Epithelial-mesenchymal Transition and Cancer Stem Cells in Endometrial Cancer

ANNA SUPERNAT[1], SYLWIA ŁAPIŃSKA-SZUMCZYK[2], HANNA MAJEWSKA[3], JACEK GULCZYŃSKI[4], WOJCIECH BIERNAT[3], DARIUSZ WYDRA[2] and ANNA J. ŻACZEK[1]

[1]*Department of Medical Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Gdańsk, Poland;*
[2]*Department of Gynaecology, Gynaecological Oncology and Gynaecological Endocrinology, Medical University of Gdańsk, Gdańsk, Poland;*
[3]*Department of Pathomorphology, Medical University of Gdańsk, Gdańsk, Poland;*
[4]*Department of Pathology and Neuropathology, Medical University of Gdańsk, Gdańsk, Poland*

Abstract. *Background/Aim: Epithelial-mesenchymal transition (EMT) and cancer stem cells (CSCs) are presumed to be key conditions for malignancy. Data concerning their role in endometrial cancer (EC) are scarce. We aimed to investigate the possible link between EMT and CSCs markers in EC samples. Materials and Methods: The study encompassed 156 primary tumour samples. Using RT-qPCR, we analyzed the expression of EMT-related genes, SNAIL and SLUG, and the CSCs marker CD133. Results: SNAIL and SLUG correlated with each other (R=0.33; p=0.00003). All the studied genes were expressed in both normal and malignant endometrial tissue. Decreased SNAIL expression was found to correlate with post-menopausal status (p=0.002). Decreased SLUG expression was associated with shorter overall survival (p=0.01). Conclusion: SLUG expression could serve as a prognostic factor in EC. No correlation between the expression of EMT and CSCs markers was found, suggesting there to be no association between the EMT and CSC phenotype in endometrial cancer.*

Endometrial cancer (EC) is the most frequent malignancy of the female genital tract in the Western world, with an estimated incidence of 10 to 20 per 100,000 women (1). Despite the high prevalence, understanding of its molecular background with regard to pathogenesis and disease progression remains unclear. Data concerning the role of cancer stem cells (CSCs) and epithelial-to-mesenchymal transition (EMT) in EC are especially scarce.

CSCs are perceived as germinative tumor cells that possess an ability to initiate and sustain tumour growth (2, 3). The presence of CSCs has been reported in many cancers, including melanoma, brain tumours, breast cancer, lung cancer, and leukaemia (4-8). Recent studies proclaim CD133 (membrane glycoprotein also known as prominin, official gene symbol *PROM1*) as a reliable marker for CSCs identification (9). During epithelial-to-mesenchymal transition epithelial cells acquire mobility as well as mesenchymal-like expression profile. This phenomenon has been discovered as a crucial mechanism which allows for invasion and metastasis of cancer (10-12). To undergo EMT, cancer cells down-regulate E-cadherin expression. This is partially achieved through the activation of transcription factors: SNAIL (*SNAI1*), and SLUG (*SNAI2*) (13).

Accumulating evidence suggests that EMT induces stem cell activity (14). This phenomenon was first observed by Mani *et al*. (15). The EMT-CSCs dependency has been confirmed in many different types of cancer (16-20). However, the data about this relationship in EC are scarce. The sole existing experimental study confirmed the relationship between EMT and CSCs in endometrial cancer cell lines, not tumour samples (21). As Tanaka *et al*. pointed to the important role of EMT in endometrial cancer specimens (22), we aimed to investigate the possible link between EMT and CSCs markers in EC tumor samples. We studied the relationship between the gene expression of EMT-related *SNAIL* and *SLUG*, and CSCs marker – *CD133* in EC specimens. To the best of our knowledge, we are the first group to present data on gene expression level of *SNAIL* and *SLUG* in relation to *CD133*. Expression levels are also compared with normal endometrium, clinical data, and patients' outcome.

Correspondence to: Anna J. Żaczek, Laboratory of Cell Biology, Department of Medical Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Dębinki 1, 80-211 Gdańsk, Poland. Tel: +48 583491438, Fax +48 583491445, e-mail: azaczek@gumed.edu.pl

Key Words: Epithelial-mesenchymal transition, cancer stem cells, molecular markers, quantitative PCR, endometrial cancer.

Materials and Methods

Patients and tissues. In the present study 156 fresh-frozen primary tumour samples retrospectively collected from a cohort of endometrial cancer patients who were operated on in the Department of Gynaecology, Gynaecological Oncology and Gynaecological Endocrinology (Medical University of Gdansk) between 2005 and 2011, were analyzed. Each patient was primarily treated by surgery, with the possible option of radiotherapy and/or chemotherapy administration. The inclusion criteria were operable endometrial cancer (stage IVB patients underwent cytoreductive surgery) confirmed by histological examination and a signed consent form. The study was accepted by the Ethics Committee of the Medical University of Gdansk.

The samples were collected from the core of the tumour by surgical excision prior to any systemic treatment and were immediately frozen and stored in –80˚C. They were kept on ice during transport. The tumour samples included all stages of endometrial carcinoma, from non-invasive stage IA to metastatic IVB cancers, as distinguished by FIGO in 2009 (International Federation of Gynecology and Obstetrics) (23). The patients' characteristics are summarised in Table I. The median age was 63 (range: 30-87) years. Patients with a body-mass index higher than 30 were classified as obese (24). The progesterone receptor (PR), oestrogen receptor-α (ERα), and Human epidermal growth factor receptor-2 (HER2) status was determined immunohistochemically (IHC) using Allred score (positivity cut-off: ≥3) for PgR and ERα and the HercepTest, with the score of 3 classified as HER2-positive. A survival analysis was performed for 94 patients. After a median follow-up of 54.5 months (range: 0-96), 16 patients (17.0%) had died. The last follow-up data were collected in May 2013. The study was performed in accordance with the REMARK criteria (25).

Controls. Normal endometrium from patients treated for ailments other than EC was collected as control samples. Control samples were accessed histopathologically; no hyperplasia was observed. For standard curve generation synthetic templates, 60-mer single-stranded oligonucleotides composed of the first (5') and last (3') 30 nucleotides of the amplicon sequence, were used.

RNA isolation. Prior to nucleic acid isolation, tissue specimens (25 mg per sample) were homogenized (1 min, 6,000 rpm) using a MagNALyser (F. Hoffmann-La Roche Ltd, Basel, Switzerland). RNA was isolated with AllPrep DNA/RNA Mini Kit (Qiagen, Hamburg GmbH, Hamburg, Germany), according to the manufacturer's instructions. After the isolation, the RNA concentration and integrity were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Median RNA concentration was 628.8 ng/μl (range: 20.5-1968.6), median RIN (RNA Integrity Number) was: 7.9 (range: 2.2-9.6). The RNA samples were stored in –80˚C. RNA was subsequently reverse-transcribed to cDNA with the Transcriptor First Strand cDNA Synthesis Kit (F. Hoffmann-La Roche Ltd, Basel, Switzerland) according to the manufacturer's instructions, using random hexamer primers. The total amount of RNA per reaction was 1000 ng.

geNorm pilot study. A geNorm pilot experiment was performed to select proper, stably-expressed reference genes for data normalization, using a set of 10 candidate reference genes on 10 representative endometrial samples (both tumors and controls). Data analysis was performed using Biogazelle's qbase+ software (Biogazelle NV, Zwijnaarde, Belgium) which is built upon a state-of-the-art and proven quantification model (26). To determine the best reference genes, the geNorm module in qbase+ was used. geNorm is the most popular algorithm to find stable reference genes (27). Three reference genes were chosen.

Genes and primers. SYBR Green I-based PrimePCR assays for the three genes of interest (*SNAIL, SLUG, CD133*), and reference

Table I. *Clinicopathological data (n=156).*

Variable	Number of cases (%)
Menopausal status	
Pre-menopausal	8 (5.1%)
Peri-menopausal	11 (7.1%)
Post-menopausal	135 (86.5%)
Missing data	2 (1.3%)
Obesity	
Absent	66 (42.3%)
Present	88 (56.4%)
Missing data	2 (1.3%)
Histology	
Endometroid	123 (78.8%)
Non-endometroid	33 (21.2%)
Stage (FIGO*)	
IA-IB	113 (72.4%)
II	21 (13.5%)
IIIA-IIIC	19 (12.2%)
IVA-IVB	3 (1.9%)
Grade	
I	60 (38.5%)
II	69 (44.2%)
III	23 (14.7%)
Missing data	4 (2.6%)
Cervical invasion	
Absent	114 (73.1%)
Present	40 (25.6%)
Missing data	2 (1.3%)
Myometrial infiltration	
≤1/2	88 (56.4%)
>1/2	67 (42.9%)
Missing data	1 (0.6%)
Metastases	
Absent	106 (67.9%)
Present	47 (30.1%)
Missing data	3 (1.9%)
PgR status	
Negative	12 (7.7%)
Positive	94 (60.3%)
Missing data	50 (32.1%)
ER status	
Negative	8 (5.1%)
Positive	98 (62.3%)
Missing data	50 (32.1%)
HER2 status	
Negative	89 (57.1%)
Positive	17 (10.9%)
Missing data	50 (32.1%)

*FIGO, International Federation of Gynecology and Obstetrics.

Table II. *Amplicon seqences*.

Gene	Ensembl Gene ID	Amplicon sequence	Amplicon length [bp]
CD133	ENSG00000007062	GTTTCCGACTCCTTTTGATCCGGGTTCTTACCTGGTGATTTGCCACAAAACCATAGAAG ATGCCAATGCTTATTATTATACAAATCACCAACAGGGAGATTGCAAAGCATTTCCTCAG GAAGGGCCCATTTTCCTTCTGTCGCTGGTGCATTTCTCCACCACATTTGTTACAGCAA	146
SNAIL	ENSG00000124216	CGCGAATCGGCGACCCCAGTGCCTCGACCACTATGCCGCGCTCTTTCCTCGTCAGG AAGCCCTCCGACCCCAATCGGAAGCCTAACTACAGCGAGCTGCAGGACTCTAATCC	82
SLUG	ENSG00000019549	CTCCTGAGCTGAGGATCTCTGGTTGTGGTATGACAGGCATGGAGTAACTCTCATA GAGATACGGGGAAATAATCACTGTATGTGTGTCCAGTTCGCTGTAGTTTGGCT TTTTGGAGGCGTTGAAATGCTTCTTGACCAGGAAGGAGCGCGGCATCTTGC	129

targets were designed using validated pipeline primerXL for design and in silico verification of high quality assays. All assays are wet-lab validated and evaluated with the gold standard method of standard curves based on a 6-point 10-fold serial dilution series using synthetic templates to create a broad detection range. The serial dilution was applied to determine assay efficiency, sensitivity and linearity. The performance and functionality of the assays was further evaluated on gDNA, cDNA and non-template controls. Subsequently, next-generation sequencing of the amplicons was performed to verify the specificity. Amplicon sequences are presented in Table II. Used PrimePCR assays met the highest quality and specificity available. All the analyses were performed by Biogazelle (Biogazelle NV, Zwijnaarde, Belgium) and were fully MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments) compliant (28). Quality control on the post-qPCR data was performed using Biogazelles's qbase+ software (Biogazelle NV, Zwijnaarde, Belgium).

qPCR-based gene expression analysis. For gene expression profiling of the genes of interest and the reference genes, validated standard operating procedures for verification of the quality of the cDNA, qPCR amplification, and data analysis were performed. All measurements were performed using CFX384 instrument (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in 384-well plates in a reaction volume of 5 µl. Reaction mixture included 10 ng cDNA, 2× SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and forward and reverse primers at a final concentration of 250 nM. Negative control (no reverse transcriptase) was always included. Each PCR reaction was performed in duplicate. Sample maximization method was implemented. For 25% of the samples (randomly selected), cDNA quality was assessed by means of three different gene expression qPCR assays (high expressed, medium expressed and low expressed gene). Gene expression analysis was performed using Biogazelle's qbase+ software (Biogazelle NV, Zwijnaarde, Belgium).

Statistical analysis. STATISTICA software (Statsoft, Inc., Tulsa, OK, USA, version 10) was used for all calculations. The tests that were used and their applications were as follows: testing normality of the data set — Shapiro-Wilk test; comparison of the expression in the normal endometrium *versus* tumour samples – box-and-whisker diagram with Mann-Whitney test used for *p*-value determination; correlations between continuous expression quantities – Spearman's correlation; correlations between continuous relative expression and clinicopathological data of the patients – Mann-Whitney test. The Kaplan-Meier estimator was employed for survival analysis, and the results were verified with the F Cox test. The end-point for the study was overall survival (OS). OS was defined as the time from sample collection to death or censoring. Censoring was defined as loss of follow-up or alive at the end of follow-up. Statistical significance was assumed when $p \leq 0.05$. Cox proportional hazards regression analysis was used to identify the independent predictors of OS. Univariate predictors significant with a value of $p \leq 0.10$ were entered into a step-wise multivariate model to identify those with independent prognostic information. For Kaplan-Meier curve generation and univariate analysis, tumors were dichotomized based on a median expression value used as a cut-off.

Table III. *Assay validation results*.

Gene	*CD133*	*SNAIL*	*SLUG*
Efficiency (%)	98	98	103
R^2	0.9996	0.9997	0.9991
cDNA Cq	23.14	23.12	20.79
cDNA Tm (Celsius)	82	85.5	81.5
Specificity (%)	100	100	100

Results

General. Out of 160 RNA samples (including 4 control samples), 160 (100%) had amplifiable cDNA.

Assay validation. Serial dilution was applied to determine assay efficiency, sensitivity, and linearity (Table III). The three most stably-expressed reference genes (*ERCC6, HMBS, UBE4A*) were retained from geNorm analysis for further gene expression normalization. High reference target stability was observed, with average geNorm M≤0.5. No indications for failed cDNA synthesis were observed. The results confirmed that the experiment was performed according to the internal quality standards: replicate variability fell within the set limit of 0.5 cycles for 87.6% of sample-target combinations. Through analyzing the samples, medium-to-low reference target stability was observed for the selected

Table IV. *Correlations between gene expression of studied genes.*

	CD133		*SNAIL*		*SLUG*	
	r	*p*-Value	r	*p*-Value	r	*p*-Value
CD133			–0.05	0.49	–0.13	0.08
SNAIL					0.33	0.00003
SLUG						

reference genes (average geNorm M value between 0.5 and 1), which is typically seen in a heterogeneous set of samples.

mRNA expression within clinical and control samples. The studied genes were expressed in both normal and malignant endometrial tissues (Figure 1a, 1b and 1c). A trend was observed towards higher mRNA *CD133* expression in EC samples compared to normal endometrium samples (p=0.06). Expression of *SNAIL* and *SLUG* was reduced in EC samples (p=0.01 and p=0.002, respectively).

Gene expression correlation and mRNA expression in clinical samples. Expression levels of *CD133, SNAIL* and *SLUG* were compared with each other (Table IV). Expression levels of *CD133, SNAIL* and *SLUG* were compared with clinical and pathological data (Table V).

Survival analysis. No prognostic impact was observed for *SNAIL* and *CD133* (Figure 2a and 2b). Decreased *SLUG* expression correlated with shorter OS (p=0.01; Figure 2c). In univariate analysis, factors predicting for shortened OS included type II histology, higher stage and grade, myometrial invasion, presence of metastases, positive PgR status and *SLUG* overexpression. Step-wise multivariate analysis showed higher FIGO stage, and decreased *SLUG* gene expression to be associated with decreased OS (Table VI).

Discussion

EMT and CSCs are presumed to be key conditions for epithelial malignancy (2, 11). Thus, the mRNA expression of the genes responsible for the aforementioned phenomena was assumed to characterize between the endometrium lacking EMT and CSCs and endometrial cancer. We found *CD133, SNAIL* and *SLUG* mRNA expression to vary greatly even within the control samples. Similar observations with regard to *CD133* mRNA levels were reported in human endometrial cancer cell lines, Hec1A, Hec1B, AN3CA and Ishikawa (29). Many EMT markers were also reported to be highly expressed in normal tissues (30). In our study, the levels of *CD133* expression in control and EC samples did not differ significantly and the expression of *SLUG* and *SNAIL* was even more reduced in EC samples compared to normal endometrium. Observations concerning *CD133* might be partially explained as the expression of this protein was reported in the endometrial glandular cells (31). An analogous study revealed CD133 to be commonly expressed not only in cholangiocarcinoma but also in many kinds of cells in normal liver (32). It was reported that CD133 exhibits a variable expression in endometrial primary tumours, ranging from 1.3% to 62.6% (33). Low *SNAIL* expression in EC samples when compared to control endometrium stays in contrast to the results of Montserrat *et al.* where *SNAIL* mRNA expression was reported to be higher in EC samples (34). This analysis, however, included a relatively small set of EC samples (42) and was based on only one control sample which might lead to confounding results, given the high variability observed in controls. Our observations are shared by the results of qPCR analysis in breast cancer cell lines that also showed decreased *SNAIL* mRNA in relation to normal mammary cells (35). Finally, reduced SLUG expression was also detected in invasive breast cancer when compared with normal breast epithelium (30).

Even though *SNAIL* and *SLUG* mRNA expression correlated with each other, we failed to find a link between these genes and *CD133*. Studies performed by Na *et al.* did indeed report such an association in hepatocellular carcinoma (36), however, they were performed on cell lines, not tumours. Several similar results were reported (36-38), but they also concerned individual cells, not actual patients' samples. Storci *et al.* managed to show a correlation between CD133 protein expression and high *SLUG* mRNA levels in breast cancer tumours, yet the study included only 21 specimens (39).

The strongest correlation was found between post-menopausal status and decreasing *SNAIL* expression (40). Results obtained by Scherbakov *et al.* (41) revealed the inverse relationship between ERα and SNAIL. The authors speculated there might be a negative feedback between ERα and SNAIL which would explain the decrease of *SNAIL* expression in post-menopausal patients. Similarly to our work, studies of Martin *et al.* (42) on *SNAIL* mRNA expression in breast cancer were often inconclusive but they also rather pointed to *SNAIL* being reduced in more advanced disease.

In our study, *SLUG* expression correlated conversely with advanced age and HER2-positivity. Similar results concerning HER2 status were reported in breast cancer (39). These authors also failed to find any association of SLUG expression with other clinicopathological data.

SLUG mRNA expression was significantly reduced in patients with poor prognosis. Similar results in EC, but concerning SNAIL mRNA expression, have been reported (42). Numerous studies confirmed CD133 overexpression to be the indicator of poor outcome in cancer (43-48) but so far there is no conclusive evidence showing such prognostic value in EC. Also, search for prognostic significance of EMT

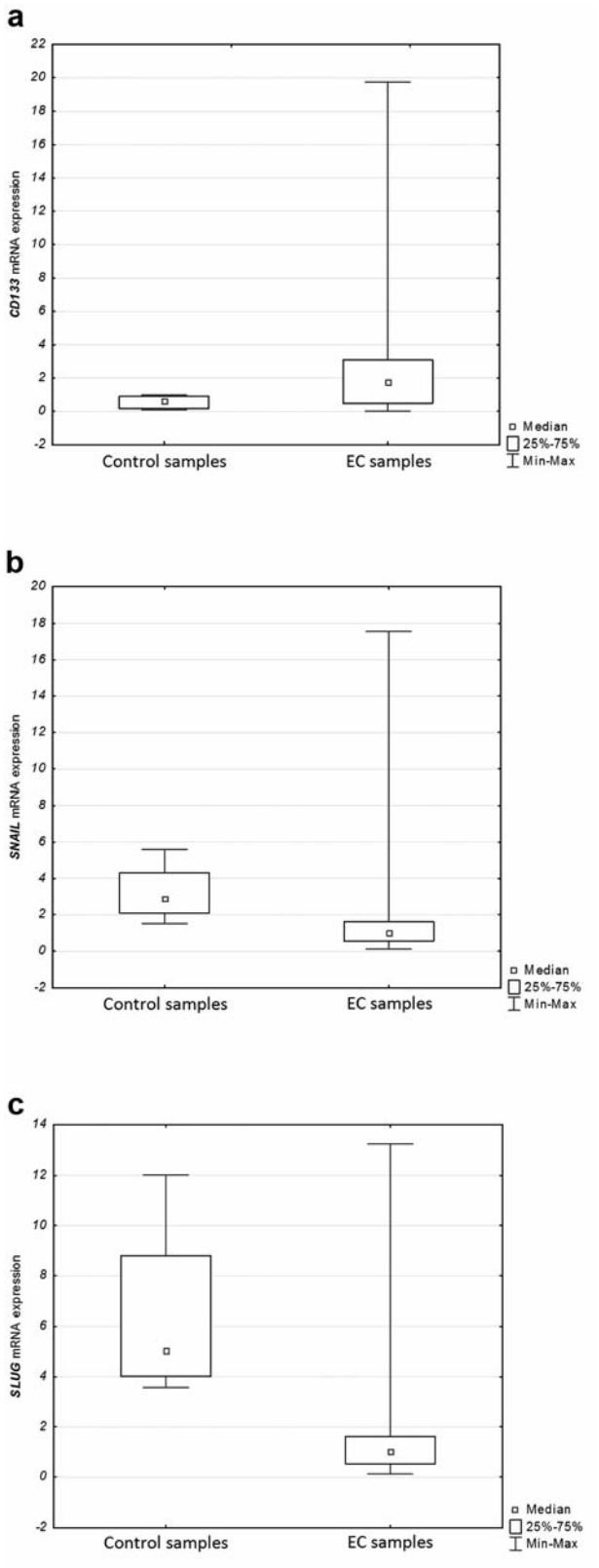

Figure 1. *a: CD133 mRNA expression in EC and control samples. b: SNAIL mRNA expression in EC and control samples. c: SLUG mRNA expression in EC and control samples.*

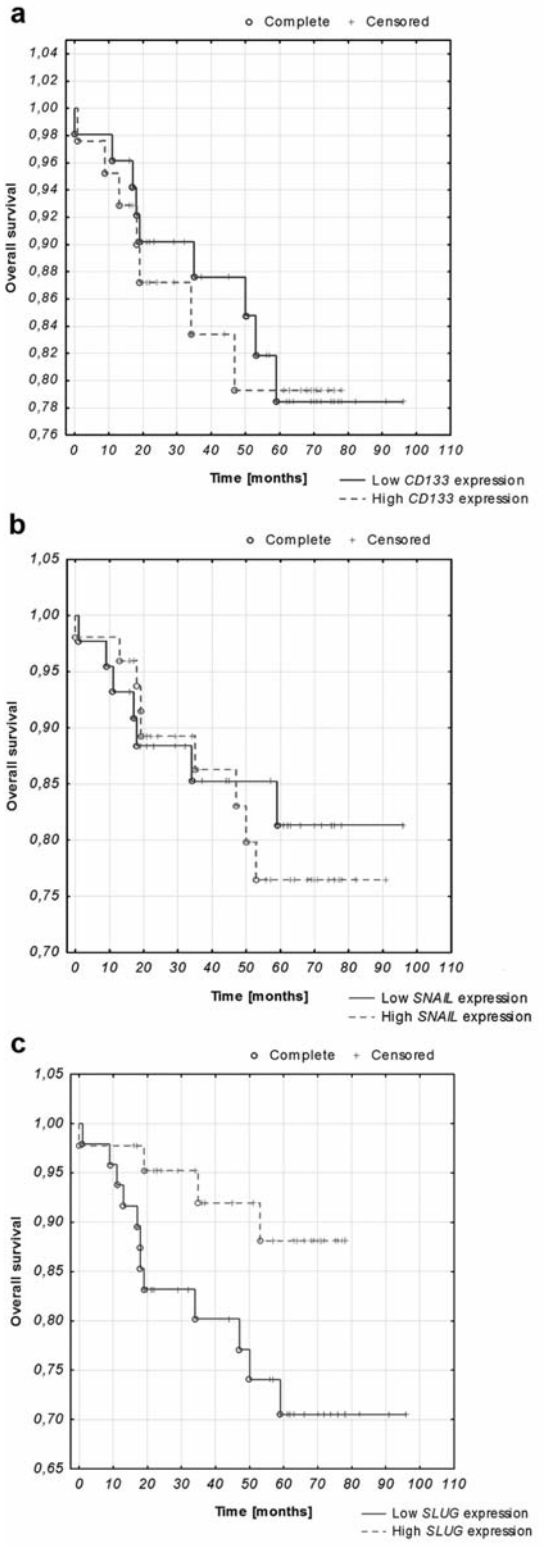

Figure 2. *a: Kaplan-Meier curves presenting OS of endometrial cancer patients stratified against CD133 status. b: Kaplan-Meier curves presenting OS of endometrial cancer patients stratified against SNAIL status. c: Kaplan-Meier curves presenting OS of endometrial cancer patients stratified against SLUG status.*

Table V. *Median expression in the context of clinicopathological data.*

			CD133		*SNAIL*		*SLUG*	
		Number of cases	Median expression	*p*-Value	Median expression	*p*-Value	Median expression	*p*-Value
Menopausal status	Pre-menopausal	19	1.17	0.09	1.71	0.002	1.62	0.02
	Post-menopausal	135	1.90		0.96		0.79	
Histology	Type I	123	1.97	0.01	0.98	0.11	0.98	0.43
	Type II	33	1.03		1.25		0.73	
Stage	I, II	134	1.67	0.63	0.99	0.05	0.93	0.68
	III, IV	22	2.30		1.12		0.98	
Grade	1, 2	129	1.97	0.01	0.90	0.13	0.90	1.00
	3	23	0.36		1.33		1.05	
Cervical invasion	Absent	114	1.45	0.04	1.05	0.55	0.88	0.98
	Present	40	2.30		0.94		0.99	
Myometrial infiltration	≤1/2	88	1.65	0.57	1.03	0.62	1.00	0.12
	>1/2	67	1.94		0.96		0.79	
Metastases	Absent	106	1.47	0.14	1.03	0.77	0.88	0.89
	Present	47	2.26		0.98		1.01	
ER status	Negative	8	1.31	0.52	1.41	0.09	0.69	0.94
	Positive	98	1.67		0.97		0.72	
PgR status	Negative	12	0.69	0.10	1.47	0.12	0.65	0.81
	Positive	94	1.71		0.97		0.72	
HER2 status	Negative	89	1.65	0.32	1.03	0.11	0.77	0.02
	Positive	17	2.05		0.60		0.50	

Table VI. *Univariate and multivariate analysis of clinicopathological and molecular parameters as prognostic factors in endometrial cancer patients.*

Parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Obesity (present vs.absent)	1.01	0.37-2.83	0.97			
Histology (Type II vs I)	2.98	1.08-8.27	0.03		NS	
Stage (III, IV vs I, II)	7.05	2.51-19.83	0.002	8.60	2.85-25.93	0.0001
Grade (3 *vs*.1,2)	2.10	1.25-3.53	0.005		NS	
Cervical invasion (present *vs*. absent)	2.20	0.80-6.07	0.12			
Myometrial infiltration (present *vs*. absent)	3.14	1.13-8.72	0.03		NS	
Metastases (present *vs*. absent)	3.29	1.23-8.84	0.01		NS	
PgR status (positive *vs*. negative)	0.29	0.10-0.84	0.02		NS	
ER status (positive *vs*. negative)	0.76	0.16-3.55	0.73			
HER2 status (positive *vs*. negative)	1.37	0.37-5.05	0.64			
CD133 status (positive *vs*. negative)	1.09	0.40-2.92	0.87			
SNAIL status (positive *vs*. negative)	1.15	0.43-3.09	0.78			
SLUG status (positive *vs*. negative)	0.34	0.11-1.06	0.06	0.28	0.09-0.91	0.03

in head and neck squamous cell carcinoma yielded no definitive conclusions (14). On the other hand, high SNAIL expression was associated with poor patient prognosis in prostate cancer (49), bladder cancer (50), or hilarcholangiocarcinoma (51). In EC, EMT status, defined as reduced E-cadherin and increased SNAIL expression, was also reported to have prognostic value but there are no survival analyses focusing on SNAIL and SLUG expression independently (22). Our data showing *SLUG* expression as the indicator of good prognosis are in compliance with the work on EMT markers in diffuse large B-cell lymphoma (52). Also, similar results, regarding TWIST expression, were obtained by Montserrat *et al.* (53). The authors suggested that this down-regulation might in fact facilitate tumour invasiveness, a possibility worthy of further exploration.

We were unable to confirm that *CD133, SNAIL* and *SLUG* expression are unambiguously implicated in cancer tumorigenicity. Nevertheless, studies supporting those facts were often based on cell lines or animal models (29, 54, 55), not tumour samples collected from patients.

One of the limitations of the study was the short follow-up times and missing survival data for 62 patients. However, the possible explanation for numerous inconclusive results may lie in tumor heterogeneity. The samples used for RNA isolation might not reflect the heterogeneity across a tumor, as they were excised manually from a single tumor region and only small tissue fragments were used. As EMT actively occurs at multiple sites and CSCs are distributed in a heterogeneous manner within the tumor, it is possible that the excised fragments were not representative for the studied phenomena. CD133-positive cells comprise only 5.7% to 27.4% of primary tumour in EC (29). The phenotype of EMT is also difficult to capture as these cells constitute minority in primary tumours (56). Furthermore, studying primary tumours-alone might not be sufficient. It has recently been reported that gene expression of SNAIL and SLUG can be easier captured in lymph node metastasis compared to matching primary tumors and provides more prognostic information (57). Metastatic deposits contain selected, more aggressive clones of tumor cells that more closely determine clinical course of disease. Therefore, analysis of prognostic markers in such a subpopulation of cancer cells might provide with more conclusive results. Unfortunately, this kind of material was unavailable for EC samples. Perhaps a micro-dissection technique of formalin-fixed paraffin-embedded EC samples might help to further overcome the problem tumor heterogeneity.

It is possible that the RT-qPCR approach is simply not able to detect EMT-related gene expression within primary tumor bulk, where tumor cells with EMT characteristics might be diluted within the rest of tumor cells. Nevertheless, tremendous effort has been put to ensure the highest possible quality of the obtained results. Quality control tests showed excellent reaction performance, specificity of the designed primers, high cDNA/RNA integrity and quality, and thus no sample loss. The developed protocol allows for highly coherent transcriptomic measurements.

The crucial role of EMT and CSCs in driving malignancy is widely described in various cancers (32, 36, 42, 48, 58, 59). Also the link between those two phenomena leading to development of CSCs due to EMT activation has been extensively investigated (14, 15, 20, 30, 60, 61). However, data concerning EMT in relation to CSC in EC are scarce. To the best of our knowledge, we are the first to present data on gene expression levels of the EMT-related markers *SNAIL* and *SLUG* in relation to the CSC marker *CD133*. Unfortunately, mRNA expression levels of *SNAIL, SLUG* and *CD133* do not provide with valuable information on EMT and CSCs in EC. We found no link between the expression of these markers, which might suggest there is no link between EMT and CSC phenotype in EC cancer or additional markers should be examined. Further studies, including larger groups of patients and probably more markers are needed to elucidate whether EMT and CSCs markers carry clinically-significant information in EC.

Acknowledgements

We would like to acknowledge Agata Łuczak for her excellent technical support. This work was supported by a grant from the National Science Centre (5715/B/P01/2010/38) and a grant from the Foundation for Polish Science Parent-Bridge Programme co-financed by the European Union within the European Regional Development Fund (DPS-424-5053/11).This research was co-financed by the European Commission in the frame of the European Social Fund, by the European Social Fund, the State Budget, and the Pomorskie Voivodeship Budget according to the Operational Programme Human Capital, Priority VIII, Action 8.2, Under-action 8.2.2: 'Regional Innovative Strategy' within the system project of the Pomorskie Voivodeship "InnoDoktorant – Scholarships for PhD students, Vth edition". The publication was also financed by the European Social Fund as a part of the project "Educators for the elite - integrated training program for PhD students, post-docs and professors as academic teachers at University of Gdansk" within the framework of the Human Capital Operational Programme, Action 4.1.1, Improving the quality of educational offer of tertiary education institutions. The sponsors had no involvement in the study design, collection, analysis, and interpretation of the data, writing the manuscript, or the decision to submit the manuscript for publication.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

References

1 Mirantes C, Espinosa I, Ferrer I, Dolcet X, Prat J and Matias-Guiu X: Epithelial-to-mesenchymal transition and stem cells in endometrial cancer. Hum Pathol, 2013.

2 Lobo NA, Shimono Y, Qian D, and Clarke MF: The biology of cancer stem cells. Annu Rev Cell Dev Biol *23*: 675-699, 2007.

3 Ward RJ and Dirks PB: Cancer stem cells: at the headwaters of tumor development. Annu Rev Pathol *2*: 175-189, 2007.

4 Fang D, Nguyen TK, Leishear K *et al*: A tumorigenic subpopulation with stem cell properties in melanomas. Cancer Res *65*: 9328-9337, 2005.

5 Hemmati HD, Nakano I, Lazareff JA *et al*: Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci USA *100*: 15178-15183, 2003.

6 Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ and Clarke MF: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA *100*: 3983-3988, 2003.

7 Kim CF, Jackson EL, Woolfenden AE *et al*: Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell *121*: 823-835, 2005.

8 Yilmaz OH, Valdez R, Theisen BK *et al*: Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature *441*: 475-482, 2006.

9 Mizrak D, Brittan M and Alison M: CD133: molecule of the moment. J Pathol *214*: 3-9, 2008.

10 Guarino M, Rubino B and Ballabio G: The role of epithelial-mesenchymal transition in cancer pathology. Pathology *39*: 305-318, 2007.

11 Thiery JP: Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer *2*: 442-454, 2002.

12 Hugo H, Ackland ML, Blick T *et al*: Epithelial – mesenchymal and mesenchymal – epithelial transitions in carcinoma progression. J Cell Physiol *213*: 374-383, 2007.

13 Moreno-Bueno G, Portillo F and Cano A: Transcriptional regulation of cell polarity in EMT and cancer. Oncogene *27*: 6958-6969, 2008.

14 Chen C, Zimmermann M, Tinhofer I, Kaufmann AM and Albers AE: Epithelial-to-mesenchymal transition and cancer stem(-like) cells in head and neck squamous cell carcinoma. Cancer Lett, 2012.

15 Mani SA, Guo W, Liao MJ *et al*: The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell *133*: 704-715, 2008.

16 Morel AP, Lièvre M, Thomas C, Hinkal G, Ansieau S and Puisieux A: Generation of breast cancer stem cells through epithelial-mesenchymal transition. PLoS One *3*: e2888, 2008.

17 Biddle A, Liang X, Gammon L *et al*: Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. Cancer Res *71*: 5317-5326, 2011.

18 Chu PY, Hu FW, Yu CC *et al*: Epithelial-mesenchymal transition transcription factor ZEB1/ZEB2 co-expression predicts poor prognosis and maintains tumor-initiating properties in head and neck cancer. Oral Oncol *49*: 34-41, 2013.

19 Li Y, Wang L, Pappan L, Galliher-Beckley A and Shi J: IL-1β promotes stemness and invasiveness of colon cancer cells through Zeb1 activation. Mol Cancer *11*: 87, 2012.

20 Fan F, Samuel S, Evans KW *et al*: Overexpression of Snail induces epithelial-mesenchymal transition and a cancer stem cell-like phenotype in human colorectal cancer cells. Cancer Med *1*: 5-16, 2012.

21 Kusunoki S, Kato K, Tabu K *et al*: The inhibitory effect of salinomycin on the proliferation, migration and invasion of human endometrial cancer stem-like cells. Gynecol Oncol *129*: 598-605, 2013.

22 Tanaka Y, Terai Y, Kawaguchi H *et al*: Prognostic impact of EMT (epithelial-mesenchymal-transition)-related protein expression in endometrial cancer. Cancer Biol Ther *14*: 13-19, 2013.

23 Pecorelli S: Revised FIGO staging for carcinoma of the vulva, cervix, and endometrium. Int J Gynaecol Obstet *105*: 103-104, 2009.

24 Consultation WE: Appropriate body-mass index for Asian populations and its implications for policy and intervention strategies. Lancet *363*: 157-163, 2004.

25 McShane LM, Altman DG, Sauerbrei W *et al*: REporting recommendations for tumor MARKer prognostic studies (REMARK). Nat Clin Pract Oncol *2*: 416-422, 2005.

26 Hellemans J, Mortier G, De Paepe A, Speleman F and Vandesompele J: qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol *8*: R19, 2007.

27 Vandesompele J, De Preter K, Pattyn F *et al*: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol *3*: RESEARCH0034, 2002.

28 Bustin SA, Benes V, Garson JA *et al*: The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem *55*: 611-622, 2009.

29 Friel AM, Zhang L, Curley MD *et al*: Epigenetic regulation of CD133 and tumorigenicity of CD133 positive and negative endometrial cancer cells. Reprod Biol Endocrinol *8*: 147, 2010.

30 Alkatout I, Wiedermann M, Bauer M, Wenners A, Jonat W and Klapper W: Transcription factors associated with epithelial-mesenchymal transition and cancer stem cells in the tumor centre and margin of invasive breast cancer. Exp Mol Pathol *94*: 168-173, 2013.

31 Schwab KE, Hutchinson P and Gargett CE: Identification of surface markers for prospective isolation of human endometrial stromal colony-forming cells. Hum Reprod *23*: 934-943, 2008.

32 Thanan R, Pairojkul C, Pinlaor S *et al*: Inflammation-related DNA damage and expression of CD133 and Oct3/4 in cholangiocarcinoma patients with poor prognosis. Free Radic Biol Med, 2013.

33 Rutella S, Bonanno G, Procoli A *et al*: Cells with characteristics of cancer stem/progenitor cells express the CD133 antigen in human endometrial tumors. Clin Cancer Res *15*: 4299-4311, 2009.

34 Montserrat N, Mozos A, Llobet D *et al*: Epithelial to mesenchymal transition in early stage endometrioid endometrial carcinoma. Hum Pathol *43*: 632-643, 2012.

35 Okubo T, Truong TK, Yu B *et al*: Down-regulation of promoter 1.3 activity of the human aromatase gene in breast tissue by zinc-finger protein, snail (SnaH). Cancer Res *61*: 1338-1346, 2001.

36 Na DC, Lee JE, Yoo JE, Oh BK, Choi GH and Park YN: Invasion and EMT-associated genes are up-regulated in B viral hepatocellular carcinoma with high expression of CD133-human and cell culture study. Exp Mol Pathol *90*: 66-73, 2011.

37 Ding Q, Yoshimitsu M, Kuwahata T *et al*: Establishment of a highly migratory subclone reveals that CD133 contributes to migration and invasion through epithelial-mesenchymal transition in pancreatic cancer. Hum Cell *25*: 1-8, 2012.

38 Cai C, Yu JW, Wu JG *et al*: [CD133 promotes the invasion and metastasis of gastric cancer via epithelial-mesenchymal transition]. Zhonghua Wei Chang Wai Ke Za Zhi *16*: 662-667, 2013.

39 Storci G, Sansone P, Trere D *et al*: The basal-like breast carcinoma phenotype is regulated by SLUG gene expression. J Pathol *214*: 25-37, 2008.

40 Blechschmidt K, Kremmer E, Hollweck R *et al*: The E-cadherin repressor snail plays a role in tumor progression of endometrioid adenocarcinomas. Diagn Mol Pathol *16*: 222-228, 2007.

41 Scherbakov AM, Andreeva OE, Shatskaya VA and Krasil'nikov MA: The relationships between snail1 and estrogen receptor signaling in breast cancer cells. J Cell Biochem *113*: 2147-2155, 2012.

42 Martin TA, Goyal A, Watkins G and Jiang WG: Expression of the transcription factors snail, slug, and twist and their clinical significance in human breast cancer. Ann Surg Oncol *12*: 488-496, 2005.

43 Okudela K, Woo T, Mitsui H, Tajiri M, Masuda M and Ohashi K: Expression of the potential cancer stem cell markers, CD133, CD44, ALDH1, and β-catenin, in primary lung adenocarcinoma--their prognostic significance. Pathol Int *62*: 792-801, 2012.

44 Woo T, Okudela K, Mitsui H *et al*: Prognostic value of CD133 expression in stage I lung adenocarcinomas. Int J Clin Exp Pathol *4*: 32-42, 2010.
45 Alamgeer M, Ganju V, Szczepny A *et al*: The prognostic significance of aldehyde dehydrogenase 1A1 (ALDH1A1) and CD133 expression in early stage non-small cell lung cancer. Thorax, 2013.
46 Shibahara I, Sonoda Y, Saito R *et al*: The expression status of CD133 is associated with the pattern and timing of primary glioblastoma recurrence. Neuro Oncol, 2013.
47 Wang K, Xu J, Zhang J and Huang J: Prognostic role of CD133 expression in colorectal cancer: a meta-analysis. BMC Cancer *12*: 573, 2012.
48 Mehra N, Penning M, Maas J *et al*: Progenitor marker CD133 mRNA is elevated in peripheral blood of cancer patients with bone metastases. Clin Cancer Res *12*: 4859-4866, 2006.
49 Whiteland H, Spencer-Harty S, Thomas DH *et al*: Putative prognostic epithelial-to-mesenchymal transition biomarkers for aggressive prostate cancer. Exp Mol Pathol, 2013.
50 Kawashima A, Takayama H, Kawamura N *et al*: Co-expression of ERCC1 and Snail is a prognostic but not predictive factor of cisplatin-based neoadjuvant chemotherapy for bladder cancer. Oncol Lett *4*: 15-21, 2012.
51 Kong D, Liang J, Li R *et al*: Prognostic significance of snail expression in hilar cholangiocarcinoma. Braz J Med Biol Res *45*: 617-624, 2012.
52 Lemma S, Karihtala P, Haapasaari KM *et al*: Biological roles and prognostic values of the epithelial-mesenchymal transition-mediating transcription factors Twist, ZEB1 and Slug in diffuse large B-cell lymphoma. Histopathology *62*: 326-333, 2013.
53 Montserrat N, Gallardo A, Escuin D *et al*: Repression of E-cadherin by SNAIL, ZEB1, and TWIST in invasive ductal carcinomas of the breast: a cooperative effort? Hum Pathol *42*: 103-110, 2011.
54 Wang Z, Ali S, Banerjee S *et al*: Activated K-Ras and INK4a/Arf deficiency promote aggressiveness of pancreatic cancer by induction of EMT consistent with cancer stem cell phenotype. J Cell Physiol *228*: 556-562, 2013.
55 Zhu LF, Hu Y, Yang CC *et al*: Snail overexpression induces an epithelial to mesenchymal transition and cancer stem cell-like properties in SCC9 cells. Lab Invest *92*: 744-752, 2012.
56 De Wever O, Pauwels P, De Craene B *et al*: Molecular and pathological signatures of epithelial-mesenchymal transitions at the cancer invasion front. Histochem Cell Biol *130*: 481-494, 2008.
57 Markiewicz A, Ahrends T, Wełnicka-Jaśkiewicz M *et al*: Expression of epithelial to mesenchymal transition-related markers in lymph node metastases as a surrogate for primary tumor metastatic potential in breast cancer. J Transl Med *10*: 226, 2012.
58 Kim A, Bae YK, Gu MJ *et al*: Epithelial-mesenchymal transition phenotype is associated with patient survival in small intestinal adenocarcinoma. Pathology, 2013.
59 Nakamura M, Kyo S, Zhang B *et al*: Prognostic impact of CD133 expression as a tumor-initiating cell marker in endometrial cancer. Hum Pathol *41*: 1516-1529, 2010.
60 Hollier BG, Tinnirello AA, Werden SJ *et al*: FOXC2 expression links epithelial-mesenchymal transition and stem cell properties in breast cancer. Cancer Res *73*: 1981-1992, 2013.
61 Turner C and Kohandel M: Investigating the link between epithelial-mesenchymal transition and the cancer stem cell phenotype: A mathematical approach. J Theor Biol *265*: 329-335, 2010.

Received September 24, 2013
Revised October 25, 2013
Accepted October 29, 2013