected in these carcinomas.

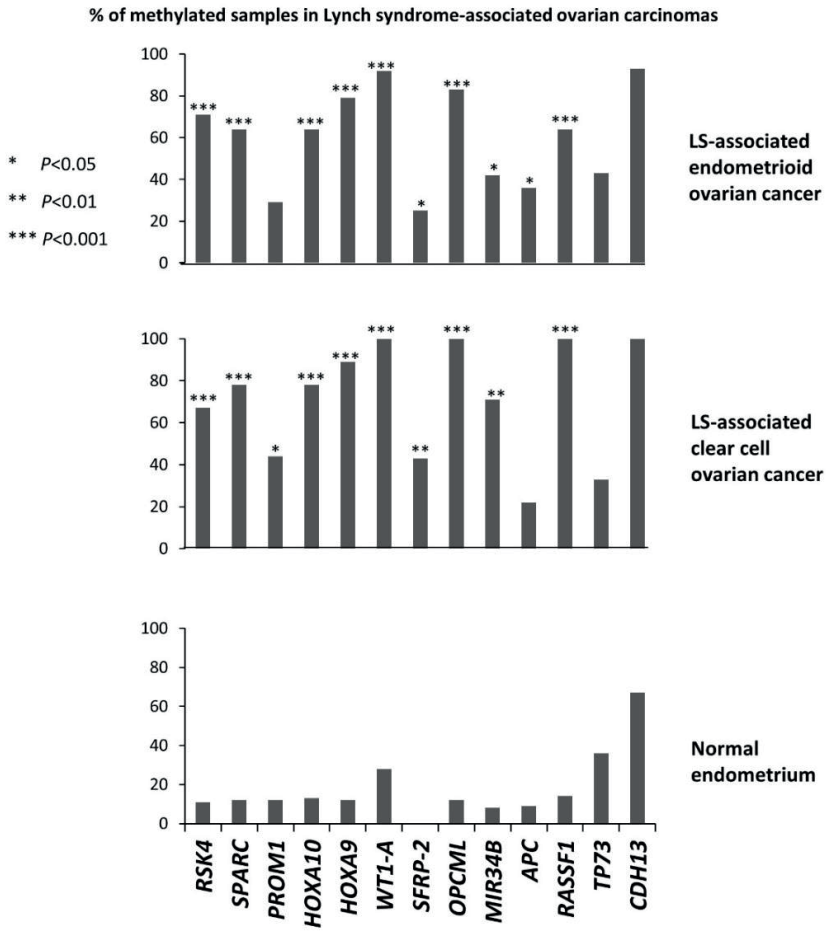


Figure 10. TSG methylation profiles of the most interesting methylation markers in LS-associated endometrioid and clear cell tumors vs. normal reference endometrial tissue. Cut-off Dm-values are gene-specific and can be found in original publications I and III. Only TSGs that showed methylation above its cut-off level in at least 35% of tumors of any histological group were included in this comparison. Asterisks denote significantly elevated methylation in tumor vs. normal endometrium by t-test for independent samples.

1.3.2 Hypomethylation is not a feature of LS ovarian tumors (I)

Decrease in methylation (hypomethylation) of highly repetitive sequences, such as LINE-1, may activate oncogenes and oncogenic microRNAs (98). Moreover, several studies have linked LINE-1 hypomethylation in hereditary and familial cancer (222, 255, 256), the level of LINE-1 hypomethylation can distinguish colorectal carcinoma subgroups (256), and it has been shown to arise early in ovarian carcinoma development (90). Motivated by these

findings, we wanted to investigate LINE-1 methylation levels in normal and tumor tissues from LS-associated and sporadic ovarian carcinoma patients by using custom MS-MLPA method designed by Pavicic et al. (222). LINE-1 is highly methylated in normal cells and often hypomethylated in tumor cells (96) and hypomethylation of this elements has been shown to increase in all histological types of ovarian cancer from normal tissue towards cancer (235, 257). However, in contrast to hypermethylation profiles, hypomethylation does not seem to differ among different histological types of sporadic ovarian cancer (258). Our findings of sporadic ovarian carcinomas are in line with the previous reports, since decreased methylation of LINE-1 compared to normal corresponding tissue was a characteristic of all histological types of sporadic ovarian cancer, and the differences between tumors of all histological types compared to the respective normal tissue were statistically significant. On the other hand, no decrease in the average level of methylation was detected between LS-associated ovarian endometrioid carcinomas (n=12, Dm=0.90) and corresponding normal endometrial tissue (n=49, Dm=0.90) and LINE-1 hypomethylation was even increased in clear cell carcinomas (n=7, Dm=0.94) compared to normal tissue from LS mutation carriers. Thus, our results emphasize that genome-wide hypomethylation is a characteristic of sporadic ovarian cancers, whereas the absence of prominent hypomethylation of LINE-1 may be a feature of LS-associated ovarian carcinoma. The same observation was made later by Sahnane et al. (256).

2 Molecular alterations in progressive endometrial specimens prior to endometrial and ovarian cancer (III)

The molecular aberrations and the sequence of events that lead to endometrial and ovarian tumorigenesis are unknown. In order to investigate early steps in these cancers, we took advantage of a lifelong surveillance program against gynecological cancer provided since 1996 in Finland (13) for women with LS. We collected all consecutive endometrial aspiration biopsy specimens, tumor tissue and respective (pre-malignant) hyperplastic and normal endometrial tissue from hysterectomy and salpingo-oophorectomy from patients identified with endometrial and/or ovarian cancer or endometrial hyperplasia as endpoint.

Altogether 213 samples were obtained from 66 LS mutation carriers and were supplemented with 197 histology-matched specimens from sporadic cohorts. The samples were examined using markers known to be involved in endometrial and ovarian tumorigenesis, including protein expression of ARID1A, L1CAM and MMR proteins, MSI analysis and TSG hypermethylation of the 24 general TSGs often methylated in cancer using commercial MS-MLPA tests (ME-001-C2) and additional 7 markers (*RSK4*, *SPARC*, *PROM1*, *WT1*, *CABLES1*, *HOXA10* and *HOXA9*) often methylated in endometrial and ovarian cancer using custom designed MS-MLPA assay.

Both LS and sporadic series of endometrial specimens revealed accumulation of genetic and epigenetic changes along with the increasing level of histological abnormality of hyperplastic lesions. **Figure 11** demonstrates that loss of ARID1A expression and MMR-deficiency were the most prominent genetic alterations, whereas increasing levels of TSG hypermethylation from low malignant potential to high malignant potential lesions illustrated epigenetic aberrations. Loss of ARID1A expression, deficient MMR and TSG hypermethylation were characteristics of early tumorigenesis, whereas L1CAM was not a particularly informative marker. The loss of ARID1A was detected already in one case of LS-associated complex hyperplasia without atypia (CH) 25% (1/4), and appeared in 20% of cases with complex atypical hyperplasia (CAH) of LS (6/30) and sporadic (4/20) origin. Even earlier changes were MMR-deficiency and TSG promoter methylation which were detected already in histologically normal endometrium of LS-associated cases (**Figure 11**). The presence of MMR-deficiency in histologically normal endometrium in 12% (12/99) of LS specimens compared to 0% (0/38) of sporadic cases was a remarkable feature of LS (the difference was statistically significant between LS and sporadic, P=0.037). An analysis of consecutive endometrial samples showed that in a few cases, MSI was detectable several years before endometrial cancer (see LEC1, original publication III), ovarian cancer (LOC20, original publication III) or endometrial hyperplasia (LCAH5, original publication III). All the cases in which CAH was detected 1 to 9 years before ovarian or endometrial carcinoma diagnosis were MMR deficient (7/7 = 100%). Interestingly, also one case with CH (1/1) and

67% (2/3) of the patients with simple hyperplasia (SH) diagnosis 1-2 years before carcinoma, were shown to be MMR deficient. Thus, MMR deficiency in hyperplastic endometrial specimens may predict endometrial or ovarian tumor development in LS patients.

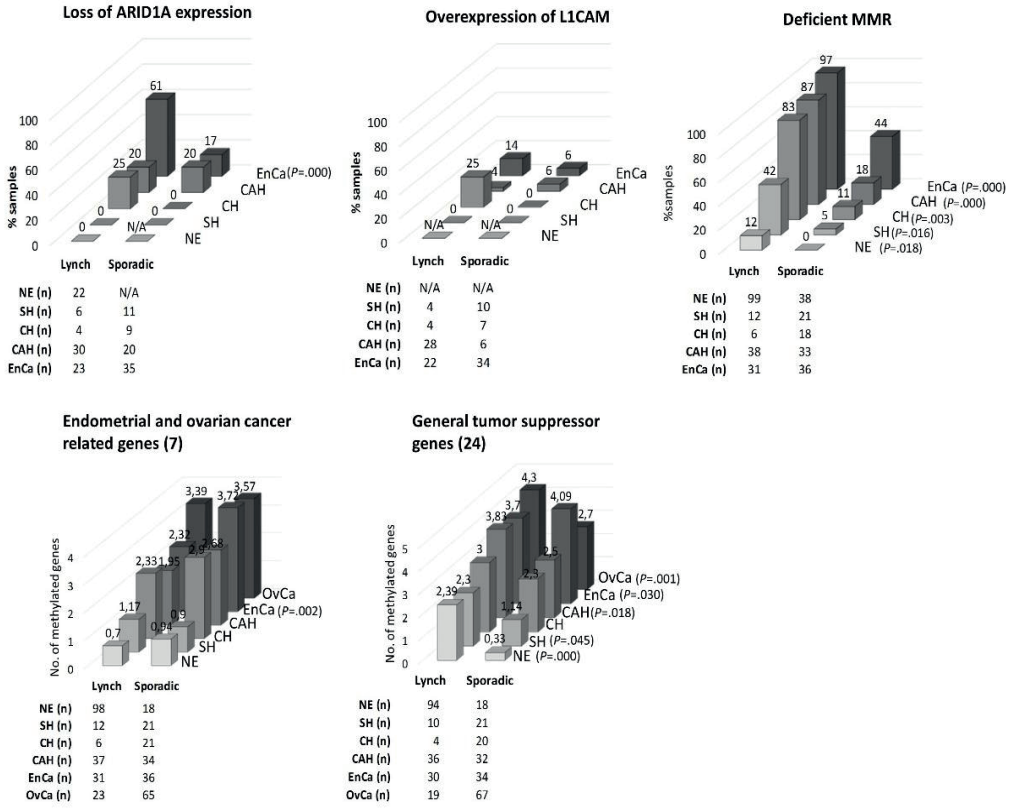


Figure 11. Occurrence of ARID1A, L1CAM and MMR aberrations (upper panel) and average numbers of methylated endometrial and ovarian cancer related TSGs and 24 general TSGs (lower panel) in LS-associated and sporadic endometrial specimens as part of progressive endometrial and ovarian tumorigenesis. Alterations in ARID1A, L1CAM and MMR in ovarian cancer are demonstrated in **Figure 8**. The number of samples studied in each category is given below the bar graphs. *P*-values by Fisher´s exact test for LS vs. sporadic comparisons test are indicated on the right. Only significant values are shown. Abbreviations: N/A, result not available; EnCa, endometrial carcinoma; OvCa, ovarian carcinoma.

Another intriguing finding made by investigating endometrial progressive specimens from LS-associated and sporadic cases was that they could be divided into three categories

according to increasing abnormalities in ARID1A expression, MMR deficiency, and TSG promoter methylation, based on the significant differences between combined groups (detailed categorization explained in original publication III). The categories comprised of normal endometrium (NE) and simple hyperplasia (SH) (I), complex hyperplasia with (CAH) or without atypia (CH) (II), and endometrial cancer (III). The current classification devised by WHO2014 combines non-atypical SH and CH and treats them as non-neoplastic forms for endometrial cancer and only considers CAH (and SAH) as potential premalignant forms of endometrial cancer. In contrast to WHO classification, our analysis with different genetic and epigenetic markers, CH and CAH were molecularly indistinguishable.

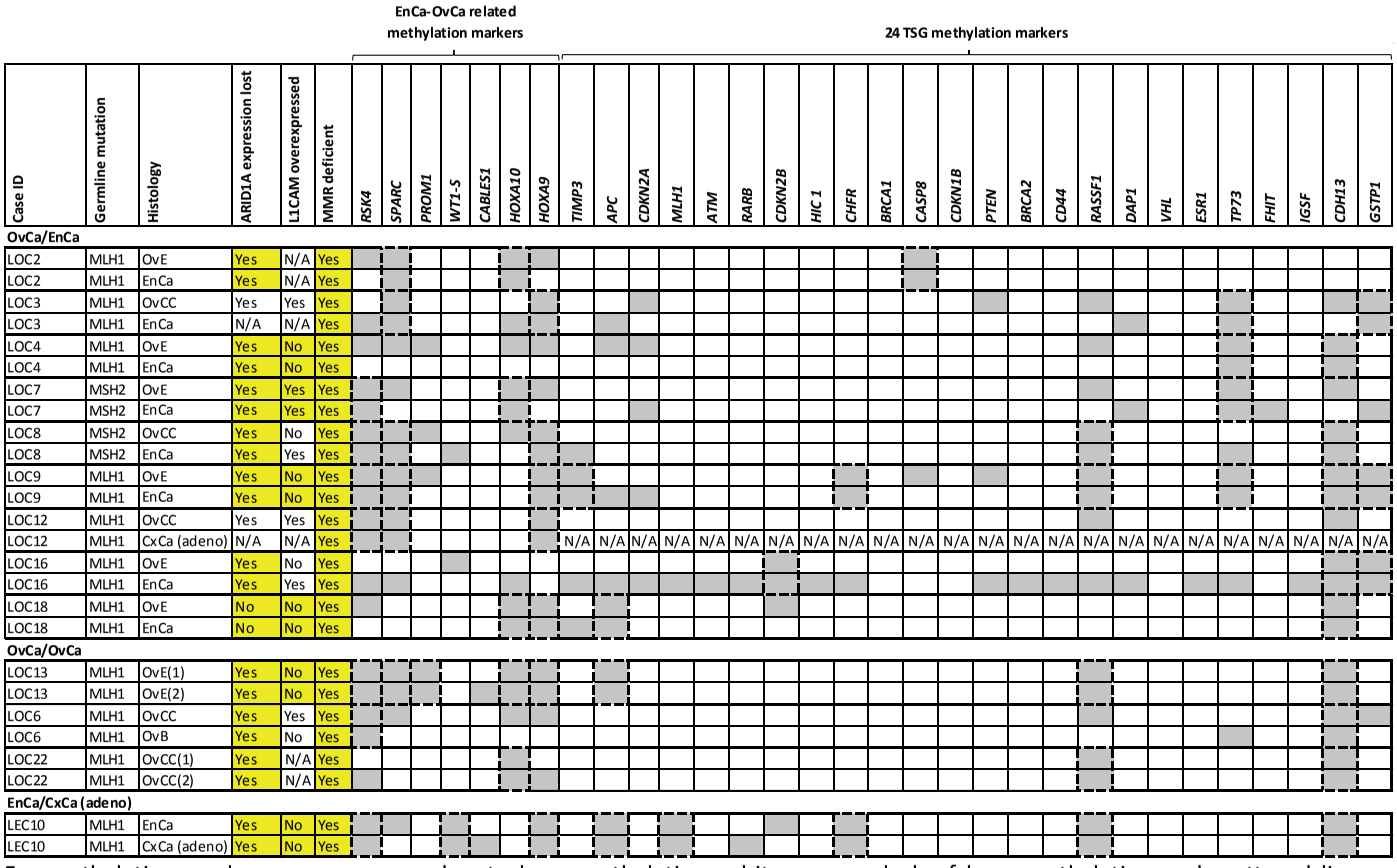
2.1 Molecular comparison of synchronous LS-associated endometrial and ovarian carcinoma specimens (III)

In approximately 20% (201, 259) of LS-associated and 5 to 10% of sporadic cases (260, 261), endometrial and ovarian carcinomas are diagnosed concurrently, raising the question of whether the two carcinomas arise independently as primary carcinomas or one is a metastasis of the other.

Among the samples collected from LS mutation carriers with endometrial and/or ovarian carcinoma, 13 cases with synchronous carcinomas were identified. We utilized this unique synchronous sample set to investigate the relationship between ovarian and endometrial carcinogenesis in LS. In total, nine pairs of synchronous endometrial and ovarian carcinomas, 3 cases with bilateral (and synchronous) ovarian carcinomas and one synchronous case of endometrial and endocervical adenocarcinoma were identified. **Figure 12** illustrates the molecular findings case by case of the synchronous tumors. Synchronous carcinomas always shared identical MMR-status (13/13) and ARID1A expression when the result was available from both cases (11/11). Moreover, TSG hypermethylation profiles showed a high intra-pair concordance and an average of 4.4 (57/13) TSGs per synchronous tumor pair were concordantly methylated among 31 TSGs investigated. The most

frequently concordantly methylated TSGs among synchronous tumor pairs were *CDH13* (75%, 9/12), *RSK4* (54%, 7/13), *SPARC* (46%, 6/13*), HOXA10* (46%, 6/13), and *RASSF1* (42%, 5/12). Interestingly, the expression of L1CAM, an adhesion molecule involved in metastasis (235, 262), was abnormal in 6/14 (43%) of the carcinomas included in synchronous ovarian and endometrial tumor pairs, compared to 3/25 (12%) of independently arisen ovarian and endometrial tumors (*P*=0.047) (A.N. et al. unpublished data). Our molecular findings from synchronous gynecological tumors in LS mutation carriers strongly suggest that each pair has a shared origin with one tumor likely to arise as the metastasis of some other tumor.

Figure 12. Molecular characteristics of synchronous carcinoma pairs from Lynch patients.

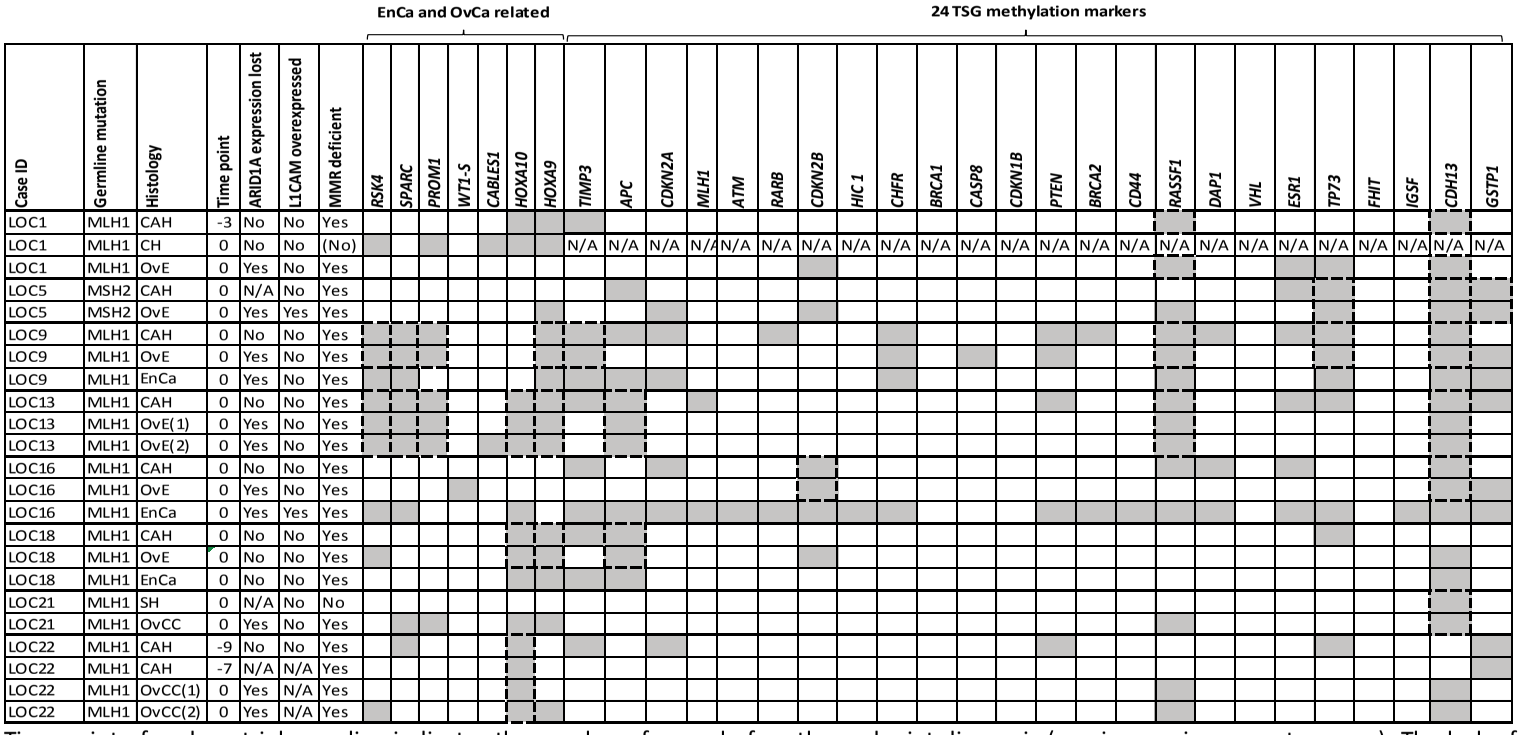


For methylation markers, gray squares denote hypermethylation, white squares lack of hypermethylation and scattered line around boxes highlights the identical hypermethylation pattern of a specific gene loci between synchronous pair. Yellow color indicates that the protein expression/MMR status is concordant. N/A, not available; CxCa, cervical adenocarcinoma; EnCa, endometrial carcinoma; OvB, borderline ovarian tumor; OvCa, ovarian carcinoma; OvCC, clear cell ovarian carcinoma; OvE, endometrioid ovarian carcinoma.

2.2 Closely entangled pathways of endometrial and ovarian tumorigenesis (III)

Molecular characteristics of endometrial hyperplasias preceding or coinciding with ovarian carcinoma from LS patients are shown case by case in **Figure 13**. In patients with endometrioid ovarian cancer CAH was diagnosed prior or concurrently with ovarian cancer in 50% (7/14) of the cases and in 22% (2/9) of the cases with clear cell ovarian cancer. The high extent of similarity of molecular markers (mainly MMR status and hypermethylation of specific genes) was detected between ovarian cancer and hyperplastic lesions, suggesting that ovarian cancer may also develop in a stepwise manner from endometrial hyperplasia. Only 38% (3/8) of the cases with ovarian cancer and concurrent CAH were diagnosed with endometrial carcinoma as well. Cases LOC1 (CAH detected three years before ovarian cancer) and LOC13 (CAH detected concurrent with ovarian cancer) highlight molecular similarity between CAH and ovarian cancer. These findings together with the common background of synchronous endometrial and ovarian cancer in LS mutation carriers as described above suggest that type I endometrial and ovarian tumorigenesis may be molecularly even more entangled than previously appreciated.

Figure 13. Molecular characteristics of endometrial hyperplasias arising prior to or concurrently with ovarian carcinoma (and synchronous endometrial carcinoma) from Lynch patients.



Case ID	Germline mutation	Histology	Time point	ARID1A expression lost	L1CAM overexpressed	MMR deficient	RSK4	SPARC	PROM1	WT1-S	CABLES1	HOXA10	HOXA9	TIMP3	APC	CDKN2A	MLH1	ATM	RARB	CDKN2B	HIC 1	CHFR	BRCA1	CASP8	CDKN1B	PTEN	BRCA2	CD44	RASSF1	DAP1	VHL	ESR1	TP73	FHIT	IGSF	CDH13	GSTP1
LOC1	MLH1	CAH	-3	No	No	Yes																															
LOC1	MLH1	CH	0	No	No	(No)								N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
LOC1	MLH1	OvE	0	Yes	No	Yes																															
LOC5	MSH2	CAH	0	N/A	No	Yes																															
LOC5	MSH2	OvE	0	Yes	Yes	Yes																															
LOC9	MLH1	CAH	0	No	No	Yes																															
LOC9	MLH1	OvE	0	Yes	No	Yes																															
LOC9	MLH1	EnCa	0	Yes	No	Yes																															
LOC13	MLH1	CAH	0	No	No	Yes																															
LOC13	MLH1	OvE(1)	0	Yes	No	Yes																															
LOC13	MLH1	OvE(2)	0	Yes	No	Yes																															
LOC16	MLH1	CAH	0	No	No	Yes																															
LOC16	MLH1	OvE	0	Yes	No	Yes																															
LOC16	MLH1	EnCa	0	Yes	Yes	Yes																															
LOC18	MLH1	CAH	0	No	No	Yes																															
LOC18	MLH1	OvE	0	No	No	Yes																															
LOC18	MLH1	EnCa	0	No	No	Yes																															
LOC21	MLH1	SH	0	N/A	No	No																															
LOC21	MLH1	OvCC	0	Yes	No	Yes																															
LOC22	MLH1	CAH	-9	No	No	Yes																															
LOC22	MLH1	CAH	-7	N/A	N/A	Yes																															
LOC22	MLH1	OvCC(1)	0	Yes	N/A	Yes																															
LOC22	MLH1	OvCC(2)	0	Yes	N/A	Yes																															

Time point of endometrial sampling indicates the number of years before the endpoint diagnosis (ovarian carcinoma, set as zero). The lack of MMR deficiency requires stable microsatellites and intact MMR protein (parentheses indicate that only one of the assays was available). For methylation markers, gray squares denote hypermethylation, white squares lack of hypermethylation, and scattered line around boxes highlights the identical hypermethylation pattern of a specific gene loci between synchronous pair. Abbreviations: CH, complex hyperplasia without atypia; CAH, complex hyperplasia with atypia; EnCa, endometrial carcinoma; N/A, not available; OvE, endometrioid ovarian carcinoma; OvCC, clear cell ovarian carcinoma; SH, simple hyperplasia.

DISCUSSION

1 Developmental model of ovarian and endometrial tumorigenesis in Lynch syndrome and sporadic cases

1.1 Overview based on currently available data

The epidemiologies of ovarian and endometrial cancer are closely entangled which is probably attributable to similarities in the hormonal, immune system-related, and inflammation mechanisms influencing the reproductive tract (155). LS offers a good model with which to study the molecular background of these gynecological cancers for three important reasons: (1) the prevalence of these cancers is significantly increased in LS mutation carriers compared to the general population, thus providing reasonable numbers of tumors and other specimens for analysis (7, 8), (2) several lesions (benign and malignant) from the same patient are common (263), and (3) consecutive specimens are available from individuals taking part in a surveillance program to detect gynecological cancers which permits an investigation of the types of changes occurring before a carcinoma is detected.

Beyond the known defects in MMR genes, LS-associated ovarian carcinomas have not been molecularly analyzed before. More is known about the endometrial carcinomas in women with LS, but there is very limited information about the molecular changes preceding endometrial cancer. Our findings revealed a novel and distinct genetic and epigenetic background of LS-associated ovarian and endometrial cancer as compared to sporadic corresponding cases, including MMR deficiency and TSG hypermethylation of specific genes appearing already in histologically normal endometrium, a high frequency of ARID1A expression alterations, and L1CAM overexpression, specifically in clear cell ovarian cancer. The novel findings of LS-associated ovarian and endometrial tumorigenesis are illustrated in **Figures 14 and 15**, respectively.

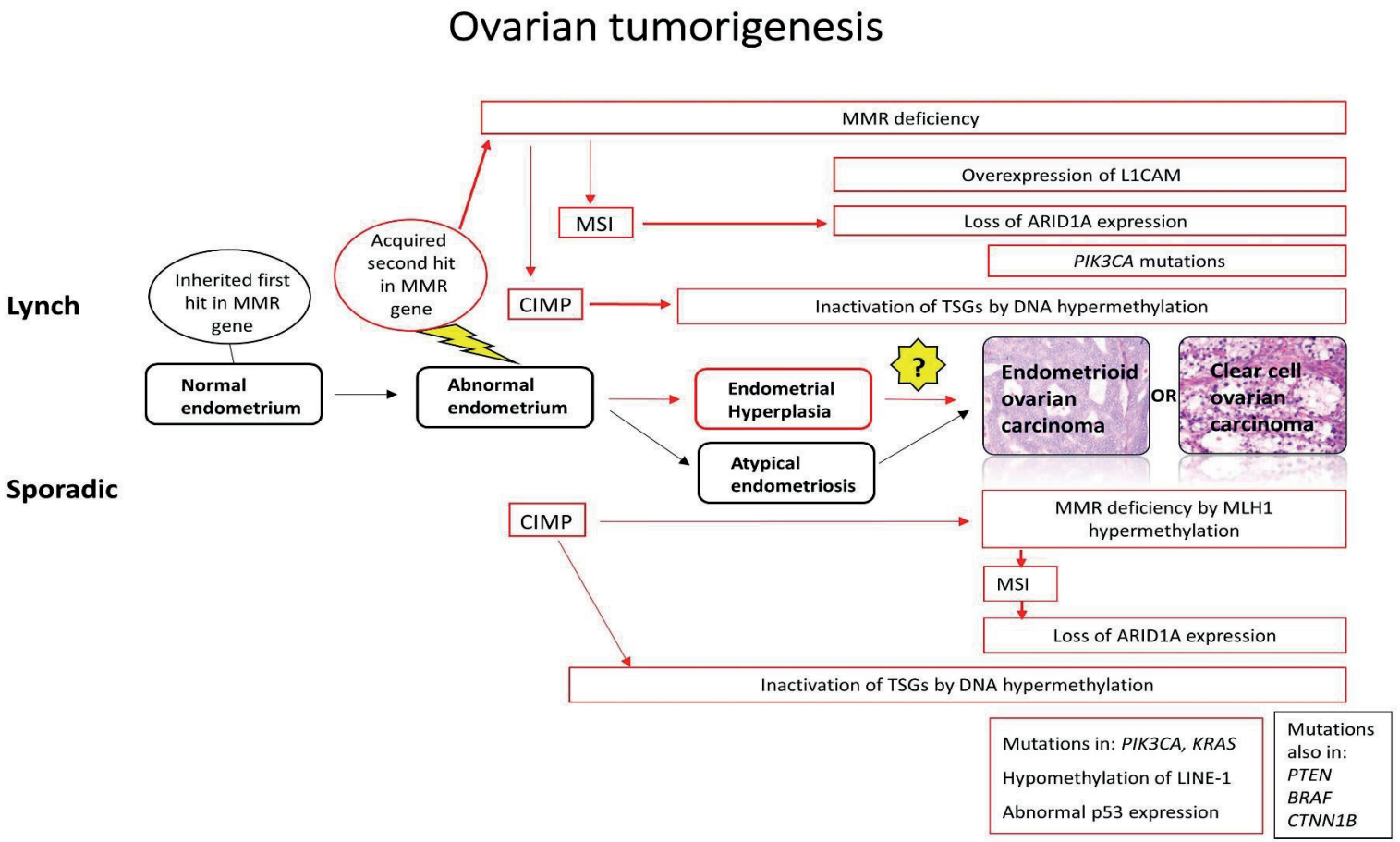


Figure 14. Molecular alterations of type I ovarian tumorigenesis in Lynch mutation carriers and sporadic cases. Horizontal bars denote the stage of tumorigenesis at which each molecular alteration occurs. A red box around indicates findings made in the course of this thesis work, whereas a black box displays information obtained from the literature. Abbreviations: CIMP, CpG island methylator phenotype.

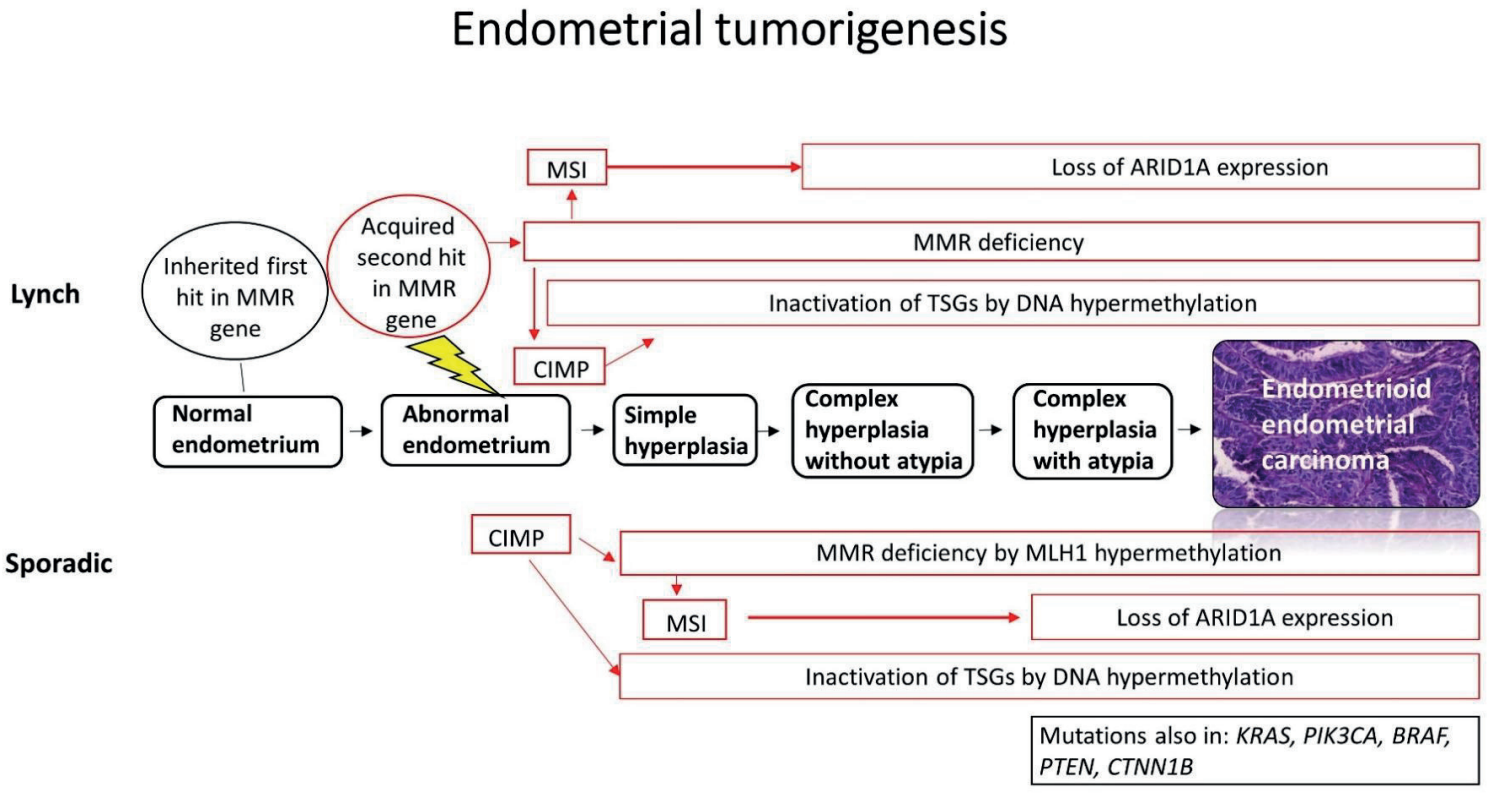


Figure 15. Molecular alterations of type I endometrial tumorigenesis in Lynch mutation carriers and sporadic cases. Horizontal bars denote the stage of tumorigenesis at which each molecular alteration occurs. A red box indicates findings made in the course of this thesis work, whereas a black box displays information obtained from the literature. Abbreviations: CIMP, CpG island methylator phenotype.

1. 2 The role of deficient MMR

Lynch mutation carriers have one defective allele in an MMR gene in every cell of their body. An acquired second hit in a particular MMR gene in a tissue of an LS mutation carrier compromises the MMR machinery and eventually leads to inactivation of MMR protein expression in the cell, conferring a growth advantage on the cell and the possibility to transform into a neoplastic cell. Thus, MMR deficiency in a pre-malignant specimen indicates an increased risk for tumor development. In fact, LS-associated ovarian and endometrial carcinomas displayed MMR deficiency already at histologically normal endometrium in 12% (12/99) of the cases compared to 0% (0/38) in sporadic cases (*P*=0.037), suggesting that this is a very early change in LS-associated tumorigenesis.

Gynecological cancers could be almost completely prevented with prophylactic total hysterectomy and bilateral salpingo-oophorectomy (THBSO), and it has been shown to effectively decrease the risk of these cancers in LS women (264, 265). Women with LS are recommended to take part in regular endometrial surveillance against gynecological cancer every 2 to 3 years, starting at 30 to 40 years of age, but currently there are no further official guidelines to screen for gynecological cancer. Moreover, the optimal timing of prophylactic THBSO is debated (5, 266). It is intriguing to speculate whether analysis of MMR deficiency of endometrial specimens from LS patients taking part in gynecological screening might be beneficial in determining the timing of prophylactic THBSO and other treatment decisions (see below).

The frequency of MMR deficiency in endometrial specimens increased with increasing histological abnormality of lesions in LS and sporadic series. The frequencies were exceptionally high in LS mutation carriers, 42% (5/12) in SH, 83% (5/6) in CH and 87% (33/38) in CAH. Moreover, an MMR deficiency was detected in all of the cases of CAH and appeared 1 to 9 years before the diagnosis of either ovarian or endometrial carcinoma (7/7 = 100%). These patients with CAH did not have THBSO at the time of diagnosis either because the patient preferred not to undergo the surgery or CAH was not originally

diagnosed at the time of biopsy. Interestingly, also 67% (2/3) of the patients with SH and one case with a CH (1/1) diagnosis 1-2 years before carcinoma diagnosis, were shown to be MMR deficient. Similar to our findings, Faquin et al. (169) detected MMR deficiency by microsatellite analysis in one non-neoplastic endometrial specimen 7 years before the development of sporadic endometrioid endometrial carcinoma and argued that MSI diagnosis in endometrial tissues could predate the carcinoma diagnosis by years. Moreover, the diagnosis of aspiration biopsy specimens may be unreliable for pathologic inspection due to the fragmented nature of specimens, especially with postmenopausal women who often have an atrophic endometrium. Accordingly, pre-malignant (CAH) and even carcinoma cases may be diagnosed as benign (SH) and therefore, molecular analysis of hyperplasias could assist in treatment decisions, especially in borderline cases.

1.3 ARID1A alterations and relationship with MMR

Mutations of *ARID1A* are known to be common in sporadic clear cell (43-57%) and endometrioid (30%) ovarian carcinomas and endometrioid endometrial (40%) carcinoma (34, 39, 267), and many studies have shown a high correlation between *ARID1A* mutation by genetic analysis and deficient ARID1A expression by IHC (34, 267). In our study, the frequency of aberrant ARID1A expression was exceptionally high in LS-associated ovarian carcinomas (100%, 9/9 in clear cell and 86%, 12/14 in endometrioid ovarian carcinomas) compared to sporadic cases (0/39 in clear cell and 11%, 3/28 in endometrioid ovarian carcinomas) ($P<0.001$ in both histological types). Only 10% (1/10) of sporadic non-serous ovarian carcinomas with deficient MMR and showing MSI had lost their ARID1A expression. The loss of ARID1A expression was also high in endometrioid endometrial carcinoma, accounting for 61% (14/23) of the cases in comparison to 17% (6/35) in sporadic cases ($P<0.001$). In contrast to the low frequency of concurrent MSI and ARID1A aberrations in sporadic ovarian carcinomas (10%, 1/10), these alterations were frequent in endometrial carcinomas (67%, 4/6) ($P=0.036$). Bosse et al. (268) also detected a high frequency of aberrant ARID1A expression in sporadic MSI-high endometrial carcinomas (75%, 24/32),

but in contrast to our findings, they detected inactivation of ARID1A in only 14% (5/36) of their LS-associated cases.

Mutations of *ARID1A* and consequent loss of protein expression have been detected in atypical endometriotic lesions adjacent to ovarian cancer, suggesting that this is an early event in ovarian tumorigenesis (267). No aberrant ARID1A expression was detected in progressive endometrial specimens (0/17, including NE, SH, CH, and CAH) from LS patients with ovarian carcinoma as the endpoint diagnosis, suggesting that ARID1A loss is a late event in LS-associated ovarian tumorigenesis or it possibly occurs only in atypical endometriosis preceding and/or adjacent to ovarian carcinoma which was not analyzed. On the other hand, a loss of ARID1A expression was detected in 15% (5/33) of complex hyperplasias (including one CH and 4 CAH) prior to or concurrent with LS-associated endometrial carcinoma. Similar findings were detected in sporadic cases of endometrial carcinoma by Werner et al. (269), who detected inactivation of ARID1A in complex atypical hyperplasia (16%) but no signs of this alteration in non-atypical hyperplasias. Our findings together with Werner et al. (269) propose that the loss of ARID1A expression is an early event in endometrial carcinoma regardless of origin (hereditary or sporadic) and could potentially be used as a biomarker in endometrial complex hyperplasia to evaluate the malignant potential of the lesion, and further to guide treatment decisions.

Our findings raise a question about the relationship between MMR deficiency and the loss of ARID1A expression, since both were always present in clear cell carcinoma of the ovary and they are very common in endometrioid ovarian and endometrial carcinomas from LS mutation carriers and also frequent in sporadic MSI-high endometrial carcinomas. Bosse et al. (268) speculated that the loss of ARID1A expression was attributable to epigenetic inactivation of the MLH1 gene instead of being inactivated by mutations resulting from MSI. Our findings from consecutive specimens preceding cancer showed that MMR deficiency arises already in histologically normal endometrium and ARID1A later in complex hyperplasia. This discovery argues against the theory of Bosse et al. (268) and proposes

that ARID1A inactivation follows MSI and not vice versa. Arguments of MMR deficiency leading to aberrant ARID1A have also been hypothesized in gastric (270) and colorectal cancer (271, 272). In the study of Wang et al. (270), the somatic *ARID1A* mutation rate was found to be 12-61 fold higher compared to the general background mutation rate in MSI associated gastric carcinomas. Although mutations affecting *ARID1A* are typically nonsense and frameshift (34), Wang et al. (270) detected mainly insertions/deletions of short mononucleotide repeats in *ARID1A* in MMR-deficient cases suggesting that for some unknown reason, *ARID1A* is specifically targeted by MSI. A similar trend of high incidence of *ARID1A* insertions/deletions mutations associated with MMR deficiency in colorectal cancer was detected by Jones et al. (271) and Chou et al. (272) indicating that MMR-deficiency often leads to aberrant ARID1A gene as the result of MSI.

1.4 Epigenetic changes as early events

Genetic modifications alone cannot explain the complex nature of LS-associated and sporadic ovarian and endometrial cancer. Epigenetics, including hypermethylation, is expected to play a major role in the LS-associated tumors, since deficient MMR machinery causes hypermutability and increases the possibility of alterations in other genes involved in cancer development (273). Some of the affected genes may be involved in epigenetic regulation which can cause hypermethylation of TSGs (274). Similar to MMR deficiency, hypermethylation of specific TSGs (frequently including *RSK4*, *SPARC*, *HOXA10*, *HOXA9*, *RASSF1* and *CDH13*) was detected even in normal endometrium several years prior to the development of ovarian or endometrial carcinoma in LS cases, suggesting that these are early alterations in tumorigenesis. Our findings, together with published reports, support the concept that detection of specific epigenetic gene profiles in premalignant lesions and in cancer tissue may facilitate early detection, classification and treatment of ovarian and endometrial cancer (see also the next Chapter) (275, 276) .

2 Three-tiered categorization system proposed for endometrial specimens

The main view at present is that type I endometrial carcinomas mainly originate from atypical hyperplasia (192). Therefore, the WHO2014 schema categorizes hyperplasias into two types, non-atypical (including SH and CH) or atypical (including SAH and CAH) (195). A correct classification of hyperplasia is highly important, since it guides treatment decisions. These decisions can be quite dramatic: Atypical hyperplasia is mainly treated with hysterectomy whereas medication is the standard treatment for hyperplasia without atypia (185).

In contrast to the WHO2014 classification, our findings of MMR-deficiency, the loss of ARID1A expression, and the methylation status of TSG promoters divided progressive endometrial samples into three categories of combined histological types: NE plus SH (category I), CH plus CAH (category II), and endometrial carcinoma as its own entity (category III). Importantly, our results suggested that in addition to CAH, CH should be considered as a pre-neoplastic form of endometrial carcinoma. In particular, the average numbers of methylated TSGs in both panels (the panel of 24 general TSGs and the panel comprising of 7 endometrial and ovarian cancer related TSGs) and regardless of origin (LS or sporadic) were able to discriminate between categories I (NE + SH) and II (CH+CAH) and III (endometrial cancer). Our results are compatible with the idea of classes with low (I) and high (II, III) malignant potential.

Similar to our observations, van der Putten et al. (190) found that the genetic profile for complex hyperplasias (both CH and CAH) differed from simple hyperplasias of both non-atypical (SH) and atypical (SAH) type and stated that complex hyperplasia, regardless of atypia, seems to be the most important precursor for endometrial carcinoma. The categorizations of hyperplasia are still largely based on the original findings of Kurman et al. in 1985 (192), who reported that only 3% of complex hyperplasia without atypia (CH) progressed to cancer and partly because of this statement, CH has not been considered as a pre-neoplastic form of endometrial cancer. However, the study of Kurman et al. (192)

included only 29 cases of CH. A recent publication by Matsuo and colleagues (277) estimated a 21% risk of concurrent endometrial cancer with CH. In addition, one may also speculate whether the transition from CH to CAH is so fast that it is often not recognized, suggesting that CH should be regarded as pre-neoplastic lesion as well.

Several biomarkers have been proposed to detect and predict the cancer risk in hyperplasias, but so far, these have not proven effective enough for application in a clinical setting (185). It is tempting to postulate that the methylation analysis of specific TSGs (and MMR status in cases of LS mutation carriers) could improve diagnostics and help to predict the malignant potential of hyperplastic lesion, either atypical or non-atypical.

3 Is endometrial hyperplasia a component of ovarian tumorigenesis?

Only a few studies have investigated the prevalence of endometrial hyperplasia occurring concurrently with ovarian cancer and the knowledge of this subject is poor (184, 278). Nonetheless, surprisingly high frequencies of atypical hyperplasia concurrent with ovarian carcinoma have been detected. Mingels et al. (184) reported that approximately half of the endometrioid and 29% of clear cell ovarian carcinomas present with concurrent atypical hyperplasia. These results were nearly identical to our findings of concurrent CAH with endometrioid (7/14, 50%) and clear cell ovarian cancer (2/9, 22%) from LS patients (**Figure 13**). It is well known that endometrial hyperplasias are significant steps in endometrial tumorigenesis (185), but our findings together with those of Mingels et al. (184), raise the question of whether endometrial hyperplasias are also connected to ovarian tumorigenesis. Moreover, the correspondence of the molecular markers between complex hyperplasias and concurrent ovarian cancer (**Figure 13**) included in our studies, indicate that endometrial hyperplasias may have a role in the development of ovarian carcinoma. These findings should act as a springboard for additional research. In particular, the epigenetic findings may help to reveal relationships within samples reflecting the common origins (279). Indeed, hypermethylation of the same loci was often detected in hyperplasia

prior to or concurrent with ovarian cancer frequently in the *HOXA10*, *HOXA9*, *RASSF1* and *CDH13* genes.

Mingels et al. (184) speculated that atypical hyperplasia could be caused by increased production of estrogen by the ovarian cancer, i.e. especially the endometrioid type ovarian cancer has been shown to increase estrogen levels. But since Mingels et al. (184) only investigated concurrently occurring samples, it is not known which abnormality occurred first, i.e. was it atypical hyperplasia or ovarian cancer? In our consecutive sample series, two LS patients, LOC1 and LOC22, were diagnosed with CAH but treated with hysterectomy only instead of THBSO. The patients ended up developing endometrioid and clear cell ovarian cancer three and seven years later, respectively. Thus, our observations of CAH arising several years before the identification of ovarian carcinoma, are compatible with the possibility of CAH being an early step in ovarian tumorigenesis.

Mingels et al. (184) hypothesized that concurrent atypical hyperplasia is a premalignant form of synchronous endometrial carcinoma. This is likely to be true, but additionally, our findings raise the question of whether a hyperplastic lesion in the endometrium could also metastasize to the ovary and thus be the precursor for ovarian cancer as well, since endometrial epithelial cells are the putative origins of endometrioid and clear cell ovarian tumors (280). Kelemen et al. (260) studied the risk factors of synchronous endometrial and ovarian tumors by multivariable models and observed that endometriosis in the ovary was associated with a decreased risk of synchronous tumors relative to clear cell and endometrioid ovarian-only tumors. Kelemen et al. (260) suggested that endometriosis is not a likely step in the development of synchronous endometrial and ovarian carcinomas, leaving room for the scenario that endometrial hyperplasia might represent this step in synchronous cases.

CONCLUDING REMARKS AND FUTURE PROSPECTS

LS-associated ovarian cancer differs from its sporadic counterpart in several ways, but the molecular alterations behind these differences remain unanswered. This thesis work describes a novel genetic and epigenetic profile of LS-associated ovarian carcinomas: There was the virtually inevitable loss of ARID1A expression, a mismatch repair deficiency, a lack of *KRAS* and *BRAF* mutations, normal p53 expression, the unique hypermethylation profile of selected tumor suppressor genes and a lack of LINE-1 hypomethylation. Additionally, the frequency of *PIK3CA* mutations and L1CAM overexpression were common and comparable to that encountered in sporadic tumors of the same histological types. Moreover, we identified different hypermethylation profiles of selected genes in non-serous histological types of Lynch and sporadic ovarian carcinomas, as compared to sporadic serous ovarian tumors. The prominent differences discovered between ovarian tumors of Lynch and sporadic origin as well as between histological types help to explain the distinct behavior of these carcinomas and emphasize the need for individualized clinical management.

Møller et al. (8) described the excellent survival in endometrial (98%) and ovarian (89%) cancer, but speculated that further investigation would be needed to determine the factors behind this favorable prognosis. Our results from genetic and epigenetic analysis indicate that LS-associated ovarian carcinomas have more favorable molecular characteristics compared to sporadic ovarian carcinomas. In addition to the novel molecular characteristics of LS-associated ovarian carcinomas, other possible explanations to account for the better prognosis need to be addressed; these will include surveillance and the consequent early detection of pre-neoplastic lesions of endometrial carcinoma and the subsequent detection of synchronous ovarian carcinoma, as well as clarifying the role of immunogenic factors and explaining the lack of metastatic features (like in colorectal cancer) (13, 183, 281).

Survival of ovarian cancer is poor, and regardless of major histological and molecular differences within different histological types of ovarian cancer, the therapy is similar in all patients. Indeed, the detection of driver mutations and epimutations between ovarian cancer subtypes may offer new opportunities in ovarian cancer management through subtype-specific care. At present, only two biomarkers, CA125 and HE4 (Human epididymis protein 4) for ovarian cancer monitoring have been approved by FDA (282-284), but more specific markers, especially biomarkers for early detection are urgently needed. In particular, epigenetic biomarkers involving DNA methylation would be ideal because of its reversible nature and the possibility of targeting a specific gene region (172). Our findings, together with other reports, suggest that alterations in DNA methylation profile are able to discriminate between non-serous and serous types of ovarian cancer. By selecting the most prominent markers, DNA methylation could be used as a biomarker to distinguish between different subtypes of ovarian cancer especially in borderline cases in which the diagnosis may be difficult by pathological inspection only.

Molecular alterations that predict ovarian and endometrial cancer risk and progression identified in this thesis project may have valuable clinical significance. As discussed earlier, information obtained from hyperplastic lesions guides treatment decisions. Although the risk-reducing effect of gynecological surveillance or early detection of these has not been proven scientifically yet, for now women with LS are recommended to take part in endometrial biopsy screening every 2 to 3 years starting at 30-40 years of age (5, 266). Endometrial biopsies obtained from this kind of surveillance program could be most beneficial when analyzed with specific markers during the interval between the diagnosis of LS and possible prophylactic surgery. Molecular changes detected in endometrial aspiration biopsies could identify those patients who would benefit from intensive screening and cancer prevention, including oral contraceptives, progestin therapy and aspirin-based chemoprevention that may be effective against LS-associated gynecological carcinomas (266, 285). Furthermore, molecular alterations could help to predict the optimal time point for prophylactic surgery.

Currently, nuclear atypia detected by histopathological inspection of endometrial hyperplasias, is the only characteristic that confers an elevated risk for endometrial carcinoma in the general population and an additional risk of ovarian cancer in Lynch mutation carriers. Important information of the histologically normal endometrial tissue of hyperplastic biopsy specimen may remain undetected if investigated by histopathological inspection alone. This warrants further investigation but already now the results strongly emphasize that molecular testing of specific markers from aspiration biopsies, such as detection of MMR-deficiency status or hypermethylation profiles of specific gene promoters can provide more information of the malignant potential of pre-neoplastic specimens and clinical behaviour of tumor specimens. The results would allow more efficient counselling, help to select cases more suitable for non-surgical treatment of endometrial hyperplasia, guide treatment options in hereditary and sporadic cases and facilitate in the selection of the optimal timing of THBSO in women with Lynch syndrome. Indeed, our findings from the consecutive sample series prior to the appearance of ovarian and endometrial cancer, suggest that the precursor for endometrial cancer (or possibly even ovarian cancer) can be detected from aspiration biopsies taken from histologically normal endometrium several years before any carcinoma diagnosis.

Our findings, together with findings from others, suggest that complex hyperplasia with and without atypia of LS and sporadic origin should be considered as equally important precursors for endometrial carcinoma progression and these findings should be included in considerations of treatment decisions. In addition, in the future, it could be investigated whether the histological classification should be combined with information from molecular markers (such as MSI status, ARID1A expression, *KRAS*) in order to predict the risk for endometrial carcinoma progression.

Our molecular analyses emphasize the shared background of LS-associated synchronous endometrial and ovarian carcinomas and the possibility of metastatic disease. Moreover, molecular analysis of endometrial hyperplasias collected as a part of long-term surveillance

program before or concomitant with endometrial and/or ovarian cancer revealed concordance in alterations (MMR-status and hypermethylation profile of selected TSGs appearing already in histologically normal endometrium and the loss of ARID1A expression being evident in endometrial hyperplasias), pointing to an early convergence of endometrial and ovarian tumor development. If one wishes to obtain a broader perspective, it would be important to supplement our findings from LS specimens with results from synchronous sporadic gynecological carcinomas.

Interestingly, the examination of consecutive specimens revealed that endometrial hyperplasias prior to or concurrent with ovarian carcinoma exhibit similar degrees of molecular alterations as compared to endometrial carcinoma. This discovery reveals that in addition to endometrial tumorigenesis, endometrial hyperplasia may also be connected to ovarian carcinogenesis. Overall, our observations of multilevel ties between endometrial and ovarian tumor development suggest that always when a pre-malignant or malignant endometrial lesion is detected, the possibility of ovarian cancer should be kept in mind and vice versa.

In the future, it would be interesting to design a more comprehensive MS-MLPA based assay to detect methylation of the most prominent epigenetic biomarkers (including *RSK4*, *SPARC*, *PROM1*, *HOXA10*, *HOXA9*, *WT1-AS*, *SFRP2*, *OPCML*, *MIR34B*, *APC*, *RASSF1*, *TP73* and *CDH13*) in a single assay and to investigate the markers in larger sample cohorts. A digital MLPA assay (286) has been recently developed to detect copy number alterations in up to a thousand target sequences, and a similar digital solution could be designed for MS-MLPA. This digital MS-MLPA test would be perfect for our purposes. In fact, if we were able to identify reliable and common epigenetic alterations preceding endometrial and ovarian cancer, these alterations could be used as epigenetic biomarkers which would not only further assist in the early detection but also help to predict prognosis and monitor the treatment response.

Genetic and epigenetic analysis of LS-associated ovarian and endometrial carcinomas on a genome-wide scale will be an obvious next step. Our truly interesting findings of the possible endometrial hyperplasia step involved in the development of ovarian carcinoma in women with LS is a springboard for additional research. For now, it remains to be resolved whether endometrial hyperplasia serves as a precursor lesion for endometrial carcinoma alone or could it also be relevant for ovarian tumorigenesis. Therefore, it would be especially important to find a suitable method to examine the whole methylome and genome of consecutive endometrial samples prior to endometrial and ovarian carcinoma. This genome-wide analysis could help to reveal the epigenetic relationships between specimens as well as identifying more specific and early genetic changes contributing to these carcinomas, and hopefully resolving whether endometrial hyperplasia is a precursor of ovarian tumorigenesis. At present, the problem is that most genome-wide methods require high quality and/or large quantities of DNA to produce reliable data and are therefore not suitable for FFPE samples. In particular, the amount and quality of DNA extracted from endometrial aspiration biopsies are mainly poor and inadequate and cannot be used to perform these whole genome analyses. Fortunately, methods are developing at a high speed and hopefully soon there will be an assay available to fulfill our needs.

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