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Original Article

High throughput silencing identifies novel genes in endometrioid endometrial cancer

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ABSTRACT

Objective: To validate the gene expression profile obtained from the previous microarray analysis and to further study the biological functions of these genes in endometrial cancer. From our previous study, we identified 621 differentially expressed genes in laser-captured microdissected endometrioid endometrial cancer as compared to normal endometrial cells. Among these genes, 146 were significantly up-regulated in endometrial cancer.

Materials and Methods: A total of 20 genes were selected from the list of up-regulated genes for the validation assay. The qPCR confirmed that 19 out of the 20 genes were up-regulated in endometrial cancer compared with normal endometrium. RNA interference (RNAi) was used to knockdown the expression of the upregulated genes in ECC-1 and HEC-1A endometrial cancer cell lines and its effect on proliferation, migration and invasion were examined.

Results: Knockdown of *MIF*, *SOD2*, *HIF1A* and *SLC7A5* by RNAi significantly decreased the proliferation of ECC-1 cells ($p < 0.05$). Our results also showed that the knockdown of *MIF*, *SOD2* and *SLC7A5* by RNAi significantly decreased the proliferation and migration abilities of HEC-1A cells ($p < 0.05$). Moreover, the knockdown of *SLC38A1* and *HIF1A* by RNAi resulted in a significant decrease in the proliferation of HEC1A cells ($p < 0.05$).

Conclusion: We have identified the biological roles of *SLC38A1*, *MIF*, *SOD2*, *HIF1A* and *SLC7A5* in endometrial cancer, which opens up the possibility of using the RNAi silencing approach to design therapeutic strategies for treatment of endometrial cancer.

Introduction

The increasing trend of endometrial cancer incidence was documented with a total of 319,605 estimated new cases and 76,155 related deaths which occurred in 2012, compared with 287,100 and 74,000 respectively, in 2008 [1]. Worldwide, it is the sixth most common cancer in women [1]. In Malaysia, 414 women were diagnosed with endometrial (approximately 4.1% from total women cancers) in 2007 [2]. Generally, the incidence of this cancer increases with age, with 75%–80% of new cases occurring among post-menopausal women [3]. The peak incidence in Malaysian women was between 55 and 59 years old [2].

Traditionally, endometrial cancer is classified into two subtypes according to the histopathological and clinical features. Type I, also known as endometrioid, is the most frequent type of endometrial cancer and has more favorable prognosis. It usually presents as a result of prolonged unopposed estrogen exposure [4–6]. Mutations of *PTEN*, *K-ras* and *β-catenin* are among the most frequent genetic alteration associated with type I [7,8]. In contrast, type II which has a non-endometrioid histological appearance is less common. These tumors are often poorly differentiated, unrelated to estrogen exposure and have poor prognosis [4–6]. Mutations of *p53* and *Her-2/neu* are found at higher rates in these cancer type [7,8].

The primary treatment for endometrial cancer is surgery. Typically, it involves hysterectomy and bilateral salphingo-oophorectomy, with loss of reproductive function especially in

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young women [9,10]. Despite complete surgical treatment, approximately 10%–15% of patients will experience recurrences, either local or distant metastases [11]. Other modalities of therapeutic interventions such as radiotherapy and chemotherapy may work in certain cases but not without toxicity to normal cells [12].

Therefore, to improve the management of this disease, it is necessary to understand the basis underlying its development. Although a number of proto-oncogenes and tumor suppressor gene have been associated with endometrial cancer, research is still ongoing to investigate and identify new genes with oncogenic potential or tumor-suppressing activities which can facilitate additional therapeutic strategies in endometrial cancer.

In an earlier study, 15 laser-captured microdissected endometrioid endometrial cancer and 15 normal endometrial samples were analyzed using microarray. We identified 146 genes that were significantly up-regulated in endometrioid endometrial cancer compared with normal endometrium [13]. In the present study, we validated a subset of genes from the list of up-regulated genes identified by the microarray. Since the role of these genes in endometrial cancer pathogenesis is yet to be fully understood, we performed further functional analysis on the candidate genes using RNA interference (RNAi) mediated loss-of-function assays.

Materials and Methods

Tissue samples

Ethics approval of the study protocol was obtained from the Universiti Malaya Medical Centre Ethics Committee (MEC Ref. No: 812.11). Written informed consent was obtained and endometrial cancer tissue samples were obtained from patients who underwent total hysterectomy with bilateral salphingo-oophorectomy (TAHBSO). Whenever possible, the normal endometrial tissues adjacent to the tumor site were taken from the same patient. In addition, samples of normal endometrium were obtained from patients undergoing hysterectomy for benign gynecological diseases (e.g. ovarian cyst, fibroid, endometriosis, endometrial hyperplasia). In total, 15 normal endometrium and 9 endometrial cancer tissue samples were used for this study. Table 1 shows the clinical information of the cancer tissue samples. Out of nine cancer samples, eight samples were at stage 1A and a single sample was at stage 1C. Five patients were of grade 2 and four of grade 1. None of the patients had received preoperative treatments such as radiation therapy or chemotherapy. Each sample was examined histologically with hematoxylin and eosin stained sections. Only samples with a consistent tumor cell content of more than 80% in tissues were used for analysis.

Cell lines

ECC-1 (Catalogue No: CRL2923) and HEC-1A (Catalogue No: HTB112) were the human endometrial cancer cell lines used in the study, obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA. ECC-1 cells were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS) (Invitrogen). HEC-1A cells were maintained in McCoy's 5A medium (Invitrogen) supplemented with 10% FBS. Cells were incubated at 37 °C in 95% humidified atmosphere containing 5% CO_2.

Table 1
Clinical information of the cancer tissue samples.

Patient ID	Ethnicity	Age	Histology	Stage	Grade
EC1	Chinese	55	Endometrioid	IA	2
EC2	Malay	41	Endometrioid	IA	2
EC3	Chinese	32	Endometrioid	IC	2
EC4	Chinese	52	Endometrioid	IA	1
EC5	Indian	61	Endometrioid	IA	1
EC6	Others	72	Endometrioid	IA	1
EC7	Malay	40	Endometrioid	IA	2
EC8	Malay	54	Endometrioid	IA	2
EC9	Chinese	45	Endometrioid	IA	1

RNA extraction

Total RNA from tissue samples and cell lines were extracted using Trizol reagent (Invitrogen) and the RNeasy Mini Kit (Qiagen, Valencia, CA) respectively according to the manufacturer's instructions. RNA concentration and purity was assessed by NanoDrop 2000c UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Extracted RNA was stored at −80 °C until further used. To ensure the highest quality results, only samples with RNA integrity number of more than 7 were analyzed for qPCR assays.

Quantitative Real-time PCR

Total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Quantitative Real-time PCR (qPCR) was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems) using the TaqMan gene expression assays (Applied Biosystems). Table 2 lists the TaqMan assays for the 20 selected genes. The thermal cycle conditions were 50 °C for 2 min, 95 °C for 20 s, 40 cycles of 95 °C for 1 min and 60 °C for 20 s. Each qPCR run included a negative control without RNA template to assess the specificity of the reaction. All assays were done in duplicates and *18S* (assay ID: Hs99999901_m1) was used as the endogenous control. For the qPCR analysis of tissue samples, normal endometrium tissue sample was used as a control. The comparative Cq method was used to calculate the relative quantification of gene expression. The

Table 2
List of TaqMan gene expression assays for qPCR.

Gene ID	Gene symbol	Name	Assays
81539	*SLC38A1*	Solute carrier family 38, member 1	Hs01562168_m1
64798	*DEPTOR1*	DEP domain containing mTOR-interacting protein 1	Hs00961900_m1
3431	*APOC1*	Apolipoprotein C1	Hs03037377_m1
83853	*ROPN1L*	Rhophilin associated tail protein 1-like	Hs00230481_m1
4065	*LY75*	Lymphocyte antigen 75	Hs00982383_m1
6772	*STAT1*	Signal transducer and activator 1	Hs01013996_m1
2713	*GKGK3P*	Glycerol kinase 3 pseudogene	Hs02340012_g1
1612	*DAPK1*	Death-associated protein kinase 1	Hs00234489_m1
6648	*SOD2*	Superoxide dismutase 2	Hs00167309_m1
85495	*RPPH1*	Ribonuclease P RNA component H1	Hs03297761_s1
7033	*TFF3*	Trefoil factor family 3	Hs00902278_m1
5296	*PIK3R2*	Phosphoinositide-3-kinase, regulatory subunit 2	Hs00178181_m1
4282	*MIF*	Macrophage migration inhibitory factor	Hs00236988_g1
3091	*HIF1A*	Hypoxia inducible factor 1, alpha subunit	Hs00153153_m1
8140	*SLC7A5*	Solute carrier family 7, member 5	Hs00185826_m1
57674	*RNF213*	Ring finger protein 213	Hs00899029_g1
65065	*NBEAL1*	Neurobeachin-like 1	Hs02517512_s1
10397	*NDRG1*	N-myc downstream-regulated gene 1	Hs00608387_m1
55971	*BAIAP2L*	Brain-specific angiogenesis inhibitor 1-associated protein 1	Hs00989192_m1
6781	*STC1*	Stanniocalcin 1	Hs00174970_m1