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Next-generation sequencing and immunohistochemistry approaches for microsatellite instability detection in endometrial cancer

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ABSTRACT

The purpose was to determine the effectiveness of immunohistochemical observation, a quick and easy method for determining Microsatellite instability MSI and/or other types of endometrial cancer (EC) based on modern classification. As molecular study takes more cost we want to compare both methods and show the possibility of doing IHC instead of molecular study. This study was designed to establish a small gene panel of Next Generation Sequencing (NGS) of endometrial cancer patients targeting 4 mismatch repair genes; MSH6, MSH2, MLH1 and PMS2. Using the DNBSEQ-G400 Platform, the Human Core Exome kit and Python software for analysis were used. At the same time, the Dako kit was used to perform IHC for six primary antibodies used to detect each of MSH6, MSH2, MLH and PMS2. The primary antibodies were applied on 5 µm formalin-fixed paraffin-embedded (FFPE). Results showed that histopathological examinations of all patients were at stage I endometrioid endometrial carcinoma. The FIGO classifications were Ia and Ib. Microsatellite instability (MIS) was observed through the IHC study. The molecular studies detected several polymorphisms that have clinical significance and some of them have conflicting interpretations. In conclusion, we considered that a simple immunoreaction staining procedure can be used as an alternative method for MSI phenotype detection rather than any type of more expensive and complex method of NGS.

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Introduction

Globally, in 2018 endometrial cancer (EC) was regarded as the sixth most commonly diagnosed cancer and the fourteenth leading cause of cancer with most 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 higher 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, however, some evidence shows elevating uterine cancer in general including endometrial cancer in some cities (2)

More than 30 years ago, based on hormonal and clinical characteristics 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 to 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 is 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). The International Federation of Gynecology and Obstetrics (FIGO) adopted a-surgical pathologic staging in 1988 and in 2009, FIGO updated the staging system and classified patients into prognostic groups based on the extent of disease (4, 5).

The new classification is based on molecular features, according to The Cancer Genome Atlas (TCGA), which established 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 (7-9), which include, the POLE (DNA polymerase ε) ultramutated group, the hypermutated/microsatellite unstable (10) group, the copy number low/microsatellite stable group and the copy number high (serous-like) group.

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 based on modern classification. As molecular study takes more cost we want to compare both methods and show the possibility of performing IHC instead of molecular study.

Materials and Methods

The Study design

This study included two techniques at the same time for comparison, NGS as molecular study and IHC as histopathological study for EC. The designed study is shown in Figure 1.

Sample collection

Samples were collected at Erbil Maternal Hospital

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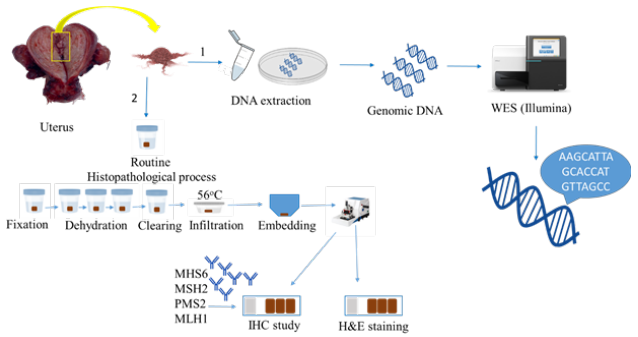

Figure 1. An illustration diagram for methodology. WES: Whole Exome Sequencing, H&E staining: Hematoxylin and Eosin staining, IHC study: Immunohistochemistry study.

from those patients who were previously diagnosed with endometrial carcinoma through their curettage biopsy examination, then gynecologists did a total abdominal hysterectomy 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 it in cold PBS for transporting to the lab for molecular study. Routinely, the whole removed organs were put in buffered Formalin for histopathological examination. EC1, EC2, EC3, EC4, EC5 and EC6 indicate the name of patients instead of their real names.

Cancer Gene Panel

Genomic DNA was extracted from the fresh tissue by using a geneaid kit. Then we established a small cancer gene panel, covering 4 mismatch repair genes for next-generation sequencing (whole exome sequencing=WES); MSH6, MSH2, MLH1, PMS2, By using DNBSEQ-G400 Platform and Human Core Exome kit and sequencing was performed by DNA Laboratuvarları Genetik Hastalıklar Tanı Merkezi. Python software was used for analysis.

Histopathological Examination

A routine histopathological examination was performed for our samples through putting them in 10% formal saline for one week to fix the tissue, then all samples underwent serial treatments including dehydration, clearing, infiltration, embedding, sectioning and finally staining with hematoxylin and eosin.

Immunohistochemical detection

Four primary antibodies were used to detect each of MSH6, MSH2, MLH1 and PMS2 by using Dako kits to detect some of the mutated genes immunohistochemically. According to the manufacturer's instructions. The primary antibodies were applied on 5 µm formalin-fixed paraffin-embedded (FFPE).

Immunohistochemical interpretations

The four slides from each tumor block staining for MLH1, MSH2, MSH6 and PMS2 were assessed. The semiquantitative scoring system was used to minimize interobserver variation (11-13). 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 the 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 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 (12, 13). 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).

Results

Whole exome sequencing WES results

The observed polymorphism in Table 1 was analyzed as clinical significance, from the current variations most of them were found in dbSNP but not found as a clinical significance for EC in the Clin Var database. About 9 of them were not found even in dbSNP, such as MSH2 (2: 47641560AAAA>-) for the second patient (EC1), see the remaining 8 variations in Table 1, these are named novel variations.

Within these polymorphisms, some of them were located in intron regions and others were located within exon regions. 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 all non-synonymous SNV, they were missense variants (MSH2 for EC2, MLH1 and PMS2 for EC3 and MSH2 for EC4).

Table 2 shows the variations which have conflicting interpretations. Conflicting interpretations are defined 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 (14, 15). Some variations in MSH2 for EC1, EC2 and EC4 and MSH6 for EC2 were found in the Clin Var database as conflicting interpretations. Three of them were found in the dbSNP database but no data was recorded for EC as clinically significant. One of them (MSH2) was novel (2:47641560AAAA>-) as not found in the dbSNP database.

Routine histopathological examination

The architecture of the endometrial layer of patients with endometrioid carcinoma was 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 are also revealed by Tavassoli and Devilee, 2003(16), Figure 2. Cytonuclear atypia which is a precursor for endometrioid carcinoma characterized by loss of polarity, rounded nuclei, anisokaryosis, hyper or hypochromasia and a more eosinophilic cytoplasm (9, 17).

Table 3 shows the FIGO classifications for all patients separately. Each of EC1 and EC2 were in Ib FIGO stage, pT1a, pN0 in TNM staging. pT1a means only affects the

Table 1. Nucleotide polymorphism identified in EC patients.

Patients	Gene	Variant coordinate	AA change	Zygosity	Mutation type	External database\ clinical significant
EC1	MSH6	NC_000002.11:g.48025764C>T	p.Y214Y	het	synonymous SNV	dbSNP/ not reported in Clin Var for EC
		NC_000002.11:g.48032572C>T		het	intron	dbSNP/ not reported in Clin Var for EC
		NM_000251.3	c.942+24_942+29del	het	intron	dbSNP/ not reported in Clin Var for EC
	MSH2	NC_000002.11:g.47690162G>T		het	intron	dbSNP/ not reported in Clin Var for EC
		NC_000002.11:g.47698179A>G	p.K>K	het	synonymous SNV	dbSNP/ not reported in Clin Var for EC
	MLH1	NC_000003.11:g.37067097A>T		het	intron	dbSNP/ not reported in Clin Var for EC
	PMS2	NM_000535.7	c.706-4del	het	intron	dbSNP/ not reported in Clin Var for EC
		NC_000007.13:g.6022626C>T		het	intron	dbSNP/ not reported in Clin Var for EC
EC2	MSH6	NC_000002.11:g.48026172C>T	p.A>A	het	synonymous SNV	dbSNP/ not reported in Clin Var for EC
	MSH2	NC_000002.11:g.47702191A>G	p.N>S	het	missense	dbSNP/ not reported in Clin Var for EC
		2: 47641560AAAA>-	Intron	het	DEL	Novel
	MLH1	NC_000003.11:g.37053549C>T	p.T>T	het	synonymous SNV	dbSNP/ not reported in Clin Var for EC
		3: 37067094 TATATATT>-	intron	het	Del	Novel
	PMS2	7: 6037058A>-	intron	het	Del	Novel
EC3	MSH6	NC_000002.11:g.48032754A>T	Intron	het	SNV	dbSNP/ not reported in Clin Var for EC
	MSH2	2: 47641560AAA>-	Intron	het	DEL	Novel
		NC_000002.11:g.47694037T>C	Intron	het	SNV	dbSNP/ not reported in Clin Var for EC
	MLH1	3: 37067094 TATATATTT>-	intron	het	DEL	Novel
		NC_000003.11:g.37067306G>A	p. S>N	het	missense	dbSNP/ not reported in Clin Var for EC
		7:6037058AA>-	Intron	het	DEl	Novel
	PMS2	NC_000007.13:g.6026384C>T	Intron	het	SNV	dbSNP/ reported in Clin Var for EC
		NC_000007.13:g.6026942G>T	p. T>K	het	missense	dbSNP/ not reported in Clin Var for EC
		NC_000007.13:g.6043386G>A	p. A>A	het	synonymous SNV	dbSNP/ not reported in Clin Var for EC
		NC_000017.10:g.41245471C>T,	p. D>N	het	missense	dbSNP/ not reported in Clin Var for EC
EC4	MSH6	NC_000002.11:g.48032717T>A	Intron	het	SNV	dbSNP/ not reported in Clin Var for EC
		NC_000002.11:g.48010558C>A,	p.R62R	het	synonymous SNV	dbSNP/ not reported in Clin Var for EC
		2:47641560AAAAA>-	intron	het	DEL	Novel
	MSH2	NC_000002.11:g.47643457G>A	p.G>D	het	missense	dbSNP/ not reported in Clin Var for EC
		NC_000002.11:g.47694037T>C	Intron	het	SNV	dbSNP/ not reported in Clin Var for EC
	MLH1	3: 37067094 TATATATTT>-	Intron	het	DEL	Novel
		NC_000003.11:g.37067097A>T	Intron	het	SNV	dbSNP/ not reported in Clin Var for EC
	PMS2	7: 6037058A>-	Intron	het	DEL	Novel
		NC_000007.13:g.6043495T>C	Intron	het	SNV	dbSNP/ not reported in Clin Var for EC

endometrium or only penetrates around half of the myometrium. pN0 means Only a small several 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, as also shown by (18). While each of EC3, EC4 and EC6 are of the same FIGO staging which are T1b, Nx, Mx, Ib. T1b

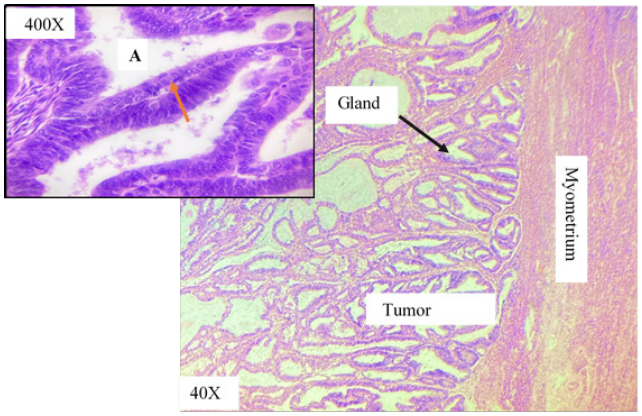

Figure 2. Endometrioid endometrial carcinoma, tumor region showing tubular differentiation of gland (black arrow) and cells are columnar, stratified and showed different cytonuclear atypia (CA) (green arrow).

signifies that the cancer is between 1-2 cm in size. Both Nx and Mx mean nothing has been identified for nearby lymph nodes and unable for mearing metastasis, respectively. Finally, EC5 was at T1a, Nx, Mx 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 are shown in Figure 3. These biomarkers had different levels of reaction intensities and had different regional reactions. For all patients, the nuclear staining for both MSH6 and MSH2 was observed in tumor regions while nuclear staining reactions of both MLH1 and PMS2 occurred in the non-tumor regions. The nuclear staining was observed for all MMR proteins. IHC study for these genes PMS2 and MLH1 expressed in the non-tumor region while MSH6 and MSH2 expressed in the tumor region.

The staining intensity score and percentage positivity

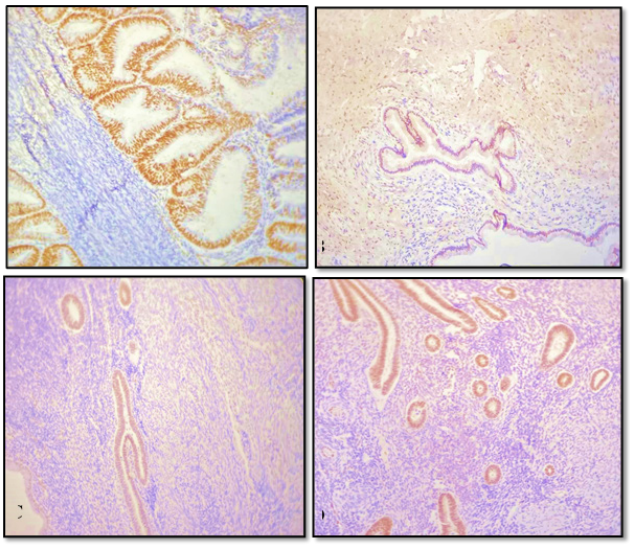

Figure 3. Nuclear immunoreaction of MMR proteins in EC patients. A: MSH6 and B: MSH2 antibodies are detected in tumor regions while C; MLH1 and D: PMS2 antibodies are detected in non-tumor regions. 100X.

Table 2. Nucleotide polymorphism with conflicting interpretation clinical significance.

Patients	Gene	Variant coordinate	AA change	Zygosity	Mutation type	CLNSIG	External database\ clinical significant
EC1	MSH2	***NM_000251.3***	c.942+24_942+29del	het	intron	Conflicting	dbSNP/ not reported in Clin Var for EC
	MSH6	NC_000002.11:g.48026172C>T	p.A>A	het	synonymous SNV	Conflicting	dbSNP/ not reported in Clin Var for EC
EC2	MSH2	NC_000002.11:g.47702191A>G	p.N>S	het	nonsynonymous SNV	Conflicting	dbSNP/ not reported in Clin Var for EC
		2:47641560AAAA>-	Intron	het	DEL	Conflicting	Novel
EC4	MSH2	2:47641560AAAAA>-	intron	het	DEL	Conflicting	Novel

Table 3. Clinical and pathologic data of patients.

		EC1	EC2	EC3	EC4	EC5	EC6
Age		51	56	70	68	56	55
Grade		2	2	2	2	2	2
FIGO staging	T	pT1a	pT1a	T1b	T1b	T1a	T1b
	N	pN0	pN0	N0	Nx	Nx	Nx
	M	Mx	Mx	Mx	Mx	Mx	Mx
	stage	Ia	Ia	Ib	Ib	Ia	Ib
Myometrium invasion		half	half	half	half	half	half

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

Patients	MSH6	MSH2	MLH1 non- tumor region	PMS2 non- tumor region
EC1	6	1	1	0
EC2	8	3	1	3
EC3	4	3	4	6
EC4	2	2	2	4
EC5	9	9	0	2
EC6	12	1	3	3

score are shown in Table 4. The scoring system for 6 antibodies was at a different level for each sample. There was no MLH1 antibody reaction for the EC5 and no PMS2 antibody reaction for the EC1 even after repetition of the procedure. The highest score was MSH6 antibodies for EC6.

Discussion

The result of histopathological examinations of all patients revealed that the cancer type may belong to type I EC according to Bokhman, 1983 which is estrogen-dependent (3). Besides the modern classification for our samples was the endometrioid subtype according to WHO classification 2020 (19).

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 (20, 21). WES provides many advantages over WGS, including lower cost, quicker data analysis, and simpler data management (22). Considering the results of WES for 4 patients we found many variations and showed them in two tables separately, table 1 including the polymorphisms which are interpreted as clinically significant, and Table 2; variations 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 (23, 24)*. 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 (23-25). An 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 located in a sequence of two together as follows; MLH1 with PMS2 and MSH2 with MSH6 (26). These results were also detected in our IHC study for these genes as PMS2 and MLH1 are expressed in the non-tumor region while MSH6 and MSH2 are expressed in the 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 (26). 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 as MSI phenotype (27-30).

Conclusions

Regarding our evaluation system in dMMR proteins determination through the IHC study, which has already been utilized to determine the MSI phenotype, we considered that a simple immunoreaction staining procedure can be used as an alternative method for MSI phenotype detection rather than any type of more expensive and complex method of NGS.

Disclosure

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.

Ethical approval was obtained from the Salahaddin University College of Science no: 4S/505.

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