Lavage of the Uterine Cavity for Molecular Detection of Müllerian Duct Carcinomas: A Proof-of-Concept Study

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ABSTRACT

Purpose
Type II ovarian cancer (OC) and endometrial cancer (EC) are generally diagnosed at an advanced stage, translating into a poor survival rate. There is increasing evidence that Müllerian duct cancers may exfoliate cells. We have established an approach for lavage of the uterine cavity to detect shed cancer cells.

Patients and Methods
Lavage of the uterine cavity was used to obtain samples from 65 patients, including 30 with OC, five with EC, three with other malignancies, and 27 with benign lesions involving gynecologic organs. These samples, as well as corresponding tumor tissue, were examined for the presence of somatic mutations using massively parallel sequencing (next-generation sequencing) and, in a subset, singleplex analysis.

Results
The lavage technique could be applied successfully, and sufficient amounts of DNA were obtained in all patients. Mutations, mainly in TP53, were identified in 18 (60%) of 30 lavage samples of patients with OC using next-generation sequencing. Singleplex analysis of mutations previously determined in corresponding tumor tissue led to further identification of six patients. Taken together, in 24 (80%) of 30 patients with OC, specific mutations could be identified. This also included one patient with occult OC. All five analyzed lavage specimens from patients with EC harbored mutations. Eight (29.6%) of 27 patients with benign lesions tested positive for mutations, six (75%) as a result of mutations in the KRAS gene.

Conclusion
This study proved that tumor cells from ovarian neoplasms are shed and can be collected via lavage of the uterine cavity. Detection of OC and EC and even clinically occult OC was achieved, making it a potential tool of significant promise for early diagnosis.

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In addition, for type II EC, a lesion that seems to play an important role in carcinogenesis has been identified, called endometrial intraepithelial carcinoma. Similar to serous EC, endometrial intraepithelial carcinoma is associated with mutations and an abnormal accumulation of the TP53 protein.7 Late diagnosis is a large contributor to the poor prognosis of these cancers. Serum CA-125 measurement and transvaginal ultrasound are the most commonly used tests for diagnosis. Specificity and sensitivity of both are low, making them ineffective for screening and early or differential diagnosis.8,9 In the United States, 5% to 10% of women undergo surgery for a suspected ovarian neoplasm at least once during their life,10 and the majority of these procedures reveal only benign diseases.11-14 In part, this is a result of difficulties in discrimination before surgery and why only 30% to 50% of women with OC receive optimum treatment by a gynecologic oncologist.15,16 Studies have shown that surgical treatment in specialized centers results in a better survival, proving the benefit of a method able to discriminate between a benign or malignant lesion.17 Familial or inherited syndromes account for 10% to 15% of HGSCs.18 BRCA1 and BRCA2 mutation carriers have a 54% and 23% estimated lifetime risk of developing HGSC, respectively.19 Even in this high-risk group, screening with serum CA-125 measurement and transvaginal ultrasound was not able to improve clinical outcomes.8 At present, risk-reducing salpingo-oophorectomy (RRSO) is the only effective approach to reduce the risk of HGSC in these women.20,21 Thus, there is a clear and unmet clinical need for earlier detection of HGSC and type II EC.

A study published by Kinde et al22 made use of the so-called liquid Pap, which is routinely used for human papillomavirus detection, and showed that cells of gynecologic Mullerian duct cancers can be present in the uterine cervix. Massively parallel sequencing for tumor-specific mutations was performed on DNA from liquid Pap smear specimens. This technique was successfully applied to 24 (100%) of 24 patients with EC. In patients with OC, the sensitivity was less, with mutations identified in nine (41%) of 22 patients.

In this article, we describe a new method based on recent insights concerning the pathogenesis and characteristics of Mullerian tumors. The ovarian surface, fallopian tube, and uterine cavity form a communicating space. Peristaltic waves allow the fallopian tube to function as a conduit, possibly transporting exfoliated cells from HGSCs or STICs into the uterine cavity. We want to address whether cells from Mullerian duct cancers can be collected with a lavage of the uterine cavity. This lavage concept could improve both the earlier detection of type II Mullerian duct cancers and the differential diagnosis of adnexal masses.

PATIENTS AND METHODS

Patient Samples

Samples were collected in accordance with the institutional review boards of the Medical University of Vienna (EK#1148_2011, EK#1766_2013), the Catholic University Leuven (B3220121486/S54406), the Klinikum Essen-Mitte (LANR_2013456), and the Charité Medical University of Berlin (EA2/ 025/14). Informed consent was obtained from each participant. Patients with the following diagnoses were included (Table 1): OC (n = 30), EC (n = 5), benign gynecologic diseases (n = 27), and other malignancies with involvement of gynecologic organs (n = 7). To increase the number of patients with malignant diagnosis, three patients (patients 196, 197, and 198) were randomly selected from the prospective running Lavage of the Uterine Cavity for the Diagnosis of Ovarian and Tubal Carcinoma and Their Premalignant Changes (LUDOC) study (ClinicalTrials.gov identifier: NCT02387645). To prove that exfoliated cells may be present even in a clinically occult stage of disease, one patient (patient 205) from the prospective running Lavage of the Uterine Cavity for the Diagnosis of Serous Tubal Intraepithelial Carcinoma (LUSTIC) study (ClinicalTrials.gov identifier: NCT02039388) was included. In all 73 patients, lavage specimens were taken for subsequent mutation analysis. Eight patients were excluded from final analysis for one of the following reasons (Table 1): two or more malignant tumors were present, tubal ligation was performed previously, patients were suspected of harboring a germline mutation, or primary tumor tissue was available but did not harbor any of the tested mutations.

Corresponding tumor tissue was available for 21 of the included patients with OC (70%), three patients with EC (60%), 19 patients with benign diseases (70.4%), and the patient with a metastatic signet ring carcinoma. Uterine lavage was performed before surgery for suspected malignant adnexal pathology, immediately after induction of anesthesia. Lavage samples were centrifuged at 300 × g for 10 minutes at room temperature. DNA was isolated from the cell pellet (QIAamp MinElute Media Kit, No. 57414; Qiagen, Hilden, Germany), and concentration was determined using a Qubit fluorometer (ThermoFisher, Waltham, MA).

Lavage Technique

First, an antiseptic lotion was used for cleaning the cervix. Using a bullet forceps, the cervix was grasped at 12 o’clock. A three-way catheter (Coloplast AH5312; Coloplast, Minneapolis, MN) was inserted into the cervical canal, and the balloon was inflated with approximately 1 mL of saline to seal the cervical canal. If the cervical canal was too narrow to pass the catheter, it was dilated to 4 mm with Hegar dilators. Two 10-mL syringes, one of them containing 10 mL of saline, were connected to two of the catheter tubes. The patient was put into an anti-Trendelenburg position. By pushing on the plunger of the syringe containing saline, the uterine cavity and fallopian tubes were slowly perfused. Simultaneously, the plunger of the empty syringe was gently pulled out. Finally, the balloon was deflated, and the catheter was removed. This procedure was partially still under optimization during sample collection within this proof-of-concept study.

Massively Parallel Sequencing

Multiplex amplification and sequencing of 136 amplicons covering approximately 13,000 base pairs were applied to lavage samples, and the corresponding tumor specimens were22 modified as described earlier.23 Amplicons comprised gene regions of AKTI, APC, BRF, CDKN2A, CTNNB1, EGFR, FBXW7, FGFR2, KRAS, NRAS, PIK3CA, PIK3R1, POLE, PPP2R1A, PTEN, and TP53 (Data Supplement). Confirmation of each detected mutation was carried out through independent singleplex amplification (safe sequencing system [SafeSeqS]) and sequencing using the same DNA sample, as described in Bettegowda et al.24

Digital Droplet Polymerase Chain Reaction

Lavage samples of six patients were analyzed by digital droplet polymerase chain reaction (ddPCR; QX100 Droplet Digital PCR System; Bio-Rad Laboratories, Hercules, CA). Mutations previously identified in tumor tissue were evaluated. For confirmation, DNA from tumor tissue was analyzed through ddPCR as well. Custom TaqMan SNP Genotyping Assays (No. 4331349; Life Technologies, Carlsbad, CA) were designed using Primer Express 3.0 software (ThermoFisher). A positive control and wild-type control were included in every polymerase chain reaction run. Ten to 20 ng of template DNA was used per amplification. All samples were analyzed at least in duplicates. To ensure specificity, all designed assays were also tested on samples obtained from three patients with benign diseases.

RESULTS

Identification of Tumor Cells in Lavage Samples

Sufficient amounts of DNA were obtained from each lavage sample (mean, 1.8 μg; range, 33 ng to 22.9 μg). Lavage samples were
| Patient No. | Age (years) | Type of Disease | FIGO Stage | Grade | Mutations in Primary Tumor | Mutations in Lavage Specimen | Notes |
|------------|-------------|-----------------|------------|-------|----------------------------|-------------------------------|-------|
| 194        | 55          | OC              | IA         | G1    | FGFR2 p.K659E, c.1975T->C | FGFR2 p.K659E, c.1975T->C (1) |       |
| 155        | 45          | OC              | IC         | G1    | PK3CA p.H1047R, c.3140A>G | PK3CA p.H1047R, c.3140A>G (3) |       |
| 152        | 50          | OC              | IIIC        | G3    | TP53 p.R248Q, c.743G>A   | TP53 p.R248Q, c.743G>A (1)   |       |
| 140        | 44          | OC              | IIIA        | G3    | TP53 p.H168R, c.503A>G   | TP53 p.H168R, c.503A>G (3)   |       |
| 149        | 57          | OC              | IIIA        | G3    | TP53 p.V272E, c.815T>A   | TP53 p.V272E, c.815T>A (3)   |       |
| 148        | 71          | OC              | IIIB        | G3    | TP53 p.V216M, c.646G>A   | TP53 p.V216M, c.646G>A (1)   |       |
| 205        | 42          | OC              | IIIB        | G2    | TP53 p.V217fs, c.650delT | TP53 p.V217fs, c.650delT (1) |       |
| 143        | 52          | OC              | IIIC        | G3    | TP53 p.C135Y, c.404G>A   | TP53 p.L194R, c.581T>G (3)   |       |
| 158        | 49          | OC              | IIIC        | G3    | TP53 p.G245V, c.734G>T   | TP53 p.G245V, c.734G>T (3)   |       |
| 130        | 72          | OC              | IIIC        | G2    | PPP2R1A p.W257C, c.771G>T | PPP2R1A p.W257C, c.771G>T (1) |       |
| 132        | 64          | OC              | IIIC        | NA    | TP53 p.M237I, c.711G>C   | TP53 p.M237I, c.711G>C (1)   |       |
| 139        | 70          | OC              | IIIC        | G3    | TP53 p.H193P, c.578A>C   | TP53 p.H193P, c.578A>C (1)   |       |
| 144        | 43          | OC              | IIIC        | G3    | TP53 p.G245V, c.734G>T   | KRAS p.G12C, c.34G>T (1)     |       |
| 160        | 35          | OC              | IIIC        | G3    | TP53 p.T170 (in-frame deletion), c.508_510delACG | TP53 p.T170 (in-frame deletion), c.508_510delACG (2) |       |
| 168        | 58          | OC              | IIIC        | G3    | TP53 p.R273H, c.818G>A   | TP53 p.R273H, c.818G>A (1)   |       |
| 179        | 73          | OC              | IIIC        | G3    | NA                         | KRAS p.G12A, c.35G>C (1)     |       |
| 137        | 61          | OC              | IIIC        | G3    | TP53 p.H179R, c.536A>G   | TP53 p.H179R, c.536A>G (1)   |       |
| 194        | 49          | OC              | IIIC        | G3    | NA                         | NRAS p.Q61R, c.182A>G (1)    |       |
| 196        | 59          | OC              | IIIC (A)    | G3    | NA                         | PK3CA p.T1025N, c.3074C>A (1) |       |
| 131        | 55          | OC              | Endometrioid, partly serous | G2    | KRA5 p.G13D, c.38G>A     | KRA5 p.G13D, c.38G>A (1)     |       |
| 147        | 60          | OC              | Endometrioid | G3    | KRA5 p.G12D, c.35G>A     | KRA5 p.G12D, c.35G>A (1)     |       |
| 204        | 61          | OC              | Clear cell  | G3    | NA                         | TP53 p.A159I, c.475_476C>G>AT (1) |       |
| 198        | 59          | OC              | Serous      | G3    | NA                         | PK3CA p.M1004V, c.3010A>G (1) |       |
| 172        | 55          | OC              | Terr      | G3    | NA                         | TP53 p.G226V, c.785G>T (1)   |       |
| 133        | 31          | OC              | LMP         | NA    | AKT1 p.E17K, c.494G>A     | AKT1 p.E17K, c.494G>A (1)    |       |
| 161        | 52          | OC meta         | Signet ring carcinoma, spread in ovaries | G3    | TP53 p.R282W, c.844C>T   | TP53 p.R282W, c.844C>T (1)   |       |
| 164        | 72          | UC              | Uterine carcinosarcoma | IIIC    | NA                         | PTEN p.L193F5, c.578delE<T (1) |       |
| 199        | 73          | EC              | IA          | G2    | PTEN p.R130*, c.388C>T   | PTEN p.R130*, c.388C>T (1)    |       |
| 200        | 70          | EC              | IA          | G2    | PK3CA p.H1047Y, c.3139C>T | PK3CA p.H1047Y, c.3139C>T (1) |       |
| 201        | 70          | EC              | IA          | G2    | APC p.Q1469*, c.4405C>T  | APC p.Q1469*, c.4405C>T (1)   |       |
| 202        | 63          | EC              | IA          | G1    | NA                         | PTEN p.Y315*, c.945T>A (1)    |       |
| 203        | 55          | EC              | IA          | G1    | NA                         | CTNNB1 p.S537F, c.110C>T (1)  |       |
| 165        | 61          | B               | Fibroma     | NA    | NA                         | FBXW7 p.R565S, c.1513C>A (1)  |       |
| 175        | 50          | B               | Ovarian cyst, teratoma | NA    | NA                         | KRAS p.G12C, c.34G>T (1)     |       |
| 177        | 31          | B               | Secondary infertility | NA    | NA                         | KRAS p.G12C, c.34G>T (1)     |       |
| 178        | 46          | B               | Uterus myomatous, ovarian cyst | NA    | NA                         | KRAS p.G12C, c.34G>T (1)     |       |
| 181        | 43          | B               | Secondary infertility, polyp of the cervix mucosa | NA    | NA                         | KRAS p.G12C, c.34G>T (1)     |       |
| 184        | 78          | B               | Ovarian cyst | NA    | NA                         | KRAS p.G12C, c.34G>T (1)     |       |
| 190        | 58          | B               | Teratoma    | NA    | NA                         | KRAS p.G12C, c.34G>T (1)     |       |
| 193        | 54          | B               | Inclusion cysts | NA    | NA                         | KRAS p.G12C, c.34G>T (1)     |       |
| 146*       | 39          | BC              | Breast cancer, uterine leiomyoma | NA    | TP53 p.C242F, c.725G>T (1) | Germline mutation |       |

(continued on following page)
| Patient No. | Age (years) | Type of Disease | FIGO Stage | Grade | Mutations in Primary Tumor | Mutations in Lavage Specimen | Notes |
|------------|-------------|-----------------|------------|-------|---------------------------|-------------------------------|-------|
| 162        | 77          | B Ovarian serous cystadenoma (previous leukemia, signet ring carcinoma, squamous cell carcinoma) | NA         |       | TP53 p.N239D, c.715A>G (1) |                               | Two or more malignant tumors |
| 129        | 82          | OC Serous       | IIIC       | G2    | TP53 c.920-1G>A, g.7576927C>T (splice site) |                               | —     |
| 150        | 51          | OC Serous       | IIIC       | G3    | TP53 p.C176Y, c.527G>A     |                               | —     |
| 163        | 67          | OC Serous       | IIIC       | G3    | TP53 p.C238R, c.712T>C     |                               | —     |
| 195        | 69          | OC Serous       | IIIC       | G2    | NA                         |                               | —     |
| 166        | 77          | OC Serous       | N          | G3    | NA                         |                               | —     |
| 174        | 69          | OC Serous       | N          | G3    | TP53 p.R342*, c.1024C>T    |                               | —     |
| 145        | 52          | B Cervix mucous polyp | NA         |       |                           |                               | —     |
| 136        | 49          | B Follicle cyst | NA         |       |                           |                               | —     |
| 138        | 47          | B Uterus myomatosus | NA         |       |                           |                               | —     |
| 151        | 33          | B Follicular cyst | NA         |       |                           |                               | —     |
| 155        | 45          | B Serous cystadenoma | NA         |       |                           |                               | —     |
| 157        | 58          | B Thecocoibroma  | NA         |       |                           |                               | —     |
| 159        | 49          | B Cystic teratoma | NA         |       |                           |                               | —     |
| 176        | 67          | B Cystic teratoma of the ovary | NA         |       |                           |                               | —     |
| 179        | 45          | B Uterus myomatosus | NA         |       |                           |                               | —     |
| 180        | 35          | B Uterus myomatosus | NA         |       |                           |                               | —     |
| 182        | 43          | B CIN III, ovarian cyst, endometriosis | NA         |       |                           |                               | —     |
| 183        | 35          | B Ovarian cyst, endometriosis | NA         |       |                           |                               | —     |
| 185        | 62          | B Ovarian cyst, cystadenofibroma, | NA         |       |                           |                               | —     |
| 186        | 36          | B Teratoma of the ovary | NA         |       |                           |                               | —     |
| 187        | 36          | B Endometriosis | NA         |       |                           |                               | —     |
| 188        | 72          | B Thecocoibroma  | NA         |       |                           |                               | —     |
| 189        | 45          | B Ovarian cyst  | NA         |       |                           |                               | —     |
| 191        | 28          | B Chronical salpingitis, oophoritis | NA         |       |                           |                               | —     |
| 192        | 52          | B Thecocoibroma  | NA         |       |                           |                               | —     |
| 141*       | 60          | OC Low-grade endometrioid adenocarcinoma | IB         | G1    |                           |                               | No mutation identified in primary tumor |
| 170*       | 67          | OC Serous carcinoma | IIIA       | G1    |                           |                               | No mutation identified in primary tumor |
| 134*       | 52          | OC Serous       | IIIC       | G3    |                           |                               | No mutation identified in primary tumor |
| 142*       | 58          | OC Serous       | IIIC       | G2    | TP53 p.Q104*, c.310C>T    |                               | Tubal ligation |
| 153*       | 62          | OC Metastatic ovarian cancer from lobular mamma carcinoma | IIIC       | G2    |                           |                               | Two or more malignant tumors |
| 128*       | 58          | BC, OC Ductal breast cancer, mucinous adenocarcinoma ovary | IIIC       | G2    |                           |                               | Two or more malignant tumors |

NOTE. Mutations detected by NGS, safe sequencing system (SafeSeqS), and digital droplet polymerase chain reaction (ddPCR).
Abbreviations: —, no mutation detected; B, benign disease; BC, breast cancer; CIN, cervical intraepithelial neoplasia; EC, endometrial cancer; FIGO, International Federation of Gynecology and Obstetrics; G, grade; LMP, low malignant potential; NA, not applicable; NGS, next-generation sequencing; OC, ovarian cancer; OC meta, cancer metastasized in ovaries; UC, uterine cancer; *Excluded from final analysis.
Lavage specimens were analyzed by massively parallel sequencing. In patients with malignant diseases, a median mutant allele fraction of 13.9% (range, 1.5% to 44.6%) was detected (Data Supplement). All samples of patients with malignant diseases were analyzed in the manner described in Figure 1.

In case a mutation was detected in lavage specimens, the results were validated twice. First, the index mutation found in the lavage sample (mutation with highest mutant allele fraction) was assessed in an independent singleplex (SafeSeqS) experiment. In all malignant cases with previously identified mutations, the precise mutation was confirmed.

Second, if primary tumor tissue was available, mutation analysis was conducted in the primary sample as well. In all but two patients, the same mutation was identified. For patient 144, a KRAS G12C mutation (4.1%) was detected in the lavage fluid only, possibly because of heterogeneity or low tumor cell content of the tissue specimen. For patient 143, a mutation (TP53 p.L194R) was found that was present in a metastatic lesion but absent in the primary tumor.

When no mutation was found in the lavage DNA, we evaluated the primary tumors by multiplex sequencing, if available. Identified mutations could be verified in the lavage fluids by SafeSeqS (n = 1) or ddPCR (n = 5) with a sensitivity of 0.1% (Fig 2).

Next-generation sequencing (NGS) identified at least one mutation in 18 (60%) of 30 patients with OC. Furthermore, a mutation was also identified in a lavage sample of a patient with a signet ring carcinoma that had spread to the ovaries, a uterine carcinoma, and a tumor with low malignant potential (Fig 2B). When no mutation was found in the lavage DNA (n = 15), we evaluated the available primary tumors by multiplex sequencing (n = 12). Identified mutations were verified in the lavage fluids by SafeSeqS (one of 12 tumors) or ddPCR (five of six tumors) with a sensitivity of 0.1% (Fig 2). Mutation types identified in tumor tissues or lavage samples by either method are listed in Table 1.

In total, 24 (80%) of 30 patients with OC were identified by either the NGS approach or singleplex analysis for the presence of a mutation previously identified in the primary tumor tissue. Applying NGS of the same panel of genes, lavage samples of five patients with stage IA EC were analyzed. In all five samples (100%), mutations were identified, with mutant allele frequencies ranging between 3.91% and 46.42% (Fig 2B).

**Analysis of a Lavage Sample of a Woman With an Occult OC**

A lavage sample was analyzed that was taken from a 41-year-old BRCA1 mutation carrier (patient 205) submitted for RRSO, who was recruited from the prospectively running LUSTIC trial. Although no signs of cancer were present (CA-125 and transvaginal ultrasound...
were inconspicuous) before surgery, laparoscopy and subsequent laparotomy revealed the presence of a few peritoneal nodules and a 15-mm peritoneal lesion on the right diaphragm as the only other site of metastatic spread. Final histopathology revealed a grade 2, pT3b, pN0, International Federation of Gynecology and Obstetrics stage IIIB serous OC. Both ovaries showed microscopic involvement, and STICs were found in both tubes. TP53 mutation analysis of the lavage sample was performed by NGS, an approach suitable for screening of a high-risk population. A frameshift mutation (TP53 p.V217fs, c.650delT) was detected and confirmed by ddPCR (16.8%) and SafeSeqS (17.3%) in DNA from the lavage and the corresponding tissue sample (Speiser et al, submitted for publication).

**Analysis of Lavage Samples From Patients With Benign Gynecologic Diseases**

Eight (29.6%) of 27 lavage samples from patients with benign gynecologic diseases were found to be mutated by NGS. In six of these patients, a KRAS mutation was detected (Table 1). No mutations were identified in 19 tested tissue samples of patients with benign diseases. Other genes affected only one or two patients (FBXW7, PIK3CA, PIK3R1, or PTEN), some of whom also harbored KRAS gene mutations. None of the lavage samples harbored TP53 mutations, the gene that was most commonly mutated in the patients with OC (Fig 3).

**DISCUSSION**

This pilot study demonstrates that cells shed from Müllerian duct cancers, including OC and EC, can be collected through a lavage of the uterine cavity, in which tumor-specific mutations can be detected through massively parallel sequencing. Sixty percent of patients with OC had mutations in their lavage samples detected with the NGS approach, a technology possibly suitable for routine application.

In patients with known mutation status of the primary tumor tissue but nondetectable mutation in the lavage by NGS, two methods with an even higher sensitivity were applied (SafeSeqS and ddPCR). Using these methods, mutation detection rates in patients with OC could be increased to 80%, demonstrating the general validity of this concept.

Because we could demonstrate that the lavage of a patient with occult OC was positive for cancer cells, shedding and transtubal transport of cancer cells seems to take place early in OC propagation. Approximately one third of lavages from patients with benign disease tested positive with the panel of genes applied. In most cases, these were a result of mutations in the KRAS gene or other genes not commonly mutated in OC. All lavages of the five patients with EC tested positive for cancer cells using the NGS approach.

New insights into the biology of high-grade serous OC led to the hypothesis that cells of these tumors and possibly even their precursor lesions can be transported into the uterine cavity. Therefore, we established the lavage technique to collect and identify these cells. As opposed to Pap smears, as used in the study by Kinde et al, the lavage permits the evaluation of cells closer to the ovaries (ie, in the uterine cavity and the fallopian tubes). The evaluation of lavage fluids resulted in higher sensitivity than previously reported in Pap smear fluids (60% vs 41%, respectively). We show here that, even in lavage samples, the fraction of mutant alleles can be below the detection limit of the NGS approach and that using individualized, singleplex methods (SafeSeqS or ddPCR) allows detection of such mutations. With the amount of DNA and depth of coverage used, the NGS approach could reliably detect mutations present in more than 1% of the total alleles examined. Only a multiplex assay such as the NGS method used here is applicable for screening of patients with OC as a result of the large number of different mutations in TP53 that can occur in OC. Therefore, advances in sequencing technologies should be able to increase detection of OC in lavage fluids in the future.

Both the lavage and liquid Pap smear suffer from the fact that few patients with early disease were analyzed because most OCs are not detected until they are advanced. The detection of early disease is one of the most important goals because of the expected greater impact on mortality along with higher chances of surgically curable disease. Still, it is encouraging that two of four stage I OCs were detected in the study by Kinde et al and two of two stage I OCs were detected in the current study. Even more promising for early diagnosis is our detection of a clinically occult OC that was missed by the current state-of-the-art diagnostic methods. As a result of transperitoneal spread, serous OC may not progress stage by stage, and earlier detection will not necessarily result in a stage shift. However, earlier detection of serous OC equates to less tumor burden at diagnosis, a substantial increase in patients who can undergo complete tumor resection, and less radical procedures necessary to achieve this result. Among women with an increased risk of HGSC who undergo RRSO, 10% to 15% are diagnosed with an STIC or an invasive neoplasm. Notably, more than 90% of STICs harbor TP53 mutations, like the more advanced lesions that evolve from them. Thus, the lavage technology has the potential to not only detect OC earlier, but also detect premalignant lesions. This is further supported by the progression time from STIC to an invasive carcinoma, opening a window for early detection or even prevention of HGSC. Cancers that the lavage procedure missed...
were International Federation of Gynecology and Obstetrics stage IIIC or IV. However, such a diagnostic method is not needed for advanced stages, and a successful detection of all of these advanced cancers was not expected as a result of physical reasons. A disadvantage of the invasive lavage method may be the theoretical risk of infection, although no such infections have been observed to date (P. Speiser, personal communication, September 2015).

Possible improvements for future follow-up studies comprise performing the lavage at specific time points during the menstrual cycle. Moreover, the lavage procedure is standardized now, and one Conformité Européenne (CE)-certified catheter is used, in contrast to the proof-of-concept study, where these issues were still being optimized. Additional information will be obtained from comparing liquid Pap smears with lavage samples from the same patients. Both are expected to be exquisitely specific for neoplasia given the nature of the biomarker assessed (somatic mutations). However, it cannot be excluded that benign tumors, which are also neoplastic, contribute mutations to the analyzed fluids. To address this for the first time, we also analyzed samples of patients with benign diseases. In eight of 27 lavage samples, a mutation was detected. Some of these cases may be explained by the fact that low-grade serous carcinoma, in contrast to HGSC, shows a slow transition from benign to malignant stage and thereby accumulates mutations in KRAS, BRAF, or PPP2R1A and other genes. Whether these cases should be considered true-positive results is debatable; a similar debate has been waged for early cervical lesions in conventional Pap smears or small adenomas in colono-copy. These results, especially the high frequency of KRAS mutations, suggest the exclusion of certain genes from mutation analysis for differential diagnosis. The clinically most relevant group is patients with HGSC, which in almost all patients can be detected via TP53 analysis. In patient 131, besides a predominant KRAS mutation, mutations in several other genes were detected, including TP53 (Data Supplement). Thus, the exclusion of KRAS will not alter the test result because of the present TP53 mutation. We were also able to reproduce the high frequency in EC detection, as published by Kinde et al. Although we analyzed lavage samples of only five patients with EC, the potential of the lavage technique is obvious.

In summary, this proof-of-concept study demonstrates the potentially high diagnostic power of the lavage approach for OC and EC detection, especially for early detection in high-risk populations. This is currently being investigated in a large study (ClinicalTrials.gov identifier: NCT02039388).

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AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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