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ORIGINAL REPORT

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

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A B S T R A C T

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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INTRODUCTION

Ovarian cancer (OC) and endometrial cancer (EC) account for almost 40% of deaths from gynecologic cancers and share some similarities.[1] Both develop from Müllerian epithelium and either show a more indolent growth pattern with a good prognosis (type I) or an aggressive phenotype with a poor prognosis (type II).[2]

Of all OCs, approximately 75% are type II, classified as high-grade serous cancers (HGSCs). HGSCs show frequent mutations in the *TP53* gene (> 90%), early transperitoneal dissemination, and a poor prognosis with a 5-year survival rate of 10% to 30%.[3] Over the last decade, the origin of OC has

been reconsidered. The weight of current evidence suggests serous tubal intraepithelial carcinomas (STICs) to be precursor lesions of HGSC. There is growing evidence that the lag time from STIC to clinically overt HGSC is approximately 5 years.[4] Recent studies demonstrated that intraluminal shedding of tumor cells from OCs and STICs occurred frequently.[5]

Of all ECs, approximately 10% are type II. Histologically, these tumors are classified as serous, clear cell, or poorly to undifferentiated endometri-oid. Because of early transperitoneal dissemination and lymph node metastasis, prognosis of type II EC is poor, accounting for approximately 40% of EC mortality and with a 5-year survival rate of 50% to 60%.[6]

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 al[22] made use of the so-called liquid Pap, which is routinely used for human papillomavirus detection, and showed that cells of gynecologic Müllerian 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 Müllerian 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 Müllerian duct cancers can be collected with a lavage of the uterine cavity. This lavage concept could improve both the earlier detection of type II Müllerian 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 (B322201214864/S54406), the Kliniken Essen-Mitte (LÄNR_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 $300 \times g$ 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 were[22] modified as described earlier.[23] Amplicons comprised gene regions of AKT1, APC, BRAF, 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.[23]

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

Table 1. Characteristics of the Study Cohort and Index Mutation Assessed in Primary Tumor Tissues and Lavage Specimens by NGS and Singleplex Approaches

Patient No.	Age (years)	Type of Disease	Histology	FIGO Stage	Grade	Mutations in Primary Tumor	Mutations in Lavage Specimen	Notes
154	55	OC	Endometrioid	IA	G1	FGFR2 p.K659E, c.1975T>C	FGFR2 p.K659E, c.1975T>C (1)	
155	45	OC	Serous	IC	G1	PIK3CA p.H1047R, c.3140A>G	PIK3CA p.H1047R, c.3140A>G (3)	
152	50	OC	Serous	IIC	G3	TP53 p.R248Q, c.743G>A	TP53 p.R248Q, c.743G>A (1)	
140	44	OC	Serous	IIIA	G3	TP53 p.H168R, c.503A>G	TP53 p.H168R, c.503A>G (3)	
149	57	OC	Serous	IIIA	G3	TP53 p.V272E, c.815T>A	TP53 p.V272E, c.815T>A (3)	
148	71	OC	Serous	IIIB	G3	TP53 p.V216M, c.646G>A	TP53 p.V216M, c.646G>A (1)	
205	42	OC	Serous	IIIB	G2	TP53 p.V217fs, c.650delT	TP53 p.V217fs, c.650delT (1)	
143	52	OC	Serous	IIIC	G3	TP53 p.C135Y, c.404G>A	TP53 p.L194R, c.581T>G (3)	
158	49	OC	Serous	IIIC	G3	TP53 p.G245V, c.734G>T	TP53 p.G245V, c.734G>T (3)	
130	72	OC	Serous	IIIC	G2	PPP2R1A p.W257C, c.771G>T	PPP2R1A p.W257C, c.771G>T (1)	
132	64	OC	Serous	IIIC	G2	NA	TP53 p.M237I, c.711G>C (1)	
139	70	OC	Serous	IIIC	G3	TP53 p.H193P, c.578A>C	TP53 p.H193P, c.578A>C (1)	
144	43	OC	Serous	IIIC	G3	TP53 p.G245S, c.733G>A	KRAS p.G12C, c.34G>T (1)	
160	35	OC	Serous	IIIC	G3	TP53 p.T170 (in-frame deletion), c.508_510delACG	TP53 p.T170 (in-frame deletion), c.508_510delACG (2)	
168	58	OC	Serous	IIIC	G3	TP53 p.R273H, c.818G>A	TP53 p.R273H, c.818G>A (1)	
197	73	OC	Serous	IIIC	G3	NA	KRAS p.G12A, c.35G>C (1)	
137	61	OC	Serous	IIIC	G3	TP53 p.H179R, c.536A>G	TP53 p.H179R, c.536A>G (1)	
194	49	OC	Malignant mixed Müllerian tumor	IIIC	G3	NA	NRAS p.Q61R, c.182A>G (1)	
196	59	OC	Malignant mixed Müllerian tumor	IIIA1(i)	G3	NA	PIK3CA p.T1025N, c.3074C>A (1)	
131	55	OC	Endometrioid, partly serous	IIC	G2	KRAS p.G13D, c.38G>A	KRAS p.G13D, c.38G>A (1)	
147	60	OC	Endometrioid	IIC	G3	KRAS p.G12D, c.35G>A	KRAS p.G12D, c.35G>A (1)	
204	61	OC	Clear cell	IIC	G3	NA	TP53 p.A159I, c.475_476GC>AT (1)	
198	59	OC	Serous	IVB	G3	NA	PIK3CA p.M1004V, c.3010A>G (1)	
172	55	OC	Serous	IV	G3	NA	TP53 p.G262V, c.785G>T (1)	
133	31	OC LMP	Serous low malignant potential		NA	NA	AKT1 p.E17K, c.49G>A (1)	
161	52	OC meta	Signet ring carcinoma, spread in ovaries			TP53 p.R282W, c.844C>T	TP53 p.R282W, c.844C>T (1)	
164	72	UC	Uterine carcinosarcoma	IIIC	G3	NA	PTEN p.L193fs, c.578delT (1)	
199	73	EC	EC	IA	G2	PTEN p.R130*, c.388C>T	PTEN p.R130*, c.388C>T (1)	
200	70	EC	EC	IA	G2	PIK3CA p.H1047Y, c.3139C>T	PIK3CA p.H1047Y, c.3139C>T (1)	
201	70	EC	EC	IA	G2	APC p.Q1469*, c.4405C>T	APC p.Q1469*, c.4405C>T (1)	
202	63	EC	EC	IA	G1	NA	PTEN p.Y315*, c.945T>A (1)	
203	55	EC	EC	IA	G1	NA	CTNNB1 p.S37F, c.110C>T (1)	
165	61	B	Fibroma			NA	FBXW7 p.R505S, c.1513C>A (1)	
175	50	B	Ovarian cyst, teratoma			—	KRAS p.G12V, c.35G>T (1)	
177	31	B	Secondary infertility			NA	KRAS p.G12C, c.34G>T (1)	
178	46	B	Uterus myomatosus, ovarian cyst			—	KRAS p.G12C, c.34G>T (1)	
181	43	B	Secondary infertility, polyp of the cervix mucosa			NA	KRAS p.G12V, c.35G>T (1)	
184	78	B	Ovarian cyst			—	KRAS p.G12V, c.35G>T (1)	
190	58	B	Teratoma			—	PIK3R1 p.R348*, c.1042C>T (1)	
193	54	B	Inclusion cysts			—	KRAS p.G12D, c.35G>A (1)	
146*	39	BC	Breast cancer, uterine leiomyoma			NA	TP53 p.C242F, c.725G>T (1)	Germline mutation

(continued on following page)

Table 1. Characteristics of the Study Cohort and Index Mutation Assessed in Primary Tumor Tissues and Lavage Specimens by NGS and Singleplex Approaches (continued)

Patient No.	Age (years)	Type of Disease	Histology	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	IV	G3	NA	—	
174	69	OC	Serous	IV	G3	TP53 p.R342*, c.1024C>T	—	
145	52	B	Cervix mucous polyp			—	—	
136	49	B	Follicle cyst	NA			—	
138	47	B	Uterus myomatosus	NA			—	
151	33	B	Follicular cyst	NA			—	
156	45	B	Serous cystadenoma			—	—	
157	58	B	Thecofibroma			—	—	
159	49	B	Cystic teratoma			—	—	
176	67	B	Cystic teratoma of the ovary			—	—	
179	45	B	Uterus myomatosus			—	—	
180	35	B	Uterus myomatosus			—	—	
182	43	B	CIN III, ovarian cyst, endometriosis	NA		—	—	
183	35	B	Ovarian cyst, endometriosis	NA		—	—	
185	62	B	Ovarian cyst, cystadenofibroma,			—	—	
186	36	B	Teratoma of the ovary			—	—	
187	36	B	Endometriosis			—	—	
188	72	B	Thecofibroma			—	—	
189	45	B	Ovarian cyst			—	—	
191	28	B	Chronical salpingitis, oophoritis			—	—	
192	52	B	Thecofibroma			—	—	
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			—	—	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;
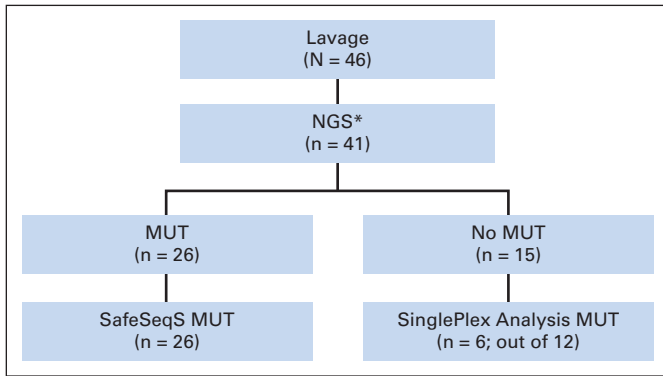
*Excluded from final analysis.

Fig 1. Scheme of the procedure applied for mutation analysis of lavage samples from patients with malignant diseases. When a mutation was detected in the lavage specimen by next-generation sequencing (NGS), it was confirmed by safe sequencing system (SafeSeqS) and analysis of the corresponding tumor tissue. If no mutation was detected by NGS, singleplex methods (SafeSeqS for all 12 patients and digital droplet polymerase chain reaction for six patients) were used to detect a specific mutation previously determined in tumor tissue of 12 patients. (*) Excluded because of previous tubal ligation, germline mutation, or two or more malignant tumors present. MUT, mutation.

classified as positive when one or more mutations were detected in one sample.

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
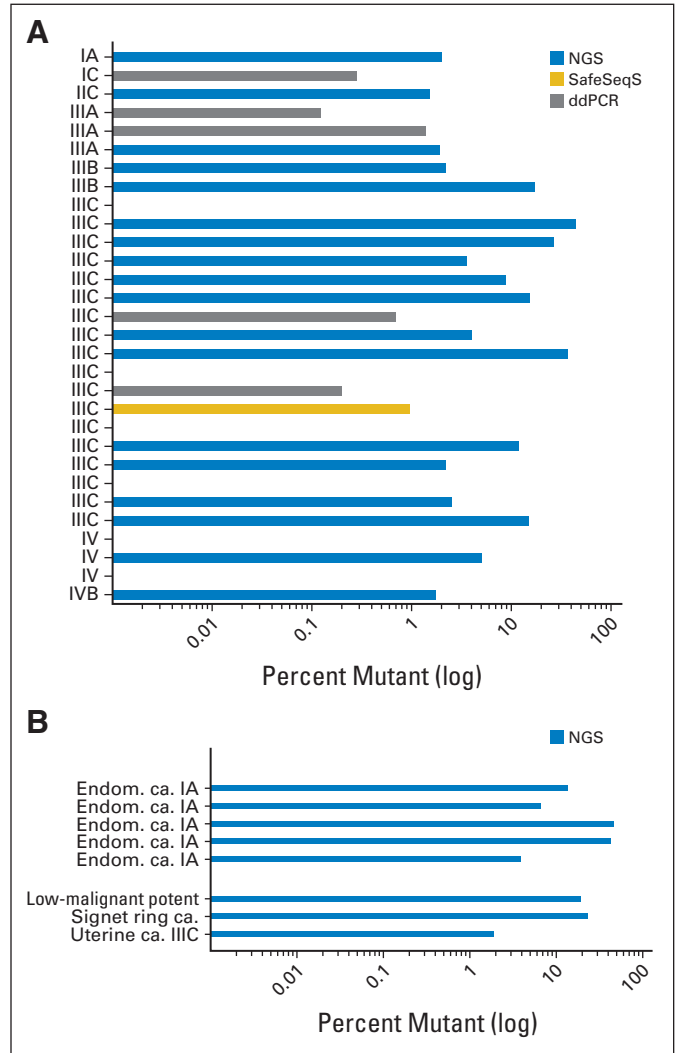

Fig 2. Mutation analysis of lavage specimens by next-generation sequencing (NGS) or singleplex approaches (safe sequencing system [SafeSeqS] and digital droplet polymerase chain reaction [ddPCR]) depicted as percentage of mutant alleles present. (A) Results obtained from analysis of patients with OC included in final analysis. All samples were analyzed by NGS, 12 initially negative samples were analyzed by SafeSeqS, and six of those were also analyzed by ddPCR. (B) Results obtained from analysis of patients with other malignancies, including low-malignant-potential ovarian tumor, uterine carcinosarcoma, and signet ring carcinoma metastasized to the ovaries, as well as five patients with endometrial carcinoma. Disease type and International Federation of Gynecology and Obstetrics stage of disease, if applicable, are listed on the y-axis. ca., carcinoma; Endom., endometrial; potent, potential.

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
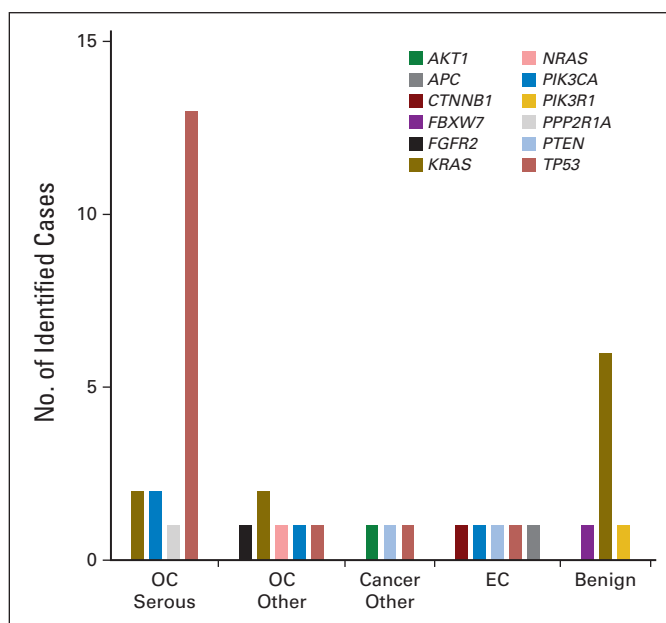
Fig 3. Distribution of genes affected by mutations, leading to the identification of different sample types (index mutation). A mutation of the *TP53* gene is the most important marker in identifying ovarian cancer (OC), whereas *KRAS* mutations can also be observed in patients with benign diseases. EC, endometrial cancer.

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.V217*fs*, 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 transportation 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,[22] 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% *v* 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[22] 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.[11,24-31] 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 Européene (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.[32] 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 colonoscopy. 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.[22] 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).

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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

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