Exploring the Diagnostic Potential of IHC and Next-Generation Sequencing Approaches for Endometrial Carcinoma Detection and Classification

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Key words: Endometrioid carcinoma, Microsatellite instability, PTEN and E-cadherin

1. Abstract

Background: Globally, in 2018 endometrial cancer (EC) regarded as the sixth most commonly diagnosed cancer and the fourteenth leading cause of cancer with more deaths in females. Nowadays classical and modern classifications can be performing for diagnosing various types of cancers including endometrial cancer. Based on this our Objective of study is to determine the effectiveness of immunohistochemical observation, a quick and easy method for determining MSI and/or other types of EC which based on modern classification. As molecular study takes more cost we want to compare the both methods and showing the possibility of doing IHC instead of molecular study.

Methods: This study designed to establish a small gene panel of Next Generation Sequencing NGS of endometrial cancer patients targeting 12 genes; MSH6, MLH3, MSH3, CDH1, PTEN, MSH2, MLH1, PMS2, FGFR2, BRCA1, MUTYH and AREL1. By using DNBSEQ-G400 Platform, Human Core Exome kit and Python software for analysis is used. At the same time, Dako kit used to performing IHC for six primary antibodies used to detect each of MSH6, MSH2, MLH, PMS2, PTEN and CDH1. The primary antibodies were applied on 5 µm formalin-fixed paraffin-embedded (FFPE).

Results: histopathological examinations showed that all patients were at stage II endometrioid endometrial carcinoma. The FIGO classification were Ia and Ib. Microsatellite instability (MSI), nuclear staining immunoreaction for PTEN antibodies and membranous E-cadherin immunoreactions observed through IHC study. The molecular studies detected several polymorphisms which have clinical significant and some of them have conflict interpretations.

Conclusions: we considered that, simply immunoreaction staining procedure can be use as alternative method for MSI phenotype detection rather than any type of more expensive and complex method of NGS. Concerning PTEN and ECAD1 antibodies reactions, also could be dependable in diagnosis and treatment and performing them if molecular diagnosis not available.
2. Introduction

Globally, in 2018 endometrial cancer (EC) regarded as the sixth most commonly diagnosed cancer and the fourteenth leading cause of cancer with more deaths in females [1]. The annual incidence is estimated at 10.8 per 100,000 women in the world. The incidence of this cancer is four times more high in the industrialized countries of Europe and America North compared to Asia (including Japan), Africa and South America [1]. There is no precise data for endometrial cancer in Iraq country, however some evidences showing elevating uterine cancer in general including endometrial cancer in some cities [2].

More than 30 years ago, based on hormonal and clinical characters Bookhman classified EC into two types, type I EC and type II EC [3]. Type I EC are estrogen-dependent, mainly low grade, hormone-receptor-positive adenocarcinomas with endometrioid morphology and are often referred as endometrioid endometrial cancers and account for approximately 85% of all EC usually diagnosed at an early stage and characterized by a good prognostic. Type II EC are characterized by non-endometrioid subtypes such as serous, clear-cell and undifferentiated carcinomas. They generally are high-grade, hormone-receptor negative, and have poor prognosis [3]. According to the International Federation of Gynecology and Obstetrics (FIGO) adopted a surgical pathologic staging since 1988 and in 2009, FIGO updated the staging system classified patients into prognostic groups based on extent of disease [4,5].

New classification is based on molecular features, according to The Cancer Genome Atlas (TCGA), establish a new molecular classification of EC by identifying 4 distinct classes of tumor listed from best to worst prognosis [6]. Efforts have been made to classify ECs into these 4 molecular subgroups using techniques available in routine [7,8,9]. Which include, the POLE (DNA polymerase ε) ultramutated group, the hypermutated/microsatellite unstable (MSI) group, the copy number low/microsatellite stable group and the copy number high (serous-like) group.

2.1 Objectives of study

The objectives of this study were to determine the effectiveness of immunohistochemical observation, a quick and easy method for determining MSI and/or other types of EC which based on modern classification. As molecular study takes more cost we want to compare the both methods and showing the possibility of doing IHC instead of molecular study.

3. Methodology

3.1 Study design

Sample collection: Samples collected at Erbil maternal hospital from those patients who previously diagnosed by having endometrial carcinoma through their curettage biopsy examination, then gynecologists did total abdominal hysterectomy TOH for them. We took a small piece of tumor region (the size of the tissue from each EC patient was ≥0.5 × 0.5 × 0.5 cm3) immediately after removing their uteri, putting in cold PBS for transporting to lab for molecular study. Routinely, the whole removed organs put in buffered Formalin for histopathological examination.

Cancer Gene Panel: Genomic DNA extracted from the fresh tissue by using geneaid kit. Then we established a small cancer gene panel, covering 12 genes for next-generation sequencing (whole exome sequencing=WES); MSH6, MLH3, MSH3, CDH1, PTEN, MSH2, MLH1, PMS2, FGFR2, BRCA1, MUTYH and ARELI. By using DNBSEQ-G400 Platform and Human Core Exome kit and sequencing was performed by DNA Laboratuvarları Genetik Hastalıklar Tanı Merkezi. Python software for analysis is used (Figure 1).

Histopathological Examination: Routine histopathological examination was performed for our samples through putting them in 10% formal saline for one week for fixing the tissue, then underwent serial treatment including dehydration, clearing, infiltration, embedding, sectioning and finally staining with hematoxylin and eosin.

Immunohistochemistry detection: Six primary antibodies used to detect each of MSH6, MSH2, MLH, PMS2, PTEN and CDH1 by using Dako kits to detect some of mutated gene immunohistochemically. According to the manufacturer’s instruction. The primary antibodies were applied on 5 µm formalin-fixed paraffin-embedded (FFPE).

Immunohistochemical interpretations: The six slides from each tumor block staining for MLH1, MSH2, MSH6, PMS2, PTEN and CDH1 were assessed. Semiquantitative scoring system a was used to minimize interobserver variation [10,11,12]. A valid result required the existence of internal control immunopositivity, the lymphocytes, endometrial stromal cells, and the epithelial cells of the nearby normal mucosa are the internal controls. On a scale from 0 to 3, the level of immunoreactivity in the malignant epithelial cells' nuclear compartment was assessed. This rating system was based on a comparison of the tumor cells' level of reactivity to the positive control cells. A score of 0 meant there was no reactivity, while a score of 3 meant there was reactivity in the...
tumor cells that was comparable to that of positive control cells. For the percentage positivity which means distribution of the genes within the tumor cells, the scoring was as follows; no tumor cell immunopositivity equal to 0 score, 1-10% positive tumor cells equals to score 1, 11- 50% positive tumor cells equals to score 2, 51-80% positive tumor cells equals to score 3 and more than 80% is score 4 [10,12]. For making one number for the semiquantitative scores the intensity and percentage of immunopositivity for each antibody were multiplied to produce a number between 0 to 12 [11].

4. Results

4.1 Whole exome sequencing WES results

The observed polymorphism in table 1 analyzed as clinical significance, from the current variations most of them found in dbSNP but not found as a clinical significance for EC in Clin Var database. About 13 of them not found even in dbSNP, such as PTEN (89720633->T) for the first patient (EC1), see remaining 12 variations in table 1, these are named novel variations.

Within these polymorphisms, some of them located at intron regions and others located within exon region. The former included deletions, insertions and deletion/insertion, while the latter has many types of mutations which means functional consequences including synonymous single nucleotide variation (SNV), and non-synonymous SNV. Within non- synonymous SNV most of them were missense variants (MLH3 for EC2, MLH1, PMS2 and BRCA1 for EC3 and MSH2 for EC4) with only one non frameshift insertion (MSH3 for EC2) (Table 1).

Table 2 showing the variation which have conflicting interpretations. conflicting interpretations come define as genetic results from multiplex panel testing utilized in clinical practice are frequently interpreted differently, which may have an impact on how a patient is managed [13,14]. Some variations in MSH2 for EC1, EC2 and EC4 and MSH6 for EC2 were found in Clin Var database as conflicting interpretations. Three of them found in dbSNP database but no data recorded for EC as clinical significant. And one of them (MSH2) was novel as not found in dbSNP database.

4.2 Routine histopathological examination

The architecture of endometrial layer of patients with endometrioid carcinoma characterized by the tubular, cribriform (a malignant epithelial growth that takes the shape of massive nests pierced by numerous, very spherical gaps of various sizes). The tumor cells are columnar, stratified and showed different cytonuclear atypia. Our results also
### Table 1: Nucleotide polymorphism identified in EC patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Gene</th>
<th>Variant coordinate</th>
<th>AA change</th>
<th>Zygosity</th>
<th>Mutation type</th>
<th>External database</th>
<th>clinical significant</th>
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<tbody>
<tr>
<td>EC1</td>
<td>MSH6</td>
<td>NC_000002.11:g.48025764C&gt;T</td>
<td>p.Y214Y</td>
<td>het</td>
<td>synonymous SNV</td>
<td>dbSNP/ not reported in Clin Var for EC</td>
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<tr>
<td>PTEN</td>
<td></td>
<td>89720633-&gt;T</td>
<td></td>
<td>het</td>
<td>intron</td>
<td>dbSNP/ not reported in Clin Var for EC</td>
<td></td>
</tr>
<tr>
<td>MSH2</td>
<td></td>
<td>NM_000251.3</td>
<td>c.942+24_942+29del</td>
<td>het</td>
<td>intron</td>
<td>dbSNP/ not reported in ClinVar for EC</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patients</th>
<th>Gene</th>
<th>Variant coordinate</th>
<th>AA change</th>
<th>Zygosity</th>
<th>Mutation type</th>
<th>External database</th>
<th>clinical significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSH6</td>
<td>NC_000002.11:g.47690162G&gt;T</td>
<td>p.Y214Y</td>
<td>het</td>
<td>intron</td>
<td>dbSNP/ not reported in Clin Var for EC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSH2</td>
<td>NC_000002.11:g.48032572C&gt;T</td>
<td>p.Y214Y</td>
<td>het</td>
<td>intron</td>
<td>dbSNP/ not reported in Clin Var for EC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLH1</td>
<td>NC_000003.11:g.37067097A&gt;T</td>
<td>p.K&gt;K</td>
<td>het</td>
<td>intron</td>
<td>dbSNP/ not reported in Clin Var for EC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLH3</td>
<td>NC_000014.8 g.75513463A&gt;G</td>
<td>p.S&gt;S</td>
<td>het</td>
<td>missense</td>
<td>dbSNP/ not reported in Clin Var for EC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Continued...
revealed by [15]. Cytonuclear atypia which are a precursor for endometrioid carcinoma characterized by loss of polarity, rounded nuclei, anisokaryosis, hyper or hypochromasia and a more eosinophilic cytoplasm [8,16] (Figure 2).

Table 3 showing the FIGO classifications for all patients separately. Each of EC1 and EC2 are in Ib FIGO stage, pT1a, pN0 in TNM staging. pT1a means only affects the endometrium or only penetrates around half of the myometrium. pN0 means Only small a number of cancer cells less than 0.2 mm in diameter (which are isolated tumor cells) or none at all in any adjacent nodes are seen, also showed by [17]. While each of EC3, EC4 and EC6 are of the same FIGO staging which are T1b, Nx, Mx, Ib. T1b signifies that the cancer is between 1-2 cm in size. Both Nx and Mx means nothing has been identified for nearby lymph nodes and unable for measuring metastasis, respectively. Finally,

<table>
<thead>
<tr>
<th>Patients</th>
<th>Gene</th>
<th>Variant coordinate</th>
<th>AA change</th>
<th>Zygosity</th>
<th>Mutation type</th>
<th>CLN SIG</th>
<th>External database\ clinical significant</th>
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</thead>
<tbody>
<tr>
<td>EC1</td>
<td>MS H2</td>
<td>NM_000251.3</td>
<td>c.942+24_942+29del</td>
<td>het</td>
<td>intron</td>
<td>Conflicting</td>
<td>dbSNP/ not reported in Clin Var for EC</td>
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<tr>
<td>EC2</td>
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<td>p.A&gt;A</td>
<td>het</td>
<td>synonymous SNV</td>
<td>Conflicting</td>
<td>dbSNP/ not reported in Clin Var for EC</td>
</tr>
<tr>
<td></td>
<td>MS H2</td>
<td>NC_000002.11.g.47702191A&gt;G</td>
<td>p.N&gt;S</td>
<td>het</td>
<td>nonsynonymous SNV</td>
<td>Conflicting</td>
<td>dbSNP/ not reported in Clin Var for EC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2: 47641560AAAAA-&gt;A</td>
<td>2: 47641560AAAAA-&gt;A</td>
<td>intron</td>
<td>DEL</td>
<td>Conflicting</td>
<td>Novel</td>
</tr>
<tr>
<td>EC4</td>
<td>MS H2</td>
<td>NC_000002.11.g.6037058AA-&gt;T</td>
<td>p.D&gt;N</td>
<td>het</td>
<td>missense</td>
<td>dbSNP/ not reported in Clin Var for EC</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Nucleotide polymorphism with conflicting interpretation clinical significance.
EC5 was at T1a, Nx, Mx clearly means this patient had endometrioid carcinoma which only affects the endometrium or only penetrates around half of the myometrium (T1a), in addition to no information about adjacent lymph nodes and metastasis, Nx and Mx respectively.

**Immunoreactions observation and interpretations:**
The reactions for four MMR antibodies showed in Figure 3. These biomarkers had different level of reaction intensities and had different regional reactions. For all patients the nuclear staining for both MSH6 and MSH2 observed in tumor regions while nuclear staining reactions of both MLH1 and PMS2 occurred at non-tumor regions.

Nuclear staining of PTEN antibodies reactions occurred in normal region and punched out in cancerous glandular region. While membranous E-CAD1 antibodies reactions observed in glandular tumor regions. The two later biomarker reactions showed in Figure 4.

The nuclear staining observed for all MMR proteins. IHC study for these genes PMS2 and MLH1 expressed in non-tumor region while MSH6 and MSH2 expressed in tumor region. PTEN also is have nuclear staining characteristics but observed at non-tumor region. And membranous antibodies reaction seen for ECAD1 under microscopic examinations.

**Table 3: Clinical and pathologic data of patients.**

<table>
<thead>
<tr>
<th></th>
<th>EC1</th>
<th>EC2</th>
<th>EC3</th>
<th>EC4</th>
<th>EC5</th>
<th>EC6</th>
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<tbody>
<tr>
<td>Age</td>
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<td>56</td>
<td>70</td>
<td>68</td>
<td>56</td>
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<tr>
<td>Grade</td>
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<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>FIGO staging</td>
<td>T</td>
<td>pT1a</td>
<td>pT1a</td>
<td>T1b</td>
<td>T1b</td>
<td>T1a</td>
</tr>
<tr>
<td>N</td>
<td>pN0</td>
<td>pN0</td>
<td>N0</td>
<td>Nx</td>
<td>Nx</td>
<td>Nx</td>
</tr>
<tr>
<td>M</td>
<td>Mx</td>
<td>Mx</td>
<td>Mx</td>
<td>Mx</td>
<td>Mx</td>
<td>Mx</td>
</tr>
<tr>
<td>Myometrium invasion</td>
<td>half</td>
<td>half</td>
<td>half</td>
<td>half</td>
<td>half</td>
<td>half</td>
</tr>
</tbody>
</table>

**Figure 2:** Endometrioid endometrial carcinoma, tumor region showing tubular differentiation of gland (black arrow) and cells are columnar, stratified and showed different cytonuclear atypia (CA) (green arrow).
The staining intensity score and percentage positivity score were showed in table 3. The scoring system for 6 antibodies were at different level for each samples. There was no MLH1 antibodies reaction for the EC5 and no PMS2 antibodies reaction for the EC1 even after repetition of procedure. The highest score was MSH6 antibodies for EC6 (Table 4).

**Figure 3:** Nuclear immunoreaction of MMR proteins in EC patients. A: MSH6 and B: MSH2 antibodies are detected in tumor regions while C; MLH1 and D: PMS2 antibodies are detected innon-tumor regions. 100X.

**Figure 4:** Nuclear immunoreaction of PTEN antibodies, in non-tumor regions (A) and membranous immunoreaction of E-CAD1 antibodies (B). 100X.
5. Discussion

The result of histopathological examinations of all patients revealed that the cancer type may belong to type I EC according Bokhman, 1983 which estrogen dependent [3].

WES is an alternative to whole genome sequencing (WGS), because the exome makes up only 2% of the human genome yet contains 85% of known disease-related mutations [18,19]. WES provides a number of advantages over WGS, including lower cost, quicker data analysis, and simpler data management [20]. Considering the results of WES for 4 patients we found many variations and showed in two tables separately, table 1 including the polymorphisms which are interpreted as clinical significant, table 2; variation that have different interpretations known to be conflicting.

Microsatellites are DNA elements composed of short repetitive motifs that are prone to misalignment and frameshift mutations during cell division. In healthy cells, the ensuing small indels or single-base mispairs are corrected by heterodimer enzyme complexes of the DNA mismatch repair (MMR) system encoded by the key MMR genes MLH1, MSH2, PMS2 and MSH6 [21,22]. DNA mismatch repair deficiency (dMMR) results in the progressive accumulation of genetic mutations with each cell replication, potentially dysregulating many oncogenes or tumor suppressor genes. The molecular hallmark of dMMR is MSI (microsatellite instability), with expansions or contractions in the number of tandem repeats throughout the genome. This phenomenon is observed in a considerable proportion of colorectal, endometrial, gastric, pancreatic, brain, biliary tract, urinary tract and ovarian tumors [21,23,22]. A MSI or dMMR was defined as the lack of at least one MMR protein [12].

The heterodimer arrangements are presented for MMR proteins as the four proteins are locating in sequence of two together as follows; MLH1 with PMS2 and MSH2 with MSH6 [24]. This results also detected in our IHC study for these genes as PMS2 and MLH1 are expressed in non-tumor region while MSH6 and MSH2 expressed in tumor region. Relay on their functional structure, it is feasible to carry out an immunohistochemical panel of PMS2 and MSH6 antibodies as early screening for MMR deficiency [24]. Based on some previous studies for immunohistochemical evaluation we consider the presence or absence of nuclear staining, assuming that a positive reaction of tumor cells is considered intact protein expression (MSS phenotype) and that lack of expression, with positive internal control, is regarded MSI phenotype [25-28].

Nuclear staining observation of PTEN showed in Figure 4. Pten normally present in the nucleus, cytoplasm, and cell membrane [29] which can appear weak and somewhat variable. True Pten loss is characterized by complete absence of nuclear and cytoplasmic expression in glands (excluding intraglandular leukocytes, which can be abundant) [30]. In normal endometria, Pten loss in scattered glands was a common occurrence, in accordance with previous landmark studies [31,32,33,32].

One of the distinguishing features of a malignant tumor is altered cell adhesion, which includes variations in the expression and distribution of adhesion molecules [34-36]. Numerous cancers of various epithelial origins have been found to express E-cadherin less frequently, a protein necessary for the formation of cell-cell interactions [37]. This cell to cell contact define as adherent junctions [38].

Homogenous distribution of E-cadherin found through immunoreactivity test for our sample in glandular epithelium, figure 4.3. Non-nuclear region for this gene expression is presented by previous studies [36]. Depending on Pećina-Slaus, 2003 evidence who revealed that low expression of E-cadherin is considering as one of the primary molecular mechanisms essential in malfunction of the cell-cell adhesion system, leading to cancer invasion and metastasis [39], and linking this observation with our results for E-cadherin, could explain that the intense interaction of this antibody may since of low grade of cancer with no metastasis.

<table>
<thead>
<tr>
<th>Patients</th>
<th>MSH6</th>
<th>MSH2</th>
<th>MLH1 non-tumor region</th>
<th>PMS2 non-tumor region</th>
<th>PTEN non-tumor region</th>
<th>ECAD1</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC1</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>EC2</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>EC3</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>EC4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>EC5</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>EC6</td>
<td>12</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 4: IHC assessment (staining intensity score · percentage positivity score).
6. Conclusions

Regarding our evaluation system in dMMR proteins determination through IHC study, which has already been utilized to determine the MSI phenotype [26,28,40,41], we considered that, simply immunoreaction staining procedure can be use as alternative method for MSI phenotype detection rather than any type of more expensive and complex method of NGS.

Considering PTEN and ECAD1 antibodies reactions, also could be dependable in diagnosis and treatment and performing them if molecular diagnosis not available.

7. Recommendations

More studies required by evaluating larger samples size at different stage of endometrial cancer.

7.1 Authorship contribution statement

Both authors made contributions to the planning and design of the study. Azhin Saber Ali and Lana Sardar Alalem prepared the material and collected and analyzed the data. Azhin Saber Ali wrote the first draft of the manuscript, and both authors provided feedback on earlier drafts. The final manuscript was read and approved by both writers.

7.2 Ethical approval

It was obtained from the Salahaddin university College of science no: 4S/505.

7.3 Funding

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