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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

relative quantity of each target gene mRNA to the endogenous control was calculated as ΔCq, where ΔCq = Cq target gene − Cq18S. The fold change of each target gene mRNA to the normal tissue sample was calculated as $2^{(-\Delta\Delta Cq)}$, where ΔΔCq = ΔCq target gene mRNA of endometrial cancer samples − ΔCq target gene mRNA of normal endometrium samples. The expression level was defined as being up-regulated in tumor tissues with a relative expression ratio more than 1, and was defined as being down-regulated in tumor tissues with a relative expression ratio less than 1.

Small interfering RNA (siRNA) library

The Reverse Transfection Format (RTF) siRNA library (Dharmacon, Thermo Fisher Scientific, Waltham, MA) was available in a series of 96-well plate with 0.5 nmol lyophilized siRNA per well. A smart pool approach incorporating four siRNAs targeting on each gene was utilized. The library was designed to include a total of nine targeted genes together with the non-targeting control siRNA (siNTC) acted as the control. All siRNAs were transfected into both cell lines using DharmaFECT 1 transfection reagent (Dharmacon) according to the manufacturer's instructions. At the end of incubation, cells were harvested and qPCR was used to determine the degree of knockdown.

Transient transfection of siRNA

siRNA corresponding to the target genes was designed and synthesized by Dharmacon. Each siRNA was transiently transfected into the cells with DharmaFECT 1 transfection reagent (Dharmacon) by the reverse transfection method. After 24 h, the transfection medium was changed with complete culture media and the cells were further incubated for another 48 h before being harvested for qPCR.

Cell proliferation assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) assay was performed to determine the extent of cell proliferation. Transfected cells were plated in a 96-well plate (2×10^3 cells/well) and incubated for 24, 48, 72 and 96 h. Then, 15 μl of MTT solution (5 mg/ml) (Amresco, Solon, OH) was added to each well and incubated for 4 h at 37 °C. After incubation, the formazan produced in the cells appeared as dark crystals in the bottom of the wells. To dissolve the formazan crystals, 100 μl of 10% sodium dodecyl sulphate (SDS) solution (Invitrogen) was added into each well, and incubated at 37 °C overnight. Finally, the absorbance was examined at 570 nm using a microplate reader (Sunrise, Tecan, Austria).

Cell migration assay

Cell migration assay was performed using the QCM™ 24-well Colorimetric Cell Migration Assay (Milipore Corporation, Billerica, MA). The assay was performed in a migration chamber, based on the Boyden chamber principle. Each insert utilizes an 8 μm pore polycarbonate filters. Transfected cells (2.5×10^5 cells) were added into the upper chamber, while the lower chamber was filled with 500 μl culture medium containing 10% FBS as a chemo-attractant. Cells were incubated in 5% CO_2 at 37 °C for 24 h. The migrated cells were stained for 20 min, followed by extraction buffer, and transferred to a 96-well plate for colorimetric measurement. The optical density was measured at 560 nm.

Cell invasion assay

Cell invasion was assessed by the QCM™ 24-well Collagen-based Cell Invasion Assay (Milipore), using inserts with 8 μm pore size polycarbonate membrane coated with a thin layer of polymerized collage. Prior to the assay, the membrane was rehydrated by adding 300 μl of serum-free medium into the insert for 30 min at room temperature. Following that, the rehydration medium was removed, and a total of 2.5×10^5 transfected cells were added into the upper chamber. The lower chamber was filled with 500 μl culture medium containing 10% FBS as a chemo-attractant. Cells were incubated in 5% CO_2 at 37 °C for 48 h. Migratory cells were stained and extracted prior to the measurement of the optical density at 560 nm.

Statistical analysis

Results are expressed as mean ± standard deviation of the mean, unless stated otherwise. GraphPad Prism 5.03 software (San Diego, CA) was used for all the statistic calculations and $p < 0.05$ was considered as the significance cut-off point. For the qPCR analysis of tissue samples, the non-parametric Mann–Whitney test was used to compare the mean values between the normal and tumor samples.

Results

Validation of a subset of up-regulated genes in endometrial cancer tissue samples

We carried out qPCR to validate the microarray data by Mokhtar et al. (2012). The candidate genes selected for the analysis were the top 20 genes from the list of significantly up-regulated genes in endometrial cancer. Our qPCR analysis showed that 19 out of 20 candidate genes were up-regulated in tumor samples compared to normal samples which were in concordance with the microarray data. Only one gene, *ROPN1L*, was found to be down-regulated by qPCR in tumor compared to normal samples. The number of tumor samples which over-expressed the candidate genes is also presented in Table 3.

Table 3
Up-regulated genes in endometrial cancer identified by microarray, and validated by qPCR. The qPCR was carried out on 15 normal and 9 tumor samples. Values for each gene were normalized to the level of endogenous gene, *18S*.

Genes	Microarray	qPCR	No. of tumor samples showing ≥ 2-fold up-regulated by qPCR
SLC38A1	3.162	2.908 ([a])	7/9 (78%)
DEPTOR1	2.583	2.211 (NS)	6/9 (67%)
APOC1	2.493	1.747 (NS)	6/9 (67%)
ROPN1L	2.304	0.906 (NS)	3/9 (33%)
LY75	2.241	1.487 (NS)	5/9 (56%)
STAT1	2.212	1.622 (NS)	4/9 (44%)
GKGK3P	2.178	2.214 ([a])	6/9 (67%)
DAPK1	2.098	3.487(NS)	6/9 (67%)
SOD2	2.095	2.042 ([a])	6/9 (67%)
RPPH1	2.092	1.223 (NS)	5/9 (56%)
TFF3	2.069	3.023 (NS)	4/9 (44%)
PIK3R2	2.004	2.179 (NS)	4/9 (44%)
MIF	1.944	2.764 ([a])	6/9 (67%)
HIF1A	1.866	1.903 (NS)	4/9 (44%)
SLC7A5	1.843	2.451 (NS)	5/9 (56%)
RNF213	1.828	3.030 (NS)	6/9 (67%)
NBEAL1	1.827	2.644 ([a])	6/9 (67%)
NDRG1	1.783	1.892 (NS)	4/9 (44%)
BAIAP2L	1.781	2.650 (NS)	7/9 (78%)
STC1	1.728	3.398 (NS)	5/9 (56%)

NS = not significant.
[a] $p < 0.05$ was accepted to be statistically significant.

Expression levels of candidate genes in endometrial cancer cell lines

The human endometrial cancer cell lines ECC-1 and HEC-1A were used in the study to investigate the roles of candidate genes in endometrial cancer. The relative expression of candidate genes was measured by qPCR. The ECC-1 cells were derived from a well-differentiated endometrial adenocarcinoma, while HEC-1A cells were derived from a moderately differentiated endometrial adenocarcinoma. As shown in Fig. 1, the qPCR identified nine genes (*SLC38A1, STAT1, SOD2, PIK3R2, MIF, HIF1A, SLC7A5, RNF213* and *BAIAP2L*) that were more highly expressed in both ECC-1 and HEC-1A cell lines compared with normal endometrial tissues. These genes were selected for further investigations.

Effect of gene knockdown on the expression levels of candidate genes in endometrial cancer cells

We used the RTF siRNA library plate as the siRNA screening platform to determine which siRNA is the most powerful in down-regulating the expression of the targeted genes. The results showed that the nine siRNAs have different abilities to alter the expression levels of corresponding genes in ECC-1 and HEC-1A cells. After 48 h, the expression of *SLC38A1*, *SOD2*, *MIF*, *HIF1A* and *SLC7A5* genes were decreased more than 85% in both ECC-1 and HEC-1A cells transfected with the specific siRNA targeting the respective genes compared to the siNTC-transfected cells (Fig. 2a). The percentage reduction of the corresponding expression levels in ECC-1 cells transfected with si*STAT1*, si*RNF213* and si*BAIAP2L* ranged between 52% and 81% compared to the control cells. Meanwhile in HEC-1A cells transferred with si*STAT1*, si*RNF213* and si*BAIAP2L*, the expression levels were reduced between 50% and 84% of the levels seen in the control cells. ECC-1 and HEC-1A cells transfected with si*PIK3R2* did not show any alteration in the expression of *PIK3R2* gene. The siRNAs targeting *STAT1*, *RNF213*, *BAIAP2L* and *PIK3R2* genes were excluded from further experiments as they did not give significant reduction of the targeted genes in both cell lines.

Reverse-transfection of ECC-1 and HEC-1A cells with the specific siRNAs targeting *SLC38A1*, *SOD2*, *MIF*, *HIF1A* and *SLC7A5* genes

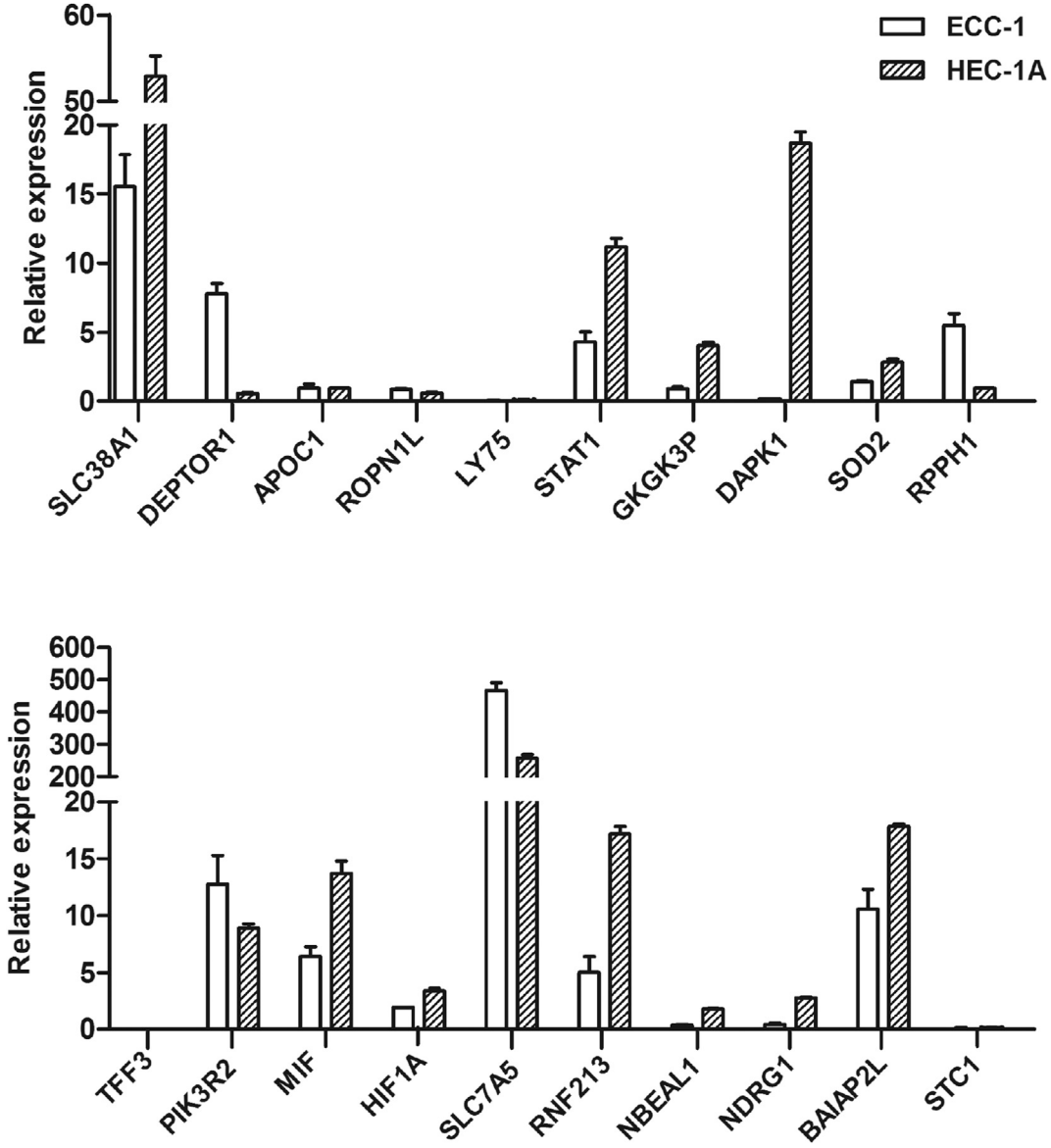

Fig. 1. Expression levels of 20 candidate genes in ECC-1 and HEC-1A endometrial cancer cell lines detected by qPCR. The mean and standard deviation of the candidate genes expression levels are shown. The data represent triplicate measurements from single RNA samples.

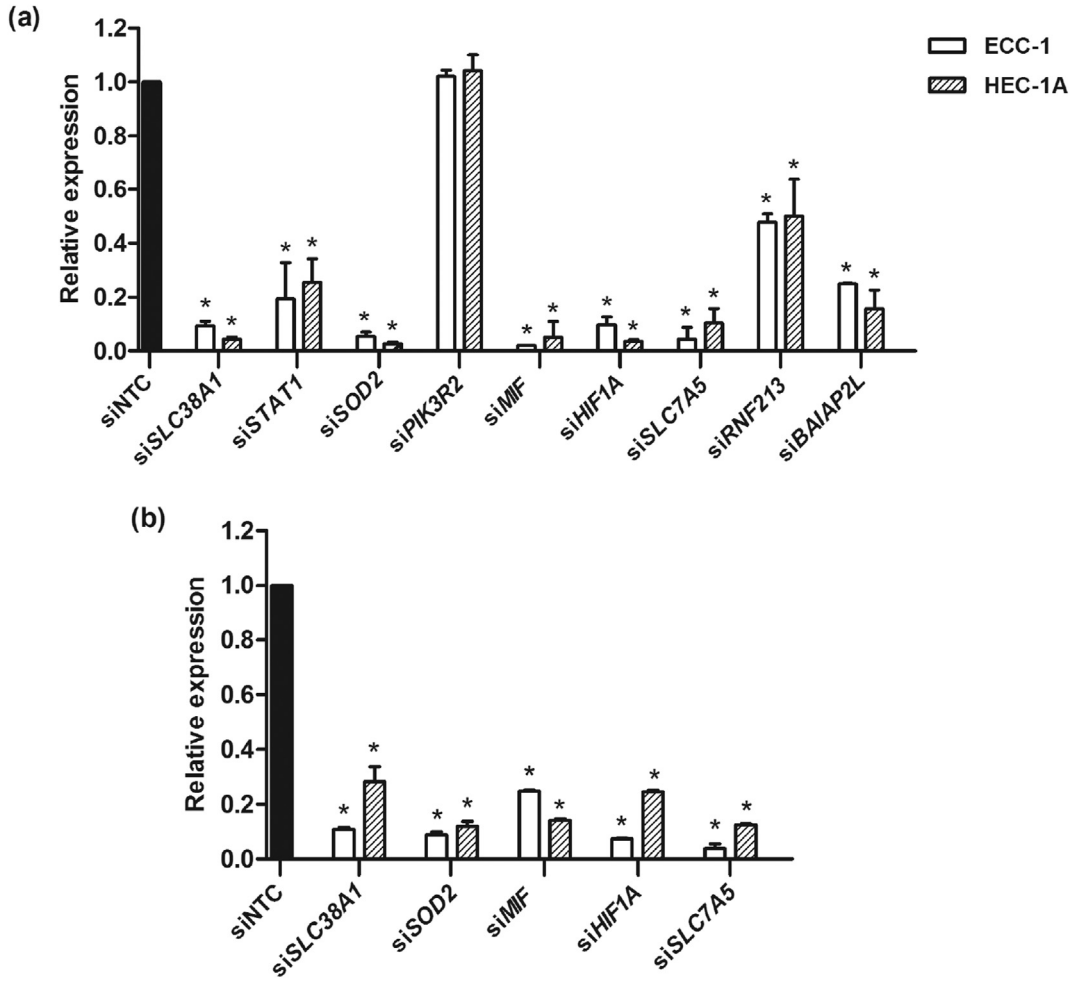

Fig. 2. Effect of siRNA knockdown on candidate genes expression levels in endometrial cancer cell lines. (a) ECC-1 and HEC-1A cells were transfected with a panel of 9 siRNAs using the RTF siRNA library plate. Expression of each gene was analyzed by qPCR 48 h after transfection. (b) Gene specific siRNA targeting *SLC38A1*, *SOD2*, *MIF*, *HIF1A* and *SLC7A5* were reverse-transfected into ECC-1 and HEC-1A cells. To determine the gene silencing efficiency associated with siRNA treatment, qPCR was performed. The values are the mean ± standard deviation of three independent experiments. Cells transfected with siNTC were used as control. $^{*}p < 0.05$, significantly different from siNTC-transfected cells.

showed more than 70% reduction of the respective gene expression levels when compared to the levels seen in siNTC-transfected cells (Fig. 2b). Thus, the results confirmed that the siRNAs targeting the *SLC38A1*, *SOD2*, *MIF*, *HIF1A* and *SLC7A5* genes were efficiently introduced into both cell lines and down-regulated the expression levels of the respective genes.

Effect of gene knockdown on the proliferation of endometrial cancer cells

To determine whether the decrease in expression levels of *SLC38A1*, *SOD2*, *MIF*, *HIF1A* and *SLC7A5* genes in ECC-1 and HEC-1A cells following siRNA transfection affected cell proliferation, MTT assay was carried out. The si*SLC38A1*-transfected cells exhibited no difference in the proliferation of ECC-1 cells, but reduced proliferation of HEC-1A cells with a significant difference observed at 96 h (Fig. 3a). As shown in Fig. 3b and d, knockdown of *SOD2* and *HIF1A* genes by RNAi significantly reduced the proliferation ability of ECC-1 and HEC-1A cells over the period tested. The knockdown of *MIF* by RNAi significantly reduced the proliferation of ECC-1 cells as early as 24 h, but in HEC-1A cells, a significant decrease in the cell proliferation was only observed at 96 h (Fig. 3c). Transfection of si*SLC7A5* led to a significant decrease in the proliferation of ECC-1 cells at 48 and 72 h, and in HEC-1A cells at 72 and 96 h (Fig. 3e).

Effect of gene knockdown on the migration and invasion of endometrial cancer cells

Next, we examined the effects of *SLC38A1*, *SOD2*, *MIF*, *HIF1A* and *SLC7A5* knockdown by RNAi on the migration and invasion of endometrial cancer cells. After transfection, cells were transferred to the upper chamber of a 24-well migration plate and incubated for 48 h as described in the Materials and Methods section. The results demonstrated that the knockdown of *SOD2*, *MIF* and *SLC7A5* genes by RNAi significantly decreased the migration ability of HEC-1A cells when compared with the siNTC-transfected cells (Fig. 4a). A decrease in the migration of ECC-1 cells transfected with si*SLC38A1*, si*SOD2*, si*MIF* and si*SLC7A5* was observed when compared to siNTC-transfected cells. However, the differences were not significant. Transfection of si*SLC38A1* did not alter the cell migration of HEC-1A cells. A similar result was also observed in ECC-1 and HEC-1A cells transfected with si*HIF1A*.

The cell invasion assay demonstrated that there is no significant differences in the cells transfected with siRNAs targeting *SLC38A1*,

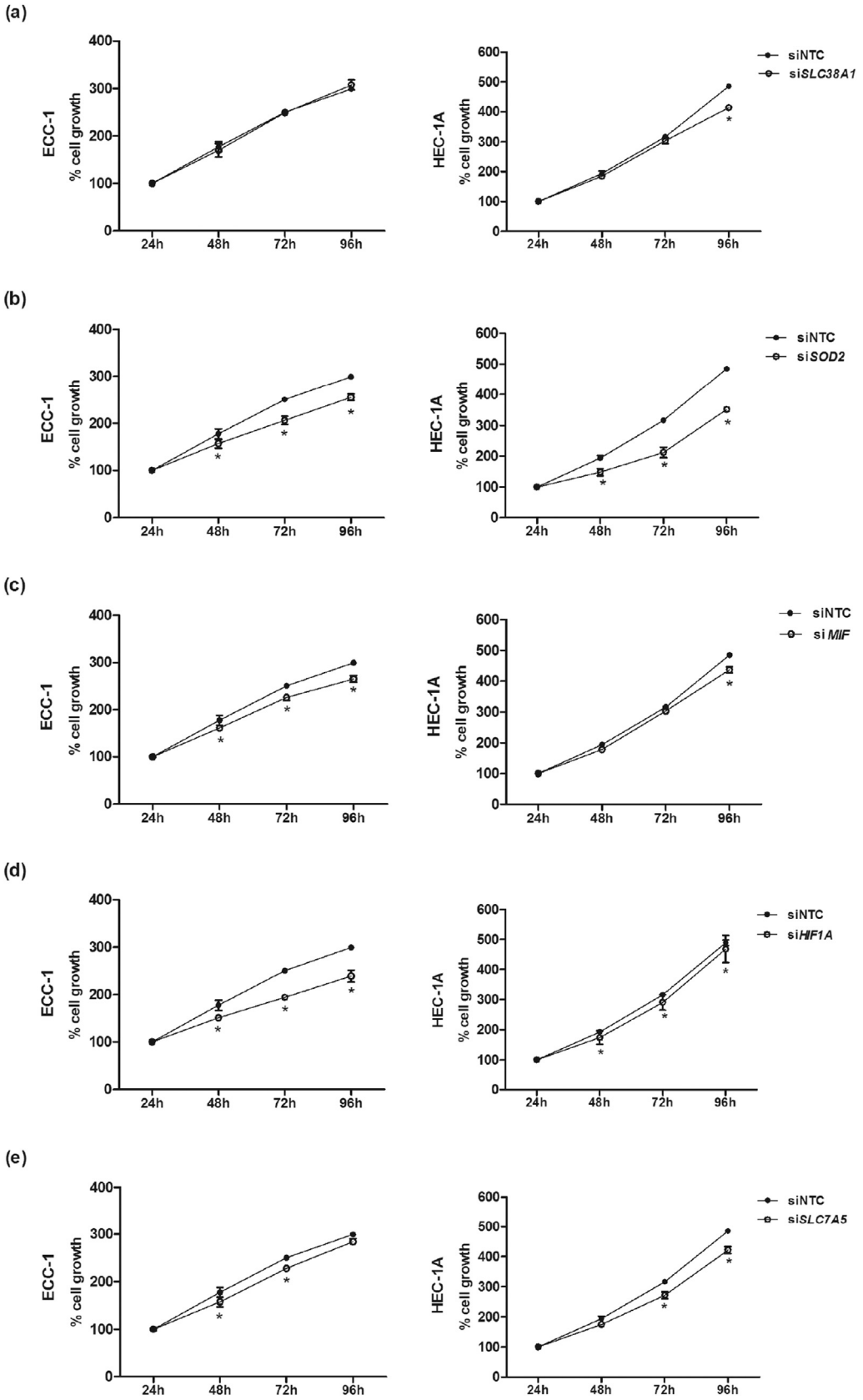

Fig. 3. Effect of (a) *SLC38A1*, (b) *SOD2*, (c) *MIF*, (d) *HIF1A* and (e) *SLC7A5* gene knockdown on endometrial cancer cell proliferation. ECC-1 cells (left) and HEC-1A (right) were transfected with siRNA targeting respective genes or siNTC. Cell proliferation was monitored at the indicated time points by MTT assay. The values are the mean ± standard deviation of three independent experiments. Cells transfected with siNTC were used as control. $^{*}p < 0.05$, significantly different from siNTC-transfected cells.

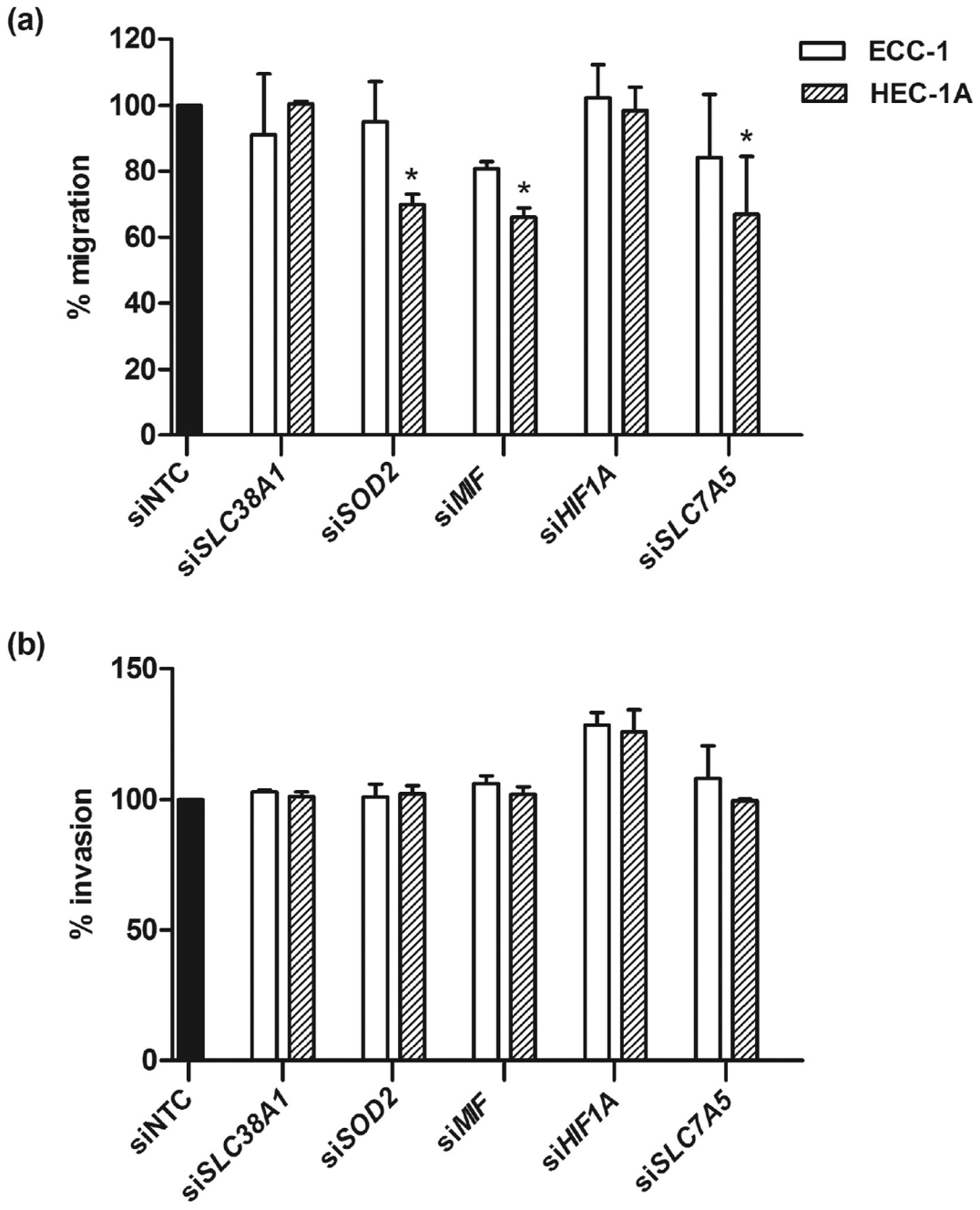

Fig. 4. Endometrial cancer cell migration and proliferation after *SLC38A1*, *SOD2*, *MIF*, *HIF1A* and *SLC7A5* gene knockdown by siRNA transfection. (a) Transfected cells were allowed to migrate towards FBS for 24 h. Migratory cells on the bottom of the polycarbonate membrane were stained and quantified at OD 560 nm after extraction. (b) Transfected cells were allowed to invade towards FBS for 48 h. Invasive cells on the bottom of the invasion membrane were stained and quantified at OD 560 nm after extraction. Values are expressed as mean ± standard deviation of triplicate determinations. Cells transfected with siNTC were used as control. $^{*}p < 0.05$, significantly different from siNTC-transfected cells.

SOD2, *MIF*, *HIF1A* and *SLC7A5* genes when compared with the siNTC-transfected cells (Fig. 4b).

Discussion

In an attempt to identify endometrial cancer-related genes, we previously performed a microarray analysis to identify the differentially expressed genes in normal endometrial and endometrial cancer tissue samples [13]. Multiple studies have already used gene expression microarrays to examine endometrial cancer and has identified a number of genes as being differentially expressed between endometrial cancer and normal endometrial tissue samples [8,13,14]. These studies provided important insights into the molecular basis of endometrial cancer. However, most of these studies did not expand their analyses to address the biological function of individual genes identified by the microarray.

In this study, we carried out qPCR on 24 endometrial tissue samples to validate the microarray results by Mokhtar et al. (2012) and to identify the relevant target genes that may be involved in the pathogenesis of the disease. A total of 20 genes from the list of significantly up-regulated genes in endometrial cancer were chosen for the analysis. A few of these genes have previously been described in the literature to be associated with endometrial cancer, thus our work validated their findings.

Among the genes we validated were the *Trefoil factor family 3* (*TFF3*). *TFF3* gene is located at 21q22.3 and is a member of the *TFF*-domain peptide family [15]. The *TFF* peptides function to maintain the mucosal surface integrity and to repair the mucosal through motogenic and antiapoptotic activities [15,16]. Up-regulation of *TFF3* in cancers promote cell migration, invasion and metastasis through reduction of cell–cell and cell–matrix interactions as well as enhancement of cell-scattering in bronchiole or other epithelia cells [17]. Activation of various pathways related to cellular proliferation, apoptosis, migration, invasion and clonogenic survival such as *PI3K*, *MAPK* and *JAK/STAT* was accompanied with up-regulation of *TFF3* in cancers [18]. A meta-analysis of gene expression in endometrial cancer identifies *TFF3* as one of the up-regulated genes in endometrioid endometrial cancer compared to non-endometrioid endometrial cancer [19]. *TFF3* has been observed to be ~8-fold up-regulated in endometrioid endometrial cancer compared to normal endometrium [8]. The microarray result was further validated using qPCR (TaqMan) and showed a ~40-fold up-regulation of *TFF3* in endometrioid endometrial cancer compared to normal endometrium [8]. Our microarray and qPCR showed a 2-fold and 3-fold up-regulation of *TFF3*, respectively (Table 2). The role of *TFF3* in endometrial cancer cells (*in vitro*) has yet been examined [15]. However, the compiling evidences makes *TFF3* an attractive target for future therapy in the management of endometrial cancer patients.

In addition, there are a few candidate genes in our study that have not been captured by previous studies. For example, *Staniocalcin 1* (*STC1*) is a glycoprotein hormone that functions in wound healing, mitochondria metabolism, macrophage chemotaxis and calcium/phosphate regulation [20,21]. Increased expression of *STC1* gene has been observed in colorectal, gastric and ovarian cancers [20–22]. A recent publication reported that silencing of *STC1* in hepatocellular carcinoma cells lead to the down-regulation of secretory *STC1* level and the suppression of lung metastasis in *in vivo* mouse model [23]. *STC1* was found to be expressed in human endometrial tissue [24]. However, there have been no reports on *STC1* expression in endometrial cancer.

Another example of candidate gene that have not been captured by previous studies is the *Lymphocyte antigen 75* (*LY75*) gene. This gene is mapped at 2q24, and belongs to a collagen-binding mannose family receptor [25]. Faddaoui et al. (2016) reported that *LY75* is over-expressed in advanced epithelial ovarian cancer (EOC) and suppression of this gene induces mesenchymal-to-epithelial transition in EOC cell lines with mesenchymal morphology. In addition, the migratory and invasive capacity was reduced *in vitro*, and the tumor cell colonization and metastatic growth was enhanced *in vivo* [26]. In an earlier study, over-expression of *LY75* was suggested to be an additional mechanism by which *interleukin-6* enhances the progression of ovarian cancer [25]. The same study also proposed that by blocking *LY75*, it could reduce the early metastasis of ovarian cancer [25].

We focused on five candidate genes for mechanistic investigation. In order to examine the roles of *SLC38A1*, *SOD2*, *MIF*, *HIF1A* and *SLC7A5* genes in endometrial cancer pathogenesis, we used RNAi to down-regulate the expression of the corresponding genes in ECC-1 and HEC-1A endometrial cancer cell lines. RNAi is a process where 21- to 25-nucleotide siRNAs are used to interfere the expression of a specific gene [27,28]. It has been demonstrated that gene expression knockdown by siRNA significantly suppressed tumor growth and metastasis [29]. Therefore, gene expression inhibition using siRNA is a suitably good approach to develop effective therapy against cancer.

Solute carrier family 38 member 1 (*SLC38A1*), a subtype of the amino acid transport system A, has been linked with cancer development and progression [30]. It was suggested that *SLC38A1* is highly activated in cancer to provide glutamine to the tumor cells, which is the primary metabolic fuel and nitrogen source for the cell metabolism and proliferation [31]. In this study, we have shown that *SLC38A1* is up-regulated in endometrial cancer tissues and cell lines. Previously, the up-regulation of the gene was significantly related with tumor size, lymph node metastasis and advanced stage of breast cancer [30]. The down-regulation of *SLC38A1* by siRNA reduced the proliferation ability in HEC-1A cells, but no effect was seen in the invasion ability of the cells. A similar finding was observed in a study using human pancreatic cancer cells [32].

We also found that *Superoxide dismutase 2* (*SOD2*) is one of the up-regulated genes in endometrial cancer tissues compared to normal endometrial tissues. Similar *SOD2* expression patterns were observed in tongue squamous cell carcinoma and ovarian cancer [33,34]. Expression of *SOD2* was also increased in endometrial cancer cell lines. Our study indicated that the knockdown of *SOD2* by siRNA reduced the endometrial cancer cell proliferation. It is documented that the *in vitro* and *in vivo* loss of *SOD2* activity induces reactive oxygen species (ROS), which trigger the signals to inhibit cell proliferation, thereby leading to cell death *via* apoptosis or necrosis [35,36]. Furthermore, RNAi targeting *SOD2* reduced the ability of migration in HEC-1A cells. Knockdown of *SOD2* by RNAi has been demonstrated to significantly reduce the cell migration of tongue squamous cancer cells [37].

Macrophage migration inhibitory factor (*MIF*) is also up-regulated in endometrial cancer and is associated with the inhibition of distant and lymphatic spread [38]. Functional studies suggested that *MIF* is able to inactivate *p53*, enhance angiogenesis or act through the *Rho*-dependent pathways to aid the development of cancer [39]. Here, we demonstrated that the *MIF* gene is up-regulated in endometrial cancer tissues and cell lines, a finding which supports that of Giannice et al. (2013). Another endometrial cancer study associated over-expression of *MIF* mRNA and protein with low histological grade, early FIGO stages and no lymphovascular invasion [40]. The overall data suggested that *MIF* up-regulation may be associated with the inhibition of metastatic spread, but the up-regulation could also promote tumor progression [40]. We also found that down-regulation of *MIF* expression in endometrial cancer cells contributed to the reduction in cell proliferation ability. In addition, knockdown of *MIF* in HEC-1A cells by RNAi led to a decrease in the migration ability. To our knowledge, this is the first report to demonstrate the association of *MIF* with endometrial cancer cell migration.

Moreover, we found that *Hypoxia inducible factor 1, alpha subunit* (*HIF1A*) is up-regulated in endometrial cancer tissues and cell lines. In normal condition, *HIF1A* protein accumulates in the cytosol [41,42]. However, during hypoxia, the protein translocates to the nucleus hence activating the hypoxia-sensitive genes such as *VEGF* [41,42]. Generally, *HIF1A* is present at low levels in cells but an elevated level of *HIF1A* was found in tumor cells [43]. The over-expression of *HIF1A* has been previously correlated with the *VEGF* pathway, increased microvessel density and poor prognosis in Stage 1 endometrioid carcinoma [44]. Here, we demonstrated that the knockdown of *HIF1A* by RNAi reduced the endometrial cancer cell proliferation. However, the knockdown does not cause any effect on the migration and invasion ability of the endometrial cancer cells.

Another amino acid transporter might also be a potential molecular target for endometrial cancer therapy. *Solute carrier family 7, member 5* (*SLC7A5*) is an L-type transporter which functions as the Na^+-independent transport of large neutral amino acids such as leucine, isoleucine and valine [45]. It was hypothesized that *SLC7A5* provides the essential amino acids that enhance the tumor cell growth through the *mammalian target-of-rapamycin* (*mTOR*)-stimulated translation [46]. Our data showed that *SLC7A5* expression is

up-regulated in endometrial cancer tissues and cell lines. In addition, we found that the knockdown of *SLC7A5* by RNAi reduced cell proliferation of endometrial cancer cells. Fan et al. (2010) reported that *SLC7A5*, as a single entity may have a limited impact on cell proliferation of human ovarian cancer [47]. The decreased expression of *SLC7A5* by RNAi also decreased the migration ability of HEC-1A cells.

In conclusion, we showed that *SOD2*, *MIF* and *SLC7A5* genes were up-regulated in endometrial cancer, and the down-regulation of these genes by RNAi decreased the proliferation and migration potential of endometrial cancer cells. In addition, the *SLC38A1* and *HIF1A* genes were also observed to be up-regulated in endometrial cancer, and its down-regulation by RNAi resulted in the decrease of endometrial cancer cell proliferation but not its migration potential. These results indicated that *SLC38A1*, *SOD2*, *MIF*, *HIF1A* and *SLC7A5* genes appear to play a role in the development of the disease. This is the first report documenting the involvement of *MIF* and *SLC7A5* genes with endometrial cancer cell proliferation and migration, as well as the involvement of *SLC38A1* genes in endometrial cancer proliferation. Further studies are necessary to understand the detailed mechanisms on how these genes regulate endometrial cancer proliferation and migration. These additional approaches will be beneficial to develop effective diagnostic and management strategies for endometrial cancer.

Conflicts of interest statement

The authors have no conflicts of interest relevant to this article.

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