Chapter 4
Traditional Approaches to Molecular Genetic Analysis

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Abstract Molecular studies of endometrial cancer have evolved with the tools available to researchers: the methods for measuring nucleic acids, protein expression, and combinations thereof. Today "molecular genetic analysis" implies a broad range of indirect and direct tests that yield molecular phenotypes or genotypes, immunotypes, or signatures that were not conceived of when the histologic and biologic heterogeneity was first fully acknowledged.

We will provide a historical perspective on molecular genetic studies of endometrial cancers focusing on candidate genes and how early foundational research shaped both our understanding of the disease and current research directions. Examples of *direct tests* (mutation, DNA methylation, and/or protein expression) will be provided along with examples of *indirect tests* that have been and continue to be central to endometrial cancer molecular biology, such as DNA content or microsatellite instability analysis. We will highlight clinically relevant examples of molecular phenotyping and direct evaluation of candidate genes that integrate direct and indirect testing as part of routine patient care. This is not intended to be an exhaustive review but rather an overview of the progress that has been made and how early work is shaping current molecular, clinical, and biologic studies of endometrial cancer.

Keywords Indirect tests • Direct tests • Mutation testing • Candidate genes • Biologic relevance • Clinical significance

Introduction

Endometrial cancer was for many years the red-headed stepchild of oncology: unwanted and neglected. Clinically focused research has led to improved detection and treatments. Molecular biologists, however, gave little attention to endometrial cancer at the time molecular tools first became available. This is somewhat surprising

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in light of the high incidence of endometrial cancer and the remarkable increase in the number of cases associated with the use of unopposed estrogens in the 1970s. The strong link between excess estrogen and risk for development of endometrial cancer did, however, provide a solid biologic framework for correlative and descriptive molecular studies. Researchers began to formulate and test hypotheses regarding the influence of steroid hormones and their receptors on endometrial cancer biology.

Initial molecular studies of endometrial cancer were largely based on observations from other cancer types (endometrial cancer remained a "me-too" subject of investigation). The candidate gene/candidate pathway approach nonetheless yielded important insights into the pathobiology of endometrial carcinoma. Over the past two decades it has become evident that the molecular complexity of these cancers is among the highest of common tumor types studied to date. Indeed the molecular heterogeneity is consistent with the histologic and clinical variability recognized today. The rapid evolution of methods for molecular biology and informatics continues to change the perception of endometrial cancer, and its ever rising incidence has garnered the attention of epidemiologists, health care providers, and health care economists (see Chaps. 1 and 2).

DNA Content Studies

Among the earliest molecular studies of endometrial cancers were DNA content analyses that began more than 60 years ago [1]. In 1902 Theodor Boveri proposed that chromosomal defects account for cancerous phenotypes [2]. Observational studies from the 1950s and 1960s proved that the total nucleic acid content of tumor cells can differ from nonmalignant cells. Aneuploidy, referring to abnormalities in the number of chromosomes, is a "mutator phenotype" [3, 4]. It was recognized early on in the study of endometrial cancers, and the clinical diagnostic and prognostic significance of DNA content has been explored repeatedly. DNA content analysis is an indirect test that can be used to measure what is referred to as a chromosomal instability (CIN) phenotype [5]. Mauland, Wik and Salvesen [6] have recently reviewed the clinical value of DNA content assessment in endometrial cancer focusing on DNA content as a potential prognostic and predictive maker. Despite more than two decades of investigation and numerous reports on positive association between abnormalities in tumor cell DNA content and factors known to portend poor outcome, the prognostic and predictive value of DNA ploidy in endometrial cancers remains controversial [7–9]. Prospective evaluation of the prognostic and predictive value of aneuploidy is ongoing. It is conceivable that an indirect test such as DNA content measurement might be replaced by what are potentially more resolving and more powerful copy number loss or gain analyses. It is equally possible that DNA ploidy assessment combined with direct tests for mutations, epigenetic marks, changes in transcription, and altered protein expression will come to the forefront of endometrial cancer management.

DNA Mismatch Repair (MMR): Molecular Phenotyping and Direct Assessment of Candidate Genes

Endometrioid endometrial cancers have one of the highest incidences of mismatch repair (MMR) defects in human cancers studied to date. Loss of DNA MMR is associated with an easily recognized tumor phenotype, microsatellite instability (MSI). MSI is a result of somatic strand slippage mutations that have been referred to as replication errors [10, 11]. MSI analysis provides a convenient way of assessing the MMR status of tumors and falls into the category of indirect testing. When the tumor phenotype was first noted in familial colon cancers members of the conserved *mutS*, *mutH*, and *mutL* families were immediately recognized as candidate genes [12]. Loss of function alleles in *mutS*, *mutH*, and *mutL* genes in bacteria and yeast were known to lead to an accumulation of strand slippage mutations [13, 14]. In 1993, with the discovery of germline mutations in patients with familial/inherited colon cancer [15, 16], direct testing for MMR defects became possible and candidate genes were credentialed as causative factors. It was immediately obvious that carriers of MMR mutations had increased risk for endometrial cancer as well as colon cancer. This in turn spurred both direct and indirect testing for MMR defects in sporadic endometrial cancers and direct testing of candidate genes: MSI and mutation analyses. Immunohistochemistry (IHC) studies directly testing for loss of MMR proteins in tumors proceeded rapidly.

The initial studies focusing on the mutation status of candidates, specifically the *MLH1*, *MSH2*, *MSH6*, and *PMS2* genes, were disappointing. Few MSI-positive tumors had mutations [17, 18]. However, methylation of MLH1 regulatory sequences, initially seen in colon cancers with MSI, was found in the majority of MSI-positive endometrial cancers and rarely in tumors with normal MMR (no MSI or so-called microsatellite stable (MSS) tumors) [19, 20]. Aberrant *MLH1* methylation was linked to epigenetic silencing of *MLH1* based on MLH1 protein measured by IHC: tumors with methylation failed to express MLH1 [20]. *MLH1* promoter methylation thus became a direct test for a cause of MMR deficiency. Work by a number of groups confirmed methylation of *MLH1* is frequent in tumors with MSI and that germline or somatic mutations in *MLH1*, *MSH2*, and *PMS2* were seen at low frequency [17, 21, 22].

Although MMR defects associated with epigenetic silencing of *MLH1* are seen frequently in endometrial cancers, the precise mechanisms by which *MLH1* is silenced remain a matter of uncertainty. One factor contributing to *MLH1* silencing is sequence variation at or near the *MLH1* locus. Again, a candidate gene approach was pursued to test the hypothesis. In 2007, Chen and colleagues [23] provided evidence for heritable predisposition to epigenetic silencing of *MLH1*. A single nucleotide polymorphism in the 5′ untranslated regions (rs1800734) was shown to be associated with aberrant methylation of *MLH1* in both endometrial and colon cancers using a nested case study design. The finding has been confirmed in several other cohorts [24, 25]. Subsequent work in colon cancer further suggested that variation in the *MLH1* locus at rs1800734 might in fact be a low penetrance risk allele

[26]. The same association with risk has also been reported for endometrial cancer [27]. It is noteworthy that association with aberrant methylation was recently reported in peripheral blood cells [28]. This discovery has important implications for normal aging and tumorigenesis.

The study of MMR defects and endometrial tumorigenesis began as a "me too" analysis. Endometrial cancers were underappreciated or seen as a minor component of the inherited colon cancer syndromes. Today endometrial cancer is recognized as a hallmark of inherited MMR deficiency and Lynch syndrome eponym has been adopted to reflect colon, endometrial, and other tumor risk [29]. The high frequency of MMR defects (tumor MSI) in endometrial cancer spurred a range of molecular studies. One of the candidate MMR genes, *MSH6*, had been considered to play a minor role in inherited susceptibility to colon cancer. *MSH6*'s possible causative role in endometrial cancer came to prominence with a report on *MSH6* mutation in a family with Lynch syndrome in which several members were affected by endometrial cancer [30]. In 2004, a search for *MSH6* mutation in endometrioid cancers revealed frequent germline *MSH6* mutations [31]. The finding was confirmed in a second cohort shortly thereafter [32]. Today, alterations in *MSH6* are recognized as perhaps the most frequent cause of inherited endometrial cancer and clinical testing for germline *MSH6* mutation has been implemented widely for endometrial cancer patients with suspected Lynch syndrome.

Molecular testing of endometrial tumors is used in the triage for genetic testing for germline mutations. A combination of indirect and direct testing has been recommended: MSI, MMR IHC, MLH1 promoter methylation, and mutation analysis [33, 34]. Universal testing of MMR defects has been recommended by gynecologic oncology in an effort to identify patients with Lynch syndrome [35].

The link between MLH1 epigenetic silencing and endometrial tumorigenesis was firmly established in the late 1990s. The importance of loss of MMR in the initiation of endometrial cancer, be it due to inherited mutation in the context of Lynch syndrome or epigenetic silencing in sporadic endometrial cancers, was clear. In colorectal cancers, loss of MMR (MSI phenotype) was shown to be prognostic and ultimately predictive of outcome [36–38]. The discoveries in colon cancer led to similar analyses in endometrial cancer. Despite many published studies, some showing that MSI is associated with improved outcomes, others suggesting an association with reduced survival, and still other showing no effect, it is still unclear if tumor MSI is a prognostic marker. It has been suggested that both clinical heterogeneity and how MMR status is assessed and categorized (molecular lumping of indirect phenotyping of MMR status as normal or defective) may explain the differences among the different studies [39]. Bilbao-Sieyro and colleagues [40] have argued that lumping tumors into two groups, MSI-positive and MSS ignores the long appreciated variation and DNA content (ploidy) that could confound outcome studies.

The similarities and differences in MMR defects in endometrial and colon cancer have helped shape our understanding of the role MMR plays in cancer susceptibility, tumor initiation, and tumor progression. Inherited *MSH6* mutations are far more common in endometrial cancer patients than colon cancer patients. On the surface

this could be taken to mean that MSH6 is the guardian of the endometrial epithelium genome, and by extension its role in colonic epithelium less critical. However, loss of MMR due to epigenetic silencing of MLH1 is the most common cause of defective MMR in both colon and endometrial cancers and it is nearly twice as frequent in endometrial cancers than colon cancers. Clearly MMR defects help drive endometrial tumorigenesis. Molecular studies of uterine cancers focused on MMR defects will continue to rely on both direct and indirect testing methods. The Cancer Genome Atlas for uterine cancers [41] recognizes MMR deficiency as a defining feature of one of the major molecularly defined classes of endometrial cancer: tumors that have MSI and many more somatic mutations than their MMR normal counterparts. The genomic landscape of endometrial cancers is discussed in greater detail in Chap. 5.

Steroid Hormone Receptors

Aberrant steroid hormone signaling has been implicated in endometrial tumorigenesis for over a half century [42–44]. Early studies exploring the relationship between hormone receptor status and clinical parameters relied largely on radiolabeled ligand binding assays. Absence of estrogen receptor (ER) and progesterone receptor (PR) has been associated with high tumor grade, advanced stage, metastasis, and recurrence [45–48]. Today it is widely accepted that estrogen excess is associated with risk for the development of endometrial cancer [49, 50], progesterone can have antitumor activities [51, 52], and absence of the receptors on tumors appears to be associated with poor outcomes for endometrial cancer patients [53].

A major technical advance is the study of steroid hormone receptors in endometrial cancer came in 1986 when Budwit-Novotny and colleagues [54] described the use of monoclonal antibodies to detect ER and PR in tissue samples. IHC methods made it possible to distinguish between glandular and stromal expression and to determine the subcellular localization of the receptors [55, 56]. IHC analysis could also be used to conveniently study large numbers of tumors. IHC confirmed earlier reports that reduced steroid hormone receptor expression is associated with factors that portend poor outcomes in endometrial cancer patients including advanced stage, high tumor grade, advanced patient age, and presence of lymphovascular space invasion [57–61]. There are many reports on the potential prognostic significance of ER and PR expression in endometrial cancers, but to date there have been no prospective, well-controlled IHC studies [53, 62–65].

Advances in molecular biology have repeatedly changed the prism through which hormone receptors are viewed. Gene cloning and new tools for molecular biology have shown how very complex steroid hormone signaling is in normal tissues and in disease. Early IHC expression studies in endometrial cancer did not account for the multiple ER and PR protein isoforms, nor did they consider ER and PR cofactors. It is clear that estrogen, progesterone, and their receptors all play critical roles in endometrial cancer biology. In some regards it appears that the more we know, the less we understand.

There are two estrogen receptor genes, *ESR1* and *ESR2*, encoding ERα and ERβ, respectively [66, 67]. Work in many different systems has led to general acceptance that ERβ acts to oppose the actions of the canonical ERα isoform in normal tissues in breast, ovarian, and endometrial cancers [68–70]. The complexity of ERα and ERβ gene regulation makes receptor analysis in primary tissue specimens extremely challenging. Although both the alpha and beta forms bind estrogen responsive elements, they recruit different cofactors to regulate different targets or have opposite effects on the same targets [71–74]. At least three ERα and five ERβ isoforms exist and all of these are likely to play unique roles in hormone signaling [75, 76].

A single *PGR* gene exists that encodes at least seven transcripts with three established isoforms, PR-A, PR-B, and the less well-studied PR-C, along with several possible other isoforms [77–80]. Like ERα and ERβ, PR-A and PR-B have distinct molecular targets.

Candidate Tumor Suppressors and Oncogenes

TP53

The tumor suppressor gene *TP53* is the most frequently mutated gene in human cancers [81]. TP53's role in endometrial cancer has been a subject of investigation for over two decades using indirect tests (testing for allelic deletion) or direct tests for mutations or overexpression of *TP53* protein. Today it is known that TP53 is mutated in over 90% of serous endometrial cancers and is infrequently mutated in low grade endometrioid endometrial tumors [41]. However, early studies did not always make clear distinctions between type I and type II endometrial cancers or histologically different tumors as the existence of distinctive biology was not yet established.

In 1991, Okamoto and colleagues [82] first reported on *TP53* abnormalities in endometrial cancers. They tested 24 tumors for evidence of loss of heterozygosity (LOH) using Southern blot-based restriction fragment length polymorphism (RFLP) analysis with a panel of 57 markers representing all chromosomes. Five tumors had LOH on the short arm of chromosome 17 involving TP*53*. Using single strand conformation analysis and Sanger sequencing of variants, Okamoto and colleagues [82] went on to demonstrate two of these five cases with LOH also harbored *TP53* mutations as would be expected for a classical "two-hit" tumor suppressor. In the same year, it was reported that *TP53* mutations were common in endometrial cancer cell lines [83]. TP53 expression measured by IHC and indicative of *TP53* mutations was observed in 21% of endometrial cancers studied by Kohler and colleagues [84]. Collectively, the analyses in the early 1990s described earlier firmly established a role for TP53 in a subset of endometrial cancers.

The relationship between *TP53* mutation and pathologic features was further explored by Enomoto et al. [85] who assessed *TP53* mutation and LOH as well as *KRAS* mutations in endometrial cancer and atypical hyperplasia samples. *TP53* alterations were seen in ~25% of samples, including atypical hyperplasias, with a higher rate of *TP53* defects in grade 3 endometrioid endometrial cancers than in

grade 1 or 2 tumors. *TP53* and *KRAS* mutation tended to be mutually exclusive, which provided some early insights into the existence of molecularly distinct subgroups of endometrial tumors [85].

In an effort to determine if *TP53* mutations occur as early events in endometrial tumorigenesis, Kohler and colleagues investigated simple, complex, and atypical endometrial hyperplasia and carcinomas for mutations using single-strand conformational variant (SSCV) analysis coupled with direct sequencing. No mutations were identified in the hyperplasias, including 41 atypical hyperplasia specimens, and based on these findings the authors postulated that *TP53* mutation is a late event in endometrial tumorigenesis [86]. The study by Kohler and colleagues [86] did not include endometrial intraepithelial carcinoma or endometrial glandular dysplasia specimens, the putative precursors of serous endometrial carcinoma. Sherman et al. [87] reported findings for *TP53* expression (IHC status) in broad range of endometrial specimens including benign endometrium, atypical endometrial hyperplasia and endometrial intraepithelial carcinoma samples, as well as endometrioid, clear cell, and serous carcinomas. They noted positive *TP53* staining (indicative of *TP53* defects) for most endometrial intraepithelial carcinoma, clear cell, and serous samples. In contrast, only 20 % of endometrioid samples were positive, and all atypical endometrial hyperplasia and benign endometrium samples were negative. This study helped to establish that *TP53* mutation is indeed an early and frequent event in serous and clear cell endometrial carcinomas, and that mutations were less common in endometrioid tumors and rare in the histologically defined precursors of endometrioid cancer [87]. Recent studies that rely on more sensitive methods have confirmed an increasing frequency of *TP53* abnormalities with progression from normal endometrium through endometrial glandular dysplasia and endometrial intraepithelial carcinoma to serous carcinoma [88].

TP53 was one of the first candidate genes studied as a prognostic marker in endometrial cancer. Several reports suggested association between mutation status and/or positive IHC staining and features associated with poor outcome including nonendometrioid histology, advanced stage, and high grade [84, 89–91]. Subsequent studies of larger cohorts revealed TP53 status is not an independent marker of poor outcome in multivariable analyses that included histologic subtype as a confounding variable [92–95]. It is noteworthy that the rates of *TP53* mutation in endometrioid cancers reported in early studies tend to be higher than what has been reported in recent years. Possible explanations for the higher mutation rates in early studies are sample bias to larger and/or higher stage and grade tumors and misclassification of nonendometrioid tumors as *TP53*-mutated endometrioid endometrial cancers [41].

PTEN

The *PTEN* tumor suppressor is the most frequently mutated gene in endometrial cancer. Its existence and importance in endometrial cancers was first suggested by the results of deletion mapping studies (indirect tests for tumor suppressor

function). Allelic loss/deletion of the genomic region including the *PTEN* locus was recognized in endometrial cancers several years before the *PTEN* gene was cloned. In 1994, Jones and colleagues reported on loss of heterozygosity (LOH) studies in endometrial cancers with a panel of 29 microsatellite markers distributed across the genome as part of an effort to map the location of tumor suppressors. More than a third of tumors had deletion of 10q [96]. The finding of frequent 10q deletion in endometrial cancers was subsequently confirmed and the minimum region of deletion mapped to 10q23-26 [97]. In 1997 the *PTEN* gene, a novel tumor suppressor mapping to 10q23, was cloned and shown to be mutated in a range of malignancies [98, 99]. Following the initial discovery, Kong et al. examined mutation (direct testing) and LOH status of *PTEN* in a panel of endometrial, colorectal, gastric, and pancreatic carcinomas [100]. They found that mutation and LOH were seen infrequently in colorectal, gastric, and pancreatic tumors. However, among the endometrial cancers tested, 48 % showed LOH and 55 % were mutated, with most mutations resulting in clear loss of function [100]. The Kong et al. study provided the first evidence that *PTEN* is frequently mutated in endometrial cancers and strongly suggested that *PTEN* is the 10q tumor suppressor for which there is strong selection for deletion in endometrial cancers.

Around the same time, Tashiro et al. examined a panel of endometrioid endometrial cancers, serous endometrial cancer, ovarian cancer, and cervical carcinomas and found that mutation in *PTEN* is specific to endometrioid endometrial cancers [101]. A follow-up study confirmed that *PTEN* mutations are much more frequent in endometrioid than serous or clear cell endometrial cancers [102]. *PTEN* became the most commonly mutated tumor suppressor gene in endometrial cancers, and endometrial cancers garnered a great deal of attention by geneticists and cancer biologists interested in *PTEN*.

A potential link between *PTEN* mutation and MMR status was established shortly after the *PTEN* gene was discovered. MSI-positive tumors appeared to have more frequent *PTEN* mutation. Furthermore, it was initially reported that outcomes were better for women with *PTEN* mutant tumors [102]. Mutter and colleagues determined that *PTEN* defects occur early in tumorigenesis by analyzing cancers and precancers [103]. It was subsequently shown that *PTEN* lesions might precede MMR defects, which were previously established as occurring early in the development of endometrial cancers [104]. With the advent or antibodies for immunohistochemical analysis of *PTEN* expression and direct testing for defects, the Mutter lab confirmed that loss of *PTEN* protein is observed in some normal endometrial glands. They speculated that concurrent loss of *PTEN* and additional critical regulators of development may be necessary for malignant transformation [105]. Given the high frequency of both mutation and deletion of *PTEN* in endometrial cancers, it was not surprising that a search for epigenetic silencing of *PTEN* was undertaken. It has been reported that *PTEN* can also be inactivated through promoter methylation [106], but how frequently this occurs is uncertain and further methylation studies in endometrial cancers using additional methods are warranted [107].

Because *PTEN* mutation is an early event in tumorigenesis many groups have investigated the utility of *PTEN* staining in precancerous lesions to predict progres-

sion to carcinoma. Several studies suggest that there is a stepwise decrease in *PTEN* expression between normal endometrium, precancerous lesions (endometrial intraepithelial neoplasia and complex atypical hyperplasia), and endometrial cancer [103, 105, 108–111]. A large study by Lacey et al. published in 2008, on the other hand, found that *PTEN* IHC is not useful for predicting progression of atypical endometrial hyperplasia to endometrioid endometrial cancer [112]. Similar reports have found that *PTEN* negativity in endometrial intraepithelial neoplasia is not sufficient to predict malignant transformation, although combining *PTEN* status with nuclear atypia increases prediction sensitivity and specificity [113, 114]. The inconsistent findings are likely attributable to etiologic heterogeneity and the reliability of the tests used.

Traditional approaches to molecular genetic analysis include generation and characterization of genetically modified animals. The functional consequences of in vivo *PTEN* loss were first examined in 1999 by Podsypanina and colleagues who developed a knockout mouse model and observed that the *Pten*+/− heterozygous animals developed neoplasms in the endometrium, as well as liver, prostate, GI tract, thyroid, and thymus [115]. By 6 months of age, 100 % of *Pten*+/− mice exhibited endometrial hyperplasia, providing evidence to the importance of *PTEN* in this tissue [116]. Early studies combining in vivo loss of *PTEN* with other genetic alterations in cancer-associated genes determined that loss of tumor suppressors such as INK4a/ARF [117], MLH1 [118], and MIG6 [119] accelerated hyperplastic growth and led to development of carcinomas. In contrast, loss of the *Akt* oncogene in *Pten*+/− mice was found to be protective, particularly in the endometrium [120]. The *Pten* +/− mouse model was later used to show in vivo that loss of *PTEN* leads to elevated Akt activation and a subsequent increase in ER signaling that drives endometrial hyperplasia/carcinoma [121]. Interestingly, neonatal estrogen exposure was also found to be protective against endometrial hyperplasia [122]. Interest in endometrial cancer and research investments in endometrial tumorigenesis grew remarkably when *PTEN*'s role in endometrial tumorigenesis was appreciated. The endometrium became a model system in which to study perturbed signaling.

In 2008, Diakoku et al. developed an inducible uterine-specific homozygous Pten knockout using a PR (progesterone receptor) (Cre+/−) *Pten*(fl/fl) system. At the time a conditional knock out was state of the art, but today it is a traditional approach in mouse genetic analysis. Diakoku and colleagues demonstrated that homozygous deletion of *Pten* led to development of carcinomas with 100 % penetrance and early onset [123]. The model has been subsequently used to further investigate other common genetic events in endometrial cancers in vivo, in the absence of Pten. These studies have shown that endometrial carcinogenesis can be accelerated through mutational activation of Pik3ca [124], loss of Apc [125], loss of Cdh1 [126], and loss of Lkb1 [127], and that knockout of Grp78 prevents carcinoma development [128]. Today the "one gene at a time" approach for mouse models for endometrial cancer seems particularly daunting given how many genes have been implicated based on candidate gene studies alone.

The use of tumor *PTEN* protein expression to predict patient outcome and/or response to therapy has been extensively studied over the past 15 years. Complete loss of *PTEN* protein and RNA (direct tests) occurs in many patient samples, although

the reported percentage of *PTEN* negative tumors varies between 7 and 65 %, depending on the methods used and patient population investigated [9, 129–131]. The frequent involvement of a gene, such as *PTEN*, in endometrial cancer makes it an attractive candidate for therapeutics, but based on frequency alone, an unlikely prognostic marker. An early report by Mutter et al. described reduced *PTEN* protein compared to normal endometrium in most cancers investigated and 13 of 33 cases had no immunodetectable protein [103]. A similar report from Salvesen et al. found that 20 % of EC tumors examined had loss of *PTEN*, and in their study *PTEN* negativity was associated with metastasis [9]. Still another study showed that *PTEN* negative tumors tend to be less well differentiated than *PTEN*-expressing EECs [132]. The high frequency of *PTEN* abnormalities combined with the many different mutations that coexist with *PTEN* defects explains why clear pictures regarding *PTEN* status and clinical features have failed to emerge. A subgroup of *PTEN* negative tumors that also lack p27 are well differentiated and have favorable outcome [133]. Recent comprehensive mutation studies that include *PTEN* and other candidates show consistent high frequency of *PTEN* mutation or deletion in endometrioid tumors, plus or minus other common and rare mutations: these next-generation studies reflect what we began to learn by studying one candidate at a time, then combinations. Studying *PTEN* alone, as was done in early studies, gave mixed results as might be expected. *PTEN* negativity was associated with poor outcome [131, 134, 135] but there are clear contrasting reports [136, 137]. Among advanced stage patients, *PTEN* negativity is associated with favorable response to chemotherapy, and although this was first reported over a decade ago, *PTEN* status has never been used in the clinic to direct treatment strategies [138, 139]. The candidate gene *PTEN* is undeniably important in endometrial cancer. At present the prognostic and predictive significance of *PTEN* defects in endometrial cancer is entirely unknown.

KRAS

The ras family of oncogenes is frequently mutated in cancers [140, 141]. Most mutations inhibit ras GTPase activity, resulting in constitutively active ras and activation of the downstream PI3-kinase and MAP-kinase pathways. The potential role for ras family members in endometrial cancer was first investigated more than a quarter of a century ago using immunohistochemistry [142, 143]. Direct testing for the known activating mutations followed [144, 145].

Ras mutations in endometrial cancers typically are in *KRAS*, with much less frequent involvement of *NRAS* and *HRAS* [146, 147].

KRAS mutations were first identified using PCR and dot plot hybridization mutational screening for a small number of tumors, half of which harbored *KRAS* mutations [145]. Shortly thereafter *KRAS* mutation was implicated as an early event in endometrial tumorigenesis based on the observation that some endometrial hyperplasias carried *KRAS* mutations [146]. With advances in methods for mutation testing, specifically PCR amplification of tumor DNAs and allele specific

oligomer dot-blot hybridization, it was possible to analyze larger numbers of specimens and to interrogate additional base substitutions. Duggan and colleagues tested *KRAS* codons 12 and 13 for mutations in 60 endometrial cancers (a sizeable number of specimens at the time) and found that mutations were present in both the carcinomas and surrounding atypical hyperplasia [148]. The use of UV radiation fractionation to interrogate the mutation status of precancerous cells firmly established a role for *KRAS* early in endometrial tumorigenesis [148]. Additional early studies on ras mutation status in smaller numbers of cases provided a wide range of mutation frequency for *KRAS* ranging from 10 % for primary tumors to 64 % for cell lines [147, 149, 150].

There were early reports on differences in *KRAS* mutation frequency in different histologic subtypes of endometrial cancer: differences in the methods for mutation detection and histological classification of tumors likely explain some of the apparently contradictory findings for early studies. The overall consensus is that *KRAS* mutations are infrequent in nonendometrioid cancers. *KRAS* mutations, predominantly involving codon 12, are present in ~20 % of endometrioid tumors with no clear difference in mutation frequencies in tumors with intact mismatch repair and MSI-positive tumors [34, 41, 151–154].

Aberrant ras activity could provide therapeutic opportunities in endometrial cancer and although ras mutations were among the first defects described, the finding has not translated to new therapies. Pharmacologically, direct targeting of the ras family remains elusive [155], although recent efforts have shown some promise [156, 157]. The use of molecules targeting downstream ras effectors (e.g., mTOC1/2, PI3-kinase, AKT) has been explored in preclinical models and clinical trials [158]. Activation of ras in endometrial cancers may ultimately factor into treatment and even prevention strategies.

FGFR2

Members of fibroblast growth factor receptor (FGFR) family (FGFRs 1–4) play important roles in development, normal cellular processes, and pathophysiology [159]. The FGFRs are classic multifunctional receptor tyrosine kinases for which combinations of receptor isoforms and multiple ligands afford tremendous functional diversity. FGFRs activate the ras, src, and PI3-kinase pathways [160]. Kinome screens (mutation analysis of a large number of kinases) were undertaken in cancer cell lines and a variety of primary cancers with the goal of identifying druggable targets [161–163]. The FGFRs were recognized as potential oncogenes, but largely lacking cancer associations. *FGFR2*, however, became a candidate oncogene/drug target for endometrial cancers when mutations were identified in uterine cancer cell lines (http://www.sanger.ac.uk/genetics/CGP/CellLines). Mutations in *FGFR2* were first reported in primary endometrial cancers in 2007 [164]. The majority of alterations seen in endometrial cancers are missense mutations that have previously been characterized as causative germline mutations in patients with congenital

craniofacial developmental disorders (S525W and N550K as two examples) [164]. Activation of ras signaling appears to mediate the oncogenicity of *FGFR2* mutations [165, 166] and not surprisingly *KRAS* and *FGFR2* mutations are nearly mutually exclusive. The therapeutic implications for activating *FGFR2* mutations in endometrial cancer were recognized by both cancer biologists and developmental biologist [167, 168]. Efficacy of *FGFR2* inhibition was shown in endometrial cancer cell lines using the FGFR/VEGF inhibitor PD173074 as a single agent [165, 169] and in combination with doxorubicin and paclitaxel [170]. *FGFR2* thus became a viable target for therapeutic intervention in endometrial cancers: the candidate gene from a cell line screen was confirmed by simple mutation analysis in primary tumors and drug testing in cells lines. Years of work in other experimental systems, driven in large part by the importance of *FGFR2* mutations in human congenital malformation syndromes, paved the way for clinical trials in endometrial cancer using anti-FGFR agents. What, if any clinical benefit for endometrial cancer patients will come from *FGFR2* inhibitor remains to be determined. The importance of discovery of *FGFR2* activation is nonetheless important. It has further highlighted the roles of multiple signaling axes in endometrial cancers and has prompted questions regarding the function that FGFR signaling plays in the normal endometrium, precancerous endometrium, and in frank carcinoma.

Combinations of Molecular Defects Explain the Biology

Early genetic studies in endometrial cancer were performed one gene/one factor at a time. The findings from those early studies have provided both conceptual and biological frameworks for multifactor molecular approaches currently being used to characterize endometrial cancers. The idiom *nanos gigantum humeris insidentes* (discovering truth by building on previous discoveries) seems particularly apt as we begin to adopt "next-generation" technologies for molecular analysis of endometrial cancers. The increasing resolution for the cancer cell genomic landscape will have meaning only if we look back to where we have come from. Doubtless some of the giants we have already discovered (*PTEN*, MMR defects, steroid hormones, and their receptors and others) will provide important vantage points as we seek to understand the genomic complexity of individual tumors and endometrial cancers in general.

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