Low-Coverage Whole Genome Sequencing Diagnoses Endometrial Carcinoma from Tampon DNA of Uterine Bleeding Patients

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Abstract

Background

Endometrial carcinoma (EC) is a disease predominantly affecting postmenopausal women. It accounts for about 5% of abnormal uterine bleeding. It is still challenging to diagnose cancers from uterine bleeding patients. Previously, chromosome aberrations were found to be frequent in EC. Here we employed a low coverage whole genome sequencing technology to investigate chromosome aberrations in tampon-collected DNA of patients with suspicious EC.

Methods

Thirty ECs and 14 benign cases with abnormal bleeding are prospectively recruited. Tampons were used to collect exfoliated cells and DNA extracted from these exfoliated cells was analyzed by a customized bioinformatics workflow, uterine exfoliated cell chromosomal aneuploidy detector (UterCAD).

Results

As our data shown, frequent chromosome copy number variations (CNV) were found in EC patients as compared to non-tumor controls, especially the chromosome 8q gain and 10q gain. Using UterCAD, CNVs were detected in tampon-derived DNA from 83.3% (25/30) EC, which were 80.8% (21/26) EECs and 100% (4/4) USCs. In EEC group, CNVs were found in 81.3% (13/16), 85.7% (6/7), and 66.7% (2/3) patients of stage IA, IB, and II/III, respectively. Moreover, all the 4 USC patients presented significant CNVs.

Conclusions

UterCAD could be a highly specific, robust uterine cancer diagnosis method, with an especially high sensitivity for the more aggressive subtype - serous carcinoma. It may be used as a non-invasive approach for diagnosis and active surveillance in endometrial cancer prior to the use of biopsy, thereby largely reducing the treatment burden on patients.

Background

Globally, endometrial cancer (EC) is the second leading gynecologic malignancy, with an estimated 382,069 new cases and nearly 90,000 deaths in 2018[1]. The life-time risk of EC for a woman is about 3%, and this rate may be even higher in some developed countries[1–3]. In recent decades, the incidence of EC has notably increased due to world-wide prevalence of obesity, elongated life expectancy, exposure to excessive estrogen and other carcinogenic factors[3]. In terms of early-stage tumors, EC is highly curable with a satisfactory 5-year overall survival (95% for these localized tumor). However, patients with regional and distant invasive lesions have a much poorer prognosis (the 1-year and 5-year overall survival rates are 69% and 16%, respectively). According to the histologic characteristics, EC was classified into type I (endometrioid carcinoma, EEC) and type II (papillary serous, clear cell, or undifferentiated carcinoma). Type I tumor usually showed a chronic progress through 5–10 or more years, pathologically classified as normal endometrium, non-atypical hyperplasia, atypical hyperplasia, and finally invasive carcinoma. Type I EC is commonly characterized with higher expression of estrogen receptor (ER), progesterone

receptor (PR), which account for 80–90% of all the EC while only 40% of the EC-related deaths[3–5]. By contrast, up to date, no precancerous conditions were identified for type II tumors, which were believed to be non-estrogen-related and lack the typical symptoms such as abnormal uterine bleeding (AUB) and postmenopausal bleeding (PMB). Remarkably, type II EC is more invasive, and the 5-year survival is much lower than type I[3, 4, 6].

For most if not all cancers, the population-based screening and detection of precancerous and early-stage cancers could be of much help to decrease the mortality and relieve the socio-economic burden[7]. Taking cervical cancer as an example, in the last decades, widely practiced human papillomavirus and cytology screening for high-risk subjects has a good efficacy on detecting cervical pre-cancerous lesions and eventually decreased the incidence of invasive cervical cancer[8]. Unfortunately, the screening and early detection of EC remains a huge challenge. The current clinical evaluation for EC hinges on women presenting symptoms such as AUB or PMB, or asymptomatic women with thickened endometrium or uterine cavity mass indicating by ultrasonography or other imaging tests[9]. Following suspicious diagnoses, more invasive procedures, such as dilation and curettage (D&C), and hysteroscopy examination, are performed to obtain the pathologic evidences. Not only does this place much more burden on the individual patient, it also introduces increased costs and additional risks of anaesthesia- or procedure-related complications[10]. More importantly, considering that only 5–10% of AUB or PMB cases were diagnosed to be EC or atypical hyperplasia, it is believed that most patients undergo unnecessary but intensive diagnostic work-up for benign diseases[10].

EC is a disease predominantly in postmenopausal women, with over 80% cases occurring in women over 50 years-old. However, the epidemiologic data showed that increasing rate of obesity may lead to a rise in the proportion of pre-menopausal EC cases[11, 12]. The high proportion of ECs diagnosed at an early stage is largely due to abnormal bleeding, which is present in 94% of such cases[13]. In postmenopausal women, the presence of PMB equates to a risk of EC of 9%. In contrast, the risk of EC in pre-menopausal women with AUB is only 0.33% (95% Confidence Interval (CI) 0.23–0.48).

According to the findings from The Cancer Genome Atlas projects (TCGA), a large portion of EC patients presented significantly Copy Number Variations (CNVs), which might be used for the diagnosis and treatment of EC[14]. In this pilot study (single-blinded), we prospectively collected the exfoliated cells using a vaginal tampon, and developed a bioinformatics protocol named “Uterine exfoliated cell Chromosomal Aneuploidy Detector” (UterCAD) to detect CNVs. The sensitivity and specificity of UterCAD for early EC diagnosis were investigated in patients presented with AUB or PMB.

Methods

Samples collection

Our study adhered to the Standards for Reporting Diagnostic Accuracy Studies (STARD) 2015 guideline. This prospective study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University and written informed consent was obtained from all participants. Fifty-one consecutive patients were enrolled in the First Affiliated Hospital of Zhengzhou University from March 2020 to January 2021.

Inclusion and Exclusion Criteria

Patients (over 40 years of age) with AUB or PMB and planned for a hysteroscope or hysterectomy were eligible for inclusion. Pregnancy or coagulopathy were excluded by testing blood β -HCG and coagulation related factors (platelet counts, PT, APTT, INR, TT, FIB and D-dimer). All participants underwent standard collection of tampon samples at

admission to examine CNVs and tumor markers. For EC cases, tumor stage was determined according to the latest edition of the International Federation of Gynecology and Obstetrics (FIGO).

DNA Extraction

Total genomic DNA was isolated from the tampon specimen using the Amp Genomic DNA Kit (TIANGEN) and QIAseq DNA Extraction kit (Qiagen), respectively. Next generation sequencing was performed as previously described. DNA was fragmented into an average size of 300bp, and then 100ng of fragmented genomic DNA was used for preparation of sequencing libraries (NEBnext Ultra II). An 8-bp-long barcoded sequencing adaptor was ligated with each DNA fragment and amplified by PCR. Purified sequencing libraries were massively parallel sequenced by Illumina HiSeq X10 platform. About 4 GB sequencing raw data per sample were filtered and aligned to the human reference genome.

Low-Coverage WGS

For low-coverage WGS, libraries were prepared using the Kapa Hyper Prep kit with custom adapters (IDT and Broad Institute) starting with 3 to 20 ng of DNA input (median, 5 ng), or approximately 1,000 to 7,000 haploid genome equivalents, was used for low-pass whole-genome sequencing. Up to 22 libraries were pooled and sequenced using 150 bp paired-end runs over 1× lane on a HiSeq X10 (Illumina). Segment copy numbers were derived via the customized workflow UterCAD. Poor-quality sequencing data would be flagged if the median absolute deviation of copy ratios (log2 ratio) between adjacent bins, genome-wide, was 0.38, and the corresponding sample would be excluded in such a case. UterCAD test results were blinded to the clinical information.

Statistical Analysis

At least 10 M paired reads were collected for each sample. The reads were mapped to human reference genome HG19. Genomic coverage was counted using the software SAMtools mpileup. Then we calculated average coverage for each 200-kb bin. Z-scores for each bin was then normalized by Z-score by using the Formula 1.

$$Z = \frac{V_{\text{tumor-average}}(V_{\text{control}})}{\text{stdev}(V_{\text{control}})} \quad (\text{Formula 1})$$

The Circular binary segmentation algorithm was then used to identify significant genomic breakpoints and copy number changed genomic segments, by using the R package 'DNACopy'.

Categorical variables were reported as frequencies and percentages, and continuous variables were described as mean and standard deviation (SD), or median with interquartile range (IQR), as appropriate. Continuous variables were analyzed using the Mann-Whitney U test, and categorical variables were analyzed by the Pearson chi-square test or Fisher's exact test, as appropriate. Missing data were discarded from analyses. All analyses were performed with the use of SPSS18.0 (SPSS Inc., Chicago, IL, USA), R software (version 3.4.3; R Foundation for Statistical Computing), and MedCalc software (version 19.1; MedCalc Software, Mariakerke, Belgium). $P < 0.05$ was considered as statistically significant. Original data and R code used in the statistical analysis will be made available upon request.

Results

Patient characteristics

From March 2020 to January 2021, 51 consecutive patients presented with AUB or PMB were admitted to our department. After the tampon use, one patient withdrew due to personal reason. After DNA extraction and surgery, 5

patients diagnosed as EC according to the biopsy from previous D&C were not found with cancer cells after hysterectomy ± lymphadenectomy, and another 1 patient was flagged with low DNA quality. Thus, these 7 patients were excluded from further UterCAD analysis. The STARD flow diagram was shown in Fig. 1. The baseline characteristics of these patients were shown in Table 1. The median ages were 57 years-old and 59 years-old in the EC group (ranged from 43 to 83 years-old) and benign group (ranged from 45 to 75 years-old), respectively. The postmenopausal rate was 83.3% (25/30) and 85.7% (12/14) ($P=0.608$), respectively. According to the final pathological reports, 26 cases were diagnosed to be EEC and the other 4 cases were USC. The benign group consisted of focal atypical hyperplasia ($n=1$), endometrial polyps ($n=5$), non-atypical hyperplasia ($n=4$), submucosal leiomyoma ($n=2$) and endometritis ($n=2$).

Table 1
Patient's characteristics.

		Benign	EEC	USC
BMI(Kg/m ²)	< 25	6	11	1
	>=25	8	15	3
Age (year)	<=55	3	13	1
	> 55	11	13	3
Postmenopause	Yes	12	21	4
	No	2	5	0

CNV Profile

For all tampon samples that passed sequencing data quality assessment, the positive rate of CNV was 83.3% (25/30) in patients diagnosed with EC, and 7.14% (1/14) in patients with benign endometrial diseases. The genome-wide landscape of CNVs in all patients was shown in Fig. 2. Representative chromosome CNVs included 8q gain ($n=6$) and 10q gain ($n=2$) in EEC (Fig. 2A), and multiple chromosome changes in USC (Fig. 2B), while none of these CNVs were found in benign lesions (Fig. 2C). Z-scores for chromosomal arms (1p, 1q, 2p, 2q, 3p, 3q, 4p, 4q, 5p, 5q, 6p, 6q, 7p, 7q, 8p, 8q, 9p, 9q, 10p, 10q, 11p, 12q, 13q, 14q, 15q, 16p, 16q, 17p, 17q, 18p, 18q, 19p, 19q, 20p, 20q, 21p, 21q, and 22q) were calculated in normalization to benign controls. As to cancer subtypes, Chromosomal 8q gain was found in both EEC and USC patients, while more frequent Chromosomal 10q gains were detected in USC ($P<0.05$). These differential CNV patterns indicated that EEC and USC potentially had different cell origins and tumorigenesis process. A heatmap of Z-scores and the overview of CNV profiles including potentially relevant genes were shown in Supplemental Table 1.

Z-scores Between Malignant and Benign Lesions

We further explored the value of Z-score of each chromosome arm in differentiating endometrial malignancies from benign diseases. The Area Under Curve (AUC) of Receiver-Operating-Characteristic curve ranged from 0.428 to 0.749 (median AUC = 0.549, Table 2). Chromosomal 8q and 10q showed high diagnostic accuracy, with AUCs of 0.749 and 0.612, respectively (Table 2). We combined all the chromosomes' information to build a diagnostic model for EC. The optimal Z-score cutoff $|Z| \geq 2.40$ was calculated by Youden Index. At this cutoff, UterCAD test showed a sensitivity of 83.3% and a specificity of 92.9% (Table 3). The AUC was 0.91 (0.83–0.99), which was better than the result from any single chromosome. A higher cutoff ($|Z|=3$) showed better specificity (100.0%), whereas compromising the

sensitivity (47.1%). The diagnosis model identified 100% (4/4) of USC and 80.8% (21/26) EEC (Table 3). As shown in Fig. 3. The AUC is 1.00, 0.933 and 0.942 for diagnosing USC, EEC, and overall, respectively.

Table 2
Performance of aneuploidy of each chromosome arm as EC
predictor.

Marker	AUC	95CI_lower	95%CI_upper	Cutoff
chr8q	0.749	0.604	0.895	0.915
chr6q	0.658	0.479	0.836	0.185
chr4p	0.643	0.456	0.829	-0.480
chr12p	0.636	0.454	0.819	0.445
chr2p	0.623	0.437	0.808	0.780
chr18q	0.623	0.442	0.804	0.285
chr14q	0.620	0.425	0.816	0.065
chr9q	0.617	0.417	0.816	0.470
chr10q	0.612	0.432	0.791	0.070
chr19p	0.603	0.434	0.772	-0.855
chr1q	0.586	0.412	0.759	-0.385
chr17q	0.586	0.399	0.772	-0.160
chrXp	0.578	0.365	0.791	0.275
chr11q	0.576	0.377	0.775	-0.575
chr21p	0.572	0.367	0.777	-0.420
chr15q	0.567	0.350	0.784	0.780
chr9p	0.565	0.362	0.767	0.295
chr13q	0.563	0.374	0.753	0.455
chr1p	0.553	0.346	0.761	0.165
chr21q	0.553	0.358	0.749	0.710
chr20q	0.552	0.358	0.747	0.140
chr5p	0.548	0.334	0.763	0.340
chr8p	0.547	0.355	0.739	0.050
chr4q	0.542	0.354	0.731	0.200
chr19q	0.532	0.346	0.718	-0.135
chr16q	0.530	0.335	0.725	0.300
chr17p	0.516	0.337	0.695	1.005
chr3p	0.512	0.324	0.701	0.635
chr11p	0.512	0.320	0.704	0.030
chr7p	0.509	0.326	0.692	0.565

Marker	AUC	95CI_lower	95%CI_upper	Cutoff
chr7q	0.509	0.331	0.687	-1.280
chr22q	0.490	0.286	0.694	0.255
chr6p	0.480	0.272	0.688	0.690
chr12q	0.480	0.294	0.667	0.600
chr10p	0.474	0.290	0.658	-0.425
chr5q	0.463	0.255	0.671	-0.065
chr18p	0.462	0.256	0.667	0.345
chr20p	0.449	0.257	0.641	-0.025
chr3q	0.448	0.267	0.629	0.135
chr16p	0.448	0.257	0.639	0.040
chr2q	0.428	0.257	0.599	-1.395

Table 3
EC prediction performance by the combined aneuploidy information from all chromosome arms.

	AUC (95% CI)	Cutoff	TN	TP	FN	FP	PPV	NPV	Specificity	Sensitivity	Accuracy
UterCAD	0.91	2	3	16	1	5	76.2%	75.0%	37.5%	94.1%	76.0%
CHR1-22	(0.83–0.99)	2.4	13	25	5	1	96.2%	72.2%	92.9%	83.3%	86.4%
		3	8	8	9	0	100.0%	47.1%	100.0%	47.1%	64.0%

Tampon DNA positive rate correlates with menopause.

We further explored the clinical parameters which may affect the tampon DNA positivity rate (Table 4). Tampon DNA positivity was found in 100% (5 of 5), 85.7% (14 of 16) and 66.7% (6 of 9), for patients before menopause, patients within 8 years of postmenopause, and patients exceeding 8 years of postmenopause, respectively (Table 4, Fig. 4, P = 0.034, trend tests). No statistical significant correlation was found for FIGO stage, tumor grade, BMI, lymph vascular space invasion, lymph node metastasis, and age (Table 4).

Table 4
Correlation between clinical parameters and UterCAD results in EC patients.

		UterCAD		%	Pvalue
		Negative	Positive		
Stage	IA	4	15	78.9%	1.000
	IB	1	5	83.3%	
	II/ III	1	4	80.0%	
Grade	1	0	6	100%	0.562
	2	3	14	82.4%	
	3	2	5	71.4%	
LN	positive	0	1	100%	1.000
	negative	5	24	82.8%	
LVSI	positive	1	6	85.7%	1.000
	negative	4	19	82.6%	
Age	< 55	2	12	85.7%	1.000
	≥ 55	3	13	81.3%	
PM	≥ 8 years	3	6	66.7%	0.034
	< 8 years	2	14	85.7%	
	No	0	5	100%	
BMI	≥ 25	3	16	84.2%	1.000
	< 25	2	9	81.8%	
Pathology	USC	0	4	100%	1.000
	EEC	4	22	84.6%	
LN: lymph node metastasis; LVSI: lymph vascular space invasion; PM: postmenopause; BMI: body mass index; USC: uterine serous carcinoma					

Discussions

As a lesion located in the uterus cavity, endometrial cancer usually causes abnormal bleeding, which is much more prevalent in women elder than 45 or postmenopausal phase[3]. During last decades, the mortality and morbidity of EC keep rising, which even surpassed cervical cancer in certain areas and significantly threatened women's health[2, 15]. Thus, an effective approach for the screening and early detection of EC is urgently needed.

According to previous studies, human cancers (including EC) are commonly characterized by a rapid growth and increased need of energy and material supplies. Due to the lack of energy and oxygen, necrosis was quite common in tumor mass and caused the fell off of cancer cells into nearby cavities like oesophagus, stomach, urinary and uterine/vaginal tracts[16–19]. This proposed an opportunity to collect the exfoliated cells from EC tumor using a

vaginal tampon, which is much less invasive than the previously reported uterine cavity brush or D&C[20, 21]. In this research, we proposed a non-invasive method, UterCAD, which relies on tampon-based whole genome DNA sequencing technology.

In this study, we used a tampon to collect the exfoliated cells from upper genital tract and investigated its CNVs for the early diagnosis of EC. As data shown, the UterCAD technology achieved a sensitivity of 83.3% and a high specificity of 96.2%, proposing it may be a powerful non-invasive method for the early detection of EC. Interestingly, 5 EEC patients (diagnosed in other hospitals via D&C) presented no cancer cells after hysterectomy in our hospital, indicating these small lesions might be only localized in endometrium. Consistently, UterCAD analysis detected no CNVs in all these 5 cases, implying the superior specificity of this technology.

Similarly, several previous studies have reported the Pap test could collect samples from vagina, cervix surface, cervical canal, uterine cavity, and even fallopian tubes, which might be an attractive approach for the non-invasive diagnosis of EC. As previously reported, Kinde et al. and Wang et al. detected EC-derived DNA in 41% and 29% of associated Pap samples in two independent patients groups, the low detection rate might be caused by the short period (just a few seconds) for sample-collecting using Pap-smear, which significantly limited the quantity of tumor cells[17, 18]. In the current study, we improved the cells-collection using a vagina tampon (6-hour protocol) and demonstrated it could harvest many more cells for UterCAD test.

In fact, the presence of chromosomal CNV may have a stronger indication for an underlying carcinogenic event, since not all mutations lead to gene dysfunction and cancer events. According with TCGA data, the application of UterCAD for EC early diagnosis is endorsed by the fact that this malignancy is particularly rich in CNVs, especially in these high grade tumors like USC and clear cell carcinoma[22]. With regards to our cohort, UterCAD detected significant CNVs in all these 4 USCs, which was in consistent with previous researches[23]. In addition, women with positive findings indicated by UterCAD might be in risk for the more aggressive USC and warrants immediate actions.

While this study was a pilot study to test DNA copy numbers, further large-sample-size validations should be performed in our future work. Some previous studies also showed a small proportion of endometrial tumors are characterized with immense gene single nucleotide mutations but negligible copy number variations[24]. The Cancer Genome Atlas provided us an overview of endometrial cancer and the over-represented point mutations. A panel of high frequent mutations may be further necessary in improving the overall diagnosis sensitivity, especially with regards to certain subtypes of EC[14].

Collectively, we firstly investigated the efficiency of UterCAD, a genome sequencing method based on tampon-collected DNA, in a group of suspicious EC. Our results proposed a special effect of UterCAD for the early detection of ECs (especially type II tumors with more frequent CNVs). The high sensitivity and specificity warrant UterCAD as a non-invasive procedure before endometrium biopsy.

Abbreviations

UteCAD

uterine exfoliated cell chromosomal aneuploidy detector; EC: endometrial carcinoma; EEC: endometrioid carcinoma; USC: uterine serous carcinoma; CNV: copy number variation.

Declarations

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Disclosure

Authors declare no conflicts of interest for this article.

Ethics approval and consent to participate:

This prospective study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University and written informed consent was obtained from all participants.

Consent for publication:

All authors are in agreement with the content of the manuscript and the submission to BMC Cancer.

Availability of data and material:

Key data were provided in supplementary Tables. All the data were available on request.

Competing interests

No.

Funding

No.

Authors' contributions:

JL, RG and HQ contributed to the conception and design of this study. JL, TC, YF and YZ contributed to the samples collection. Data analysis was carried out by LZ and ZQ.

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Figures

Figure 1

Overview of patients selection.

Figure 2

Representative UterCAD results of endometrioid endometrial carcinoma (EEC), uterine serous carcinoma (USC) and benign cases. CNVs were indicated by red arrows.

Figure 3

ROC curve of UterCAD model by incorporating all chromosome aberrations in endometrial carcinoma (EC), endometrioid endometrial carcinoma (EEC) and uterine serous carcinoma (USC) groups.

Figure 4

Correlations between UterCAD detection results and the postmenopausal period.

Supplementary Files

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- [TableS1UterCADCNV.xlsx](#)