Deregulation of *RAD21* and *RUNX1* expression in endometrial cancer

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Received March 7, 2012; Accepted June 25, 2012

DOI: 10.3892/ol.2012.794

Abstract. Cohesins and cohesin-regulated genes are deregulated in numerous types of human cancer. However, data concerning their status and role in endometrial cancer are scarce. This study aimed to determine the clinical significance of *double-strand-break repair protein rad21 homolog* (*RAD21*) and *runt-related transcription factor 1* (*RUNX1*) gene dosage and mRNA expression in endometrial cancer. RAD21 is a component of the cohesin complex, crucial for chromosome segregation and DNA repair. RUNX1 is the transcription factor implicated in RAD21 regulation. The study group included 144 endometrial cancer patients. *RAD21* and *RUNX1* expression profiles were measured by reverse-transcription quantitative PCR. *RAD21* gene dosage was determined by quantitative PCR. *RAD21* gene dosage was associated with *RAD21* mRNA expression (ϱ=0.22; p=0.009). Furthermore, *RAD21* expression strongly correlated with *RUNX1* expression (ϱ=0.43; p<0.0000001). Increased *RAD21* gene dosage correlated with more advanced tumor stage (p=0.021), higher grade (p=0.021), cervical involvement (p=0.01) and the absence of obesity (p=0.025), while *RAD21* mRNA expression correlatd with cervical involvement (p=0.027). The mRNA expression of *RAD21* and *RUNX1* was found to be deregulated and co-dependent in endometrial cancer. *RAD21* gene dosage is associated with unfavorable tumor characteristics. However, elucidating the role of these molecular markers in endometrial oncogenesis requires further investigation, including functional studies and survival analysis.

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Key words: endometrial cancer, molecular markers, RAD21, RUNX1, gene expression, gene dosage

Introduction

Endometrial cancer is the most frequent malignancy of the female genital tract, with an estimated 46,470 cases and 8,120 mortalities expected to be recorded in 2011 in the USA (1). Despite such high prevalence, the understanding of its molecular background in terms of genesis, growth and progression remains insufficient. Furthermore, little is known concerning factors which would allow for the differentiation between types I and II endometrial cancer, which differ substantially in prognosis.

In view of the poor understanding of the molecular background of endometrial cancer we have attempted to identify new markers which may: i) correlate with patients' clinicopathological features; ii) further elucidate molecular pathways of endometrial carcinogenesis; iii) aid the differentiation of types I and II.

One of the key elements to be investigated in this particular context are cohesins: multisubunit protein complexes which are highly conserved and play canonical roles in processes such as chromatin regulation, chromosome segregation and DNA damage response (2-4). Is has been shown that cohesin-defective cells possess features known to be crucial drivers of oncogenesis. These features include genomic instability, impaired DNA repair and anomalies concerning gene expression (4-7). The deregulation of cohesin expression and cohesin-regulated genes is common in numerous types of human cancer (8-13), including endometrial cancer (14). Furthermore, cohesin-defective cells have been discovered to be sensitive to ionizing radiation and DNA-damaging drugs (8,15).

RAD21 (double-strand-break repair protein rad21 homolog), a mammalian ortholog of Mcd1p, is one of the four core proteins comprising a cohesin ring in sister chromatid cohesion (SSC), a physical linkage between sister chromatids. SSC allows for cell cycle checkpoint control and homologous repair of DNA double-strand breaks (16). Experiments performed on zebrafish revealed *rad21* to be a regulator of *runx1* (6). RUNX1/AML1 (runt-related transcription factor 1/acute myeloid leukemia 1) belongs to the family of RUNX transcription factors which, when complexed with other

proteins, activates or represses the transcription of regulators involved in cell differentiation, growth and survival. The *RUNX* genes function as tumor suppressors and dominant oncogenes, depending on the context (17).

RAD21 and RUNX1 actions are crucial for sustaining basic functions in healthy cells. These two markers have been found to be deregulated in different types of tumors, including endometrioid, prostate, breast and oral squamous carcinoma together with acute lymphoblastic leukemia (8,9,11,12,14,18-25). The present study was designed to address the hypothesis of cohesin deregulation in endometrial cancer. It aimed to investigate *RAD21* and *RUNX1* mRNA expression profiles with the use of reverse transcription quantitative PCR in endometrial cancer tumors. Additionally, *RAD21* mRNA expression was compared with *RAD21* gene dosage measured by quantitative PCR, as it has been shown that copy number variations (CNVs) are common in various types of cancer (26) and that some of them may contribute to aberrant cohesin expression in cancer (9,16).

Materials and methods

Patients and tissues. The retrospective study encompassed 144 frozen tumor samples collected from a cohort of endometrial cancer patients treated at the Department of Gynaecology, Gynaecological Oncology and Gynaecological Endocrinology (Medical University of Gdańsk, Gdańsk, Poland) between 2005 and 2011. The inclusion criteria were operable endometrial cancer confirmed by histological examination and a signed consent form. The characteristics of the patients are summarized in Table I. The mean age was 63.3 years (range, 30-87). The study was accepted by the Ethics Committee of the Medical University of Gdańsk.

Tumor samples were collected by surgical excision prior to any systemic treatment and were immediately frozen and stored at -80˚C. Tissue samples covered the spectrum of pathological stages of endometrial carcinoma, from non-invasive IA to metastatic IVB cancer according to the staging by FIGO in 2009 (International Federation of Gynecology and Obstetrics) (27). A Ca-125 level between 0 and 35 U/ml was considered normal (28). Patients with a body mass index >30 were classified as obese (29).

DNA and RNA isolation. Prior to nucleic acid isolation, tissue specimens (25 mg per sample) were homogenized (1 min, 6,000 rpm) with the use of MagNA Lyser (Roche, Basel, Switzerland). DNA and RNA were isolated with AllPrep DNA/RNA Mini kit (Qiagen, Hilden, Germany) using the tissue protocol, in accordance with the manufacturer's instructions. After the isolation, DNA/RNA concentration and purity were determined by Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Good quality DNA was defined as an $A_{260\ nm/280\ nm}$ ratio between 1.70 and 1.90. Good quality RNA was defined as an $A_{260\ nm/280\ nm}$ ratio of ~2.

RNA was subsequently reverse transcribed to cDNA with the Transcriptor First Strand cDNA Synthesis kit (Roche), according to the manufacturer's instructions, with the use of random hexamer primers. There was 1000 ng of total RNA per reaction.

Table I. Clinicopathological data (n=144).

Variable	Number of cases (%)
Menopausal status	
Premenopausal	9 (6.3)
Postmenopausal	135 (93.7)
Obesity	
Absent	43 (29.9)
Present	54 (37.5)
Missing data	47 (32.6)
Ca-125 status	
Negative	86 (59.7)
Positive	11 (7.6)
Missing data	47 (32.6)
Histology	
Endometrioid	135 (93.7)
Nonendometrioid	9 (6.3)
Stage (FIGO)	
IA-IB	107 (74.3)
II	19 (13.2)
IIIA-IIIC	14 (9.7)
IVA-IVB	3 (2.1)
Missing data	1 (0.7)
Grade	
I	53 (36.8)
II	61 (42.4)
III	22 (15.3)
Missing data	8 (5.6)
Lymph node status	
Negative	39 (27.1)
Positive	8 (5.6)
Missing data	97 (67.4)
Myometrial infiltration	
≤1/2	81 (56.3)
>1/2	62 (43.1)
Missing data	1 (0.7)
Cervical invasion	
Absent	104 (72.2)
Present	39 (27.1)
Missing data	1 (0.7)
Metastases	
Absent	102 (70.8)
Cervix	18 (12.5)
Cervix and other organs	13 (9)
Other organs	9 (6.3)
Missing data	2 (1.4)
ESR1 status	
Positive	37 (25.7)
Negative	103 (71.5)
Missing data	4 (2.8)

FIGO, International Federation of Gynecology and Obstetrics; *ESR1*, estrogen receptor 1 gene.

Quantitative PCR. Control DNA and RNA from five frozen samples of healthy donors were isolated, pooled and used for qPCR assay optimization as well as a calibrator. Analysis was performed with StepOnePlus™ Instrument (Applied Biosystems, Carlsbad, CA, USA). Each new set of the master mix was verified by a standard curve. The thermal profiles used were the default settings of the manufacturer, dedicated to either SYBR-Green or TaqMan probe assays. Results were analyzed and reported with the use of StepOne Software v2.1.

Gene dosage analysis. *RAD21* and *ESR1* (estrogen receptor 1) gene copy numbers were determined by qPCR with Power SYBR-Green Master mix (Applied Biosystems), using the *APP* (amyloid precursor protein) gene as a reference. *APP* was selected as a reference gene upon a search performed in the Atlas of Genetics and Cytogenetics in Oncology and Haematology (http://www.atlasgeneticsoncology.org/). Its stability against *3P* (RNA, U4 small nuclear pseudogen) and *SOD2* (superoxide dismutase 2) genes was verified using geNorm software (30). The primer sequences were as follows: APP F, 5'-AGC CCA GAA GGT GTC AAA CA-3'; APP R, 5'-CAT CTT CAT GTC CGT TGC AT-3'; RAD21 F, 5'-GGC ACT GTT ACC ACA AAC CTT TGG-3'; RAD21 R, 5'-GGG GAC ATT TGA ATG CTG ACT GGC-3'; ESR1 F, 5'-ACA TGG ACA CCT CCC AGT C-3'; ESR1 R, 5'-ACA GAC TAA CAC AGC CCA TC-3'. The quantity of DNA used per well was 100 ng.

RAD21 and *ESR1* copy number was calculated using the ΔΔCt quantification method (31), which relates the gene dosages of a studied gene and a reference gene in the tumor tissue and a calibrator. The reactions were performed in duplicate on 96-well plates; a negative control for each gene and three calibrators were included on each plate.

We used experimentally determined cut-off values calculated using the critical difference parameter, as described previously (32). The amplification of *RAD21* and *ESR1* was classified as a relative quantity >1.36 and 1.14, respectively.

mRNA expression analysis. *RAD21* and *RUNX1* RNA expression levels were determined by qPCR with TaqMan® Universal PCR Master mix (Applied Biosystems), using *HPRT1* (hypoxanthine phosphoribosyltransferase 1) as a reference. *HPRT1* gene expression stability was verified against the expression of *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) and *ACTB* (β-actin) genes. TaqMan® Expression Assays (Applied Biosystems) used were as follows: *HPRT1* Endogenous Control Hs99999909_m1; *RAD21* Gene Expression Assay Hs01085854_mH and *RUNX1* Gene Expression Assay Hs01021967_m1. The quantity of cDNA per well was 75 ng.

RUNX1 and *RAD21* expression was also calculated using the ΔΔCt quantification method. Reactions were performed in triplicate on 96-well plates; on each plate two negative controls for each gene and four calibrators were included. *RUNX1* and *RAD21* overexpression was classified as a value 2-fold higher than the value in the calibrator sample.

Statistical analysis. All statistical analyses were performed using the STATISTICA software, version 10. Logarithmized relative quantities of *RAD21* gene dosage together with *RUNX1* and *RAD21* expression levels were assessed by Spearman correlation and Crosstabs statistics with Pearson's chi-square test. Various comparisons of the results and clinicopathological data were performed with the nonparametric statistics, including the Mann-Whitney U test (Table II). $P<0.05$ was considered to indicate a statistically significant result.

Results

An increased level of *RAD21* gene dosage was identified in 18/141 samples. The average gene dosage was 1.103±0.345. *RAD21* overexpression was found in 23/144 samples, with an average of 1.524±0.931. Increased *RUNX1* expression was observed in 58/141 samples, with an average of 3.656±8.805. *RAD21* gene dosage was significantly associated with *RAD21* mRNA expression ($\varrho=0.22$; $p=0.009$; Fig. 1A). Furthermore, *RAD21* expression markedly correlated with *RUNX1* expression ($\varrho=0.43$; $p<0.0000001$; Fig. 1B).

Increased *RAD21* gene dosage correlated with more advanced tumor stage ($p=0.021$), higher grade ($p=0.021$), cervical involvement ($p=0.01$) and the absence of obesity ($p=0.025$), while *RAD21* mRNA expression was correlated with cervical involvement ($p=0.027$; Table II). In the case of *RUNX1* mRNA expression only a trend was observed, with a higher expression level in the nonendometrioid histological type ($p=0.073$) and in the tumors with negative lymph node status ($p=0.083$; Table II). Menopausal status, level of Ca-125, myometrial infiltration and *ESR1* status did not correlate with any of the examined molecular markers.

Discussion

Although endometrial cancer is well characterized at the level of clinicopathological features, its molecular background to date has received far less attention. As RAD21 and RUNX1 actions are crucial for sustaining basic functions in healthy cells and their expression tends to be deregulated in various types of cancer (2-4,8,9,11,12,14,18-25), we analyzed the role of these two markers in the context of endometrial cancer formation using the qPCR method. The examined markers correlated with each other, partially unraveling the network of genes involved in endometrial tumorigenesis. Findings of previous studies have suggested that the decreased expression of cohesins results in an inappropriate increase in homologous recombination which may drive tumorigenesis through the promotion of genomic instability, such as loss of heterozygosity (15,33,34).

In the present study, a marked correlation between mRNA expression of *RAD21* and *RUNX1* was observed. Furthermore, *RAD21* copy number variations were significantly associated with *RAD21* mRNA expression and *RAD21* and *RUNX1* status correlated with the clinicopathological features of the endometrial cancer patients.

Aberrant expression of RAD21 in cancer has been documented by several authors (8,9,11,18,19). RAD21 was found to be especially overexpressed in undifferentiated cancers of the breast, lung, bladder, brain and ovaries (18). Its suppression by siRNA reduced the proliferation of breast cancer cells (8). RAD21 overexpression has also been reported to confer poor prognosis in breast cancer patients (9,19). Notably, RAD21 was downregulated in oral squamous cells with high metastatic potential (11).

Table II. *RAD21* and *RUNX1* status with regard to clinicopathological data.

	RAD21 gene dosage			*RAD21* expression			*RUNX1* expression		
Variable	n	Average ± SD	P-value	n	Average ± SD	P-value	n	Average ± SD	P-value
Menopausal status									
Premenopausal	9	1.15±0.27	0.569	9	1.90±1.21	0.269	9	5.10±5.42	0.286
Postmenopausal	132	1.10±0.35		135	1.50±0.91		132	3.56±9.00	
Obesity									
Absent	41	1.18±0.30	**0.025**	43	1.39±0.82	0.069	40	4.81±15.34	0.611
Present	53	1.08±0.44		54	1.66±1.09		54	3.07±3.90	
Ca-125									
Negative	83	1.09±0.26	0.284	86	1.57±1.04	0.629	84	3.87±10.90	
Positive	11	1.41±0.86		11	1.28±0.35		10	3.26±4.73	0.718
Histology									
Endometrioid	133	1.11±0.35	0.969	135	1.51±0.91	0.975	132	3.61±9.05	0.073
Nonendometrioid	7	1.11±0.26		8	1.76±1.37		8	4.83±3.68	
Stage									
I, II	125	1.07±0.23	**0.021**	126	1.55±0.97	0.564	124	3.77±9.28	0.527
III, IV	15	1.41±0.77		17	1.35±0.53		16	2.92±4.00	
Grade									
I, II	113	1.09±0.35	**0.021**	114	1.44±0.71	0.589	112	3.72±9.69	0.691
III	20	1.22±0.32		22	1.92±1.68		21	3.57±4.19	
Lymph node status									
Negative	38	1.10±0.24	0.197	39	1.61±1.31	0.543	37	2.77±2.72	0.083
Positive	8	1.56±1.03		8	1.28±0.45		7	2.41±4.99	
Myometrial infiltration									
≤1/2	79	1.07±0.23	0.353	81	1.53±0.79	0.432	80	4.23±11.32	0.934
>1/2	59	1.14±0.46		60	1.53±1.12		59	2.78±3.23	
Cervical invasion									
Absent	102	1.05±0.22	**0.01**	104	1.60±0.97	**0.027**	104	3.94±9.99	0.306
Present	38	1.24±0.54		39	1.32±0.82		36	2.65±3.76	
ESR1 status									
Negative	103	1.09±0.38	0.269	103	1.40±0.64	0.085	102	3.73±10.06	0.213
Positive	37	1.13±0.26		37	1.87±1.45		35	4.59±4.20	

SD, standard deviation; *ESR1*, estrogen receptor 1 gene; RAD21, double-strand-break repair protein rad21 homolog; RUNX1, runt-related transcription factor 1. Significant P-values are presented in bold. P-values were calculated using the Mann-Whitney U test.

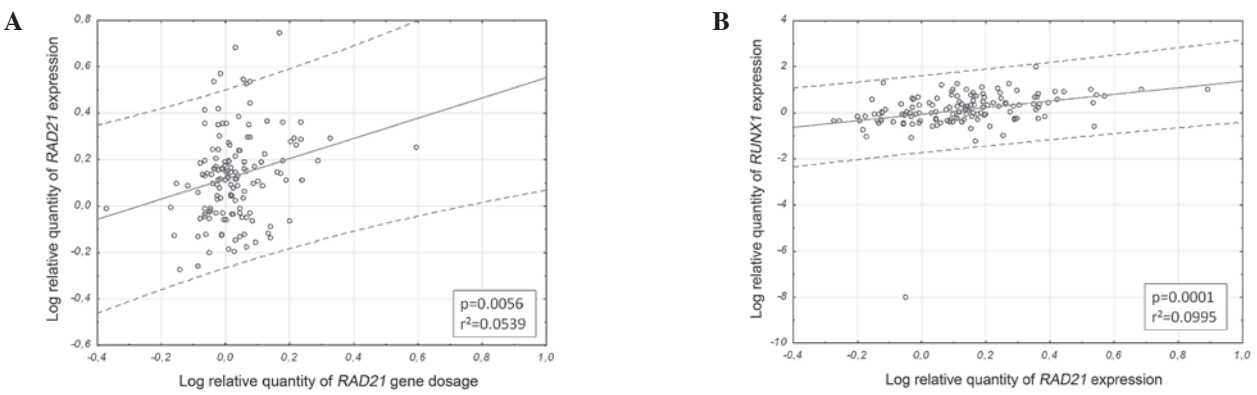

Figure 1. (A) Correlation of *RAD21* expression and *RAD21* gene dosage. (B) Correlation of *RAD21* and *RUNX1* gene expression. RAD21, double-strand-break repair protein rad21 homolog; RUNX1, runt-related transcription factor 1.

The aberrant expression of RUNX1 in cancer has also been documented in the literature (14,25), in particular *RUNX1* amplification has been reported to be implicated in the development of leukemia (22,35,36). The upregulation of *RUNX1*, as measured by qPCR, has been reported in invasive endometrioid carcinoma (14). On the contrary, in breast cancer *RUNX1* may act as a tumor suppressor gene. RUNX1 downregulation is a component of a 17-gene signature predicting metastasis (37). It has also been shown that *RUNX1* expression decreases as the breast tumor grade increases (38). Notably, experiments performed on neuroblastoma cell lines have shown that high and low RUNX1 levels disrupt proliferation, inducing cell death (23).

Our analyses did not reveal any link between *RAD21*/*RUNX1* gene expression status and the stage of the tumor, however, *RAD21* gene dosage was found to correlate with more advanced tumor stage, grade and cervical involvement. This suggests that *RAD21*-positive status is correlated with unfavorable clinical characteristics. Therefore, *RAD21* amplification may serve as a marker of poor prognosis in endometrial cancer, as in breast cancer (9,19). Furthermore, similarly to breast cancer (9), we have observed a significant association between *RAD21* expression and *RAD21* gene dosage. This suggests that in case of endometrial cancer CNVs contribute to the deregulation of *RAD21* expression. Comparative genomic hybridization revealed *RAD21* to be within the region which is prone to high-level chromosomal gains (www.progenetix.net/progenetix).

The qPCR analyses of Abal *et al* revealed *RUNX1* upregulation in endometrial cancer. The authors postulated that RUNX1 plays a crucial role during early stages of endometrial carcinogenesis and is responsible for the switch to myometrial infiltration (39). The findings of Doll *et al* indicated that RUNX1 overexpression, measured by immunohistochemistry and RT-qPCR, is associated with distant metastasis in an orthotopic endometrial cancer model in nude mice in which the endometrial cancer cell line HEC1A was used (21). Our results do not confirm these two particular hypotheses, showing a correlation only between *RUNX1* mRNA expression and tumor histological type. This, however, is in agreement with the findings of Planagumà *et al* who measured the expression levels of 53 genes, including *RUNX1*, with cDNA array hybridization. *RUNX1*, additionally verified with RT-qPCR, was reported to be the most upregulated gene among those studied in endometrioid carcinoma (14). Unfortunately, the authors did not perform such analyses for nonendometrioid carcinoma.

Our results clearly demonstrate that the mRNA expression of *RAD21* and *RUNX1* is deregulated and co-dependent in endometrial cancer cells. This is in accordance with analyses performed by Horsfield in zebrafish, in which *rad21* gene dosage reduction resulted in the decrease of *runx1* transcription, suggesting that *rad21* is a regulator of *runx1* (6). This reveals another gene to be dependent on RAD21 function. Furthermore, the correlation between *RUNX1* and ERM/ETV (40) as well as $p21^{WAF1/CIP1}$ has been reported (41), partially unraveling the network of molecular interactions occurring in endometrial cancer. Nevertheless, the exact molecular background of these changes requires further elucidation in a broader context which would include a larger number of potential molecular markers whose role could be additionally investigated at the protein level, measured by immunohistochemistry and through correlation with patients' outcome.

Given the possibility of *RAD21* status being a prognostic factor, we assume that a correlation between the gene amplification and patients' outcome is worth investigating. This is to be performed as soon as we gather necessary information concerning the patients' survival. The role of RAD21 should also be verified in the context of therapy selection as *in vitro* experiments have demonstrated that cohesin depletion leads to higher sensitivity to DNA-damaging agents and ionizing radiation (5,8,42,43). As RAD21 downregulation increases the sensitivity to certain drugs used in breast cancer therapy, the inhibition of this gene may facilitate the more effective eradication of cancer cells (8,9). This may allow prognostic and predictive analyses of endometrial tumor response to radiation and drugs.

Acknowledgements

This study 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).

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