SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 12: 473-478, 2016

Identification of key genes associated with cervical cancer by comprehensive analysis of transcriptome microarray and methylation microarray

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Received March 30, 2015; Accepted May 17, 2016

DOI: 10.3892/ol.2016.4658

Abstract. Cervical cancer is the second most commonly diagnosed type of cancer and the third leading cause of cancer-associated mortality in women. The current study aimed to determine the genes associated with cervical cancer development. Microarray data (GSE55940 and GSE46306) were downloaded from Gene Expression Omnibus. Overlapping genes between the differentially expressed genes (DEGs) in GSE55940 (identified by Limma package) and the differentially methylated genes were screened. Gene Ontology (GO) enrichment analysis was subsequently performed for these genes using the ToppGene database. In GSE55940, 91 downregulated and 151 upregulated DEGs were identified. In GSE46306, 561 overlapping differentially methylated genes were obtained through the differential methylation analysis at the CpG site level, CpG island level and gene level. A total of 5 overlapping genes [dipeptidyl peptidase 4 (*DPP4*); endothelin 3 (*EDN3*); fibroblast growth factor 14 (*FGF14*); tachykinin, precursor 1 (*TAC1*); and wingless-type MMTV integration site family, member 16 (*WNT16*)] between the 561 overlapping differentially methylated genes and the 242 DEGs were identified, which were downregulated and hypermethylated simultaneously in cervical cancer samples. Enriched GO terms were receptor binding (involving *DPP4*, *EDN3*, *FGF14*, *TAC1* and *WNT16*), ameboidal-type cell migration (*DPP4*, *EDN3* and *TAC1*), mitogen-activated protein kinase cascade (*FGF14*, *EDN3* and *WNT16*) and cell proliferation (*EDN3*, *WNT16*, *DPP4* and *TAC1*). These results indicate that *DPP4*, *EDN3*, *FGF14*, *TAC1* and *WNT16* may be involved in the pathogenesis of cervical cancer.

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Key words: cervical cancer, differentially expressed genes, differentially methylated genes, gene ontology enrichment analysis

Introduction

Among women, cervical cancer is the second most commonly diagnosed type of cancer and the third leading cause of cancer-associated mortality (1). Worldwide, ~500,000 cervical cancer cases are diagnosed and 230,000 mortalities occur due to this disease annually (2). DNA methylation, a type of epigenetic event, may increase the risk of cervical cancer through regulating gene expression and chromatin structure (3). Methylated carcinogenic human papillomavirus (HPV) DNA may be used as a predictive and/or diagnostic biomarker for risk of cervical cancer (4). Sood *et al* (3) demonstrated that methylation of the genes myogenic differentiation 1, telomerase reverse transcriptase and Ras association domain family member 1 could predict a more favorable outcome in patients with invasive cervical carcinoma treated with standard chemoradiation therapy. Kalantari *et al* (5) reported that methylation of L2 and L1 genes in HPV16, 18, 31 and 45, and of the cellular death-associated protein kinase gene were considered biomarkers of the progression of cervical neoplasia. Furthermore, Nye *et al* (6) revealed that infection with high-risk HPV types was associated with differentially methylated regions in the paternally expressed 3 (*PEG3*) gene, and that *PEG3* methylation status may have potential as a molecular marker for screening of cervical intraepithelial neoplasia. Numerous studies have reported that methylation of certain genes is associated with the pathogenesis of cervical cancer, while the mechanisms of development and progression of cervical cancer remain unclear (7,8).

Recently, microarray analyses have been performed to identify gene methylation, gene expression and RNA regulation in cervical cancer. Sun *et al* (9) analyzed differentially expressed long non-coding RNAs (lncRNAs) and mRNAs in the microarray data set GSE55940, and revealed that the enhancer of zeste 2 polycomb repressive complex 2 subunit (*EZH2*) gene and EZH2-binding lncRNA in cervical cancer (lncRNA-EBIC) may play roles in the repression of E-cadherin, which could contribute to the metastasis of cervical cancer. Ye *et al* (10) also analyzed the data in GSE55940, and determined that microRNA-145 expression was decreased in cervical cancer tissues, and was associated with advanced cancer stages and moderate/poor differentiation. In addition, Burris *et al* (11)

analyzed GSE46306 and revealed that cervical DNA methylation of the prostaglandin E receptor 2 and long interspersed nuclear element-1 *Homo sapiens*-specific genes was associated with the length of gestation in humans. Although many genes are associated with the progression of cervical cancer, comprehensive analyses of transcriptome microarrays and methylation microarrays have rarely been reported.

It is of interest to further explore the mechanisms of cervical cancer in a comprehensive manner. In the current study, a comprehensive analysis of the GSE55940 and GSE46306 data sets was performed to identify genes involved in the pathogenesis of cervical cancer. Overlapping differentially expressed genes (DEGs) in GSE55940 and differentially methylated genes in GSE46306 were identified, and Gene Ontology (GO) enrichment analysis for these genes was performed.

Materials and methods

Data preprocessing. GSE55940 was downloaded from the Gene Expression Omnibus database (GEO; http://www.ncbi.nlm.nih.gov/geo/); this data set included 5 separate cervical cancer tissues and 5 paired adjacent non-tumor samples. The platform of GSE55940 was GPL16238: [hGlue_3_0_v1] Glue Grant Human Transcriptome Array version 3.0 (GG-H) [transcript-level]. GSE46306 was also downloaded from the GEO database. In this study, 20 normal cervical samples (HPV-negative) and 6 cervical cancer tissues (HPV-positive) were included in the subsequent analysis. GPL13534 Illumina HumanMethylation450 BeadChip (HumanMethylation450_15017482) was used to detect the methylation level of the CpG sites in GSE46306.

GSE55940 was preprocessed using affy package (www.bioconductor.org/packages/release/bioc/html/affy.html) in R language. Background correction, quantile normalization and probe summarization were performed using the Robust Multi-array Average algorithm. For the data set GSE46306, the CpG sites with P>0.05 and the samples [CpG sites (P>0.05)/all CpG sites >1/100,000] were first excluded. Peak standardization was then performed for the remaining CpG sites and samples using the Illumina Methylation Analyzer (IMA) package (12).

Identification of DEGs and differentially methylated genes. The normalized data GSE55940 were further analyzed by Limma package (13), and genes with P<0.05 and |log[fold change (FC)]|>0.5 were defined as DEGs.

For the data GSE46306, the differentially methylated genes were identified based on three levels: CpG site level, CpG island level and gene level. For CpG site level, the average β value (β_a) in normal cervical and cervical cancer samples was calculated for every CpG site. Limma method in IMA was used to identify differentially methylated CpG sites that met the criteria $|\Delta\beta_a|>0.2$ and P<0.05. Genes containing the differentially methylated sites were obtained based on the annotation file of the microarray platform.

For CpG island level, the β values of five region categories [CpG islands, upstream 2,000 bp of CpG islands (N-shore), upstream 2,001-4,000 bp of CpG islands (N-shelf), downstream 2,000 bp of CpG islands (S-shore), and upstream 2,001-4,000 bp of CpG islands (S-shelf)] were calculated based on the β_a value of the CpG sites in those regions, and opensea referred to the annotations associated with UCSC islands. Additionally, Limma method in IMA was used to identify differentially methylated regions with the cut-off of $|\Delta\beta|>0.2$ and P<0.05. The genes located in the differentially methylated regions were obtained based on the annotation file of the microarray platform.

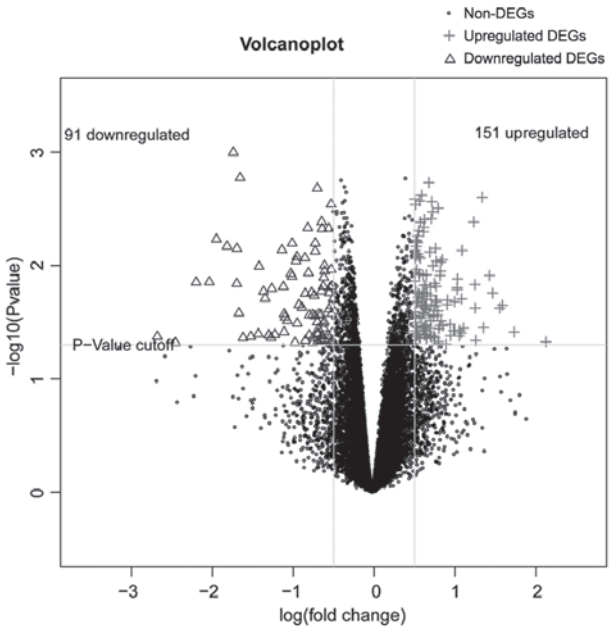

Figure 1. For GSE55940, the distribution of differentially expressed genes (DEGs) between the cervical cancer group and the adjacent non-tumor group.

For the gene level, the β values of the upstream 200 bp of genes (TSS200), the upstream 201-1,500 bp of genes (TSS1500), 5' untranslated region (UTR), the first exon region, the gene body regions and 3'-UTR were calculated using a *t*-test, and the gene regions with and $|\Delta\beta|>0.2$ and P<0.05 were considered to be differentially methylated.

Comprehensive analysis of DEGs and differentially methylated genes. For GSE46306, the overlapping genes that were identified based on CpG site level, CpG island level and gene level were screened. Subsequently, the overlapping genes of the overlapping differentially methylated genes in GSE46306 and the DEGs in GSE55940 were also screened, and GO enrichment analysis was performed for these genes using the ToppGene database (toppgene.cchmc.org/).

Results

Analysis of DEGs. In the analysis of GSE55940, a total of 242 DEGs between the cervical cancer group and the adjacent non-tumor group were identified, including 91 downregulated and 151 upregulated DEGs. The distribution of DEGs is shown in Fig. 1.

Analysis of differentially methylated genes. For GSE46306, 15,515 differentially methylated CpG sites were identified, involving 3,064 genes, 2,772 differentially methylated CpG island regions containing 1,082 genes, and 1,857 differentially methylated gene regions that related to 1,012 genes, between

Table I. Number of differentially methylated CpG sites, differentially methylated regions at the CpG island level and differentially methylated regions at the gene level between the cervical cancer group and the control group.

Type	Region	Count	Gene count
CpG sites	Whole genome	15,515	3,064
Gene region	TSS1500	259	1,012
	TSS200	497	
	5'-UTR	382	
	3'-UTR	91	
	Gene body	121	
	Exon 1	507	
CpG island region	Island	1,505	1,082
	N-shore	607	
	N-shelf	78	
	S-shore	520	
	S-shelf	62	

TSS1500, upstream 201-1,500 bp of genes; TSS200, upstream 200 bp of genes; UTR, untranslated region.

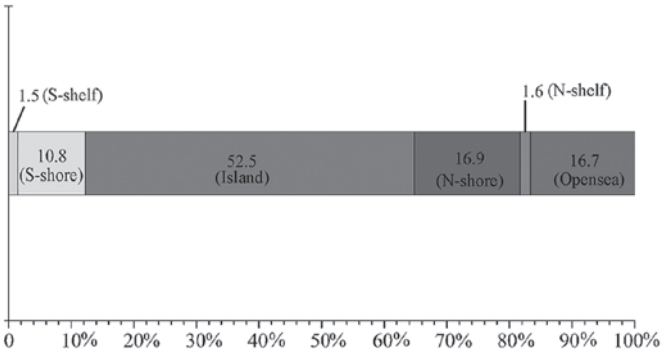

Figure 2. Distribution locations and percentages of differentially methylated regions between the cervical cancer group and the control group at CpG island level. S-shelf, upstream 2,001-4,000 bp of CpG islands; S-shore, downstream 2,000 bp of CpG islands; N-shore, upstream 2,000 bp of CpG islands; N-shelf, upstream 2,001-4,000 bp of CpG islands.

the cervical cancer group and the control group (Table I). Distribution locations and percentages of differentially methylated regions at the CpG island level are shown in Fig. 2; the distribution was 52.5% CpG islands, 16.9% N-shore, 16.7% opensea, 10.8% S-shore, 1.6% N-shelf and 1.5% S-shelf. In addition, the distribution locations and percentages of differentially methylated regions at the gene level are shown in Fig. 3, comprising 33.9% intergenic, 24.6% gene body, 13.4% TSS1500, 11.2% TSS200, 8.9% 5'-UTR, 6.5% 1st exon and 1.5% 3'-UTR.

In GSE46306, 561 overlapping differentially methylated genes were identified through the differential methylation analysis at the CpG site level, CpG island level and gene level (Fig. 4). In addition, 5 overlapping genes between the 561 overlapping differentially methylated genes and the 242 DEGs were identified (Table II): dipeptidyl peptidase 4 (*DPP4*); endothelin 3 (*EDN3*); fibroblast growth factor 14 (*FGF14*); tachykinin, precursor 1 (*TAC1*); and wingless-type MMTV integration site family, member 16 (*WNT16*). All of the 5 genes were downregulated and hypermethylated in cervical cancer samples.

Table II. Overlapping genes between the 561 overlapping differentially methylated genes in GSE46306 and 242 differentially expressed genes in GSE55940.

Gene	logFC	CpG ID	Δβ
DPP4	-0.567	cg09601770	0.210
		cg10112391	0.208
		cg12335708	0.212
		cg18892517	0.234
		cg19350270	0.274
		cg25912827	0.248
EDN3	-1.227	cg04048259	0.345
		cg08318212	0.238
		cg09005679	0.286
		cg13919285	0.213
		cg16205854	0.226
		cg17146570	0.231
		cg20910807	0.233
		cg21163415	0.395
		cg21512644	0.325
FGF14	-0.558	cg02491276	0.202
		cg05210258	0.377
		cg08597761	0.410
		cg09896622	0.277
		cg16398329	0.259
		cg20335672	0.244
		cg22583065	0.245
		cg22809871	0.292
		cg23809442	0.215
		cg24172416	0.280
		cg25317585	0.370
TAC1	-0.648	cg01287975	0.359
		cg09236284	0.253
		cg10997627	0.244
		cg11873482	0.333
		cg16288089	0.297
		cg17437939	0.387
		cg19212224	0.276
WNT16	-0.728	cg05470554	0.273
		cg14448169	0.265
		cg16868298	0.274
		cg18579879	0.223
		cg25608490	0.235
		cg26690075	0.280

logFC, $\log_2$(fold change) between the cervical cancer and normal samples; Δβ, difference of average β value of the CpG sites in cervical cancer and normal samples; *DPP4*, dipeptidyl peptidase 4; *EDN3*, endothelin 3; *FGF14*, fibroblast growth factor 14; *TAC1*, tachykinin, precursor 1; *WNT16*, wingless-type MMTV integration site family, member 16.

Table III. Top ten GO terms of *DPP4*, *EDN3*, *FGF14*, *TAC1* and *WNT16*.

Category	ID	Terms	P-value	Count	Genes
GO: MF	GO:0005102	Receptor binding	$2.63x10^{-6}$	5	*FGF14*, *EDN3*, *WNT16*, *DPP4*, *TAC1*
GO: BP	GO:0001667	Ameboidal-type cell migration	$2.66x10^{-5}$	3	*EDN3*, *DPP4*, *TAC1*
GO: BP	GO:0002027	Regulation of heart rate	$1.79x10^{-4}$	2	*EDN3*, *TAC1*
GO: BP	GO:0050886	Endocrine process	$2.22x10^{-4}$	2	*EDN3*, *TAC1*
GO: BP	GO:0046887	Positive regulation of hormone secretion	$3.73x10^{-4}$	2	*EDN3*, *TAC1*
GO: BP	GO:0000165	Mitogen-activated protein kinase cascade	$4.16x10^{-4}$	3	*FGF14*, *EDN3*, *WNT16*
GO: BP	GO:0007610	Behavior	$4.84x10^{-4}$	3	*FGF14*, *EDN3*, *TAC1*
GO: BP	GO:0023014	Signal transduction by phosphorylation	$4.96x10^{-4}$	3	*FGF14*, *EDN3*, *WNT16*
GO: BP	GO:0008283	Cell proliferation	$4.98x10^{-4}$	4	*EDN3*, *WNT16*, *DPP4*, *TAC1*
GO: BP	GO:0051050	Positive regulation of transport	$5.42x10^{-4}$	3	*FGF14*, *EDN3*, *TAC1*

GO, Gene Ontology; *DPP4*, dipeptidyl peptidase 4; *EDN3*, endothelin 3; *FGF14*, fibroblast growth factor 14; *TAC1*, tachykinin, precursor 1; *WNT16*, wingless-type MMTV integration site family, member 16; MF, molecular function; BP, biological process.

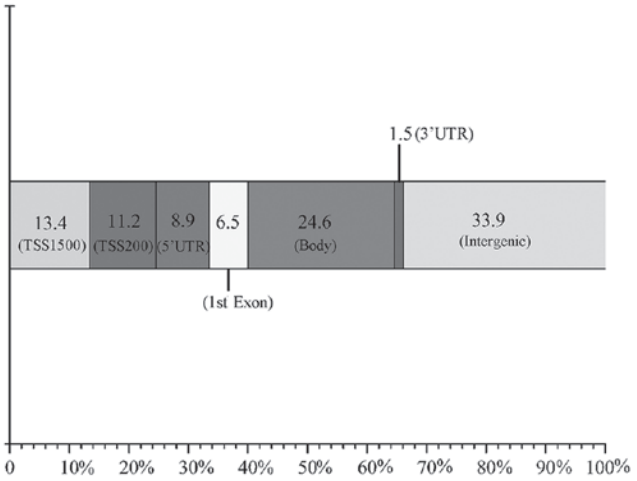

Figure 3. Distribution locations and percentages of differentially methylated regions between the cervical cancer group and the control group at gene level. TSS1500, upstream 201-1,500 bp of genes; TSS200, upstream 200 bp of genes; UTR, untranslated region.

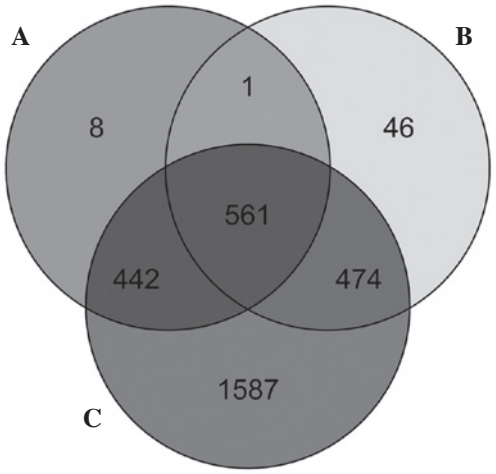

Figure 4. In GSE46306, 561 overlapping differentially methylated genes, based on the differentially methylated analysis at CpG site level, CpG island level and gene level, were screened. (A) Number of differentially methylated genes at gene level; (B) number of differentially methylated genes at CpG islands level; (C) number of differentially methylated genes at CpG site level.

Enrichment analysis of DEGs. The genes *DPP4*, *EDN3*, *FGF14*, *TAC1* and *WNT16* were enriched in 86 GO terms. The top ten GO enrichment terms are shown in Table III. In particular, these genes were predominantly enriched in cell migration and cell proliferation, including receptor binding (*DPP4*, *EDN3*, *FGF14*, *TAC1* and *WNT16*), ameboidal-type cell migration (*DPP4*, *EDN3* and *TAC1*), mitogen-activated protein kinase (MAPK) cascade (*FGF14*, *EDN3* and *WNT16*) and cell proliferation (*EDN3*, *WNT16*, *DPP4* and *TAC1*).

Discussion

DNA methylation is one of the most common epigenetic events and can regulate the expression of certain genes through preventing the binding of transcription factors with the genes (14). A number of studies have investigated the relationship between the occurrence and development of cervical cancer and DNA methylation or gene expression (15,16). However, comprehensive studies of DNA methylation and gene expression are rare.

In the current study, 5 genes (*DPP4*, *EDN3*, *FGF14*, *TAC1*, and *WNT16*) that were overlapping between the 561 overlapping differentially methylated genes and the 242 DEGs were identified; these genes were downregulated and hypermethylated simultaneously in cervical cancer samples. The predominant enriched GO terms included receptor binding, ameboidal-type cell migration, MAPK cascade and cell

proliferation. The comprehensive analysis of transcriptome and methylation microarrays indicated that *DPP4*, *EDN3*, *FGF14*, *TAC1* and *WNT16* may contribute to the development of cervical cancer.

DPP4 [also known as cluster of differentiation (CD) 26)], is a membrane-bound enzyme that serves functions in metabolism, the immune and endocrine systems, cancer growth and cell adhesion (17). *DPP4* has been shown to act as a tumor suppressor or activator by associating with fibroblast activation protein α, adenosine deaminase, CD45 and C-X-C motif chemokine receptor 4, and could be considered as a potential therapeutic target in cancers expressing *DPP4* (18,19). In addition, Buffon *et al* (20) demonstrated that *DPP4* was involved in processes of cervical cancer by regulating cell migration and adhesion. In the current study, *DPP4* was identified as a differentially methylated gene and DEG associated with the pathogenesis of cervical cancer and involved in receptor binding, ameboidal-type cell migration and cell proliferation. Taken together, these findings indicate that *DPP4* is closely associated with the development of cervical cancer by regulating cell migration and adhesion.

EDN3 is a member of endothelin family, and the *EDN3* pathway has been shown to be essential for the proliferation, survival and migration of melanocyte precursor cells (21,22). Garcia *et al* (23) demonstrated that *EDN3* exhibited an tumor-angiogenic response in a melanoma mouse model. Furthermore, *EDN3* may be a target of epigenetic inactivation, affecting the endothelin signalling pathway in human breast cancer, and hypermethylation of the *EDN3* promoter could lead to gene silencing (24). In addition, Espinosa *et al* (25) revealed that the expression of *EDN3* was downregulated in cervical cancer, and Chen *et al* (26) revealed that DNA methylation of *EDN3* could be considered a cancer biomarker in cervical cancer. The current findings were consistent with these previous reports; *EDN3* was identified as differentially methylated gene and DEG related to cervical cancer, and was indicated to be involved in ameboidal-type cell migration, MAPK cascade and cell proliferation. Based on these findings, it may be speculated that *EDN3* is involved in the development of cervical cancer.

FGF14 is a member of the fibroblast growth factor (FGF) family, which includes four homologous factors: *FGF11*, *FGF12*, *FGF13* and *FGF14* (27). Upregulated expression of *FGF13* has been demonstrated to mediate the resistance to platinum-based drugs in cervical cancer cells (28). Thus, as a homolog of *FGF13*, it is possible that *FGF14* is involved in cervical cancer development. However, few studies have reported on the correlation between *FGF14* and cervical cancer (28,29). In the current study, using a comprehensive analysis of microarray data, *EDN3* was identified to be a differentially methylated gene and DEG associated with cervical cancer. These findings suggest that *FGF14* may be involved in the development of cervical cancer.

Previously, methylation of *TAC1* has been demonstrated to have a potential correlation with prognosis in cervical cancer (2). Consistently, the results of the current study implied that *TAC1* was associated with the pathogenesis of cervical cancer. In addition, *WNT16* is important in oncogenesis, and the WNT signalling pathway is related to β-catenin signaling and prostate cancer development (30,31). However, few studies have reported on the participation of *WNT16* in the development of cervical cancer. In the current study, *TAC1* and *WNT16* were identified as differentially methylated genes and DEGs in cervical cancer. Taken together, it may be speculated that *TAC1* and *WNT16* are involved in cervical cancer development.

In summary, the present study identified 91 downregulated and 151 upregulated DEGs in the GSE55940 data set. In GSE46306, 561 overlapping differentially methylated genes based on a differential methylation analysis at the CpG site level, CpG island level and gene level were screened. A total of 5 overlapping genes (*DPP4*, *EDN3*, *FGF14*, *TAC1* and *WNT16*) of the 561 overlapping differentially methylated genes and 242 DEGs were identified, which were downregulated and hypermethylated in cervical cancer samples. *DPP4*, *EDN3*, *FGF14*, *TAC1* and *WNT16* may be involved in the pathogenesis of cervical cancer. However, the results must be confirmed by further experiments.

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