Transcript levels of ten-eleven translocation type 1-3 in cervical cancer and non-cancerous cervical tissues

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Abstract. Decreased expression of ten-eleven translocation (TET1, TET2 and TET3) proteins has been reported in various types of cancer. However, the expression levels of TET proteins in cervical cancer (CC) remain to be elucidated. The present study determined the levels of TET1, TET2 and TET3 transcripts in cancerous (n=80) and non-cancerous cervical tissues (n=41). The results revealed a significant reduction in TET1 transcripts (P=0.0000001) in cervical tissue samples from patients with primary CC compared with samples from control patients. Significantly decreased TET1 transcript levels, as compared to non-cancerous cervical tissues, were also observed in tissue samples with the following characteristics: Stage I (P=0.016), II (P<0.0001), III (P=0.00007) and grade of differentiation G1 (P=0.026), G2 (P=0.00006), G3 (P=0.0007) and Gx (P=0.0004) and squamous histological type (P<0.00001). TET1 transcript levels were significantly lower in patients aged 45-60 years (P=0.0002) and patients age >60 years (P=0.003), as compared with non-cancerous cervical tissues. TET2 transcript levels were lower in cervical cancer tissues classified as stage II (P=0.043) and TET3 transcript levels were lower in stage III samples (P=0.010), tissue samples with a grade of differentiation of G3 (P=0.025) and tissue with squamous type histology (P=0.047), all compared with non-cancerous cervical tissues. The present study demonstrated a significantly reduced level of TET1 transcripts in cancerous cervical tissues, as compared with non-cancerous tissues. Furthermore, decreased TET1-3 transcript levels were identified when patients with CC were stratified by clinicopathological variables, as compared with non-cancerous cervical tissues.

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Introduction

Cervical cancer (CC) is one of the most common solid tumors in females worldwide, and has a high mortality rate (1-3) due to asymptomatic development of the disease delaying diagnosis (4,5). CC is a gynecological malignancy associated with oncogenic human papillomavirus (HPV) infection (6,7). In addition to HPV infection, other factors affect the development of CC, including immunological disorders and genetic malfunctions such as point mutations, deletions, amplifications and rearrangements of DNA (6). Previous studies have suggested that epigenetic changes may significantly impact cervical carcinogenesis (6-9).

Epigenetic alterations are heritable traits that impact the regulation of gene expression without altering the DNA sequence (10). These traits control genetic and transcriptional activity during growth, differentiation or organism adaptation to environmental changes (6). One epigenetic mechanism of DNA methylation consists of cytosine methylation in cytosine-phosphate-guanine (CpG) dinucleotide islands, located in the promoter region of numerous genes (6,11,12). During malignant transformation, CpG islands become hypermethylated, silencing the expression of suppressor genes and leading to a loss in the control of cell proliferation (13,14). By contrast, the hypomethylation of oncogenes increases cell division and enhances the metastasis of cancer cells (14). The process of methylation has been well characterized in recent years, but the underlying mechanism of demethylation, particularly during carcinogenesis, remains to be elucidated (6,15,16). Ten-eleven translocation (TET) proteins have an important role in DNA demethylation, with reduced expression observed in various tumors (6,13,17-22).

The TET protein family includes TET1, TET2 and TET3 (10,23). The TET1 and TET3 proteins use the CXXC zinc motif to bind to5-methylcytosine (5-mC) in CpG islands (16,17,24). The TET proteins have been revealed to catalyze the oxidation of 5-mC to 5-hydroxymethylcytosine

(5-hmC) (25). Subsequently, 5-hmC is oxidized to 5-formylocytosine (5-fC) and 5-carboxycytosine (5-caC), eventually converting 5-mC to cytosine (13,17,26). This transformation may contribute to unlocking the promoter regions of suppressor genes and facilitating the development of cancer (8,13,16). Low TET expression levels are correlated with decreased 5-hmC levels in malignant tissues and with clinicopathological features in various primary cancer tissues (13,17,20,22,27). However, little is understood regarding the levels of TET expression in cervical cancerous and non-cancerous tissue. Therefore, the present study evaluated the expression levels of TET1, TET2 and TET3 transcripts in cervical cancerous (n=80) and non-cancerous (n=41) tissues. Furthermore, the TET1, TET2 and TET3transcript levels were compared in patient groups stratified by clinicopathological variables in primary CC and non-cancerous cervical tissues.

Materials and methods

Patients and tissue samples. Primary CC tissue samples were collected following surgical resection between June 2013 and August 2015 from 80 female Caucasian patients, which is representative of the female Polish population. Patients were treated at the Department of Radiotherapy and Gynecological Oncology Greater Cancer Center (Poznań, Poland). Non-cancerous cervical tissues were obtained from 41 women with uterine fibroids undergoing uterine surgical resection in the Division of Gynecological Surgery, Poznań University of Medical Sciences (Poznań, Poland).

At the time of surgery, the mean age of patients in the cancer and control groups was 58.6±11.4 and 49.9±9.0 years, respectively. Of the 80 females in the study group, 11 patients were classified as <45 years, 48 were aged 45-60 and 21 were aged >60 years. Among the 41 women in the control group, 12 patients were classified as <45 years of age, 24 were aged 45-60 and 5 were >60 years. Among the 80 patients with CC, 4 patients were classified as stage I, 26 as stage II, 43 as stage III and 7 as stage IV, based on the International Federation of Gynecology and Obstetrics (FIGO) classification system and World Health Organization (28). Cancerous and non-cancerous cervical tissue samples were obtained following protocol approval by the Local Ethics Committee of Poznań University of Medical Sciences. Oral and written informed consent were obtained from all participants in the study. A portion of the tissue sample was immediately snap-frozen in liquid nitrogen and stored at -80˚C until RNA isolation. The remaining portion was used for histopathological assessment, which was performed by an experienced pathologist (Greater Poland Cancer Centre, Poznań, Poland).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of TET transcript levels. Frozen tissue was homogenized and total RNA was isolated according to the protocol of Chomczyński and Sacchi (29). RNA quality was determined spectrophotometrically using a BioPhotometer® from Eppendorf AG (Hamburg, Germany) and 2% agarose gel electrophoresis. RNA samples were reverse transcribed to cDNA using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. RT-qPCR was performed using a Light Cycler1480 real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using EvaGreen® (Solis BioDyne, Tartu, Estonia) as the detection dye. The thermal cycling conditions were as follows: 15 min activation, followed by 40 cycles consisting of 10 sec denaturation at 95˚C, 10 sec annealing at 58˚C, 10 sec at 72˚C. The transcript levels for patients and controls were quantified by the relative quantification method using a calibrator, which is a standard curve described in the Relative Quantification Manual, Roche Diagnostics GmbH (Mannheim, Germany). The calibrator was prepared as a cDNA mix from all samples. For amplification, 1 μl (total 20 μl) of cDNA using 9 μl of 5X HOT FIREPol® EvaGreen® qPCR Mix Plus (no ROX) (Solis BioDyne) was used. Primer sequences are presented in Table I. A total of 1 μl of 10 μM primer was used per reaction. All analyses included a negative control without cDNA, each experiment was repeated three times for all samples. The quantity of TET1, TET2 and TET3 transcripts in each sample was corrected by the measurement of porphobilinogen deaminase cDNA levels and expressed as a multiple of the copies in the calibrator.

Statistical analysis. Statistical analysis was performed with STATISTICA version 12 software (StatSoft, Inc., Tulsa, OK, USA) and Cytel Studio version 10.0 (Cytel Software Corporation, Cambridge, MA, USA). The data were presented as mean ± standard deviation and median with range. For the comparison of variables with a normal distribution, the unpaired *t*-test was used; in other cases, the non-parametric Mann-Whitney U test or the Kruskal-Wallis test was used to calculate statistically significant differences between the compared mean values. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Significantly fewer TET1 transcripts (P=0.0000001) were present in primary CC tissue from all patients, compared with non-cancerous tissue from the control group (Table II; Fig. 1). However, there were no significant differences in TET2 (P=0.084040) and TET3 (P=0.068636) transcript levels between these groups (Tables III and IV; Fig. 1). Stratification of patients based on age, the FIGO classification system, grade of differentiation and histological features was performed to evaluate the differences in theTET1, TET2 and TET3 transcript levels between cancerous and non-cancerous tissues. Significantly fewer TET1 transcripts were observed in stage I (P=0.016), II (P<0.0001), III (P=0.00007), grade of differentiationG1 (P=0.026), G2 (P=0.00006), G3 (P=0.0007) and Gx (P=0.0004) and squamous histological type (P<0.00001) cervical tissue samples, compared with non-cancerous tissue (Table II). TET1 transcript levels were significantly higher in CC tissue samples from patients aged 45-60 (P=0.0002) and patients aged >60 years (P=0.003), compared with controls (Table II). Furthermore, lower TET2 transcript levels were observed in CC tissue samples characterized as stage II (P=0.043), and lower TET3 transcript levels were detected in samples characterized as stage III (P=0.010), with a grade of differentiation of G3 (P=0.025) and a squamous histological type (P=0.047),

Table I. Primers used in reverse transcription-quantitative polymerase chain reaction analysis.

Transcript	Forward (5'-3')	Reverse (5'-3')	Product size, bp	UCSC position (GRCh37/hg19) of genes
TET1	ATACAATGGGCACCCTACCG	GGGCTTGGGCTTCTACCAAA	159	chr10:70 320 117-70 454 239
TET2	GCTGACAAACTCTACTCGG	CTTCTGGCAAACTTACATCC	188	chr4:106 067 842-106 200 960
TET3	CCCAAAGAGGAAGAAGTG	GCAGTCAATCGCTATTTC	129	chr2:74 273 405-74 335 302
PBGD	GCCAAGGACCAGGACATC	TCAGGTACAGTTGCCCATC	160	chr11:118 468 348-118 468 864

UCSC, University of California Santa Cruz; TET, ten-eleven translocation; PBGD, porphobilinogen deaminase.

Table II. Statistical analysis of TET1 transcript levels in cervical cancer and non-cancerous tissues from patients stratified by age, FIGO stage, grade of differentiation and histological type of cancer.

	No. of cases		Transcript		P-value	
Variables	Patients (%)	Controls (%)	Cancerous tissue, median (range)	Non-cancerous tissue, median (range)	Patients vs. controls[e]	Patients vs. patients[f]
Total no. of cases	80	41	2.563 (1.740-4.273)	3.262 (2.212-3.957)	0.0000001[c]	-
Age[a]						
>45	11 (13.75)	13 (31.70)	-	-	0.157000[b]	-
45-60	31(38.75)	23 (56.10)	2.522 (1.740-4.274)	3.378 (2.441-3.957)	0.000200[c]	-
>60	38 (47.50)	5 (12.20)	2.561 (2.033-3.782)	3.523 (2.997-3.927)	0.003000[c]	-
FIGO stage						
I	4 (5.00)	-	2.385 (2.272-3.122)		0.016000[c]	0.380176[d]
II	26 (32.50)	-	2.507 (1.999-3.311)		<0.000100[c]	
III	43 (53.75)	-	2.578 (1.740-4.274)		0.000070[c]	
IV	7 (8.75)	-	2.827 (2.096-3.667)		0.075000[c]	
Grade of differentiation						
G1	5 (6.25)	-	2.412 (2.033-3.508)	3.262 (2.212-3.957)	0.026000[c]	0.464650[d]
G2	36 (45.00)	-	2.582 (2.074-3.782)		0.000060[c]	
G3	11 (13.75)	-	2.418 (1.999-3.812)		0.000700[c]	
G_x	28 (35.00)	-	2.577 (1.740-4.274)		0.000400[c]	
Histological type						
Squamous	78 (97.50)	-	2.564 (1.740-4.274)		<0.000010[c]	
Adenocarcinoma	2 (2.50)	-	3.394 (2.514-4.274)		0.840000[c]	0.295441[c]

[a]Age at first diagnosis; [b]Student's *t*-test; [c]Mann-Whitney U test; [d]Kruskal-Wallis test; [e]comparison of TET expression between patients and controls for all clinicopathological data; [f]Comparison of TET expression within selected groups of patients. FIGO, International Federation of Gynecology and Obstetrics; TET, ten-eleven translocation.

Table III. Statistical analysis of TET2 transcript levels in cervical cancer and non-cancerous tissues from patients including age, FIGO stage, grade of differentiation and histological type of cancer.

Variables	No. of cases		Transcript		P-value	
	Patients (%)	Controls (%)	Cancerous tissue, median (range)	Non-cancerous tissue, median (range)	Patients vs. controls[e]	Patients vs. patients[f]
Total no. of cases	80	41	3.075 (2.246-4.962)	3.185 (1.256-3.675)	0.084040[c]	-
Age[a]						
>45	11 (13.75)	13 (31.70)	-	-	0.763000[b]	-
45-60	31 (38.75)	23 (56.10)	3.075 (2.246-4.781)	3.185 (1.256-3.625)	0.239000[c]	-
>60	38 (47.50)	5 (12.20)	3.099 (2.362-4.962)	3.243 (3.122-3.675)	0.116000[c]	-
FIGO stage						
I	4 (5.00)	-	3.077 (2.887-3.675)		0.735000[c]	0.804017[d]
II	26 (32.50)	-	3.015 (2.489-4.183)		0.043000[c]	
III	43 (53.75)	-	3.100 (2.246-4.962)		0.256000[c]	
IV	7 (8.75)	-	3.086 (2.625-3.905)		0.539000[c]	
Grade of differentiation						
G1	5 (6.25)	-	2.899 (2.647-4.781)	3.185 (1.256-3.675)	0.230040[c]	0.809431[d]
G2	36 (45.00)	-	3.095 (2.362-4.962)		0.264000[c]	
G3	11 (13.75)	-	3.029 (2.489-3.394)		0.093000[c]	
G_x	28 (35.00)	-	3.099 (2.246-3.694)		0.243000[c]	
Histological type						
Squamous	78 (97.50)	-	3.075 (2.246-4.962)		0.085000[c]	
Adenocarcinoma	2 (2.50)	-	3.089 (2.833-3.345)		0.708000[c]	0.902289[c]

[a]Age at first diagnosis; [b]Student's *t*-test; [c]Mann-Whitney U test; [d]Kruskal-Wallis test; [e]comparison of TET expression between patients and controls for all clinicopathological data; [f]Comparison of TET expression within selected groups of patients. FIGO, International Federation of Gynecology and Obstetrics; TET, ten-eleven translocation.

compared with samples from the non-cancerous control group (Tables III and IV). However, there were no significant differences between TET1, TET2 and TET3 transcript levels for I vs. II, III or IV, II vs. III or IV and III vs. IV FIGO stage (data not shown). Additionally, there were no significant differences between these transcript levels for G1 vs. G2, G3 or Gx, G2 vs. G3 or Gx and G3 vs. Gx for grade of differentiation, as well as for the histological type (data not shown).

Discussion

The involvement of TET1, TET2 and TET3 proteins in active demethylation at CpG islands in DNA has been previously documented (14,16,30). A previous study reported that the three mouse Tet proteins (Tet1, Tet2 and Tet3) may catalyze a similar reaction (12). TET1 is crucial for mouse embryonic stem (ES) cell maintenance via the regulation of methylation and the expression of the gene *Nanog,* which encodes a transcription factor essential for self-renewal of undifferentiated ES cells (12). A loss of TET proteins in ES cells has been demonstrated to be involved in the maintenance of DNA methylation patterns at several other DNA methylation regions (31).

The role of TET proteins in malignant transformation has been reported in animal models (12,32). Removal of TET function induces the development of aggressive myeloid leukemia in a mouse model (32). TET1 expression is responsible for DNA

Table IV. Statistical analysis of TET3 transcript levels in cervical cancer and non-cancerous tissues according to age, FIGO stage, grade of differentiation and histological type of cancer.

Variables	No. of cases		Transcript		P-value	
	Patients (%)	Controls (%)	Cancerous tissue, median (range)	Non-cancerous tissue, median (range)	Patients vs. controls[e]	Patients vs. patients[f]
Total no. of cases	80	41	2.720 (1.985-4.102)	2.803 (2.443-4.432)	0.068636[b]	-
Age[a]						
>45	11 (13.75)	13 (31.70)	2.782 (2.341-3.966)	2.294 (2.443-4.014)	0.242000[b]	-
45-60	31 (38.75)	23 (56.10)	2.675 (2.055-3.742)	2.761 (2.446-4.432)	0.077000[b]	-
>60	38 (47.50)	5 (12.20)	2.742 (1.986-4.102)	2.725 (2.616-3.022)	0.955000[b]	-
FIGO stage						
I	4 (5.00)	-	3.061 (2.617-3.638)		0.984000[b]	0.222237[c]
II	26 (32.50)	-	2.912 (2.268-4.102)		0.676000[b]	
III	43 (53.75)	-	2.621 (1.986-4.048)		0.010000[b]	
IV	7 (8.75)	-	2.904 (2.368-3.780)		0.726000[b]	
Grade of differentiation						
G1	5 (6.25)	-	2.396 (2.331-3.742)	2.803 (2.443-4.432)	0.138000[b]	0.498179[c]
G2	36 (45.00)	-	2.846 (1.994-4.102)		0.453000[b]	
G3	11 (13.75)	-	2.680 (2.341-3.404)		0.025000[b]	
G_x	28 (35.00)	-	2.756 (1.986-3.731)		0.158000[b]	
Histological type						
Squamous	78 (97.50)	-	2.703 (1.986-4.102)		0.047000[b]	0.109049[b]
Adenocarcinoma	2 (2.50)	-	3.542 (3.466-3.618)		0.215000[b]	

[a]Age at first diagnosis; [b]Student's *t*-test; [c]Mann-Whitney U test; [d]Kruskal-Wallis test; [e]comparison of TET expression between patients and controls for all clinicopathological data; [f]comparison of TET expression within selected groups of patients. FIGO, International Federation of Gynecology and Obstetrics; TET, ten-eleven translocation.

methylation of tissue inhibitors of metalloproteinase proteins 2 and 3 (TIMP2, TIMP3) in prostate and breast cancer (19). Reduced levels ofTET1, TET2 and TET3 have been associated with decreased 5-hmC levels in human breast, liver, lung, pancreatic and prostate cancer compared with the surrounding non-cancerous tissue (20). Decreased TET1 expression levels correspond to reduced 5-hmC levels in breast, prostate and hepatocellular cancer compared with normal tissue (19,22). Du *et al* (27) demonstrated that the loss of 5-hmC in tumors is correlated with the downregulation of TET1 expression.

Rawłuszko-Wieczorek *et al* (13) observed reduced levels of TET1, TET2 and TET3 mRNA in in colorectal cancer tissue compared with non-cancerous tissue. The decreased TET1, TET2 and TET3 mRNA levels were associated with various groups, including age, gender, cancer localization, histological grade, tumor node and metastasis classification. Furthermore, this study also demonstrated that patients with elevated TET2 transcript levels have more favorable overall survival (13). Another previous study demonstrated significantly lower levels of TET1 transcripts and protein in gastric cancer, which was correlated with gender, age and certain clinicopathological features including tumor localization, depth of invasion, lymph node metastasis, histological grade and histological type (17). Du *et al* (27) used liquid chromatography mass

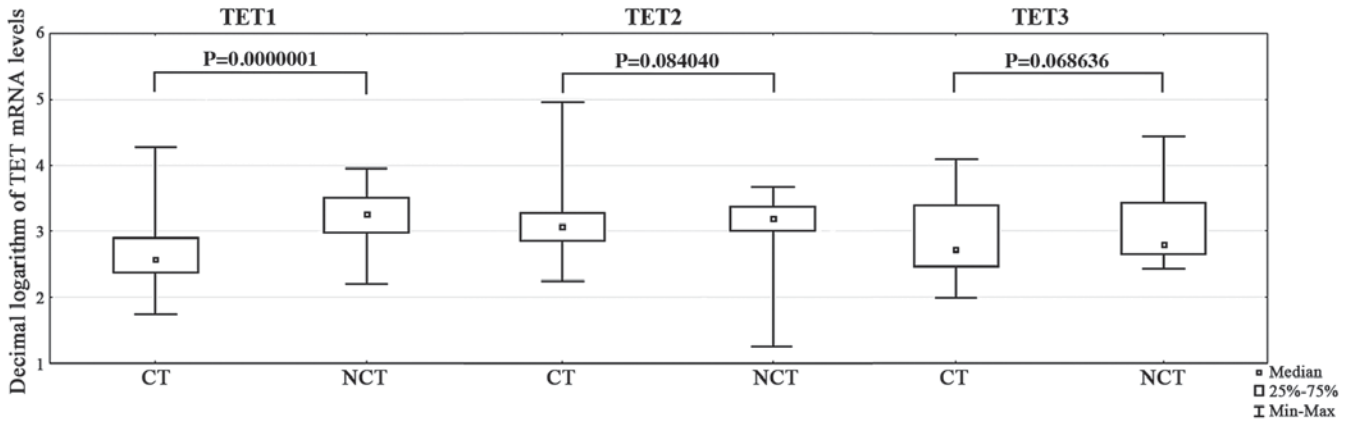

Figure 1. Comparison of TET1, TET2 and TET3 transcript levels in cervical cancer and non-cancerous tissue. CT was obtained from 80 patients with primary cervical cancer, and NCT were obtained from 41 women with uterine fibroids undergoing uterine surgical resection. Frozen tissue was homogenized and total RNA was isolated and reverse-transcribed to cDNA. The TET transcript levels for patients and controls were quantified by reverse transcription-quantitative polymerase chain reaction using the relative quantification method. The quantity of TET1, TET2 and TET3 transcripts in each sample was corrected to porphobilinogen deaminase cDNA levels and expressed as a multiplicity of these copies as a calibrator. The P-value was calculated using the Mann-Whitney test. P<0.05 was considered to be statistically significant. TET, ten-eleven translocation; CT, cancerous tissue; NCT, non-cancerous tissue.

spectrometry/mass spectrometry to demonstrate very low levels of 5-fC and 5-caC and decreased levels of 5-hmC in gastric cancer tissue compared with adjacent non-cancerous tissue. In addition, the authors revealed that the reduction of 5-hmC in gastric cancer was primarily associated with decreased TET1 expression (27). Using immunochemistry analysis, Müller *et al* (21) demonstrated that the exclusion ofTET1 from nuclei was associated with a loss of 5-hmC in the genomic DNA of gliomas. The depletion ofTET1 in prostate and breast cancer tissues has also been observed. TET1 deficiency promotes tumor growth, cell invasion and cancer metastasis in prostate xenograft mouse models (19). Furthermore, TET1 reduction corresponds to a poor survival outcome in patients with breast cancer (19). Decreased TET1 levels are responsible for maintaining the methylation of TIMP2 or TIMP3, which correlates with advanced node status in clinical samples (19).

In conclusion, to the best of our knowledge the current study is the first to demonstrate a significant reduction in the levels of TET1 transcripts in cancerous tissues compared with non-cancerous samples. In addition, TET1, TET2 and TET3 transcript levels were revealed to be reduced in patients with primary CC stratified according to their clinicopathological data, in comparison with non-cancerous tissues. The present study did not evaluate TET protein in conjunction with 5-hmC levels. Therefore, further studies are required to evaluate the potential correlation between 5-hmC levels and TET expression in CC tissues, and their associations with clinical characteristics.

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