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ARTICLE

Detection of *ERBB2* amplification in uterine serous carcinoma by next-generation sequencing: an approach highly concordant with standard assays

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Abstract

Uterine serous carcinoma is an aggressive subtype of endometrial cancer that accounts for fewer than 10% of endometrial carcinomas but is responsible for about half of deaths. A subset of cases has HER2 overexpression secondary to *ERBB2* gene amplification, and these patients may benefit from anti-HER2 therapies, such as trastuzumab. HER2 protein overexpression is currently assessed by immunohistochemistry (IHC) and *ERBB2* gene amplification by fluorescence in situ hybridization (FISH). Targeted next-generation sequencing (NGS) is increasingly used to routinely identify predictive and prognostic molecular abnormalities in endometrial carcinoma. To investigate the ability of a targeted NGS panel to detect *ERBB2* amplification, we identified cases of uterine serous carcinoma ($n = 93$) and compared HER2 expression by IHC and copy number assessed by FISH with copy number status assessed by NGS. *ERBB2* copy number status using a combination of IHC and FISH was interpreted using the 2018 ASCO/CAP guidelines for breast carcinoma. *ERBB2* amplification by NGS was determined by the relative number of reads mapping to *ERBB2* in tumor DNA compared to control nonneoplastic DNA. Cases with copy number ≥6 were considered amplified and copy number <6 were non-amplified. By IHC, 70 specimens were classified as negative (0 or 1+), 19 were classified as equivocal (2+), and 4 were classified as positive (3+). Using combined IHC/FISH, *ERBB2* amplification was observed in 8 of 93 cases (9%). NGS identified the same 8 cases with copy number ≥6; all 85 others had copy number <6. In this series, NGS had 100% concordance with combined IHC/FISH in identifying *ERBB2* amplification. NGS is highly accurate in detecting *ERBB2* amplification in uterine serous carcinoma and provides an alternative to measurement by IHC and FISH.

Introduction

Uterine serous carcinoma (USC) is an aggressive subtype of endometrial cancer which predominantly affects post-menopausal patients and arises in the setting of atrophy. Although it accounts for fewer than 10% of endometrial carcinomas, it is responsible for 40–80% of deaths [1–3]. Clinically, serous carcinoma is characterized by an aggressive disease course, often with extra-uterine disease even in the absence of myometrial invasion [4, 5]. Genetically, the hallmark of serous carcinoma is loss-of-function mutations in *TP53*, which are present in over 90% of cases [6, 7]. Chemotherapy is the mainstay of treatment for even early stage disease, although relapses and metastases are still common [3].

HER2 is a cell surface receptor in the epidermal growth factor receptor family and is encoded by *ERBB2*, which maps to 17q12. The protein is composed of an intracellular

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tyrosine kinase domain, a transmembrane domain, and an extracellular ligand binding domain [8]. Receptor activation triggers a number of pathways implicated in cell growth, apoptosis, and differentiation [9]. HER2 is overexpressed in several cancer types [8], and *ERBB2* amplification correlates with a poorer prognosis in breast [10, 11], gastrointestinal [12–14], and endometrial carcinomas [15–20]. In USC, overexpression of HER2 by immunohistochemistry (IHC) ranges from 14 to 80% [15–18, 20–24] and gene amplification in 3–42% [6, 16–18, 20–23, 25, 26].

There are two categories of targeted therapies for tumors with HER2 overexpression: monoclonal antibodies against the extracellular domain of HER2, such as trastuzumab, and tyrosine kinase inhibitors of the intracellular domain, such as neratinib. Both approaches are used routinely in breast [27–30] and gastroesophageal tumors [31, 32]. Early reports described cases of USC with HER2 overexpression responding to trastuzumab therapy [24, 33, 34]. However, a subsequent phase II trial (GOG181B) failed to demonstrate any benefit [35]. This trial was subsequently criticized for a number of perceived limitations: it included a high number of non-serous subtypes, only 45% of cases had *ERBB2* amplification, and it was statistically underpowered to detect clinically meaningful response rates [36]. A second phase II trial, however, addressed these shortcomings and demonstrated a 4-month increase in progression-free survival when patients with advanced stage or recurrent USC with HER2 overexpression were treated with trastuzumab [37]. Since then, anti-HER2 therapies have become standard of care for patients with advanced stage, recurrent, or metastatic USC [38].

Currently, HER2 overexpression and amplification in USC is assessed by IHC and in situ hybridization (ISH), respectively [39]. Next-generation sequencing (NGS) is frequently used to assess molecular alterations in endometrial carcinomas, including targetable point mutations, *POLE* mutation status [40], and microsatellite instability [41]. However, its ability to reliably detect *ERBB2* amplification in endometrial carcinoma has not been previously studied. To address this question, we examined a cohort of USC and compared *ERBB2* amplification as measured by NGS, IHC, and ISH.

Materials and methods

Case selection

Cases of USC, which had been previously tested by a targeted hybrid-capture NGS assay between 2014 and 2019 at Brigham and Women's Hospital (Boston, MA), were identified by a retrospective search. Cases were included for study only if additional material was available from the same anatomic site for IHC, and if applicable, ISH. When possible, the same paraffin block was used for IHC, ISH, and NGS. However, for some cases which were seen in consultation, the only material available from the hysterectomy for subsequent IHC/FISH studies was from a hysterectomy block different than the one tested by NGS. This study was approved by the Brigham and Women's Hospital Institutional Research Ethics Board.

Immunohistochemistry (IHC)

IHC for HER2 (SP3 clone; 1:75 dilution; Cell Marque, Rocklin, CA) was performed on 5-µm thick, full-slide sections of formalin-fixed paraffin-embedded tissue. IHC was scored independently by at least two pathologists using the 2018 ASCO/CAP clinical practice guideline for HER2 in breast cancer [42]. As per the guidelines, a positive (3+) result required intense, completely circumferential membranous staining in a contiguous focus representing at least 10% of tumor cells. In addition, intense basolateral staining was considered positive, as this pattern has been shown to correlate with amplification in USC [21]. Heterogeneity was assessed as a spatially discrete tumor population with either 2+ staining with amplification confirmed by FISH, or 3+ staining, on a background of negative tumor cells (0 or 1+). Discrepancies in interobserver scoring were resolved via consensus. Appropriate immunohistochemical controls were examined.

Fluorescence in situ hybridization (FISH)

All cases that scored 2+ (equivocal) by IHC were assessed by FISH for *ERBB2* amplification. A subset of cases that scored 3+ by IHC was confirmed by FISH if material was available. Two 3–5-mm regions of tumor with the highest expression by IHC were marked for FISH evaluation. In cases with heterogeneous expression, the target probe was applied and assessed in both IHC-positive and - negative areas for confirmation of amplified and non-amplified populations. Five-micrometer sections of formalin-fixed paraffin-embedded tumor were tested with the Vysis PathVysion HER2 DNA Probe kit (Abbott, Abbott Park, IL), which includes the locus-specific *ERBB2* probe (17q12) and the CEP17 (D17Z1) centromeric probe (17p11.1-q11.1). The previously marked areas of maximum HER2 IHC expression were evaluated by two observers examining at least 30 nuclei in a contiguous portion of the tumor, and the entire region marked by a surgical pathologist was scanned for heterogeneity in HER2 copy number (CN) status. Only cases with amplification in 10% or more of the tumor cells were considered positive [42]. Evaluation of each specimen followed the algorithm described in the 2018 ASCO/CAP clinical practice guideline for breast cancer [42]. Those

guidelines define the dual-probe FISH groups as follows: group 1 is *ERBB2*/CEP17 ratio ≥2.0 and ≥4.0 *ERBB2* signals/cell; group 2 is *ERBB2*/CEP17 ratio ≥2.0 and <4.0 *ERBB2* signals/cell; group 3 is *ERBB2*/CEP17 ratio <2.0 and ≥6.0 *ERBB2* signals/cell; group 4 is *ERBB2*/CEP17 ratio <2.0, and ≥4.0 and <6.0 *ERBB2* signals/cell; and, group 5 is *ERBB2*/CEP17 ratio <2.0 and <4.0 *ERBB2* signals/cell. Group 1 is positive, group 5 is negative, and groups 2–4 require correlation with IHC to determine the overall HER2 status.

Targeted NGS

Targeted NGS was performed as previously described [43, 44]. Briefly, areas of tumor were macrodissected from unstained slides and DNA was isolated from the tissue. The fraction of tumor nuclei in the area selected for study was estimated from hematoxylin and eosin stained slides, and only samples with a tumor percentage >20% were tested. Sequencing libraries were generated for the coding regions of at least 275 genes, including *ERBB2*, using solution-based hybrid capture (Agilent SureSelect; Agilent Technologies, Santa Clara, CA). Sequencing was performed using an Illumina HiSeq 2500 (Illumina, Inc, San Diego, CA). Cases included for study passed quality control metrics. The median average target coverage was 286.9 (range 68.3–635.1), and the median percentage of bases with >30× coverage was 98.4% (range 85.6–99.4%). *ERBB2* CN by NGS was determined by the relative number of reads mapping to *ERBB2* in tumor DNA compared to pooled normal (unmatched) control nonneoplastic DNA. *ERBB2* CN was calculated using the estimated tumor percentage

$$\mathrm{CN} = \left(2 \times \frac{\text{Median } \textit{ERBB2} \text{ copy ratio} - 1}{\text{Tumor cell fraction}}\right) + 2.$$

A receiver operating characteristic curve analysis, using FISH as the gold standard, demonstrated an AUC of 1.0 between a NGS CN cutoff of 5.4 and 7. For simplicity, we chose to use the same threshold of 6.0 as the 2018 ASCO/CAP clinical practice guideline for reporting of breast cancer single probe HER2 ISH [42]; an *ERBB2* CN ≥ 6 was considered amplified, and *ERBB2* CN < 6 was non-amplified.

Statistical analysis

A post hoc power analysis was completed using the methods described by Liu et al. [45] and Tang [46] for a matched pair non-inferiority trial with binary outcomes. A sample size of $n = 93$ had 62% power at $\alpha = 0.05$ to detect non-inferiority if the minimum ratio of amplification detection sensitivities between the two techniques resulting in non-inferiority was 0.8. With a minimum non-inferiority ratio of 0.9, the power to detect non-inferiority was 44%.

Results

In total, specimens from 93 patients were included for the study, including 71 hysterectomies, 8 endometrial biopsies, 1 curetting, and 13 metastases.

By IHC, 70 of 93 cases (75%) were negative (0 or +1), 19 (20%) were equivocal (2+), and 4 (4%) were positive (3+, Fig. 1 and Table 1). Of 9 cases (10%) with heterogeneous HER2 expression, two tumors were 3+, with intense staining in 20 and 70% of the tumor cells. In six tumors, the heterogeneity was of limited extent, with intense staining limited to an area representing <10% of the tumor, and were classified as equivocal (2+). In one additional tumor classified as IHC equivocal (2+), there was a discrete area of weak-to-moderate membranous staining (80% of tumor), which was found to be amplified by FISH.

FISH was performed on a subset of cases ($n = 24$), which had IHC scores of 1+ ($n = 3$), 2+ ($n = 19$), and 3+ ($n = 2$) (Tables 2 and 3). The median *ERBB2* copies per cell determined by FISH were 3.4 (mean 7.0, range 1.8–53.8). The median *ERBB2*/CEP17 ratio was 1.4 (mean 2.8, range 0.6–18.1). When classified according the 2018 ASCO/CAP ISH groupings, 6 (25%) were group 1, 1 (4%) group 2, 1 (4%) group 3, 2 (8%) group 4, and 14 (58%) group 5. FISH performed on tumors with heterogeneous IHC expression confirmed amplification in areas with 3+ pattern of staining, and absence of amplification in areas of tumor with 0 or 1+ pattern staining. A single case with 1+ expression was amplified by FISH, with *ERBB2* CN 13.9 and ratio 3.9.

Using the combined interpretation of the IHC and FISH assays, 8 (9%) cases showed *ERBB2* amplification and 85 (91%) were non-amplified.

By NGS, the median *ERBB2* log2 ratio for all cases was 0.035 (Fig. 2 and Supplementary Fig. 1). Using the estimated tumor fraction, *ERBB2* CN was calculated, giving a median CN of 2.1 for all cases (mean 3.7, range 0.2–62.8). Eight (9%) cases were amplified (CN ≥ 6, mean 20.0, range 7.0–62.8), and eighty-five (91%) cases were non-amplified (CN < 6, mean 2.1, range 0.2–5.4). The concordance between NGS and the combined IHC and FISH interpretation was 100% (Table 1 and Fig. 3). A least squares linear regression, including all cases with FISH, of NGS CN as a function of FISH CN yielded a slope of 0.25, with an R^2 of 0.509 (Fig. 4, gray line). When cases with heterogeneous amplification were excluded, the slope was 0.64 with an R^2 of 0.758 (Fig. 4, black line), indicating that the CN in cases with heterogenous amplification was systematically underestimated by NGS compared to FISH.

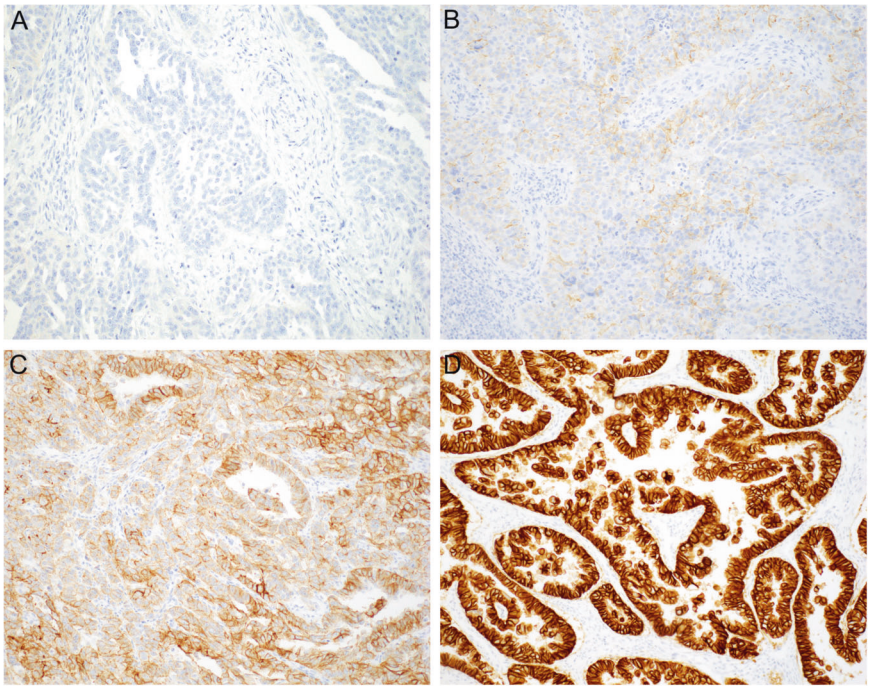

Fig. 1 HER2 immunohistochemical staining in uterine serous carcinoma. **A** Score of 0 (negative), with faint staining in ≤10% of tumor cells. **B** Score of 1+ (negative), in which there is faint, incomplete membranous staining in >10% of tumor cells. **C** Score of 2+ (equivocal), with strong, circumferential staining in ≤10% of tumor cells. **D** Score of 3+ (positive), with circumferential, complete membrane staining that is intense and >10% of tumor cells.

Table 1 Immunohistochemical (IHC) expression of HER2, *ERBB2* amplification status determined by FISH and combined IHC/FISH assays in cases of uterine serous carcinoma compared to *ERBB2* copy number as determined by next-generation sequencing (NGS).

	ERBB2 copy number by NGS	
	<6	≥6
HER2 IHC		
0	44	0
1+	25	1
2+	16	3
3+	0	4
ERBB2 FISH interpretation		
Negative	18	0
Positive	0	6
HER2 IHC/FISH combined interpretation		
Negative	85	0
Positive	0	8

Table 2 Immunohistochemical (IHC) expression of HER2 compared to *ERBB2* amplification status determined by FISH in 24 cases of uterine serous carcinoma.

	IHC HER2 expression			
	0	1+	2+	3+
ERBB2 FISH interpretation				
Negative	0	2	16	0
Positive	0	1	3	2

Discussion

Since trastuzumab was recently shown to have a progression-free survival benefit in *ERBB2*-amplified USC, it has become critical to correctly identify patients for targeted therapy. HER2 overexpression and amplification in USC has been studied for over 20 years using IHC, fluorescence [16, 18, 21–23, 33, 47, 48] and chromogenic [49, 50] ISH, and polymerase chain reaction [17, 18]. Endometrial carcinomas are routinely interrogated by NGS for targetable treatments, including point mutations and microsatellite instability. This is the first report which demonstrates that *ERBB2* amplification in USC can be accurately detected using NGS. It follows previous work that has shown excellent concordance between *ERBB2* amplification detection by NGS and IHC overexpression in breast [51], gastroesophageal [51], and colorectal carcinomas [52].

The frequency of *ERBB2* amplification found here (9%) is in the lower range of that previously reported (3–42% [6, 16–18, 20–23, 25, 26]), and this discrepancy is likely partially due to a selection bias. The cases selected for inclusion in this study were previously tested using our in-house targeted NGS assay, which is performed by clinician request. Often, this testing is performed to identify a targeted treatment when a patient has recurrent or metastatic disease. If a case of USC was found to have overexpression of HER2 during routine clinical testing, the clinician may have opted to forgo NGS testing as a targeted therapy was already available to the patient. We are aware of several

Table 3 Cases ($n = 24$) with *ERBB2* amplification determined by both fluorescence in situ hybridization (FISH) and next-generation sequencing (NGS).

Case #	HER2 IHC	HER2 IHC heterogeneity	FISH *ERBB2*: CEP17 ratio	FISH *ERBB2* CN	FISH CEP17 CN	FISH group	NGS *ERBB2* CN
5	3+	Present	8.1	13.8	1.7	1	14.9
7	2+	Absent	1.0	1.8	1.7	5	0.2
10	2+	Absent	1.1	1.9	1.7	5	1.4
14	2+	Present	3.1	8.5	2.8	1	8.6
15	2+	Absent	1.6	2.9	1.8	5	1.5
16	2+	Absent	1.5	3.8	2.5	5	2.0
17	2+	Absent	1.6	4.2	2.7	4	5.3
20	2+	Absent	1.1	1.9	1.7	5	2.2
25	2+	Present	3.5	7.3	2.1	1	1.7
26	2+	Absent	1.2	2.9	2.4	5	2.3
27	2+	Present	1.0	2.9	2.8	5	4.3
28	2+	Present	0.9	2.2	2.4	5	4.1
29	2+	Absent	1.1	3.4	3.0	5	2.8
32	2+	Absent	0.6	1.9	3.1	5	1.4
41	2+	Present	1.3	3.4	2.7	5	2.2
42	3+	Present	18.1	53.8	3.0	1	13.0
45	2+	Present	2.2	3.8	1.7	2	2.3
49	2+	Absent	1.9	8.1	4.3	3	7.0
53	2+	Absent	1.0	2.7	2.7	5	3.0
61	1+	Absent	1.4	3.1	2.2	5	4.6
64	2+	Absent	1.1	2.4	2.2	5	1.7
67	1+	Absent	1.5	5.0	3.3	4	5.3
76	2+	Present	6.5	12.5	1.9	1	7.1
86	1+	Absent	3.9	13.9	3.5	1	8.4

FISH group is defined by the 2018 ASCO/CAP guidelines for HER2 in breast carcinoma [42].

CN copy number.

such cases that showed HER2 overexpression and were not referred for genomic profiling, thereby enriching the sequenced cohort for *ERBB2* amplification negative cases. Other possible explanations for a lower proportion of positive cases include differences in the underlying patient population, the HER2 antibody clone used, or the scoring system selected. In any case, both the small fraction of HER2 positive cases and total sample size of this cohort are significant limitations, as this study was not sufficiently powered to conclusively demonstrate non-inferiority of NGS compared to IHC/FISH. Additional studies are necessary to confirm these findings.

In this study, HER2 overexpression and amplification in USC was assessed using the 2018 ASCO/CAP clinical practice guidelines for HER2 expression in breast cancer [42], an approach recommended by the College of American Pathologists [39]. This is in contrast to previous studies, including the recent phase 2 trial [37], which have used other criteria for evaluating HER2 expression in USC, including the modified 2007 ASCO/CAP guidelines, or the original US FDA criteria [53]. This decision will not affect the interpretation of most cases with straightforward positive and negative results. However, one advantage of the updated guidelines is that non-classical FISH results in groups 2–4 (i.e., those that are not clearly positive [*ERBB2*: CEP17 ratio ≥ 2 and *ERBB2* CN ≥ 4] or negative [*ERBB2*: CEP17 ratio < 2 and *ERBB2* CN < 4]) are adjudicated by the IHC interpretation, with the final HER2 status typically correlating better with the level of HER2 expression and *ERBB2* CN [54]. Furthermore, the 2018 guidelines eliminate the equivocal ISH categories and reduce the false positive rate compared to the 2013 guidelines [55, 56]. It appears that our choice of guidelines may have helped to facilitate the excellent correlation observed between IHC/FISH and gene amplification determined by NGS. For example, of the four cases in groups 2–4 in our study, only the group 3 case (*ERBB2*:CEP17 ratio < 2 and *ERBB2* CN ≥ 6) with an IHC score of 2+ was considered FISH-positive and amplified by NGS. It should be noted that according to the modified 2007 criteria used in the recent phase 2 clinical trial, cases with an *ERBB2*:CEP17 ratio ≥2 were considered positive (irrespective of CN) [21, 37]. If these group 2–4

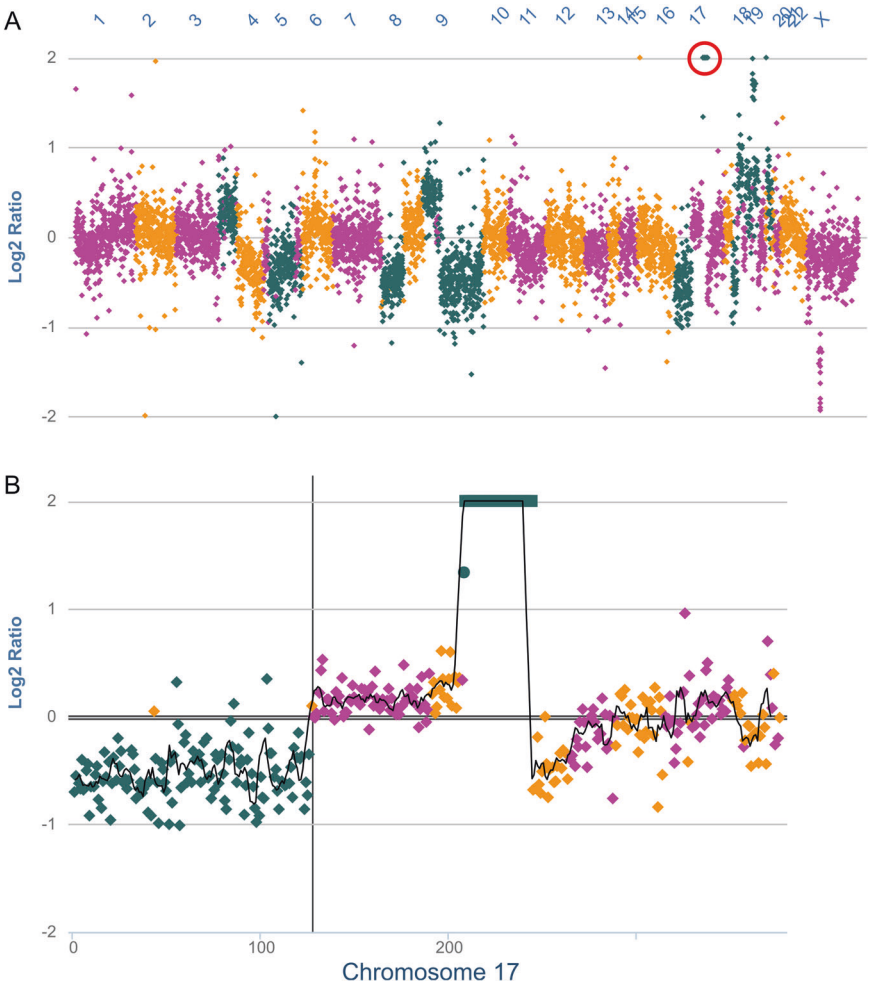

Fig. 2 *ERBB2* amplification detected by NGS. A Copy number (CN) plot of all chromosomes for a case of serous carcinoma with several copy number gains and losses, including *ERBB2* amplification (red circle). The *y*-axis is the log2 ratio of the number of reads in the tumor sample compared to a set of normals. **B** *ERBB2* amplification (CN = 13).

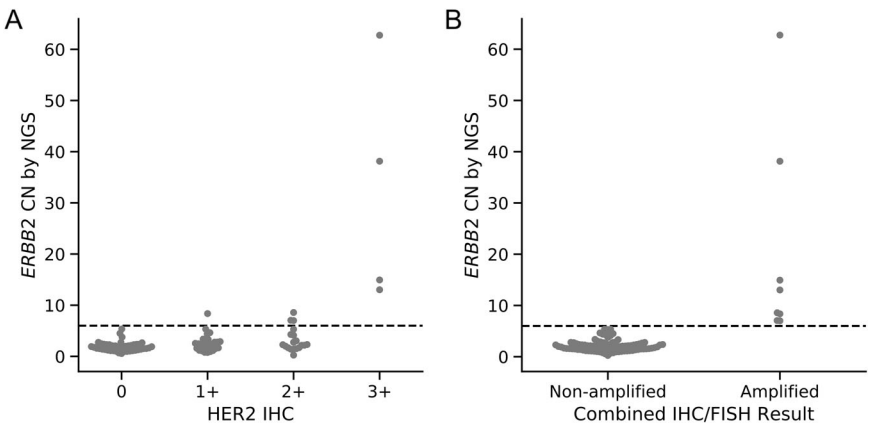

Fig. 3 *ERBB2* copy number (CN) in uterine serous carcinoma determined by next-generation sequencing (NGS) compared to traditional assays. A NGS compared to immunohistochemistry (IHC), and **B** NGS compared to combined IHC and fluorescence in situ hybridization (FISH). Dashed line is CN = 6.

cases had been assessed using this modified 2007 criteria, the group 2 case (ratio ≥ 2 and *ERBB2* CN < 4) would have been considered positive by FISH but negative by NGS, while the group 3 case (ratio < 2.0 and *ERBB2* CN ≥ 6.0) would be negative by FISH but positive by NGS, leading to discordances with our NGS results. Although these group 2–4 cases are relatively rare in breast cancer (~5%), they represent 17% of the cases tested by FISH in our small study of USC, suggesting that an appropriate strategy for how to approach these cases may be needed. Another difference between the 2018 and 2007 guidelines is the IHC criteria for an interpretation of positive (3+), which reduced the required proportion of tumor cells with intense staining from 30 to 10%. From our limited experience, it does not appear that the lower threshold for IHC-positive results had an impact on the sensitivity of NGS, as all four

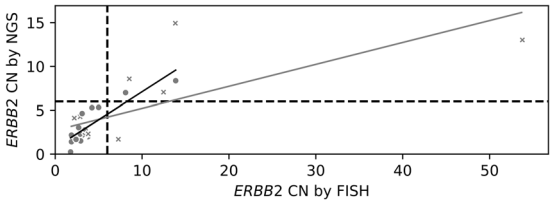

Fig. 4 Linear regression of *ERBB2* copy number measured by NGS and FISH in uterine serous carcinoma ($n = 24$). Cases with heterogeneity in HER2 expression ($n = 9$) are indicated by ×, those without heterogeneity ($n = 15$) are marked by •. Linear regression including all cases is a gray line, while the regression excluding cases with heterogeneity is a black line. Dashed line is CN = 6.

IHC-positive (3+) cases were NGS-amplified, including two IHC-positive heterogeneous cases with a 3+ pattern of staining in 20–70% of tumor cells. Although we recommend using the 2018 ASCO/CAP guidelines for breast cancer for HER2 testing in USC, we understand that some groups may be hesitant to do so given that the modified 2007 ASCO/CAP guidelines were used in the clinical trial, and that is the only scoring system which has demonstrated clinical response in a randomized trial. As such, select cases may be best managed by multidisciplinary discussion. Additional studies are needed to determine the optimal expression and amplification levels which correlate to response to anti-HER2 therapies.

One case demonstrated 1+ (negative) expression by IHC but was amplified by both FISH and NGS. This IHC result may represent a technical failure due to loss of antigenicity, as the only material available for testing was an old, archived unstained slide. This specimen may also have had inadequate fixation, as pathologists are generally not as mindful of preanalytical factors such as fixation time and cold ischemic time in hysterectomies, as compared to breast specimens. Another possible explanation is that this case had greater expression of a truncated variant of HER2 (p95HER2) that our extracellular antibody (SP3) does not detect. This shedding of the extracellular domain has been reported to be a more significant phenomenon in endometrial cancer than in breast cancer [57].

Detection of gene amplification by NGS has several advantages. We demonstrated a perfect concordance between *ERBB2* amplification by NGS and the current gold standard, FISH. FISH is usually only performed if HER2 IHC is equivocal (2+), while a targeted NGS panel is increasingly routinely performed for advanced stage USC. This raises the possibility that additional actionable information can be extracted from an NGS assay that was already performed. Currently, patients can only receive trastuzumab therapy if their tumors show overexpression by IHC or amplification by ISH; amplification by NGS can act as a trigger for a second confirmatory study. As our collective experience with NGS-based tests increase, it may eventually be accepted in place of IHC or FISH assays. Although the optimal IHC scoring system for USC has yet to be established [39], the CN measured by NGS shows excellent concordance with FISH.

Assessment of gene amplification by NGS has several limitations. The calculated CN is directly proportional to the estimated tumor percentage, and therefore an accurate assessment of the proportion of tumor nuclei in the sample is critical to accurately determine CN. Also, both IHC and (F)ISH allow for assessment of single cells and therefore identification of subclones, whereas NGS provides an average measurement over a larger tumor area. Consequently, an *ERBB2*-amplified subclone, which comprises a small proportion of the tumor, may not be detected by NGS. In this series, all amplified cases ($n = 8$) were successfully identified by NGS, including an IHC-positive case with a 3+ pattern in 20% of tumor cells. Of note, there were six cases in this series with heterogeneity of limited extent (3+ patten in <10% of cells), which are best considered as negative according to the 2018 ASCO/CAP guidelines, and which were correctly classified as negative by NGS (CN < 6). Given these results, although the limit of detection was not rigorously evaluated in this study, it appears that NGS may have the capability to discriminate between clinically relevant and insignificant amplified populations.

Tumor sequencing using clinical NGS panels may be performed either with or without analysis of paired normal DNA from the same patient. Paired sequencing allows for more accurate filtering of germline variants and may improve detection of CN variants in some situations. Despite its advantages, paired tumor-normal sequencing is not routinely performed at most institutions, because of its increased cost and workload. While patient-matched analysis offers several benefits, we demonstrated here that unmatched sequencing offers satisfactory performance in the detection of *ERBB2* CN variants in the vast majority of cases of USC.

NGS is highly specific for gene amplification, however it is less sensitive than IHC or FISH in assessing tumors with focal or low levels of *ERBB2* amplification. The predictive and prognostic significance of this subclonal amplification in USC is unknown. Heterogeneous HER2 expression has been documented in a number of tumor types; while unusual in breast carcinoma [58–60], heterogeneity is more common in gastric [61–63], bladder [64], and lung [65] tumors with *ERBB2* amplification. Intratumoral heterogeneity may be a relatively common phenomenon in USC as well, ranging from 11% of cases in this cohort to 31% of cases in a previous report [21]. Heterogeneity of *ERBB2* amplification is one possible mechanism of resistance to HER2 therapies. Another important consequence of such heterogeneity is that HER2 status may vary between biopsy, hysterectomy, and metastases from the same patient [66].

We became aware of five such possible cases which were initially considered for inclusion in this study because they had been previously tested by NGS during routine clinical work. However, only limited additional material from a different site which was not tested by NGS was available for further IHC and FISH studies. In these five cases, the CN determined by NGS at one site differed from the combined IHC/FISH interpretation at a different site. In three of the cases, NGS was performed on a metastasis but only unstained slides were available from the hysterectomy (or vice-versa), and in two of the cases, NGS was performed on one block from the hysterectomy but additional material was only available from a different block from the hysterectomy specimen. In four of the cases, there was insufficient material available to perform confirmatory IHC, FISH, and sequencing on tissue from the same site. In the fifth case, sequencing was attempted on the specimen that already had IHC and FISH but failed sequencing due to poor quality control metrics.

At our institution, NGS testing is usually performed on the hysterectomy specimen, and therefore a non-amplified result (or negative HER2 IHC on the hysterectomy) should prompt consideration of retesting of a metastasis. Consequently, we suggest testing should be performed on a metastatic site if a patient is being considered for adjuvant therapy and material is available. Similarly, we recommend confirmatory IHC or FISH studies should be performed on the same block as the NGS testing. Ultimately, while the analytical validity of this technique is high, the clinical utility, likely determined by the implications of HER2 heterogeneity, is still unknown. Further work is needed to determine the predictive and prognostic implications of heterogeneous HER2 expression in USC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

1. Hamilton CA, Cheung MK, Osann K, Chen L, Teng NN, Longacre TA, et al. Uterine papillary serous and clear cell carcinomas predict for poorer survival compared to grade 3 endometrioid corpus cancers. Br J Cancer. 2006;94:642–6.
2. Cote ML, Ruterbusch JJ, Olson SH, Lu K, Ali-Fehmi R. The growing burden of endometrial cancer: a major racial disparity affecting black women. Cancer Epidemiol Biomark Prev. 2015;24:1407–15.
3. Zhang L, Kwan SY, Wong KK, Solaman PT, Lu KH, Mok SC. Pathogenesis and clinical management of uterine serous carcinoma. Cancers. 2020;12:686.
4. Hanley KZ, Fadare O, Fisher KE, Atkins KA, Mosunjac MB. Clinical significance of positive pelvic washings in uterine papillary serous carcinoma confined to an endometrial polyp. Int J Gynecol Pathol. 2016;35:249–55.
5. Semaan A, Mert I, Munkarah AR, Bandyopadhyay S, Mahdi HS, Winer IS, et al. Clinical and pathologic characteristics of serous carcinoma confined to the endometrium: a multi-institutional study. Int J Gynecol Pathol. 2013;32:181–7.
6. Getz G, Gabriel SB, Cibulskis K, Lander E, Sivachenko A, Sougnez C, et al. Integrated genomic characterization of endometrial carcinoma. Nature. 2013;497:67–73.
7. Tashiro H, Isacson C, Levine R, Kurman RJ, Cho KR, Hedrick L. p53 gene mutations are common in uterine serous carcinoma and occur early in their pathogenesis. Am J Pathol. 1997;150:177–85.
8. Moasser MM. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene. 2007;26:6469–87.
9. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001;2:127–37.
10. Slamon D, Clark G, Wong S, Levin W, Ullrich A, McGuire W. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987;235:177–82.
11. Seshadri R, Firgaira FA, Horsfall DJ, McCaul K, Setlur V, Kitchen P. Clinical significance of HER-2/neu oncogene amplification in primary breast cancer. J Clin Oncol. 1993;11:1936–42.
12. Chan DSY, Twine CP, Lewis WG. Systematic review and meta-analysis of the influence of HER2 expression and amplification in operable oesophageal cancer. J Gastrointest Surg. 2012;16:1821–9.
13. Hechtman JF, Polydorides AD. HER2/neu gene amplification and protein overexpression in gastric and gastroesophageal junction adenocarcinoma: a review of histopathology, diagnostic testing, and clinical implications. Arch Pathol Lab Med. 2012;136:691–7.
14. Gravalos C, Jimeno A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. Ann Oncol. 2008;19:1523–9.
15. Togami S, Sasajima Y, Oi T, Ishikawa M, Onda T, Ikeda S-I, et al. Clinicopathological and prognostic impact of human epidermal growth factor receptor type 2 (HER2) and hormone receptor expression in uterine papillary serous carcinoma. Cancer Sci. 2012;103:926–32.
16. Morrison C, Zanagnolo V, Ramirez N, Cohn DE, Kelbick N, Copeland L, et al. HER-2 is an independent prognostic factor in endometrial cancer: association with outcome in a large cohort of surgically staged patients. J Clin Oncol. 2006;24:2376–85.
17. Odicino FE, Bignotti E, Rossi E, Pasinetti B, Tassi RA, Donzelli C, et al. HER-2/neu overexpression and amplification in uterine serous papillary carcinoma: comparative analysis of immunohistochemistry, real-time reverse transcription-polymerase chain reaction, and fluorescence in situ hybridization. Int J Gynecol Cancer. 2008;18:14–21.
18. Slomovitz BM, Broaddus RR, Burke TW, Sneige N, Soliman PT, Wu W, et al. Her-2/neu overexpression and amplification in

uterine papillary serous carcinoma. J Clin Oncol. 2004;22:3126–32.
19. Lapińska-Szumczyk S, Supernat A, Majewska H, Gulczyński J, Luczak A, Biernat W, et al. HER2-positive endometrial cancer subtype carries poor prognosis. Clin Transl Sci. 2014;7:482–8.
20. Saffari B, Jones LA, El-Naggar A, Felix JC, George J, Press MF. Amplification and overexpression of HER-2/neu (c-erbB2) in endometrial cancers: correlation with overall survival. Cancer Res. 1995;55:5693–8.
21. Buza N, English DP, Santin AD, Hui P. Toward standard HER2 testing of endometrial serous carcinoma: 4-year experience at a large academic center and recommendations for clinical practice. Mod Pathol. 2013;26:1605–12.
22. Woo JS, Apple SK, Sullivan PS, Rao J-Y, Ostrzega N, Moatamed NA. Systematic assessment of HER2/neu in gynecologic neoplasms, an institutional experience. Diagn Pathol. 2016;11:102.
23. Grushko TA, Filiaci VL, Mundt AJ, Ridderstråle K, Olopade OI, Fleming GF, et al. An exploratory analysis of HER-2 amplification and overexpression in advanced endometrial carcinoma: a Gynecologic Oncology Group study. Gynecol Oncol. 2008;108:3–9.
24. Villella JA, Cohen S, Smith DH, Hibshoosh H, Hershman D. HER-2/neu overexpression in uterine papillary serous cancers and its possible therapeutic implications. Int J Gynecol Cancer. 2006;16:1897–902.
25. Santin AD, Bellone S, Van Stedum S, Bushen W, De Las Casas LE, Korourian S, et al. Determination of HER2/neu status in uterine serous papillary carcinoma: comparative analysis of immunohistochemistry and fluorescence in situ hybridization. Gynecol Oncol. 2005;98:24–30.
26. Peiró G, Mayr D, Hillemanns P, Löhrs U, Diebold J. Analysis of HER-2/neu amplification in endometrial carcinoma by chromogenic in situ hybridization. Correlation with fluorescence in situ hybridization, HER-2/neu, p53 and Ki-67 protein expression, and outcome. Mod Pathol. 2004;17:277–87.
27. Pegram MD, Konecny G, Slamon DJ. The molecular and cellular biology of HER2/neu gene amplification/overexpression and the clinical development of herceptin (trastuzumab) therapy for breast cancer. Cancer Treat Res. 2000;103:57–75.
28. Higgins MJ, Baselga J. Targeted therapies for breast cancer. J Clin Invest. 2011;121:3797–803.
29. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001;344:783–92.
30. Chan A, Delaloge S, Holmes FA, Moy B, Iwata H, Harvey VJ, et al. Neratinib after trastuzumab-based adjuvant therapy in patients with HER2-positive breast cancer (ExteNET): a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol. 2016;17:367–77.
31. Pazo Cid RA, Antón A. Advanced HER2-positive gastric cancer: current and future targeted therapies. Crit Rev Oncol Hematol. 2013;85:350–62.
32. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet. 2010;376:687–97.
33. Santin AD, Bellone S, Buza N, Schwartz PE. Regression of metastatic, radiation/chemotherapy-resistant uterine serous carcinoma overexpressing HER2/neu with trastuzumab emtansine (TDM-1). Gynecol Oncol Rep. 2017;19:10–12.
34. Jewell E, Secord AA, Brotherton T, Berchuck A. Use of trastuzumab in the treatment of metastatic endometrial cancer. Int J Gynecol Cancer. 2006;16:1370–3.
35. Fleming GF, Sill MW, Darcy KM, McMeekin DS, Thigpen JT, Adler LM, et al. Phase II trial of trastuzumab in women with advanced or recurrent, HER2-positive endometrial carcinoma: a Gynecologic Oncology Group study. Gynecol Oncol. 2010;116:15–20.
36. Santin AD. Letter to the Editor referring to the manuscript entitled: "Phase II trial of trastuzumab in women with advanced or recurrent HER-positive endometrial carcinoma: a Gynecologic Oncology Group study" recently reported by Fleming et al., (Gynecol Oncol., 116. Gynecol Oncol. 2010;118:95–96.
37. Fader AN, Roque DM, Siegel E, Buza N, Hui P, Abdelghany O, et al. Randomized phase ii trial of carboplatin-paclitaxel versus carboplatin-paclitaxel-trastuzumab in uterine serous carcinomas that overexpress human epidermal growth factor receptor 2/neu. J Clin Oncol. 2018;36:2044–51.
38. National Comprehensive Cancer Network. Uterine neoplasms (Version 1.2020). 2020. https://www.nccn.org/professionals/physician_gls/pdf/uterine.pdf.
39. Fitzgibbons PL, Bartley AN, Longacre TA, Broaddus R, Chuang LT, Cohen MB, et al. Template for reporting results of biomarker testing of specimens from patients with carcinoma of the endometrium, v1.2.0.1. Northfield, Illinois: College of American Pathologists; 2019.
40. León-Castillo A, Britton H, McConechy MK, McAlpine JN, Nout R, Kommoss S, et al. Interpretation of somatic POLE mutations in endometrial carcinoma. J Pathol. 2020;250:323–35.
41. Dong F, Costigan DC, Howitt BE. Targeted next-generation sequencing in the detection of mismatch repair deficiency in endometrial cancers. Mod Pathol. 2019;32:252–7.
42. Wolff AC, Hale Hammond ME, Allison KH, Harvey BE, Mangu PB, Bartlett JMS, et al. Human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline focused update. J Clin Oncol. 2018;36:2105–22.
43. Sholl LM, Do K, Shivdasani P, Cerami E, Dubuc AM, Kuo FC, et al. Institutional implementation of clinical tumor profiling on an unselected cancer population. JCI Insight. 2016;1:e87062.
44. Garcia EP, Minkovsky A, Jia Y, Ducar MD, Shivdasani P, Gong X, et al. Validation of OncoPanel: a targeted next-generation sequencing assay for the detection of somatic variants in cancer. Arch Pathol Lab Med. 2017;141:751–8.
45. Liu J, Hsueh H, Hsieh E, Chen JJ. Tests for equivalence or non-inferiority for paired binary data. Stat Med. 2002;21:231–45.
46. Tang ML. Matched-pair noninferiority trials using rate ratio: A comparison of current methods and sample size refinement. Control Clin Trials. 2003;24:364–77.
47. Buza N, Hui P. Marked heterogeneity of HER2/NEU gene amplification in endometrial serous carcinoma. Genes Chromosomes Cancer. 2013;52:1178–86.
48. Xu M, Schwartz P, Rutherford T, Azodi M, Santin A, Silasi D, et al. HER-2/neu receptor gene status in endometrial carcinomas: a tissue microarray study. Histopathology. 2010;56:269–73.
49. Mentrikoski MJ, Stoler MH. HER2 immunohistochemistry significantly overestimates HER2 amplification in uterine papillary serous carcinomas. Am J Surg Pathol. 2014;38:844–51.
50. Singh P, Smith CL, Cheetham G, Dodd TJ, Davy MLJ. Serous carcinoma of the uterus-determination of HER-2/neu status using immunohistochemistry, chromogenic in situ hybridization, and quantitative polymerase chain reaction techniques: its significance and clinical correlation. Int J Gynecol Cancer. 2008;18:1344–51.
51. Ross DS, Zehir A, Cheng DT, Benayed R, Nafa K, Hechtman JF, et al. Next-generation assessment of human epidermal growth factor receptor 2 (ERBB2) amplification status. J Mol Diagn. 2017;19:244–54.
52. Cenaj O, Ligon AH, Hornick JL, Sholl LM. Detection of ERBB2 amplification by next-generation sequencing predicts HER2 expression in colorectal carcinoma. Am J Clin Pathol. 2019;152:97–108.
53. Buza N, Roque DM, Santin AD. HER2/neu in endometrial cancer: a promising therapeutic target with diagnostic challenges. Arch Pathol Lab Med. 2014;138:343–50.

54. Yang S-R, Bouhlal Y, De La Vega FM, Ballard M, Kuo CJ, Vilborg A, et al. Integrated genomic characterization of ERBB2/HER2 alterations in invasive breast carcinoma: a focus on unusual FISH groups. Mod Pathol. 2020;33:1546–56.
55. Murray C, D'Arcy C, Gullo G, Flanagan L, Quinn CM. Human epidermal growth factor receptor 2 testing by fluorescent in situ hybridization: positive or negative? ASCO/College of American Pathologists guidelines 2007, 2013, and 2018. J Clin Oncol. 2018;36:3522–3.
56. Martin V, Valera A, De Joffrey M, Banfi S, Mazzucchelli L. Implementation of the 2018 human epidermal growth factor receptor 2 guidelines by ASCO/College of American Pathologists will reduce false-positive tests. J Clin Oncol. 2018;36:3523–4.
57. Growdon WB, Groeneweg J, Byron V, DiGloria C, Borger DR, Tambouret R, et al. HER2 over-expressing high grade endometrial cancer expresses high levels of p95HER2 variant. Gynecol Oncol. 2015;137:160–6.
58. Hanna W, Nofech-Mozes S, Kahn HJ. Intratumoral heterogeneity of HER2/neu in breast cancer? A rare event. Breast J. 2007;13:122–9.
59. Greer LT, Rosman M, Mylander WC, Hooke J, Kovatich A, Sawyer K, et al. Does breast tumor heterogeneity necessitate further immunohistochemical staining on surgical specimens? J Am Coll Surg. 2013;216:239–51.
60. Hanna WM, Rüschoff J, Bilous M, Coudry RA, Dowsett M, Osamura RY, et al. HER2 in situ hybridization in breast cancer: clinical implications of polysomy 17 and genetic heterogeneity. Mod Pathol. 2014;27:4–18.
61. Van Cutsem E, Bang YJ, Feng-yi F, Xu JM, Lee KW, Jiao SC, et al. HER2 screening data from ToGA: targeting HER2 in gastric and gastroesophageal junction cancer. Gastric Cancer. 2015;18:476–84.
62. Hicks DG, Whitney-Miller C. HER2 testing in gastric and gastroesophageal junction cancers. Appl Immunohistochem Mol Morphol. 2011;19:506–8.
63. Hofmann M, Stoss O, Shi D, Büttner R, Van De Vijver M, Kim W, et al. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. Histopathology. 2008;52:797–805.
64. Sauter G, Moch H, Moore D, Carroll P, Kerschmann R, Chew K, et al. Heterogeneity of erbB-2 gene amplification in bladder cancer. Cancer Res. 1993;53:2199–203.
65. Grob TJ, Kannengiesser I, Tsourlakis MC, Atanackovic D, Koenig AM, Vashist YK, et al. Heterogeneity of ERBB2 amplification in adenocarcinoma, squamous cell carcinoma and large cell undifferentiated carcinoma of the lung. Mod Pathol. 2012;25:1566–73.
66. Halle MK, Tangen IL, Berg HF, Hoivik EA, Mauland KK, Kusonmano K, et al. HER2 expression patterns in paired primary and metastatic endometrial cancer lesions. Br J Cancer. 2018;118:378–87.