PRELIMINARY STUDY ON THE OCCURRENCE OF PTEN AND PIK3CA GENE MUTATIONS IN ENDOMETRIAL CANCER AMONG MALAYSIAN WOMEN

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ABSTRACT

Genetic mutations in endometrial cancer (EC) have been extensively studied in the Western population but not much in Asian cohorts. This study has demonstrated that *PTEN* and *PIK3CA* mutations are commonly found in EC among Malaysian women. Following RNA extraction from 20 cancerous and 18 non-cancerous tissues, the presence of mutations in 9 exons of *PTEN* and 3 exons of *PIK3CA* genes were detected using real-time PCR, accompanied by High Resolution Melt (HRM) analysis. Sequencing confirmed specificity of each PCR product. The mutations for both genes were detected in the samples with varying frequencies. Notably, all samples expressed mutation of *PTEN* at exon 7 but none in exon 4. Further analysis demonstrated that strong concurrent mutations occurred between exons 7 of *PTEN* with exon 20 region 1 of *PIK3CA* gene (90%). Our data showed mutations are present in EC and not the non-cancerous tissues. Larger samples are being collected to validate this observation.

Keywords: *Uterine cancer, Malaysian, genetic abnormalities*

Introduction

Worldwide, endometrial cancer (EC) ranks sixth among commonly diagnosed female cancer with 288,000 new cases and mortality rate from 1.7 to 2.4% per 100,000 women in 2008 (1). It is also the top gynecological malignancy in the United States, making it the 8th leading cause of cancer death among women worldwide. American Cancer Society has estimated about 49,560 new cases of EC and 8,190 women succumbing to this disease in 2013 (2) . In Malaysia, National Cancer Incidence reported that EC contributed to 4.1% of total cancer cases involving women in 2007. This was a rise from 3.3% in 2003. There are 2 types of EC namely Type 1 and Type 2, with the former having better prognosis and survival rate of 83% compared to 25% for the latter (3). Several factors are thought to be linked to the rising trend of EC incidence worldwide, including the increase in obesity incidence, unopposed exposure to estrogen due to hormonal treatment after menopause and nullparity (4).

In addition to the environmental and hormonal factors, genetics may represent an important key regulator in EC cancer occurrence and progression. Oncogenes and tumor suppressor genes are the two gene classifications in which their mutations affect the development of cancer cells (5). Activation of oncogenic genes such as catalytic subunit α of phosphatidylinositol-4,5-bisphosphate 3-kinase (*PIK3CA*) gene, and inactivation of tumor suppressor genes such as Phosphatase and tensin homolog (*PTEN*) gene, are thought to be the key genetic changes involved in endometrial cancer development (6,7).

PTEN is responsible for controlling cell growth by regulating the cell cycle at G_1/S checkpoint, and loss of *PTEN* gene function was reported in 83% of EC cases (8). *PTEN* often acts with phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) gene to control the activity of AKT signaling, which is important in proliferating cancer cells. Up to 26% of tumors harboring *PTEN* mutation also have mutation in *PIK3CA*. Mutation in *PIK3CA* can lead to an additive effect on PI3K signaling activation. In fact, it was found that the mutation of *PIK3CA* were more common in tumor with *PTEN* mutations compared to those without (7). Additionally, contribution of *PIK3CA* and *PTEN* gene mutations are often implicated

with endometrial cancer, both individually as well as by co-existing together (7,9-14). Given the importance of these genes in EC progression, we aim to investigate in this preliminary study whether such mutations can be detected in a small cohort of patients with endometrial cancer admitted to University Malaya Medical Center (UMMC).

Methodology

Ethics statement

The study was approved by the Ethical Committee of University Malaya Medical Center (Ref No. 865.19). Written informed consent was obtained from all participants.

Tissue inclusion and exclusion criteria

All the cancer tissues used in this study were from patients with confirmed endometrial cancer, while the control (non-tumor) tissues were from patients with non-tumor conditions such as post-menopausal bleeding, and from dilation and curettage samples. All the cancer tissues were confirmed by pathologist to be type 1 endometrial cancer of endometriod adenocarcinoma. Patients who were pregnant, under-age and had been diagnosed for other types of cancer were excluded from this study.

Human endometrium tissue processing and RNA extraction

All 38 snap frozen tissue samples (20 cancer and 18 controls) were collected from the University of Malaya, Faculty of Medicine, Biobank Unit. These tissues were cut to 2 mm length and transferred to 1.5 ml microcentrifuge tubes containing 100 µl phosphate buffered saline (Life Technologies, NY, USA). Next, equivalent amount of stainless steel beads with diameter of 1.6 mm (Next Advance, New York, USA) were added into the tubes. The tissues were then homogenized by using a bullet blender (Next Advance, New York, USA). Total RNA were extracted from homogenized tissues using TRIsure (Bioline, London, UK) according to the manufacture's protocol, and the yield of the RNA was quantified using NanoDrop ND-2000 (Thermo Fisher Scientific Inc, *Massachusetts, USA). T*otal RNA was converted into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc, *Massachusetts, USA).*

Real Time PCR and analysis of High Resolution Melt (HRM)

Real Time PCR was performed using ABI StepOne Plus (Applied Biosystem, California, USA) in 40 cycles. Each PCR reaction included5x HOT FIREPol EvaGreen qPCR Mix (Solis Biodyne, Tartu, Estonia), 10 pmol/µl forward and reverse primers, 10 ng/µl cDNA template and PCR grade H_2O prior to HRM analysis using High Resolution Melt Software v3.0.1 (Applied Biosystem, California, USA). Exons 1-9 of *PTEN* gene and exons 9 and 20 of *PIK3CA* gene were analyzed for mutation. All PCR reactions were performed in duplicates, and data shown were from at least 2 independent experiments. Primers used are shown in Table 1.

Table 1: List of primer sequences

Exon	Forward (3'→5')	Reverse (3'→5')
PTEN		
1	CAGAAGAAGCCCCGCCACCAG	AGAGGAGCAGCCGCAGAAATG
2	TTTCAGATATTTCTTTCCTTA	AACAAGAATATAAAACATCAA
3	TAATTTCAAATGTTAGCTCAT	AAGATATTTCAAGCATACAA
4	GTTTGTTAGTATTAGTACTTT	ACAACATAGTACAGTACATC
5 (1)	ACCTGTTAAGTTTGTATGCAAC	CTTTCCAGCTTTACAGTGAA
5 (2)	GCTAAGTGAAGATGACAATCA	TCCAGGAAGAGGAAAGGAAA
6	CATAGCAATTTAGTGAAATAACT	GATATGGTTAAGAAAACTGTTC
7	TGACAGTTTGACAGTTAAAGG	GGATATTTCTCCCAATGAAAG
8 (1)	TTAAATATGTCATTTCATTTCTTTT	TTGCTTTGTCAAGATCATT
8 (2)	GTGCAGATAATGACAAGGAATA	TCATGTTACTGCTACGTAAAC
9	TTCATTTTAAATTTTCTTTCT	TTTTCATGGTGTTTTATCCCTC
PIK3CA		
9	GATTGGTTCTTTCCTGTCTCTG	CCACAAATATCAATTTACAACCATTG
20 (1)	TGGGGTAAAGGGAATCAAAAG	CCTATGCAATCGGTCTTTGC
20 (2)	TTGCATACATTCGAAAGACC	GGGGATTTTTGTTTTGTTTTG

Sequencing of PCR Products

PCR products were purified using MSB Spin PCRapace kit (Stratec, Berlin, Germany) and the validity of each product is confirmed with sequencing analysis (AIT Biotech Pte Ltd, Singapore).

Results

Patient demographic distribution

Cancer tissues used in this study were all from type 1 endometrial cancer with varying stages and histograde. Stage 1A and Grade 2 were the predominant classifications, with 8 and 10 cases, respectively (Table 2a). Control tissues were collected from non-tumor conditions: post-menapausal bleeding (10 cases), endometrial hyperplasia (3 cases) and endometrial fibroid (5 cases) (Table 2b). The patient cohort in this study comprised of 3 ethnic groups (Malays, Chinese and Indians) and their age ranged from 30 to 79 years old. As shown in Table 3, the Malays represented the majority (50%) of the cancer patients compared to 22.2% in the control group. Approximately 30% and 20% of the remaining cancer cases were Chinese and Indian, compared to about 56% and 22% in control cases. Stratification analysis according to age showed that almost 80% of cancer patients were above 50 years old, consistent with the aetiology of this disease that mostly affecting post-menopausal women (Table 4).

Table 2(a): Staging and grading information for cancer tissues in this study

CANCER TISSUES		Histograde			
		1	2	3	**Total**
Tumor stages	1A	3	5	0	**8**
	1B	0	3	1	**4**
	2	1	2	0	**3**
	3C1	1	0	0	**1**
	4A	1	0	1	**2**
	4B	0	0	2	**2**
	Total	**6**	**10**	**4**	**20**

Table 2(b): Control tissues classifications for tissues used in this study

CONTROL TISSUES	
Conditions	**No.**
Post-menapausal bleeding	10
Hyperplasia	3
Fibroid	5
Total	**18**

Table 3: Patients demography according to race distribution among cases and controls

	Race			**Total**
	Malay	**Chinese**	**Indian**	
Cases	10 (50)	6 (30)	4 (20)	**20**
Control	4 (22.2)	10 (55.6)	4 (22.2)	**18**
Total	**14**	**16**	**8**	**38**

*Data are given as frequency (percentage)

Table 4: Patients demography according to age distribution among cases and controls

	Age group					**Total**
	30-39	**40-49**	**50-59**	**60-69**	**70-79**	
Cases	3 (15)	1 (5)	6 (30)	5 (25)	5 (25)	**20**
Control	5 (27.7)	7 (38.9)	5 (27.8)	1 (5.6)	0 (0)	**18**
Total	**8**	**8**	**11**	**6**	**5**	**38**

*Data are given as frequency (percentage)

Occurrence of tumor mutation

We screened for presence of mutations in the tissues as summarized in Table 5(a) and (b). Our findings were verified by capillary sequencing. Analysis of *PIK3CA* gene mutations showed highest occurrence in exon 20 region 1 (90%) followed by exon 9 (65%) and lastly in exon 20 region 2 (55%). Exons of *PTEN* gene exhibited different percentages of mutation occurrences with all samples being mutated in exon 7 (100%) compared to no samples being mutated in exon 4 (0%). None of these mutations were detected in the control tissues (0%).

Table 5(a): Frequency of mutations occurrence in cases and controls of PIK3CA gene

PIK3CA	Cases (n=20)	Controls (n=18)
	n (%)	**n (%)**
PIK3CA-9	13 (65)	0 (0)
PIK3CA 20-1	18 (90)	
PIK3CA 20-2	11 (55)	

*Data are given as frequency (percentage). RNA from cases and controls were extracted and subjected to real time PCR followed by HRM analysis. Frequency above represents total number of patients with mutation.

Table 5(b): Frequency of mutations occurrence in cases and controls of PTEN gene

PTEN	Cases (n=20)	Controls (n=18)
	n (%)	**n (%)**
PTEN 1	15 (75)	0 (0)
PTEN 2	2 (10)	
PTEN 3	2 (10)	
PTEN 4	0 (0)	
PTEN 5-1	16 (80)	
PTEN 5-2	14 (70)	
PTEN 6	13 (65)	
PTEN 7	20 (100)	
PTEN 8-1	5 (25)	
PTEN 8-2	18 (90)	
PTEN 9	6 (30)	

*Data are given as frequency (percentage). RNA from cases and controls were extracted and subjected to real time PCR followed by HRM analysis. Frequency above represents total number of patients with mutation.

Simultaneous PIK3CA and PTEN gene mutations

We further analyzed the trends of simultaneous mutations occurrence between exons of a gene as well as between genes (Table 6). For *PTEN*, the highest concurrent mutation was between exon 7 and exon 8 region 2 (18/20 cases), while for *PIK3CA*, frequent concurrent mutation were found between exon 9 and exon 20 region 1 (11 / 20 cases). Simultaneous mutations analysis was also performed between the two genes. Exon 7 of *PTEN* gene showed strong co-occurrence with exon 20 region 1 of *PIK3CA* gene (18/20 cases).

Table 6: Simultaneous occurrence of mutation between exons and genes

		PTEN										***PIK3CA***		
		1	**2**	**3**	**5 (1)**	**5 (2)**	**6**	**7**	**8 (1)**	**8 (2)**	**9**	**9**	**20 (1)**	**20 (2)**
PTEN	**1**		2	2	**12**	**11**	9	**15**	5	**13**	6	**10**	**14**	6
	2			1	2	2	2	2	2	2	2	1	2	1
	3				2	1	2	2	2	2	2	1	2	2
	5 (1)					**12**	**10**	**16**	5	**14**	5	9	**15**	7
	5 (2)						9	**14**	3	**12**	4	8	**13**	7
	6							**13**	5	**13**	4	**11**	12	9
	7								6	**18**	6	**13**	**18**	**11**
	8 (1)									5	3	4	5	2
	8 (2)										5	**13**	**16**	**11**
	9											4	5	4
PIK3CA	**9**												**11**	9
	20 (1)													9
	20 (2)													

*Data shown are frequency of concurrent occurrence; Bolded numbers represent more than 50% mutation co-occurrence

Discussion

In this study, we screened for various mutations of *PTEN* and *PIK3CA* genes in 20 endometrial cancer and 18 non-cancerous endometrial tissues collected from Malaysian women. In addition, we also analyzed the patterns of mutation co-occurrence between exons in these genes. While contribution of these genes in endometrial cancer has been studied in depth in western population (15), such information are not available for Malaysian women. Such information may provide a significant clinical implication, as status of mutations in tumors may predict resistance of tumor cells against selected drug therapy.

PIK3CA gene mutation was highly expressed in our study, where it demonstrated at least 55% frequency in all three different exons examined. *PIK3CA* is an important catalytic subunit of the phosphatidylinositol 3-kinases (PI3Ks) that regulates cell proliferation, adhesion and survival (10). Many investigative agents, such as rapamycin, RAD001 and evorolimus that primarily blocks mTOR (a downstream molecule of PI3K pathway) are also known to inhibit the action of *PIK3CA* (16,17). Determining the status of mutation in genes is crucial before making any clinical decision, considering the recent report that suggested mutation in another oncogenic gene, the K-*ras* gene may be the cause of cancer cells developing resistance towards mTOR inhibitors (16). This emphasizes the importance of mutation screening prior to decisions of therapeutic interventions. Interestingly, it was demonstrated that mutations in *PIK3CA* gene can lead to constant activation of PI3K pathway, and therefore blocking the effect of mTOR inhibitors (12,17). It was also shown recently, mutation in exon 20 of this gene is associated with high-grade endometrial cancer and that another mutation site in this gene, H1047R correlated with shorter survival (18).

Our analysis further demonstrated that this gene (*PIK3CA* exon 2 region 1) occurred in the presence of *PTEN* gene mutations (exon 7), consistent with previous findings (7,12). It is worthwhile to note that co-existence of these two gene mutations seems to be frequent in endometrial cancer but is quite rare in other cancers (13). It was also reported that *PTEN* mutations are observed specifically in endometrial cancer but not in other gynecological malignancies (14) and that it is frequently found in type 1 endometrial cancer (18). In contrast to cancer types, where *PTEN* mutation translates to increased metastatic potential, such mutation in endometrial cancer may be associated with a favorable survival (9).

Endometrial cancer is in part a genetic-driven process, and many clinical decisions are now being made based on the genetic profiling of the tumor. While the sample size in this study is relatively small, our data strongly suggests that mutations in *PTEN* and *PIK3CA* genes can be detected in the endometrial cancer samples presented by Malaysian women but not in controls, and this may imply that the etiology of this tumor in this region share similarities with those from the Western countries. A more comprehensive subsequent analysis is warranted to validate this finding in a larger sample cohort and to investigate the association of these mutations with the prognosis of these women. Determination of these mutations may allow for a more informed clinical diagnosis and to make a choice of the therapy required.

Conclusion

This preliminary study showed the presence of mutations in *PTEN* and *PIK3CA* among patients with endometrial cancer admitted to UMMC, with strong co-occurrence of

mutations between exon 7 of *PTEN* with exon 20 region 1 of *PIK3CA* gene.

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