The genomic landscape and evolution of endometrial carcinoma progression and abdominopelvic metastasis

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Recent studies have detailed the genomic landscape of primary endometrial cancers, but the evolution of these cancers into metastases has not been characterized. We performed whole-exome sequencing of 98 tumor biopsies including complex atypical hyperplasias, primary tumors and paired abdominopelvic metastases to survey the evolutionary landscape of endometrial cancer. We expanded and reanalyzed The Cancer Genome Atlas (TCGA) data, identifying new recurrent alterations in primary tumors, including mutations in the estrogen receptor cofactor gene NRIP1 in 12% of patients. We found that likely driver events were present in both primary and metastatic tissue samples, with notable exceptions such as ARID1A mutations. Phylogenetic analyses indicated that the sampled metastases typically arose from a common ancestral subclone that was not detected in the primary tumor biopsy. These data demonstrate extensive genetic heterogeneity in endometrial cancers and relative homogeneity across metastatic sites.

Endometrial cancer is the most common pelvic gynecological malignancy in industrialized countries, partially owing to the obesity epidemic1. Seventy-five percent of patients present with type I, endometrioid, tumors2, often with adjacent regions containing complex atypical hyperplasia (CAH), considered precursor lesions. Type I tumors are often responsive to estrogen and portend a good prognosis. Type II tumors comprise the non-endometrioid subtypes, including the serous, carcinosarcoma, clear cell and undifferentiated histologies. These tend to occur in older, non-obese women, are rarely responsive to estrogen and carry a poor prognosis.

Recent large-scale sequencing studies of primary endometrioid and serous tumors have indicated that the difference in phenotype is reflected in distinct molecular subgroups, with further molecular subclustering3–5. Although those studies detailed the patterns of somatic alterations across primary tumors, a comparative study of samples from endometrial CAH, primary tumors and paired metastases has not been performed to our knowledge. It is not known whether metastases derive from the same or multiple lineages within the primary tumor or whether cancer cells require specific mutations that enable metastasis. The extent to which the genetic events observed in a primary tumor biopsy represent the full diversity of subclones across a metastatic cancer is also unknown. Such information would be helpful in understanding the biological underpinnings of endometrial cancer progression and to determine treatment strategies that target features that are homogenous throughout individual cancers.

Here we address these questions using a collection of 98 extensively clinically annotated fresh-frozen samples ranging from precursor lesions to primary tumors and paired abdominopelvic metastases. We analyzed somatic mutations and allelic copy number profiles for different biopsies from the same individual to reconstruct phylogenetic relationships and annotate putative cancer drivers across sites of disease. We also reanalyzed data from TCGA using updated methods, leading us to identify recurrent mutations in NRIP1 and patterns of heterogeneity within biopsies that mimic heterogeneity across multiple tumor sites.

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RESULTS

Patient and sample cohort

Our cohort consisted of a population-based patient series with extensive clinical annotation including complete follow-up information. We obtained fresh-frozen tumor tissue from 52 individuals: 12 with CAH and 40 with metastatic endometrial cancer. We analyzed 98 biopsies: 12 CAHs, 32 primary tumors and 54 abdominopelvic metastases (Fig. 1, Supplementary Figs. 1 and 2, and Supplementary Table 1). Twenty-six primary tumors were associated with one or more paired metastases. We analyzed samples using whole-exome sequencing and/or Affymetrix SNP 6.0 arrays (Supplementary Fig. 1). For all samples subjected to whole-exome sequencing, we also tested for microsatellite instability, enabling classification according to the integrated molecular subgroups established by TCGA3 (Fig. 2a and Supplementary Fig. 2).

Significantly mutated genes and hotspots

The burden of somatic genetic alterations in our primary tumors was consistent with endometrial cancers profiled by TCGA. We observed similar rates of somatic mutation (minimum 40 to maximum 13,717 mutations per exome) and somatic copy number alternation (SCNA; Supplementary Fig. 1, Fig. 2a and Supplementary Fig. 3a,b), and an inverse correlation between them (P = 0.005; Fig. 2b). However, the mutation rates of some of the most frequently altered genes differed, with higher mutation rates for PPP2R1A, FGFR2, PIK3CA and ARID1A and lower rates for PIK3R1 in our data set as compared to TCGA data. These differences may reflect different sample inclusion strategies (Supplementary Fig. 4).

The burden of small indels detected among primary endometrial cancers was higher than previously noted, particularly among microsatellite-unstable (MSI) carcinomas. Microsatellite instability, prevalent among endometrial carcinomas, leads to high rates of these indels. However, these events are often observed at low allelic fractions in paired normal samples owing to sequencing error and are typically discarded as non-somatic. We applied recently developed methods to ‘rescue’ highly recurrent indels that were enriched in tumor samples (Supplementary Fig. 5a). We identified an average of 156 and 21 indels per MSI tumor and non-MSI tumor, respectively, as compared to 16 and 4 indels in previous analyses. We subjected the indels detected by our rescue strategy to additional technical validation using six approaches and achieved a 96% validation rate (Online Methods and Supplementary Table 2).

We used these new calls and the combined data set of our primary tumors and those in TCGA (274 primary tumors in total) to catalog the significantly mutated genes in primary endometrial cancer. We identified 49 genes with significantly recurrent rates of mutation (Supplementary Fig. 5b and Supplementary Table 3), including 21 that, to our knowledge, have not been previously described in endometrial cancer. Of the 21 new genes, 4 (NFE2L2, ERBB2, U2AF1 and ALPK2) have been found to be recurrently mutated in other primary cancer types using similar analytic methods to those used here.

The other 17 significantly recurrently mutated genes included both ESR1, encoding estrogen receptor α, and NRIP1, which encodes its binding partner. Alterations in the estrogen pathway are considered to be risk factors for endometrioid endometrial cancer10, and recurrent rearrangements involving ESR1 have recently been identified in breast cancer11. However, significantly recurrent point mutations in the estrogen pathway have not been previously described in cancers that had not been subjected to anti-estrogen therapy.

We found NRIP1 mutations in 12.5% of patients, concentrated in two highly recurrent sites, Lys728 (n = 11) and Asn516 (n = 4) (Fig. 2c and Supplementary Fig. 5c,d). All but two of these indels were in MSI samples (20% of MSI samples). In addition, 14% of colorectal MSI samples analyzed by TCGA exhibited NRIP1 indels. The NRIP1 protein binds to the AF2 domain of the estrogen receptor and is essential for its transcriptional activity12,13.

Figure 1 Samples assessed. (a) Anatomical sites from which samples were obtained. (b) Histological subtype (EN, endometrioid carcinoma; CC, clear cell carcinoma; S, serous carcinoma; U, undifferentiated carcinoma; CS, carcinosarcoma), grade, International Federation of Gynecology and Obstetrics (FIGO) 2009 stage at primary diagnosis, time of metastatic lesion sampling and treatment after primary diagnosis. Asterisks indicate four cases that were clinically difficult to distinguish as metastatic or independent synchronous primary cancers at the time of resection.
We detected *ESR1* mutations in 4% of cancers, and they clustered in the region encoding the ligand-binding domain (Supplementary Fig. 5e,f). These included mutations resulting in p.Tyr537Cys, p.Tyr537Asn and p.Tyr537Ser substitutions (three patients) that have been shown to cause constitutive activation and resistance to tamoxifen therapy in breast cancer.\(^{14,15}\) However, the only patient in our cohort with a change at Tyr537 never received anti-estrogen treatment. Moreover, the endometrial cancers profiled by TCGA were untreated, and previous malignancies were an exclusion criterion. These observations indicate that mutations encoding p.Tyr537Cys, p.Tyr537Asn or p.Tyr537Ser can occur in endometrial cancers without previous anti-estrogen treatment.

Additional genes included MAX, which encodes the binding partner of proteins encoded by *MYC* family members. We identified two recurrently mutated sites in MAX, encoding p.His28Arg (n = 5) and p.Arg60Gln (n = 2; Supplementary Fig. 6a), which also occur in other cancers and appear to interface with DNA\(^{16,17}\). We also observed and validated significantly recurrent mutations in *MYCN*, as previously noted\(^5\), with a hotspot at p.Pro44Leu (n = 5; Supplementary Fig. 6b,c).

Mutations in MAX and MYCN never occurred with each other or with amplifications of MYC or MYCN (P = 0.36, binomial test; Supplementary Fig. 6d). MYCN was also recurrently amplified in endometrial cancers (Supplementary Fig. 6e).

Even among genes previously noted to harbor significantly recurrent alterations, we often detected higher rates of alteration than previously noted, likely due to rescued indels (Supplementary Table 3). For instance, we observed ARIDIA mutations in at least one biopsy from 49% of patients, a 40% increase over previous estimates. Genes in which polymerase-slippage-associated indels have previously been identified, such as RPL22 (refs. 18, 19), RNF43 (ref. 8) and JAK1 (ref. 20), showed even more dramatic gains (370%, 206% and 163% increases, respectively; Fig. 2f). Conversely, the number of patients with biopsies exhibiting PIK3CA mutations increased by only 5.6% and there was no increase in the number of patients with CTNNB1 mutations. Overall, we called 39% more mutations (all indels) across all genes and 54% more mutations in recurrently altered genes (P = 0.28).

The higher rates of alteration detected in these recurrently mutated genes motivated us to reevaluate their relationship among TCGA data

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**Figure 2** Somatic genetic alterations in CAHs and primary and metastatic endometrial carcinomas. (a) Number of exonic mutations (top) and SCNASs (middle) detected in each tumor biopsy. *PTEN*, *TP53* and 1q amplification status, histological subtype, TCGA annotation and tissue type are also indicated (bottom). (b) Number of mutations versus fraction of the genome affected by SCNAS acrossCAHs and primary lesions from endometrioid endometrial carcinoma and non-endometrioid endometrial carcinoma, as indicated. (c) Number of mutations detected in the primary tumor as compared to the metastatic counterpart. (d) Percentage of the genome affected by SCNASs across primary tumors from endometrioid and non-endometrioid endometrial carcinoma, as indicated. (e) Stick plot depicting detected mutations in *NRIP1*. (f) Impact of indel rescue on the percentage of patients harboring mutations in known driver genes.
mutations) were excluded from the analysis. Hypermutated samples (>1,000 mutations) were clonal for the indicated genes. Bars indicate 95% confidence intervals.

Among the 186 arm-level SCNAs (comprising most of a chromosome arm) detected, 90 (48%) were heterogeneous across biopsies. Arm-level losses were more likely than gains to be shared (58% versus 40%; \( P = 0.02 \); Supplementary Fig. 9a). Losses of 10q, harboring \( PTEN \), and 17p, harboring \( TP53 \), were shared more often than other arm-level losses (\( P = 0.019 \) and 0.035, respectively). The most common arm-level gain, 1q, was truncated in only 2 of 10 cases (Supplementary Fig. 9b). The heterogeneous events included both gains and losses of alternate homologous chromosomes in PB and MBs (Supplementary Fig. 9c–f), suggesting convergent evolution of these SCNAs. These observations imply that arm-level losses, at least some of which result from in homozygous knockout of tumor-suppressor genes, generally occur before arm-level gains in endometrial cancer evolution.

**Primary biopsies lack half of the alterations in metastases**

Although the overall somatic genomic alteration burden was similar for primary tumors and their matched metastases, only an average of 48% of specific mutations (Fig. 3a) and 56% of SCNAs (Supplementary Fig. 9) found in the MB were shared with the PB. Conversely, an average of 51% and 48% of mutations and SCNAs in the PB, respectively, were shared with each MB. The fraction of MB-specific mutations tended to increase with the anatomical distance of the metastasis site from the endometrium (\( \rho = 0.27; \ P = 0.13 \); Supplementary Fig. 10), consistent with data in prostate tumors.

**PI3K pathway alterations predominate in hyperplasias**

In comparison to primary tumors, CAHs exhibited few somatic mutations with two exceptions, which were highly mutated (median of 35 mutations per sample, range of 17–348; Fig. 2b and Supplementary Fig. 3a). CAHs also exhibited less copy number alteration (median of 3.0% of the genome altered versus median of 11.9% in primary tumors, \( P = 0.005 \); Supplementary Fig. 3b).

Mutations of at least one of \( PTEN \) and \( PIK3CA \) (usually \( PTEN \)) were present in all 12 samples (Supplementary Fig. 7). Loss of heterozygosity of chromosome 10q, containing \( PTEN \), and amplification of chromosome 1q were the only recurrent copy number alterations, detected in four and three cases, respectively. Other genes that were significantly mutated in primary endometrial cancers were also mutated at lower frequency in CAHs; mutations of \( ARID1A \), \( CTCF \), \( RNF43 \), \( ARHGAP35 \) and \( MYO10 \) were each mutated in two CAHs. Phosphatidylinositol 3-kinase (PI3K) pathway gene mutations have previously been shown to be prevalent in CAHs. Although it is possible that smaller fractions of CAH exhibit additional driver mutations, these results indicate that no other genes are mutated at a similar rate to \( PTEN \) and \( PIK3CA \).

**Whole-genome doubling events (WGDs)**

Whole-genome doubling events (WGDs; detected using the software ABSOLUTE\textsuperscript{22,23}) accounted for the largest differences in aneuploidy between paired MBs and PBs. Three PB–MB pairs exhibited WGD in only the MB; two of these MBs were from a single metastasis (Supplementary Fig. 8). These MBs were therefore non-diploid (aneuploid) across most of their genomes. They also exhibited increased rates of localized SCNA, a feature that has previously been associated with WGD in model systems\textsuperscript{24} and primary tumors\textsuperscript{23,25,26}.

**Primary tumors and metastases have similar levels of alteration**

Biopsies from primary tumors (PBs) and biopsies from paired metastases (MBs) exhibited similar overall burdens of somatic genomic alteration (\( P = 0.81 \)). Metastases exhibited a median of 94.5 mutations per biopsy versus 93 mutations in primary tumors (Supplementary Fig. 3a). The number of mutations typically varied by 10.5% between a primary tumor and its paired metastasis (Fig. 2c). Metastases exhibited a median of 12 SCNAs versus 11 per primary tumor (\( P = 0.53 \)); the number of SCNs typically varied by 15.2% (Fig. 2a). The fraction of the genome altered was also similar for most primary tumors and paired metastases (Fig. 2d).

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**Distinguishing multiple clonally independent synchronous primary tumors from the metastatic spread of a single primary tumor has important implications for treatment**

We identified four cases (one in Supplementary Fig. 10b) in which biopsies of different disease sites exhibited substantially different morphology, resulting in clinical calls of synchronous primary tumors. Sequencing identified shared mutations, however, indicating a common clonal origin. Conversely, previous analyses of other cancer types have identified multiple clonally unrelated cancers in individual patients\textsuperscript{22,26}. These results suggest that genetic evaluation is necessary to assess the clonal independence of cancer lesions. 

Among the 186 arm-level SCNAs (comprising most of a chromosome arm) detected, 90 (48%) were heterogeneous across biopsies. Arm-level losses were more likely than gains to be shared (58% versus 40%; \( P = 0.02 \); Supplementary Fig. 9a). Losses of 10q, harboring \( PTEN \), and 17p, harboring \( TP53 \), were shared more often than other arm-level losses (\( P = 0.019 \) and 0.035, respectively). The most common arm-level gain, 1q, was truncated in only 6 of 12 cases (Supplementary Fig. 9b). The heterogeneous events included both gains and losses of alternate homologous chromosomes in PB and MBs (Supplementary Fig. 9c–f), suggesting convergent evolution of these SCNAs. These observations imply that arm-level losses, at least some of which result from in homozygous knockout of tumor-suppressor genes, generally occur before arm-level gains in endometrial cancer evolution.
**Figure 4** Phylogenetic trees for tumors with more than one metastasis. (a) The labeled alterations constitute a subset of the alterations that distinguish between the indicated branches. Hash symbols indicate trees that were derived from SNP 6.0 array data. cnLOH, copy-neutral loss of heterozygosity. (b) Two-dimensional phylogenetic illustrations comparing subclonal structures between biopsies for a patient with endometrial cancer, EC-007. A subclone in the biopsy of the primary tumor shared a common ancestor with the two metastases, not detected in the dominant MBs. The overlap among drivers exceeded the overlap in the overall number of mutations between primary tumors and metastases (mean of 83% versus 51%; \( P = 5.1 \times 10^{-6} \)). This suggests that the fraction of new mutations that we identified as significantly recurrent decreases along the length of the evolutionary tree.

The rate at which driver mutations were shared across all biopsies varied by gene, ranging from 0% to 100% (Fig. 3c). For five genes, we had adequate power to determine whether mutations affecting them were truncal more or less often than the average rate among drivers (here referred to as ‘trunk biased’ and ‘branch biased’, respectively; of 83% versus 51%; \( P = 5.1 \times 10^{-6} \)). This suggests that the fraction of new mutations that we identified as significantly recurrent decreases along the length of the evolutionary tree.

**Rates of intratumoral heterogeneity among common drivers**

An average PB had 83% of its driver mutations in common with its paired MB (Fig. 3b). We defined driver mutations as non-silent mutations of genes in Supplementary Table 3; we identified 1–27 (median of 3) truncal driver mutations per patient. Among the 26 PBs, 15 (57.6%) contained driver mutations not detected in the paired MBs. The overlap among drivers exceeded the overlap in the overall number of mutations between primary tumors and metastases (mean of 83% versus 51%; \( P = 5.1 \times 10^{-6} \)). This suggests that the fraction of new mutations that we identified as significantly recurrent decreases along the length of the evolutionary tree.

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Fig. 3d). Mutations of PTEN, TP53 and PPP2R1A were trunk biased (Fisher’s two-tailed $P = 0.006, 0.03$ and 0.04, respectively), suggesting that they were early events, in the cases of PTEN and PIK3CA consistent with the prevalence of mutations in these genes among CAHs. PIK3CA did not exhibit significant bias in either direction. Mutations in ARID1A were only trunkal in 25% of phylogenies, whereas 60% of mutations were trunkal for other drivers, indicating significant branch bias ($P = 0.03$). Immunohistochemical staining of ARID1A has also suggested subclonal loss 29, and we confirmed across 54 samples that ARID1A mutations were associated with loss of ARID1A immunoreactivity (Supplementary Fig. 11).

Analysis of heterogeneity in individual biopsies supports the finding of frequent heterogeneity of mutations in ARID1A. We calculated the likely fractions of sampled cancer cells (cancer cell fraction; CCF) that carried each mutation as previously described 23,30,31. Among TCGA data, mutations of PTEN and TP53 were almost exclusively clonal (81% and 92% of cases, respectively), whereas only two-thirds of ARID1A mutations were clonal (dissimilar rates from PTEN; $P = 1.6 \times 10^{-11}$; Fig. 3e,f).

Among seven phylogenies with PPP2R1A mutations, five also exhibited TP53 mutations ($P = 0.02$; Supplementary Fig. 12a), and in all cases the PPP2R1A and TP53 mutations were trunkal. We validated the association between PPP2R1A and TP53 mutations in the TCGA data set using an approach that took into account varying degrees of genomic instability across cancers 25,32 (Supplementary Fig. 12b and Supplementary Table 5). The only gene whose mutation was positively correlated with that of TP53 was PPP2R1A ($P = 0.0001$; $q = 0.08$), and mutations in both these genes were enriched in non-endometrioid tumors. Combined, these two genes formed an isolated network corresponding to a subset of non-endometrioid tumors.

Mutations of PPP2R1A clustered in two hotspots: p.Pro179Arg ($n = 11$) and p.Ser256Phe ($n = 4$; Supplementary Fig. 12c), as previously noted 33. The association between PPP2R1A and TP53 mutations among the 274 primary tumors was also primarily due to PPP2R1A hotspot mutations: 17 of 19 tumors with PPP2R1A hotspot mutations exhibited TP53 mutation ($P = 5.8 \times 10^{-8}$; Supplementary Fig. 12d), whereas only 3 of 16 tumors with non-hotspot PPP2R1A mutations exhibited TP53 mutations ($P = 0.56$). The co-occurrence of PPP2R1A and TP53 mutations mirrors the dual mechanism of transformation by the SV40 oncovirus, wherein small T antigen binds PPP2R1A to disrupt association with PP2A regulatory subunits and large T antigen mediates TP53 inactivation (Supplementary Fig. 12e).

Most metastases have a common ancestor absent in the primary biopsy

We determined phylogenetic relationships between tumor biopsies using both mutations and allelic copy number alterations. In six of seven cases with multiple MBs, all the MBs were more closely related to each other than they were to the PB (monophyly). This observation is consistent with these metastases having arisen from a limited fraction of these cancers (possibly even a single cell). In the seventh case, however, one of the MBs was more closely related to the PB than to the other MBs (polyphyly; Fig. 4a and Supplementary Figs. 13 and 14).

These results suggest that most metastases arise from one branched subclone of the cancer. If different metastases had no clonal relationship beyond deriving from the same cancer (‘independent branched subclones’), each sampled tissue would be equally likely to be most closely related to any of the other samples in a given patient. In that case, we would have expected one or two (expectation value of 1.7) cases of monophyly in our data, a significantly different result from the six of seven cases of monophyly observed ($P = 0.001$; Online Methods). Even the existence of two independent branched subclones, each the ancestor of half of the observable metastases in each patient, would have been expected to produce monophyly in only four of the seven phylogenies, a significantly different result from that observed ($P = 0.018$; Online Methods).

We performed similar calculations on phylogenies from prostate and pancreatic cancers for which genome-level sequencing had been performed on two to ten MBs and one to nine paired PBs 27,34,35. Prostate and pancreatic cancers exhibited polyphyly in one of five ($P = 0.05$) and three of five ($P = 0.98$) cases, respectively, consistent with the biopsied metastases arising from a limited fraction of the prostate cancers but perhaps not the pancreatic cancers.

No evidence of ubiquitous metastasis-specific mutations

We did not discover significantly recurrent metastasis-specific mutations. It is possible that metastasis-specific drivers remain undetected. To assess our power to detect metastasis-specific drivers, we ‘spiked’ hypothetical driver mutations into our data set and then assessed whether we recovered them (Supplementary Fig. 15). Our power exceeded 90% for genes mutated in at least 50% of metastases and remained greater than 50% for genes mutated in at least 20% of metastases. These results indicate with a probability of 0.9 that there are no metastasis-specific exomic mutations that recur in greater than 50% of abdminopelvic metastases. However, drivers of metastasis may include features not detectable through whole-exome sequencing or combinations of mutations that we were not powered to detect.

We observed no significant excess of known driver mutations among metastases. Among our 26 phylogenies, 22 exhibited the same number of driver mutations in the primary and metastatic biopsies, 3 exhibited more drivers in the metastasis and 1 exhibited more drivers in the primary tumor ($P = 0.63$).

Detection of a metastasis-related subclone in the primary tumor

We determined the CCF for each mutation as described above. Different mutations clustered around similar CCFs, indicating the presence of subclonal populations. We detected subclonal mutations in every PB and MB. An average of 20% and 26% of mutations in MBs and PBs, respectively, had CCF <1 ($P = 0.26$).

We focused on mutation clusters that were detected in more than one biopsy but had CCF <1 in at least one of them, as these may indicate seeding patterns from one biopsy to another 22,30,36 (Fig. 4b). It is possible that metastasis-specific drivers remain undetected. In one patient, this analysis identified a subclone in the PB that was closely related to an ancestor of the MBs (Fig. 4c and Supplementary Fig. 16). We did not, however, find evidence of either oligoclonal seeding of metastases or seeding of either metastases or primary tumors. These results are consistent with a nearly ubiquitous ‘branched sibling’ relationship between primary tumor samples and paired brain metastasis samples observed previously 22.

DISCUSSION

We analyzed genome-wide changes through endometrial cancer progression, including CAHs, primary tumors and paired metastases. We observed notable heterogeneity between biopsies of paired primary tumors and metastases, with only half of mutations common to any two biopsies, on average. The biopsies did not fully capture the tumors, implying higher levels of heterogeneity than what we measured in both metastatic and primary tissues. As a result, some of the mutations detected only in metastases may have been present in unsampled regions of their paired primary tumors.

Across primary endometrial cancers, we identified 21 new significantly mutated genes, owing in part to indel rescue in MSI tumors.
Among these was NRIP1, which was mutated in 12.5% of tumors. NRIP1 is an obligate cofactor of the estrogen receptor13, and germline SNPs near NRIP1 have been associated with estrogen-receptor-positive breast cancer27. These data suggest that NRIP1 alterations are common drivers of endometrial cancer oncogenesis. However, variations in indel rates across the genome are not well understood, and NRIP1 alterations were also seen in MSI colorectal cancers. Further characterization of the functional effects of NRIP1 alterations is necessary.

The varying rates in heterogeneity across mutations in different genes indicate the order in which these mutations are typically acquired during tumor evolution. In particular, likely drivers of primary oncogenesis are generally more homogenous than likely passengers. Among the drivers, mutations in PIK3CA, PTEN, TP53 and PPP2R1A occurred earlier on average in tumor evolution. In the case of PIK3CA and PTEN, these findings from advanced cancers mirror the findings in CAHs, which almost exclusively exhibited PI3K pathway mutations21.

Conversely, mutations of ARID1A, encoding a member of the BAF chromatin-remodeling complex, were frequently subclonal. This heterogeneity was mirrored by heterogeneity of mutations across BAF complex members in other cancers. Mutations of genes encoding BAF complex members displayed the most phylogenetic heterogeneity of all known driver mutations in multiregion sequencing studies, including mutations of PBRM1 in renal cell carcinomas38, SMARCA4 in gliomas39, and ARID1A or SMARCB1 in meningiomas40,41. An exception is malignant pediatric rhabdoid tumors, in which SMARCB1 mutation seems to be the sole oncogenic driver42. These observations suggest that, in many cancers, BAF complex perturbations may alter the epigenetic landscape of already established tumors rather than initiate tumor formation. The heterogeneity of ARID1A mutations also raises questions regarding the likely efficacy of ARID1A-directed therapies such as EZH2 inhibition43. However, we identified convergent evolution involving ARID1A mutations, and other mechanisms of ARID1A inactivation might also converge to generate phenotypic homogeneity. Indeed, homogeneous patterns of ARID1A loss have been observed by immunostaining advanced lesions44,45.

We did not identify examples of tumor self-seeding, which have been observed in human prostate cancers and breast cancer models27,36,46. However, our data are insufficient to reject the hypothesis that these events occurred but only involved small fractions of cancer cells or cancer tissues that were not sampled.

Notably, we did not identify recurrent metastasis-specific driver mutations. Drivers of metastasis may be intergenic, epigenetic or environmental events that are not well assessed by whole-exome sequencing47. It is also possible that a great diversity of genetic events or combinations of events contribute to metastasis, each in a small subset of metastatic cancers, and that we had insufficient power to detect them. In this case, genomic analysis of metastases from many more patients will be required.

The observation of significantly recurrent monophony is consistent with metastatic endometrial cancer cells sharing a feature that is associated with genetic ancestry. This may be a cryptic genetic event that enables metastasis, but it is also consistent with other explanations. For example, members of a lineage may happen to be located in an environment that is conducive to metastasis37. Alternatively, seeding the first metastasis may be a rate-limiting step, after which it is more likely that this metastasis will seed further metastases, a mode of spread that has been previously described in mouse models of small-cell lung cancer36 and in human prostate cancer27.

Although our data suggest that large, clinically resected abdominopelvic metastases generally arise from a limited fraction of endometrial cancer primary tumors, it is possible that metastases to other anatomical sites may exhibit different evolutionary relationships, and some histological subtypes may be more likely to generate metastases from independent branched subclones. Only a single biopsy of the primary tumor was sampled in each case, and we could not infer how many independent metastatic lineages existed in the primary tumor or what fraction of the tumor's cells they comprised. Indeed, the single observed case of polyphyle might represent a cancer with more than one independent metastatic branched subclone or a case in which the PB happened to sample descendants of the metastatic subclone within the primary tumor. Sampling more regions of primary tumors, in addition to multiple metastases from the same patient, should help resolve these issues.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** The sequencing data have been deposited in the database of Genotypes and Phenotypes (dbGaP) under accession code phs001127.v1.p1.

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**AUTHOR CONTRIBUTIONS**

E.A.H. and H.B.S. initiated the study, and W.J.G., E.A.H., S.L.C., R.B. and H.B.S. designed the study. E.A.H., M.K.H., A.B., H.M.I.W., I.M.S., K.K.M., J.T., K.W., L.B. and H.B.S. performed sample collection, annotation and curation. W.J.G., E.A.H., A.T.-W., A.D.C., E.H., T.I.Z., K.M.S., K.K., J.A.W., M.S.L., S.L.C., R.B. and H.B.S. performed the data analyses. E.A.H., K.M.S. and C.K. performed validation and microsatellite instability and immunohistochemistry experiments. W.J.G., E.A.H., M.R., A.C., K.K.M., J.T., C.K., M.G.H., E.K.V., M.S.L., G.G., S.L.C., R.B. and H.B.S. contributed reagents and algorithms. All authors critically revised the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS
Sample collection and description. Investigations in this study were approved by the Norwegian Social Science Data Services (15501) and by the local institutional review boards at Haukeland University Hospital, Bergen, Norway (REK number 2009-2315) and the Broad Institute, Cambridge, Massachusetts, USA (DFCI_12_049B). All patients consented to inclusion in this study. Samples were collected from patients from western Norway from September 2002 until September 2012.

Biopsies were snap frozen in liquid nitrogen and stored at −80 °C. Tumor purity was assessed using hematoxylin-stained sections before DNA extraction. After sequencing, purity was also calculated by ABSOLUTE. If biopsies from primary tumors had equivalent purity to those from metastases (61.3% versus 59.9%, respectively; \( P = 0.76 \)), Blood samples were collected for reference as normal controls. Clinical information on all cases is presented in Supplementary Table 1.

Our cohort included 12 patients with CAH and 40 patients with metastatic endometrial cancer. Of this latter group, 26 had endometrioid endometrial carcinoma and 14 had non-endometrioid endometrial carcinomas. Time interval and treatment given between resection of the primary tumor and metastatic lesions are detailed in Figure 1 and Supplementary Table 1. All histopathological diagnoses were subjected to formal histopathological revision and/or established in a tumor board setting as previously reported.48,49

We performed genomic characterization of 98 biopsies from these 52 individuals. We performed whole-exome sequencing on 81 biopsies from 45 individuals, including 26 with paired primary and metastatic lesions, 5 with more than one metastasis and 8 metastases without paired primary tumors, along with DNA from paired blood in all cases. We also analyzed SCNAS in 76 samples from 37 patients using Affymetrix SNP 6.0 arrays (Supplementary Fig. 2). These included 59 samples from 30 patients that had also undergone whole-exome sequencing, 10 additional metastases with paired primary tumors (from 6 cases, including 3 cases with more than one metastasis) and 1 unpaired metastasis (Supplementary Fig. 1).

Assessment of microsatellite instability status. Microsatellite instability testing was performed on all samples subjected to whole-exome sequencing using the marker set used by TCGA. DNA underwent whole-genome amplification using the GenomePlex Complete Whole-Genome Amplification Kit (Sigma-Aldrich). The probe set consisted of BAT25, BAT26, BAT40, TGFBR1I, D2S123, D5S346 and D17S250. No markers were positive in the normal controls (blood). None of the patients in this study were diagnosed with hereditary non-polyposis colorectal cancer (HNPPCC).

Exome sequencing and SNP array profiling. Genomic DNA was isolated from frozen tissues using the Qiagen DNAamp kit or a standard proteinase K protocol. Samples were sequenced on an Illumina HiSeq 2000 to an average of 77× depth (85.6% targeted reads with >20× coverage). The average rate of read alignment was 98.6%. Affymetrix SNP 6.0 arrays were used for a subset of seven samples. CAHs, out of 19 profiled, had both an exceptionally low purity (less than 25% per ABSOLUTE analysis)22 and low burden of mutations. Upon manual review, the mutations whose allelic fractions were greater than 10% were enriched in regions with low mapping quality. These seven samples were therefore excluded from further analyses.

Validation sequencing. We subjected the indels detected by our rescue strategy to additional technical validation using six approaches. First, we tried calling indels in the normal samples using the paired tumor as the control, resulting in a mean of 0.16 indels called per normal sample, as opposed to a mean of 62 indels called per tumor. Second, we applied an independent indel caller, Strelka25, which detected 86.5% of the indels we had rescued. Third, we conducted Sanger sequencing to validate rescued indels across 88 genomic loci, including 77 loci across a variety of genes and 11 loci in genes with recurrent indels: NRIP1, ARID1A and RPL22 (Supplementary Table 2). We sequenced a total of 127 indel events from the 77 loci, 123 (97%) of which were validated. Among the 11 sites in NRIP1, ARID1A and RPL22, we sequenced a total of 25 indels, of which 23 (92%) were validated. The exceptions were two samples with events of low allelic fraction (0.02 and 0.05). None of the indels were detected in the paired normal samples. Fourth, we performed Sanger sequencing across these 25 indel events to 10,000× depth and detected all 25, including the 2 low-allelic-fraction events. Fifth, we performed Sanger sequencing from subcloned PCR products for the sample with the NRIP1 mutation with an allelic fraction of 0.05 and validated the mutation in the tumor DNA but not in the matched normal DNA. Sixth, we detected two NRIP1 p.Lys728Ile indels by Sanger sequencing across an independent cohort of 37 endometrial cancer samples (Supplementary Fig. 5d). Of the 78 rescued indels subjected to validation sequencing, we validated 75 (96%) in at least one assay.

Sanger sequencing was performed by PCR with subsequent sequencing in both directions using forward and reverse M13 primers (Supplementary Table 2) and whole-genome-amplified (as described above) or original DNA, and samples were analyzed on an Applied Biosystem 7300XL Instrument as previously described40. Chromatograms were analyzed with FinchTV or Sequence Scanner. For selected NRIP1 mutations with low allelic fraction, the PCR product was subcloned into the pGEM-T Easy vector (Promega) for sequencing of single colonies.

A selection of indel variants were also amplicon sequenced on a MiSeq instrument (Illumina) to a coverage of >10,000× (Supplementary Table 2). DNA that had been subjected to whole-exome sequencing and not to whole-genome amplification underwent PCR to amplify the relevant sites for DNA input. All mutations detected by whole-exome sequencing of non-TCGA samples and presented in any stick plot figure were subjected to validation sequencing.

Immunohistochemistry. Immunohistochemistry was performed on all sections of formalin-fixed, paraffin-embedded tissue and three representative cylinders mounted in tissue microarrays (TMAs) as described previously44, using rabbit monoclonal antibody to ARID1A (Abcam, 182560, clone EPR13501; 1:2,000 dilution). The staining index was assessed in TMA samples, as a product of staining intensity (0–3) and area of positive tumor cells (1, ≤10%; 2, 10–50%; 3, ≥50%), providing a scale from 0 (negative staining) to 9 (full positive staining)45.

Somatic mutation calling. Somatic mutations were called with MuTect. One source of false positive somatic mutations is contamination with foreign DNA. We estimated this by CONTEST and found the mean level of contamination to be 0.4%. This information was used by MuTect to determine the baseline for accepting potential mutations with low variant allele frequency. OxoG artifacts were removed using the Broad Institute OxoG3 filter. Indels were called with Indelocator. Additional indels were rescued according to the following previously established criteria: at least 50 reads in both the tumor and normal samples, >0.2 allelic fraction for the variant read in the tumor and <0.05 allelic fraction for the variant read in the normal sample. These indels often occurred in microsatellites; among 4,752 microsatellites covered by exome sequencing, 1,196 exhibited indels in at least one sample, and these accounted for 1,882 of the 30,096 indels we detected. All of these recurrent-site indels were supported by small numbers of supporting reads in the paired normal samples; this accounts for their absence in previous analyses. To ensure the fidelity of this approach, we swapped tumor and normal labels to determine the false positive rate of indel calls. A median of zero indels and maximum of two indels were falsely called across the exome in this approach (Supplementary Fig. 5a).

In contrast, we rescued more than 100 mutations in 13 tumor samples using this approach. The variance in the number of mutations in PBs and MBs was approximately equal (\( P = 0.22, F \) test).

Copy number analysis. Relative copy number profiles from Affymetrix SNP 6.0 arrays were determined as previously described23. Relative copy number profiles from exome sequencing data were determined by normalizing exome coverage data to values from blood controls and generating segmented copy number profiles. These were paired with germline heterozygous sites to obtain allele-specific relative copy number profiles, as previously described22,26. The relative allele-specific copy number profiles were paired with exome mutation data for each tumor sample as input to ABSOLUTE for final determination of discrete allele-specific copy number profiles. The sequence of events that led to each allele copy number profile was inferred using a maximum-parsimony approach25.
Mutation correlation analysis. The mutations detected in primary cancers from this cohort were combined with the mutations detected in endometrial cancers profiled by TCGA to detect correlations and anticorrelations between mutated genes, using a previously described approach that maintains the marginal counts of both the number of mutations in each sample and the number of events within each gene. Ultramutated samples and rescued indels in MSI tumors were excluded from this analysis. P values were calculated using 10,000 permutations of the observed data. The network of correlated interactions was plotted using Cytoscape where the negative log of the q value for positive correlation is proportional to the spring constant of an edge between two nodes.

Associations with survival were determined by a Kaplan–Meier analysis using the R package survival. P values were computed by the log-rank test.

Detection of cancer subclones within biopsies. For each mutation, CCFs were calculated by ABSOLUTE by integrating information from local allelic copy number, biopsy purity and variant allele counts. Posterior distributions over CCF values across cancer tissues from a given case were then subjected to a clustering procedure to identify subpopulations of cells and reduce the uncertainty over CCF estimates. We determined from this analysis that most detected subclones with CCF $< 1$ were restricted to individual tissue samples (data not shown). This enabled us to construct biopsy-level phylogenetic trees without further consideration of cancer tissue heterogeneity. One exception occurred where a subclone was detected in the primary tumor sample of case EC-007 that was an ancestor of the paired metastases.

For the analysis of the CCF of mutations in TCGA data, we performed ABSOLUTE analysis across all tumor samples in the TCGA endometrial data set. ABSOLUTE computes a probability distribution of the CCF for each mutation and includes a probability that each mutation is subclonal. To exclude the possibility that passenger mutations in hypermutated samples could confound our analysis, we excluded hypermutated samples (>1,000 detected mutations) from this analysis.

Phylogenetic tree reconstruction. To improve the sensitivity of mutation calls in each biopsy, we used a previously described ‘forced calling’ strategy. This procedure effectively rescues mutations that failed to reach the evidence threshold of MuTect in a given biopsy, provided that they were confidently detected in another sample from the same case.

Phylogenetic trees were constructed using an implementation of clonal ordering. Force-called mutations were converted into a binary incidence matrix depending on their absence/presence in a set of paired biopsies. We calculated the power to detect each mutation in each biopsy on the basis of local allelic copy number and purity. Where a mutation was not detected in one biopsy but the power to detect it was less than 0.95, the mutation was excluded from the incidence matrix and separately annotated. A distance matrix was computed from the final incidence matrix using the following distance metric

$$d_{a,b} = \frac{1}{1 + m_a \cdot m_b}$$

where $m_a$ corresponds to the binary vector of mutations in biopsy a and $m_b$ is the vector describing biopsy b. Hierarchical clustering of this distance matrix was performed using the complete linkage method in R.

Homologous chromosome tracking across tumor biopsies. Germline heterozygous sites were determined from exome sequencing of the normal blood control sample. The allelic fraction of these sites was determined at each site in the exome in all paired tumor samples (primary tumors and metastases). Purity estimates (p) from ABSOLUTE were used to generate the purity-adjusted minor allelic fraction (mAF) at each site.

$$\text{mAF}_{\text{corrected}} = \frac{\text{mAF} - \frac{1}{2} (1 - p)}{p}$$

These allelic fractions were multiplied by the local total copy number (CN$_T$) by ABSOLUTE to graph a point estimate for each SNP of the major and minor tumor alleles. A point estimate for the minor allelic copy number (mACN) at each site was calculated as follows:

$$\text{mACN} = \text{CN}_T \times \text{mAF}_{\text{corrected}}$$

The major allele in the reference tumor for each site was defined as whichever allele for which the allele count was greater (variant versus reference). The expected major allele at each SNP was colored red in the resulting plot. In the test tumor, the same major and minor alleles estimated from the reference (primary) tumor were used and colored accordingly. Homologous chromosome tracking was performed across every pairwise comparison in the cohort. Resulting plots were manually reviewed for discordant tumor haplotype alterations.

Instances in which the chromosome undergoing copy loss or gain in the test tumor was opposite the homologous chromosome undergoing loss or gain in the reference tumor indicate separate events in the genetic history of the tumor. Raw plots for selected chromosomes for case EC-022 are shown in Supplementary Figure 9.

Significance analysis of phylogenies. Under the null hypothesis that evolutionary distances are randomly distributed among pairs of cancer tissues from a given patient, we expected all configurations of phylogenetic trees involving biopies of the primary tumor and metastases to be equally probable. In cases of two MBs and one PB, the phylogeny could have three configurations: either the PB was the most distantly related biopsy or either of the MBs was the most distantly related biopsy. Therefore, monophyly would be observed in one-third of cases. In cases of three MBs and one PB, the phylogenetic tree could include two clades with two members each (with a one-third probability) or clades with one and three members, respectively (with a two-thirds probability). Only the latter is consistent with monophyly, and this is only the case if the PB is in the clade by itself (with a probability of one-quarter), for a one-sixth probability of monophyly overall. We used these probabilities to calculate P values indicating the likelihood of obtaining the observed or a greater rate of monophyly.

If two independent subclones each gave rise to half of the observed metastases, any two metastases would have a 50% chance of deriving from different subclones, with a probability of monophyly of one-third (as above) and a 50% chance of deriving from the same subclone. We assume that, if the MBs derive from the same subclone, they necessarily exhibit monophyly (a conservative assumption: it is possible that the PB would by chance represent that same subclone within the primary tumor, in which case polyphyly would still be possible). We used these and similar considerations for phylogenies with three MBs to calculate P values indicating the likelihood of obtaining the observed or a greater rate of monophyly, assuming an equivalent number of patients and PB and MB samples in each patient.

Significance analysis of mutations in primary endometrial cancers. We combined the force-called mutation lists (without indel rescue) from our primary tumors with the mutations from TCGA. We applied MutSig2CV to this list of mutations. Genes with q values less than 0.1 were considered significantly mutated.

Separately, we combined the force-called mutation lists (with indel rescue) from our primary tumors with the mutations from TCGA that included indel rescue. We considered any genes that were mutated in greater than 10% of samples and whose q value was less than 10$^{-3}$ as significant.

Significance analysis of metastasis-associated driver mutations. For each phylogeny in our data set, we selected the set of mutations that were detected in every paired MB that were not detected in the PB. We applied MutSig2CV to this set of mutations and considered any mutations whose q value was less than 0.25 as significant.

Power to detect metastasis-associated drivers. We used an empirical approach to determine our power to detect mutations that conferred the ability to
metastasize. We used the list of metastasis-specific mutations that we previously constructed as a pool into which we spiked hypothetical driver gene mutations at decreasing frequencies. We then assessed the rate at which these hypothetical driver genes were recovered as significant ($q < 0.25$).

The spiking procedure used non-silent mutations randomly selected from 585,491 exomic mutations detected in TCGA tumors to ensure consistency with mutational background rates and genomic covariates observed in human tumors. For every gene, the number of patients selected for spiking was randomly drawn from the binomial distribution with a probability of success equal to the proposed frequency of driver gene mutation in metastases. When each hypothetical driver gene was proposed, the gene was set to exhibit hotspot mutations with a probability of $1/3$ such that the same mutation was spiked in for each patient selected. After a mutation was spiked into the genome of a given patient, a randomly selected mutation previously observed in the patient was removed to preserve the total number of mutations.

**Percentage of mutations in driver genes found in all biopsies.** To calculate the percentage of mutations in each driver gene that were truncal, we used force-called mutation lists annotated with detection power from ABSOLUTE$^{22,23,26}$. We then determined whether the mutation was detected in all biopsies from the same patient. If the mutation was present in all biopsies, then the number of truncal mutations was incremented by one. If a mutation was not detected in a given biopsy and the power to detect the mutation was greater than 0.8, the number of branch mutations was incremented by one. If there was not sufficient power to detect the mutation in one or more biopsies lacking the mutation, then the mutation was not counted toward the truncal or branch counts. To exclude the possibility that passenger mutations in driver genes could confound our analysis, two phylogenies with POLE exonuclease mutations and ultramutated genomes (15,095 and 30,601 mutations detected) were excluded from this analysis.

**Code availability.** Software packages used for sequence analysis are publicly available at the URLs listed at the end of the main article: Mutect, Indelocator, Recapseg, ABSOLUTEv1.2.