A new strategy in molecular typing: the accuracy of an NGS panel for the molecular classification of endometrial cancers

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Background: Multiplatform molecular subtyping has been put into clinical practice as an alternative for The Cancer Genome Atlas (TCGA)-based classification for endometrial cancer (EC), which proved a tool for predicting prognosis and guiding treatment. The traditional methods for the molecular classification of EC only based on pathological indicators are not accurate. The present study aimed to classify EC on a molecular level and explored the possibility of a one-time solution to guide clinical treatment and prognosis determination by utilizing data from a next-generation sequencing (NGS) panel. The ultimate aim was to utilize multiplatform testing to overcome disadvantages of long detection periods and limitations in the information regarding genetic variation.

Methods: An NGS-panel was produced using FFPE samples isolated from 86 patients pathologically diagnosed with EC, and molecular subtyping was performed according to the recommended criteria. In addition, 45 matched samples from 86 patients were randomly selected for immunohistochemical (IHC) staining of *P53*, *MLH1*, *MSH2*, *PMS2*, and *MSH6*. Another 41 samples were not analyzed due to incomplete IHC staining results. SPSS (V26.0; IBM Corp., Armonk, NY, USA) was used for receiver operating characteristic (ROC) curve analysis.

Results: The molecular typing ratio of the 86 cases of endometrial carcinoma was calculated to be 16.28% for POLE type, 17.44% for MSI-H type, 47.67% for CN-L type, 12.79% for CN-H type, 5.81% for unclassified case. A comparison between IHC ProMisE-based subtyping and NGS-based subtyping of the 45 cases revealed that 3 cases were classified as MSI-H by IHC but as MSS by NGS. Among these cases, 1 case was deficient in *MLH1* expression and PMS2 protein expression but had wild-type P53 protein, and the *P53* sequencing data of this sample showed a missense mutation. Good overall consistency between the 2 determination methods was shown. Receiver operating characteristic (ROC) analysis showed that NGS had particularly high specificity and sensitivity for detecting the MSI and CN subtypes [area under the curve (AUC) =0.893>0.5, P=0.000029<0.01].

Conclusions: The present study suggested that NGS-based subtyping could serve as an effective approach for the molecular typing of EC. Both NGS and IHC bear their own unique advantages and challenges in clinical practice.

Keywords: Endometrial cancer (EC); molecular typing; high-throughput sequencing; The Cancer Genome Atlas (TCGA); molecular typing

Introduction

Next-generation sequencing (NGS), a high-throughput sequencing technology that can identify genomic alterations occurring in any region of a target gene, can detect frequencies as low as one mutated copy in thousands of wild-type copies and elucidate many Types of Mutational Landscapes of Tumors. NGS has become an important aspect of accurate tumor diagnosis and treatment, and has a variety of uses, such as tumor targeted therapy-related driver gene detection, drug resistance mechanism analysis, tumor metastasis and prognosis assessment, molecular classification diagnosis (1).

Endometrial cancer (EC) is the seventh most prevalent cancer among women and is second only to cervical cancer in terms of reproductive system malignancies (2-5). EC can be classified into 2 different types, namely type I and type II. Type I tumors include estrogen-related endometrioid endometrial adenocarcinomas (EECs), which are typically low grade. By contrast, type II tumors include non-endometrioid endometrial carcinomas (NEECs), which mainly consist of serous and clear-cell EC (6). In 2013, The Cancer Genome Atlas (TCGA) performed an integrated genomic, transcriptomic, and proteomic characterization of 373 endometrial carcinomas using array- and sequencing-based technologies, before conducting a typing study on the EC genome (7). The results revealed 4 distinct molecular subgroups. Group 1 (~7% of patients) consisted of patients with EEC exhibiting polymerase ε (*POLE*) somatic mutations at an abnormally high mutation rate (hypermutation). This group of patients typically exhibited a superior prognosis. Group 2 (~28% of patients) consisted of patients with microsatellite instability (MSI) EEC exhibiting high MutL homolog 1 (*MLH1*) promoter methylation levels with a high mutation rate. Group 3 (~39% of all patients) consisted of patients with low copy number (CN-L) EEC. Group 4 (~26% of all patients) consisted of patients with high copy number (CN-H) EC, including most cases of serous carcinoma. These patients harbored *TP53* gene mutations with low mutation rates and typically exhibited a poor prognosis. The 4 subgroups of EC benefit from different treatments strategies and have distinct clinical outcome profiles, in addition to manifesting different clinicopathological characteristics (8).

Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) typing is a classification method that is suitable for clinical application but inferior to TCGA multiomics molecular typing (9). However, simple methods, such as immunohistochemistry (IHC) and *POLE* next-generation sequencing (NGS), are able to produce comparable profiles to TCGA typing. This is especially the case for *POLE*-mutated, mismatch repair-deficient (MMR-d), *P53*-abnormal (*P53*-abn), and *P53*-wild-type EC. In 2020, León-Castillo *et al.* proposed the TransPORTEC classification system for patients with high-risk EC (10). This system produced 4 categories, namely *P53*abn, *POLE*-ultramutated, MMR-d, and no specific molecular profile, which proved to be a useful tool for predicting prognosis and guiding treatment. In addition, the 2020 edition of the National Comprehensive Cancer Network (NCCN) guidelines recommended the routine molecular subtyping of EC to predict treatment outcome and prognosis of early EC while also guiding the design of adjuvant treatment strategies (11).

However, it is not accurate to classify EC into subgroups based on pathological indicators only (12). Although the majority of EC cases are in the *P53*-abn group, low-grade endometrioid carcinomas with no specific molecular profile also exist. In addition, high-grade endometrioid carcinomas can fall into any of the 4 molecular subtypes. Therefore, it is important to distinguish tumor aggressiveness while subtyping to more accurately classify EC for clinical treatment development (13). It should also be noted that stratification of high-grade EC cancers by pathological morphology alone is challenging, which limits its value in clinical applications (14).

An EC molecular typing technology platform has previously been developed that incorporates data derived from IHC and the Sanger/NGS methods. However, its data interpretation, test cycle, subjective judgment parameters, and lack of specimens restrict the accuracy of this platform in clinical application (15-17). Therefore, in the present study, NGS was performed to detect and analyze the profiles of associated genes prior to typing. Subsequently, the effectiveness and feasibility of the clinical application of this NGS-based method was assessed by comparing it with

Table 1 Patient characteristics

Patient characteristics	N (%)/mean ± SD
Age (years)	
<60	61 (70.93)/51.61±6.59
≥60	25 (29.07)/65.68±5.14
FIGO classification	
I	34 (39.53)
II-III	31 (36.05)
NA	21 (24.42)
The full depth of the infiltrate uterine wall	
<1/2	43 (50.00)
≥1/2	29 (33.72)
NA	14 (16.28)
Pathological type	
Endometrioid carcinoma	67 (77.91)
Mucinous carcinoma	4 (4.60)
Serous carcinoma	5 (5.80)
Clear cell carcinoma	4 (4.65)
NA	6 (6.98)
Remote metastasis	7 (8.16)

FIGO, International Federation of Gynecology and Obstetrics; NA, uncertain.

the existing consensus method recommended by the current guidelines. We present the following article in accordance with the TRIPOD reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3446/rc).

Methods

Clinical research design

EC from 86 patients, were molecularly subclassified using two different methods; by performing the next generation sequencing (NGS) panel and using the Proactive Molecular Risk Classifier for Endometrial Cancer (NCCN guidelines) classifier and performing immunohistochemical staining for MMR proteins and P53. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Henan Cancer Hospital affiliated with Zhengzhou University (No. 2017407), and informed written consent was obtained from all participants.

Patient and tissue selection

In total, ≥2 paraffin samples were obtained from each tissue sample surgically removed form 93 patients with EC between January 2019 and December 2020 at the Affiliated Cancer Hospital of Zhengzhou University (Zhengzhou, China). Among the 93 confirmed cases, 7 cases were excluded because the number of tumor cells in the tissues was observed to be <20%. In addition, patients who had undergone preoperative chemotherapy or other treatments were excluded. Finally, 86 specimens were obtained for subsequent experiments. The pathomorphological diagnosis of the patients was evaluated by 2 professional pathologists in a double-blinded manner. The detailed clinicopathological data are shown in Table 1.

NGS-panel detection

Pathologists first evaluated the tumor cell content before sample processing for NGS. Excess noncancerous normal tissues were excised from samples with <20% tumor cell content. A total of 86 of the 93 samples were treated to procure the proportion of tumor cells required for sequencing. Samples from all patients were sequenced using a panel containing 36 genes associated with EC. First, an EC molecular typing detection kit (Novogene Co., Ltd., Tianjin, China) was used for hybridization capture. This kit covers the detection of associated genes recommended by the NCCN guidelines for EC. In addition, clinical studies that tested a large cohort of clinical samples have found that the kit only requires a total of <1 G data to accurately detect variations at a low abundance of 1% and analyze the MSI status of the samples.

After the data were unloaded, Fastp software (V0.19.4) (18) was used for quality control and preprocessing, and the Burrows-Wheeler Aligner software (V0.7.8) (19) was used to align clean reads with the hg19 reference genome. VarScan (V2.4.3) (20) and Genome Analysis Toolkit (GATK; V4.1) (19) analyses were performed to detect somatic and germline variants, respectively, in the tumor and normal samples. Single nucleotide/indel variants were annotated and filtered using snpEff (21) + ANNOVAR (V4.3) (19,21) and Ensembl Variant Effect Predictor (ensembl-vep 90.6) + ANNOVAR. The distribution difference between 94 microsatellite fragments in case-control was analyzed using the MANTIS software (V1.0.5) (22), which contributed to the MSI class calculations. The interpretation of variants was performed by referring to the Catalogue Of Somatic

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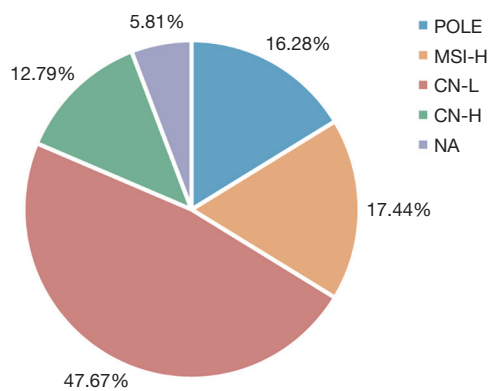
Figure 1 Distribution of molecular typing in the 86 patients with endometrial cancer. POLE, polymerase ε; MSI, microsatellite-instability; CN, copy number; H, high; L, low; NA, unclassified.

Mutations In Cancer (COSMIC) database (https://cancer. sanger.ac.uk/cosmic) and the self-built database. Any disease-causing or potential disease-causing mutations found were judged as having biological significance. Patients were then divided into 4 molecular subtypes according to the NCCN (11) and Chinese gynecological evaluation guidelines. In all cases, the *POLE* exonuclease mutation status was determined according to the León-Castillo and other criteria (10). The remaining cases were annotated for their MSI status based on the NGS results and were classified as either MSI-high (MSI-H) or microsatellite stability (MSS). Those with harmless *TP53* mutations were sorted into the CN-L group, whereas those with pathogenic *TP53* mutations were considered to be of the abnormal *P53*-abn type and were sorted into the CN-H group.

IHC analysis

Each formalin-fixed paraffin-embedded (FFPE) sample from the enrolled cases was cut into 3.5 µm slices for IHC staining. Roche reagent standard operating procedures were used. The primary antibodies used were as follows: CONFIRM anti-P53 (clone DO-7, product code 790-2912, Roche Diagnostics, Figure S1), VENTANA anti-*MLH1* (clone M1, product code 790-5091, Roche Diagnostics), mutS homolog 2 (*MSH2*; clone G219-1129, product code 790-5093, Roche Diagnostics), postmeiotic segregation increased 2 (*PMS2*, clone A16-4, product code 790-5094, Roche Diagnostics) and VENTANA anti-*MSH6* (clone SP93, product code 790-5092, 1:300, Roche Diagnostics; Figure S2). Two pathologists independently evaluated the

immunostaining results after dyeing. If strong positive staining was observed in >75% of tumor cells (nucleus or cytoplasm), then the P53 IHC was considered mutant. Otherwise, the section was considered wild type.

The expression of MLH1, MSH2, MSH6, and PMS2 proteins was detected according to the Consensus on The Detection of Microsatellite Instability in Colorectal Cancer and Other Related Solid Tumors in China published in 2019 (23). If there was no staining in the tumor cell nucleus, the sample was considered negative; if the proportion of stained cells was <5%, the sample was considered focally positive. Negative expression of any of the aforementioned proteins was considered to be loss of expression. For P53 and MMR proteins, abnormal subclonal expression was considered to occur if abnormal staining patterns were detected in the adjacent areas of the tumor (24). Nonmalignant regions and stromal tissues in the tumor specimens were used as the internal reference for the negative control of all proteins. The evaluation was performed double-blinded. After IHC staining, patients with loss of MMR protein expression were classified as MMR-d. If *P53* IHC staining was negative, the sample was considered to be wild type and of the CN-L type. If P53 IHC showed positive abnormal staining, the sample was considered to be of the CN-H type.

Statistical analysis

Classification variables were described as counts and percentages where κ-values were calculated (κ>0.75). Continuous variables were described as median and range. SPSS software (V26.0; IBM Corp., Armonk, NY, USA) (25) was used for receiver operating characteristic (ROC) curve and area under the curve (AUC >0.5) analysis.

Results

Molecular typing performed through NGS

The molecular typing of 86 EC tissue samples was performed using NGS. The proportions of *POLE* mutant, MSI-H, CN-L, CN-H, and unclassified samples were 16.28%, 17.44%, 47.67%, 12.79%, and 5.81%, respectively (*Figure 1*). The sequencing data of the 2 unclassified cases mainly revealed mutations in histone-lysine N-methyltransferase 2A and cyclin D1, which are associated with the stability of the DNA helical structure and cell cycle regulation, respectively. However, no mutations in *POLE*, *TP53*, or *PTEN* genes
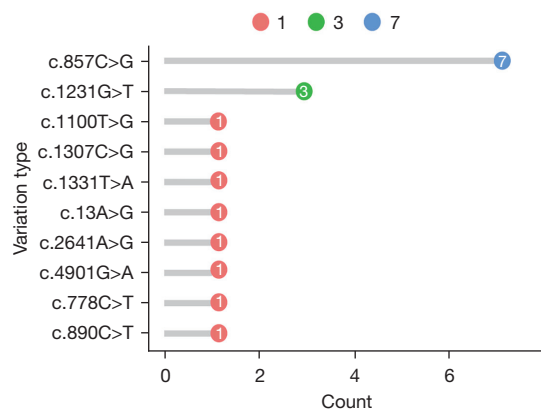
Figure 2 Distribution of POLE gene variant subtypes in the 86 patients. c.857C>G, c.1231G>T, c.1100T>G, c.1307C>G, c.1331T>A, c.13A>G, c.2641A>G, c.4901G>A, c.778C>T, c.890C>T correspond to different variation sites in the POLE gene. POLE, polymerase Ɛ.

were found. The main variation site in the *POLE* gene was NM_006231.2 exon 9 c.857C>G p.P286R. The majority of variants found were consistent with those reported in Europe and America (26-28) (*Figure 2*).

Distribution of the 4 subgroups in the different pathological subtypes

We analyzed the distributions of the 4 subgroups in the different pathological subtypes (*Figure 3*). The results showed that the CN-L group accounted for 54.41% of all endometrioid carcinomas, whereas the CN-H group comprised only 8.82%. By contrast, the CN-H group constituted the largest proportion of those with serous endometrial carcinoma, while the other 3 types were not found in this pathological type. The proportion of MSI-H was particularly high in clear cell carcinoma and mucinous endometrial carcinoma, but the *POLE* mutant group was not found in these pathological types. These result suggested that different pathological types may mediate different mechanisms in the developmental process of EC.

Classification results of 45 patients with EC by IHC and NGS-based technologies

In total, 45 FFPE samples were randomly selected from the 86-patient NGS typing cohort for IHC staining of MMR proteins and P53. Molecular typing was performed according to the recommended typing standard of Chinese

gynecology, before being compared with the typing results classified by NGS (*Table 2*).

The comparison results showed that 3 cases were sorted into the MSI-H group by IHC staining, while the NGS typing classified these cases as MSS and sorted them into the CN-L group. The specific IHC results are shown in *Table 3*. Among these 3 cases, 1 case had deficient expression of MLH1 and PMS2 proteins, although the expression of the P53 protein was normal. However, *P53* gene sequencing in this sample found a missense mutation. The IHC results of P53 in the other 2 samples were consistent with the NGS results.

Consistency check of the 2 methods

Due to the inconsistent results in the study cohort of 45 matched samples, a consistency test was conducted for the 2 diagnostic methods. The statistical results showed that κ=0.902>0.75, P<0.01, which suggested that the 2 diagnostic methods exhibited good overall consistency (*Table 4*). To understand whether the difference between the MSI-H and the CN-L group could affect the sensitivity and specificity of NGS typing, ROC curve analysis was performed. The results showed that the NGS method had statistical efficacy and good diagnostic performance in the classification of MSI-H in patients with EC [area under the curve (AUC) =0.893, P<0.01] (Figure S3).

Discussion

In 2013, a large-scale, comprehensive, integrated TCGA genome analysis of 373 cases of EC was performed according to the tumor's clinicopathological and molecular characteristics. This study divided EC into the following subtypes: *POLE* mutant (7%), MSI-H (28%), CN-L (39%), and CN-H (26%) (7). Previous studies found that the molecular typing of EC plays a potentially important role in guiding the clinical treatment of EC (13,29). Adverse effects due to excessive adjuvant chemotherapy can be avoided in patients with EC harboring *POLE* mutants, while patients with MMR-d EC can benefit from radiotherapy but not chemotherapy. By contrast, patients with *P53*-abn EC can typically benefit from chemotherapy (10,30). At present, the consensus recommendation for the molecular typing of EC is an integrated multiplatform approach using IHC and molecular detection methods to detect and classify the 4 subtypes of EC (17).

The purpose of the present study was to compare the

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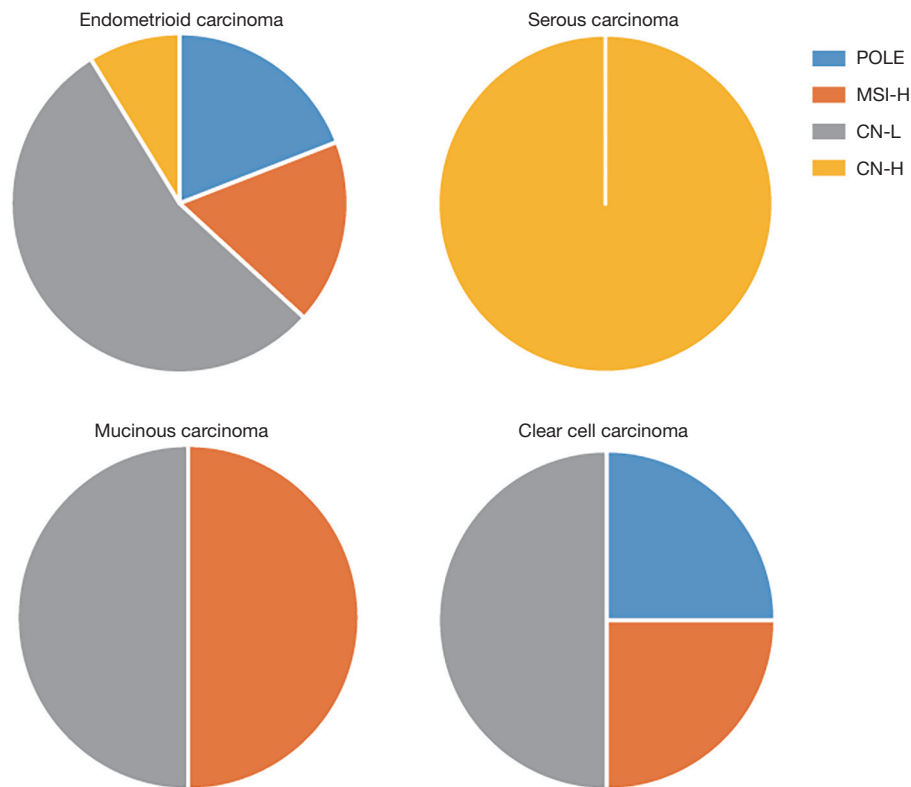
Figure 3 Distribution of the NGS-based molecular subtypes among the four pathological types. NGS, next-generation sequencing; POLE, polymerase ε; MSI, microsatellite-instability; CN, copy number; H, high; L, low.

Table 2 Classification results of 45 EC patients by IHC and sequencing-based technologies

Groups	POLE	MSI-H/MMRd	CN-L	CN-H	Total
IHC group	16	14	14	1	45
Sequencing group	16	11	17	1	45

EC, endometrial cancer; IHC, immunohistochemistry; POLE, polymerase ε; MSI-H/MMRd, microsatellite instability-high; CN-L, the low copy number; CN-H, the high copy number.

Table 3 Results of three cases with inconsistent IHC and sequencing methods

Case name	IHC group					Sequencing group	
	MLH1	MSH2	MSH6	PMS2	TP53	MSI	TP53
Case 1	−	+	+	−	wt	MSS	+
Case 2	+	+	−	+	wt	MSS	−
Case 3	−	+	+	−	wt	MSS	−

IHC, immunohistochemistry; MLH1, MutL homolog 1; MSH2, mutS homolog 2; MSH6, mutS homolog 6; PMS2, postmeiotic segregation increased 2; TP53, tumor protein p53; MSI, microsatellite instability; MSS, microsatellite stability; wt, wild type.

Table 4 Consistency check of the two methods

Sequencing group	IHC group				Total
	POLE	MSI-H	CN-L	CN-H	
POLE	16	0	0	0	16
MSI-H	0	11	3	0	14
CN-L	0	0	14	0	14
CN-H	0	0	0	1	1
Total	16	11	17	1	45

IHC, immunohistochemistry; POLE, polymerase ε; MSI-H, microsatellite instability-high; CN-L, the low copy number; CN-H, the high copy number.

consistency and potential real-world clinical application value of the 2 genotyping methods: multiplatform combined assay and NGS panel. The results revealed that the 2 methods were highly consistent but also had differences. The rate of overall agreement was 91%, with an agreement rate of 100% in the *POLE* group, 78.57% in the MSI-H group, 78.57% in the CN-L group, and 100% in the CN-H group. The classification results of MSI-H tumors using NGS showed the most difference. IHC staining showed a lack of *MLH1* and *PMS2* expression, while NGS revealed MSS status. Several previous large studies have reported individual inconsistencies between gene-based MSI results and protein-based MMR results (31-34). It is possible that the loss of expression of MMR proteins is compensated by other genes. There have been reports showing that the MSS phenomenon in tissues without MLH1 and PMS2 protein expression is associated with hypermethylation of the *MLH1* promoter (35). Another possible explanation is that there are excessive numbers of nuclei (particularly in the normal endometrium and a large number of tumor-infiltrating lymphocytes) that allow the MSI or protein expression of MMR to be used to indicate if patients with EC can benefit from immune checkpoint inhibitor therapy or radiotherapy (36). In addition, the result that MSI or protein expression of MMR is also a referring indicator for the diagnosis of Lynch syndrome.

The results of the present study suggested that patients classified into the MSI-H group by NGS all showed a loss of expression of 1 or 2 mismatch repair proteins (MLH1, MSH2, MSH6, and PMS2). There were high levels of consistency in detecting MSI/MMR-d status between NGS and IHC. Therefore, if MSI status remains unclear, it can instead be verified using IHC. However, for the diagnosis of hereditary tumors, such as Lynch syndrome, the use of these

2 methods alone is currently not sufficient. The results of the 2 methods for detecting MSI were inconsistent mainly when detecting somatic mutations, epigenetic events, or germline mutations. Since IHC detects expression at the protein level, it cannot be reliably used to detect genome mutations. By contrast, the NGS method can be used to identify MSI status and detect MMR-related gene mutations. Therefore, cases of MSI-H status without MMR mutations must be validated using an additional method, such as NGS. However, NGS cannot be used to detect gene methylation.

IHC methods are mainly used to determine the expression of MMR proteins. The molecular subtype diagnostic method using IHC has the advantage of being easy to obtain and cost-effective with fast turnaround times, even on small biopsy specimens. Although P53 and MMR protein expression can be clearly distinguished using IHC, tumor heterogeneity can result in protein expression being detected in other clonal subtypes, and weak IHC staining and background interference can make accurate diagnose difficult in some cases. In addition, improper postoperative specimens from total hysterectomy may also impact IHC results. At present, there are no alternative markers and methods for detecting point mutations based on IHC. This is one of the main limitations of IHC for the clinical detection of *POLE* or other genetic mutations. Therefore, gene sequencing is an area that requires further development.

POLE is a DNA polymerase ε catalytic subunit that is involved in nuclear DNA replication and repair and plays an important role in nuclear DNA identification and repairing after base mismatches. When *POLE* gene mutations occur, mismatched bases cannot be recognized and repaired, resulting in an abnormal increase in the number of

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mutations in the genome and leading to the occurrence of tumors (26,37). A mechanism of hypermutation caused by *POLE* defect was originally proposed, as the reported *POLE* mutation preferentially affects the conserved amino acid residues in the Pol ε exonuclease domain, leading to replication errors. The *POLE* subgroup exhibits an abnormally high tumor mutational burden, and the genes that are commonly found to be mutated include phosphatase and tensin homolog (*PTEN*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (*PIK3CA*), phosphoinositide-3-kinase regulatory subunit 1 (*PIK3R1*), F-Box and WD repeat domain-containing 7 (*FBXW7*), AT-rich interaction domain 1A (*ARID1A*), KRAS proto-oncogene, GTPase (*KRAS*), and AT-rich interaction domain 5B (*ARID5B*). The present study showed that there are *P53* and MMR gene mutations in the *POLE* subgroup apart from *PTEN*. A number of cases in the present study lacked functional validation despite missense mutations in the *POLE* gene. One was classified as MSI-H, while 2 were classified as CN-H and 6 were classified as CN-L. Although NGS can provide important analytical evidence for subtyping, it can also produce irrelevant or even misleading data, such as nonpathogenic *POLE* mutations and *TP53* passenger mutations that are not important for function. In the present study, 20 nonpathogenic mutations were found. Unlike pathogenic mutations, these mutations are independent of tumorigenesis. MMR gene mutations were also found in the *POLE* group, which may be passenger mutations of these hypermutated tumors rather than pathogenic mutations per se. Therefore, it is particularly important to carefully interpret the different variant data of NGS in combination with the clinicopathological characteristics to make a more comprehensive judgment. Previous accounts have proposed that the detection of *POLE* gene mutation is not in sequence with the detection of MSI and must be performed before CN-type detection. However, others have suggested that *POLE* gene mutation detection should be performed first, because MSI and *TP53* mutations may occur secondary to *POLE* mutations. There is no consideration of the detection order when using the NGS method, and at the same time detecting a large amount of information can provide effective supplementary information for data interpreters. In addition, Stelloo *et al.* (38) previously analyzed the feasibility of molecular typing detection methods on intratumoral heterogeneity. They discovered an inaccuracy rate of 10.2% in molecular classification due to intratumoral heterogeneity, the risk of which can be reduced by selecting representative wax

blocks. As *PTEN* is frequently accompanied by *POLE* changes, it has the potential to serve as an auxiliary diagnostic marker in detecting *POLE* gene mutations and *PTEN* mutation status (*PTEN* IHC results show deletion). Although *PTEN* mutations are partially present in MSI and CN-L, it is more common for *PTEN* and *POLE* mutations to occur synchronously in the POLE group.

CN-L tumors are predominantly low-grade endometrioid adenocarcinomas with a low mutational burden, and their prognosis is slightly superior to that of MSI-H tumors. CN-L tumors rarely harbor *TP53* gene mutations, but genes in the Wnt pathway (including *β-catenin*, *KRAS*, and *SRY-box transcription factor 17*), in addition to those encoding *PTEN*, *PIK3CA*, and *ARID1A*, have all been reported to be frequently mutated. A previous study found that (39) patients in the CN-L group with *P53*-wt had neither *POLE* gene mutations nor mismatch repair gene functional defects and thus might be the biggest beneficiary of endocrine therapy. The results of the present study are consistent with those previously reported.

CN-H tumors include almost all serous cancers, mixed cancers, and high-grade endometrioid cancers. These tumors have the worst prognosis among the 4 molecular classifications. CN-H tumors mostly harbor *TP53* gene mutations, while some high-grade endometrioid carcinomas have somatic copy number alterations with gene mutation profiles that are similar to serous carcinomas (40). This suggests that the CN-H group may benefit from treatments similar to those of serous cancer. *TP53* gene mutations can cause 2 functional results, namely increase or loss of function. Nonsynonymous mutations can lead to increased function, because ubiquitination by mouse double minute 2 homolog fails to degrade the P53 protein. This leads to the accumulation of *P53* in the nucleus, which promotes potent and diffuse P53 protein overexpression in the nucleus. This is easily recognized by IHC staining. Loss of function can occur following nonsense mutations, insertion, and splicing mutations, which can in turn interfere with correct protein translation. In fact, these mutations are associated with the complete loss of P53 protein expression (the 'zero mode') or rare intracytoplasmic aggregation. In addition, *PIK3CA*, *protein phosphatase 2*, and *FBXW7* gene mutations, coupled with human epidermal receptor 2 gene amplifications, have been frequently reported in patients with copy number variations. By contrast, *PTEN*, *ARID1A*, and *KRAS* gene mutations are rare, and their molecular changes mainly correspond to type II EC. For patients harboring the *P53* mutant, standard staging surgery and/or adjuvant

treatment is recommended, since conservative treatment is not suitable (41). In the present study, when comparing the 2 typing methods, we found a 100% agreement rate in the *P53* group. Two cases analyzed using the NGS method were different from the recommended method. It is likely that there was a deviation in the judgment of *P53* functional inactivation by NGS. This suggests that clinical testing should be repeatedly performed and that ambiguous cases should be considered for IHC verification.

At present, exploration of the molecular classification of EC is mainly focused on the associated signaling pathways and genes (42-44). The majority of ECs can be classified by NGS based on the varying statuses of *POLE*, *TP53*, *PTEN*, and *MSI* in addition to those associated with genotyping. In terms of panel selection, although the conventional large panel contains genes associated with the molecular type of EC, it produces a large number of irrelevant detection results, resulting in data redundancy and the loss of resources. Therefore, the smaller panel used in the present study for the molecular classification of EC appears to have greater clinical applicability and is more suitable for clinical promotion. MSI detection using NGS relies on the proportion of unstable sites in the selected MSI sites, where the MSI status of the patients is determined by comparing with the cutoff value. The selection of MSI sites and the delineation of cutoff values are core for ensuring accurate detection. The MSI sites in the NGS panel used in the present study have undergone strict screening and verification, in a manner that is consistent with the PCR method (45). Single NGS tests can maximize the use of samples, avoid excessive sample consumption caused by multiple tests, and avoid the problem of long routine and sequential test cycles. In addition, this method can assist clinical decision-making, improve patient yield, greatly shorten the test cycle, and save medical costs.

The present study had some limitations. The number of patients was relatively small, especially in the *POLE* and MSI-H groups. Therefore, the results may not accurately reflect the distribution of global EC molecular typing. Due to the lack of clinical follow-up results, clinical verification of this EC molecular typing is required. In the future, determination criteria of NGS detection in the *POLE* and MSI groups need to be established. Previous studies have shown that tumors with *POLE* mutations generally have superior clinical outcomes despite their high-risk histopathological features. Therefore, in this case, treatment reduction can benefit patients clinically and avoid overtreatment.

Conclusions

In general, NGS and IHC provide equal information for molecular subtype diagnosis. Both methods have unique advantages and challenges in application and clinical practice.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the TRIPOD reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3446/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3446/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3446/coif). LM and SS are from Novogene Co., Ltd. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of Henan Cancer Hospital affiliated with Zhengzhou University (No. 2017407), and informed written consent was obtained from all participants.

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References

1. Sabour L, Sabour M, Ghorbian S. Clinical applications of next-generation sequencing in cancer diagnosis. Pathol Oncol Res 2017;23:225-34.

2. Bray F, Dos Santos Silva I, Moller H, et al. Endometrial cancer incidence trends in Europe: underlying determinants and prospects for prevention. Cancer Epidemiol Biomarkers Prev 2005;14:1132-42.

3. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68:394-424.

4. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 2021;71:209-49.

5. Kasius JC, Pijnenborg JMA, Lindemann K, et al. Risk Stratification of Endometrial Cancer Patients: FIGO Stage, Biomarkers and Molecular Classification. Cancers (Basel) 2021;13:5848.

6. Urick ME, Bell DW. Clinical actionability of molecular targets in endometrial cancer. Nat Rev Cancer 2019;19:510-21.

7. Cancer Genome Atlas Research Network; Kandoth C, Schultz N, et al. Integrated genomic characterization of endometrial carcinoma. Nature 2013;497:67-73.

8. McAlpine J, Leon-Castillo A, Bosse T. The rise of a novel classification system for endometrial carcinoma; integration of molecular subclasses. J Pathol 2018;244:538-49.

9. Kommoss S, McConechy MK, Kommoss F, et al. Final validation of the ProMisE molecular classifier for endometrial carcinoma in a large population-based case series. Ann Oncol 2018;29:1180-8.

10. León-Castillo A, de Boer SM, Powell ME, et al. Molecular Classification of the PORTEC-3 Trial for High-Risk Endometrial Cancer: Impact on Prognosis and Benefit From Adjuvant Therapy. J Clin Oncol 2020;38:3388-97.

11. NCCN Guidelines Version 4.2021 Uterine Neoplasms

12. Hussein YR, Broaddus R, Weigelt B, et al. The Genomic Heterogeneity of FIGO Grade 3 Endometrioid Carcinoma Impacts Diagnostic Accuracy and Reproducibility. Int J Gynecol Pathol 2016;35:16-24.

13. Da Cruz Paula A, DeLair DF, Ferrando L, et al. Genetic and molecular subtype heterogeneity in newly diagnosed early- and advanced-stage endometrial cancer. Gynecol Oncol 2021;161:535-44.

14. Hoang LN, Kinloch MA, Leo JM, et al. Interobserver Agreement in Endometrial Carcinoma Histotype Diagnosis Varies Depending on The Cancer Genome Atlas (TCGA)-based Molecular Subgroup. Am J Surg Pathol 2017;41:245-52.

15. Pettersson E, Lundeberg J, Ahmadian A. Generations of sequencing technologies. Genomics 2009;93:105-11.

16. Chmielecki J, Meyerson M. DNA sequencing of cancer: what have we learned? Annu Rev Med 2014;65:63-79.

17. Kim G, Lee SK, Suh DH, et al. Clinical evaluation of a droplet digital PCR assay for detecting POLE mutations and molecular classification of endometrial cancer. J Gynecol Oncol 2022;33:e15.

18. Chen S, Zhou Y, Chen Y, et al. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 2018;34:i884-90.

19. Li H. and Durbin R.Fast and accurate short read alignment with Burrows-Wheeler Transform. Bioinformatics, 2009;25:1754-60.

20. Koboldt DC, Zhang Q, Larson DE, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res 2012;22:568-76.

21. Yen JL, Garcia S, Montana A, et al. A variant by any name: quantifying annotation discordance across tools and clinical databases. Genome Med 2017;9:7.

22. Kautto EA, Bonneville R, Miya J, et al. Performance evaluation for rapid detection of pan-cancer microsatellite instability with MANTIS. Oncotarget 2017;8:7452-63.

23. Committee of Colorectal Cancer, Chinese Society of Clinical Oncology; Genetics Group of The Committee of Colorectal Cancer, China Anti-Cancer Association; Genetics Committee of The Committee of Colorectal Cancer, Chinese Medical Doctor Association. Consensus on the detection of microsatellite instability in colorectal cancer and other related solid tumors in China. Zhonghua Zhong Liu Za Zhi 2019;41:734-41.

24. Singh N, Piskorz AM, Bosse T, et al. p53 immunohistochemistry is an accurate surrogate for TP53 mutational analysis in endometrial carcinoma biopsies. J Pathol 2020;250:336-45.

25. Liu Y, Wu A, Li X, et al. A retrospective analysis of eleven gene mutations, PD-L1 expression and clinicopathological characteristics in non-small cell lung cancer patients. Asian J Surg 2022;45:367-75.

26. McConechy MK, Talhouk A, Leung S, et al. Endometrial Carcinomas with POLE Exonuclease Domain Mutations Have a Favorable Prognosis. Clin Cancer Res 2016;22:2865-73.

27. Conlon N, Da Cruz Paula A, Ashley CW, et al. Endometrial Carcinomas with a "Serous" Component in Young Women Are Enriched for DNA Mismatch Repair Deficiency, Lynch Syndrome, and POLE Exonuclease Domain Mutations. Am J Surg Pathol 2020;44:641-8.

28. Stasenko M, Tunnage I, Ashley CW, et al. Clinical outcomes of patients with POLE mutated endometrioid endometrial cancer. Gynecol Oncol 2020;156:194-202.

29. Jamieson A, Bosse T, McAlpine JN. The emerging role of molecular pathology in directing the systemic treatment of endometrial cancer. Ther Adv Med Oncol 2021;13:17588359211035959.

30. Henry CE, Phan K, Orsman EJ, et al. Molecular Profiling of Endometrial Cancer: An Exploratory Study in Aotearoa, New Zealand. Cancers (Basel) 2021;13:5641.

31. Loughrey MB, McGrath J, Coleman HG, et al. Identifying mismatch repair-deficient colon cancer: near-perfect concordance between immunohistochemistry and microsatellite instability testing in a large, population-based series. Histopathology 2021;78:401-13.

32. Moreira L, Balaguer F, Lindor N, et al. Identification of Lynch syndrome among patients with colorectal cancer. JAMA 2012;308:1555-65.

33. Yan WY, Hu J, Xie L, et al. Prediction of biological behavior and prognosis of colorectal cancer patients by tumor MSI/MMR in the Chinese population. Onco Targets Ther 2016;9:7415-24.

34. Hampel H, Frankel WL, Martin E, et al. Feasibility of screening for Lynch syndrome among patients with colorectal cancer. J Clin Oncol 2008;26:5783-8.

35. Trabucco SE, Gowen K, Maund SL, et al. A Novel Next-Generation Sequencing Approach to Detecting Microsatellite Instability and Pan-Tumor Characterization of 1000 Microsatellite Instability-High Cases in 67,000 Patient Samples. J Mol Diagn 2019;21:1053-66.

36. McEachron J, Zhou N, Spencer C, et al. Adjuvant chemoradiation associated with improved outcomes in patients with microsatellite instability-high advanced endometrial carcinoma. Int J Gynecol Cancer 2021;31:203-8.

37. Yu S, Sun Z, Zong L, et al. Clinicopathological and molecular characterization of high-grade endometrial carcinoma with POLE mutation: a single center study. J Gynecol Oncol 2022;33:e38.

38. Stelloo E, Nout RA, Osse EM, et al. Improved Risk Assessment by Integrating Molecular and Clinicopathological Factors in Early-stage Endometrial Cancer-Combined Analysis of the PORTEC Cohorts. Clin Cancer Res 2016;22:4215-24.

39. Talhouk A, McConechy MK, Leung S, et al. A clinically applicable molecular-based classification for endometrial cancers. Br J Cancer 2015;113:299-310.

40. Piulats JM, Guerra E, Gil-Martín M, et al. Molecular approaches for classifying endometrial carcinoma. Gynecol Oncol 2017;145:200-7.

41. Vogelstein B, Papadopoulos N, Velculescu VE, et al. Cancer genome landscapes. Science 2013;339:1546-58.

42. Stelloo E, Bosse T, Nout RA, et al. Refining prognosis and identifying targetable pathways for high-risk endometrial cancer; a TransPORTEC initiative. Mod Pathol 2015;28:836-44.

43. Winterhoff B, Thomaier L, Mullany S, et al. Molecular characterization of endometrial cancer and therapeutic implications. Curr Opin Obstet Gynecol 2020;32:76-83.

44. Dou Y, Kawaler EA, Cui Zhou D, et al. Proteogenomic Characterization of Endometrial Carcinoma. Cell 2020;180:729-748.e26.

45. Zhao L, Shan G, Li L, et al. A robust method for the rapid detection of microsatellite instability in colorectal cancer. Oncol Lett 2020;20:1982-8.