Am J Cancer Res 2018;8(7):1249-1261
www.ajcr.us /ISSN:2156-6976/ajcr0079808

Original Article

Downregulation of *CEACAM6* gene expression in laryngeal squamous cell carcinoma is an effect of DNA hypermethylation and correlates with disease progression

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Received May 17, 2018; Accepted May 27, 2018; Epub July 1, 2018; Published July 15, 2018

Abstract: We have turned our attention to *CEACAM6* gene, already described as deregulated in various types of cancer. By using the expression microarrays performed on the set of 16 laryngeal squamous cell carcinoma (LSCC) samples: 11 cell lines and 5 primary tumors we have shown downregulation of *CEACAM6* gene as compared to non cancer controls from head and neck region. *CEACAM6* gene downregulation, further confirmed by quantitative PCR on 25 LSCC cell lines, was observed in cell lines derived from recurrent tumors in comparison to controls. A significant gene downregulation was observed in cell lines derived from advanced, high grade tumors in comparison to controls. Intrigued by the recurrent transcriptional loss of *CEACAM6* we searched for the mechanism potentially responsible for its downregulation and hence we analyzed DNA copy number changes (a-CGH), promoter DNA methylation status and occurrence of gene mutations (*in silico*). Neither the analysis of gene copy number, nor the mutation screen has shown recurrent deletions or mutations, that could contribute to the observed downregulation of the gene. However, by using bisulfite pyrosequencing, we have shown DNA hypermethylation (mean DNA methylation > 78%) of *CEACAM6* promoter region in 9/25 (36%) LSCC cell lines. Importantly, the 5-aza-2-deoxycytidine-induced inhibition of DNA methylation resulted in restoration of *CEACAM6* expression in the two LSCC cell lines on mRNA level. In summary, we have shown that recurrent downregulation of *CEACAM6* in LSCC is dependent on the gene's promoter DNA methylation and is observed predominantly in large, poorly differentiated tumors and recurrences.

Keywords: Laryngeal squamous cell carcinoma (LSCC), head and neck squamous cell carcinoma (HNSCC), DNA methylation, tumor suppressor gene, *CEACAM6*

Introduction

Understanding the relationship between changes in gene expression and tumor stage is important for extending the knowledge of cancer biology and consequently helps to improve current diagnosis and therapy. There is a strong need to provide for clinicians a panel of biomarkers which changes in expression level would precisely indicate particular stages of disease. Therefore, identification of novel potential tumor suppressor genes and oncogenes involved in laryngeal cancer development is important from a scientific and clinical point of view.

In our previous studies we have performed a microarray-based expression analysis on the set of laryngeal squamous cell carcinoma (LSCC) cell lines [1, 2]. By analyzing the gene expression profiles, we found a significant downregulation of *CEACAM6* gene in LSCC cell

Table 1. Clinico-histological characteristics of cell lines and primary tumor samples

Cell line number	Sex	Age (years)	Primary tumor location	TNM	Specimen site	Type of lesion	Grade
UT-SCC-6A	F	51	Supraglottic larynx	T2N1M0	Larynx	Rec	G1
UT-SCC-6B	F	51	Supraglottic larynx	T2N1M0	Neck	Met	G1
UT-SCC-8	M	42	Supraglottic larynx	T2N0M0	Larynx	Pri	G1
UT-SCC-11	M	58	Glottic larynx	T1N0M0	Larynx	Rec	G2
UT-SCC-13	M	53	Supraglottic larynx	T3N0M0	Larynx	Rec	G2
UT-SCC-19A	M	44	Glottic larynx	T4N0M0	Larynx	Pri	G2
UT-SCC-19B	M	44	Glottic larynx	T4N0M0	Larynx	Pri (per)	G2
UT-SCC-22	M	79	Glottic larynx	T1N0M0	Larynx	Rec	G2
UT-SCC-23	M	66	Scc transglottica	T3N0M0	Larynx	Pri (per)	G1
UT-SCC-29	M	82	Glottic larynx	T2N0M0	Larynx	Pri	G1
UT-SCC-34	M	63	Supraglottic larynx	T4N0M0	Larynx	Pri	G1
UT-SCC-35	M	50	Glottic larynx	T2N0M0	Larynx	Resid	G2
UT-SCC-38	M	66	Glottic larynx	T2N0M0	Larynx	Pri	G2
UT-SCC-42B	M	43	Supraglottic larynx	T4N3M0	Neck	Pri	G3
UT-SCC-49	M	76	Glottic larynx	T2N0M0	Larynx	Pri	G2
UT-SCC-50	M	70	Glottic larynx	T2N0; rT2N0	Larynx	Rec	G3
UT-SCC-57	M	76	Glottic larynx	T2N0M0	Larynx	Rec	G1-G2
UT-SCC-75	M	56	Supraglottic larynx	T2N2BM0	Larynx	Pri	G2
UT-SCC-106A	M	59	Plicae vocalis	T1AN0M0	Larynx	Pri	G2
UT-SCC-106B	M	59	Plicae vocalis	rT3N0M0	Larynx	Rec	G3
UT-SCC-107	M	46	Supraglottic larynx	T4N2CM0	Larynx	Pri	G2
UT-SCC-108	M	68	Supraglottic larynx	T2N0M0	Larynx	Pri	G3
UT-SCC-113	M	50	Supraglottic larynx	T3N0M0	Larynx	Pri	G3
UT-SCC-116	M	60	Supraglottic larynx	T4N1M0	Larynx	Pri	G2
UT-SCC-117	M	71	Larynx	rT2N0M0	Larynx	Rec	G2
Primary tumor sample number	Sex	Age (years)	Primary tumor location	TNM	Specimen site	Type of lesion	Grade
TS32	M	61	Larynx	T4N0M0	Larynx	NA	G2
TS36	M	53	Larynx	T4N1M0	Larynx	NA	G2
TS42	M	69	Larynx	T2N2cM0	Larynx	NA	G2
TS59	M	59	Larynx	T3N2M0	Larynx	NA	G2
TS60	M	71	Larynx	T4N3M0	Larynx	NA	G2

All cell lines were obtained from the University of Turku (Finland). M: Male; F: Female; TNM: TNM classification (T-tumor; N-lymph nodes involvement; M-distance metastases); Pri: Primary tumor; Rec: Recurrence; Met: Metastasis; Per: Persistent tumor; G: Tumor grade, NA: Data not available.

lines as compared to non cancer controls. Interestingly, overexpression of *CEACAM6* was formerly shown in pancreatic adenocarcinoma and gastric cancer [3-5], but also in head and neck cancers [6, 7]. These contradictory data triggered our interest in investigating this gene in detail. Therefore, we have further validated the change of its expression in LSCC cell lines with RT-qPCR and correlated these data with tumor staging (TNM) and grading (G). To identify the underlying mechanism responsible for the observed transcriptional deregulation we have analyzed the DNA copy number status and gene promoter DNA methylation level. Additionally, we screened *in silico* for mutations inactivating *CEACAM6* using cBioPortal and COSMIC databases.

Materials

Cancer cell lines

Twenty-five cell lines established from the whole range of LSCC tumors, representing glot-

tic, supraglottic and transglottic disease were used for this study. The cell lines characteristics is presented in **Table 1** and elsewhere [8, 9].

Tumor specimens

Five laryngeal tumor samples were used for the microarray-based expression analysis. The patients and tumor samples characteristics are shown in **Table 1**. The tissue samples were obtained from Department of Otolaryngology and Laryngological Oncology, Poznan University of Medical Sciences, Poznan, Poland. The study was approved by the local Ethical Board of the University of Medical Sciences in Poznan (number 164/10). Written consent was obtained from all patients.

Control samples

For the microarray expression analysis, the following control samples were used: commercially available human total RNA derived from healthy larynx (Stratagene, Agilent Technologies, Waldbronn, Germany), RNA derived from bronchial airway epithelia reconstituted *in vitro* (two donors-pooled, Epithelix Sarl, Geneve, Switzerland) and normal mucosa derived from surgical margin during laryngectomy.

For reverse transcription and quantitative PCR of LSCC cell lines Total RNA from healthy larynx, RNA isolated from normal human bronchial/tracheal epithelial cells (Lonza, Verviers, Belgium) and human tracheal epithelial cells (PromoCell, Heidelberg, Germany) were used. Total RNA collected from different adult human tissues (qPCR Human Reference Total RNA, Clontech Laboratories, Mountain View, USA) was used as a positive control of *CEACAM6* expression and was analyzed in parallel with LSCC cell lines during qPCR after inhibition of DNA methylation with 5-aza-2'-deoxycytidine (DAC).

For the DNA methylation analysis ten DNA samples isolated from oral cavity epithelium (buccal swabs) from healthy volunteers were used. Additional controls: fully methylated standard-MET (Millipore, Hilden, Germany) as well as unmethylated DNA (UM): the whole genome amplified DNA from peripheral blood lymphocytes (GenomePlex® Whole Genome Amplification Kit) samples were used.

Methods

Cell line culturing

LSCC cell lines were grown in 25 cm^2 flasks in Dulbecco's Modified Eagle Medium, supplemented with 10% FBS. All cultures were kept in 37°C, in atmosphere enriched with 5% CO_2.

DNA and RNA isolation

For nucleic acids isolation, cell lines were cultured until they reached 80% confluence. For DNA isolation the cells were harvested with 0.1% trypsin and 0.2% EDTA. DNA from cell lines and peripheral blood lymphocytes was isolated according to the standard method: phenol/chloroform extraction and ethanol precipitation. The DNA from the buccal swabs was extracted with High Pure PCR Template Preparation Kit (Roche, Diagnostics GmbH) with slight modification, as described elsewhere [10]. For RNA isolation Chomczynski's method was applied [11]. The cells were treated with trizol directly in the cell culture dish after removing the culture medium.

The laryngeal tumor tissue specimens designated for RNA extraction were stored in RNA *later* (Sigma-Aldrich, Saint Louis, USA) until RNA isolation. The tissue was disrupted in liquid nitrogen, and the RNA was isolated as described above. Each specimen used in this study was reviewed by a pathologist and contained at least 60% of tumor cells.

DNA purity and concentration were quantified by NanoDrop (ND-1000 spectrophotometer) and the A260/280 ratio ≥1.8 was considered as acceptable. RNA samples were analyzed using RNA 6000 NanoKit on Agilent 2100 BioAnalyzer (Agilent).

Expression microarrays

Gene expression levels were established according to LSCC cell lines expression data (GeneChip Human Genome U133 Plus 2.0 Array, Affymetrix) acquired in our previous study [1]. Eleven cell lines and five primary tumor specimens were compared to three non cancer controls. Gene expression was established by verifying all probe sets (henceforth called tags) of particular gene, as annotated in the UCSC Genome Browser database (NCBI36/hg18).

Table 2. Primer sequences and reaction conditions: RT-qPCR and bisulfite pyrosequencing

RT-qPCR				
Gene name and accession number	Primer sequence (5'-3')	Amplicon length (bp)	Annealing temperature (°C)	PCR efficiency (%)
CEACAM6 NM_002483	F: CGTCGGCATCACGATTGG R: TGGGATTGGAGGAGCTAGAAG	127-i	61	100
UBC NM_021009	F: TCGCAGTTCTTGTTTGTG R: GATGCCTTCCTTATCTTGG	150-i	55	100
ARNT NM_001668	F: TTGGCAGCACACTCTATG R: C--CTCATTCGGCAAATAAACG	191-i	55	100
GAPDH NM_002046	F: GTCGGAGTCAACGGATT R: CCTGGAAGATGGTGATGG	220	55	96
PCR reaction conditions: Initial polymerase activation 95°C for 15 min, next 40 cycles as follows: Denaturation: 95°C for 20 sec, Annealing: 55 or 61 for 10 sec, Elongation and fluorescent data collection: 72°C for 20 sec. Melt curve: 50-95°C in 0.5°C/10 sec. increments + fluorescence data collection.				
Bisulfite pyrosequencing				
Gene name and amplicon position	Primer sequence (5'-3')	Amplicon length (bp)	Annealing temperature (°C)	Number of analyzed CGs
CEACAM6 chr19: 42260536-42260626	F: #ATTTTGGGGTAGGTTGTGGG R: CACCACTACCAAACTCACTAT S: ACCAAACTCACTATTAAATC	91	65	2
LINE-1 genome-wide	F: TTTTGAGTTAGGTGTGGGATATA R: #AAAATCAAAAAATTCCCTTTC S: GGGTGGGAGTGAT	146	55	4
PCR reaction conditions: Initial heat activation: 95°C for 15 min, next 45 cycles as follows: Denaturation: 94°C for 30 sec, Annealing: 55 or 65°C for 30 sec, Elongation: 72°C for 30 sec ended with final elongation 72°C for 10 min.				

Letter "i" after the amplicon length indicates that the exon/exon boundary was inside the amplified sequence. The double hyphen in the primer sequence denotes the exon/exon boundary. #denotes primer with biotin. F: forward primer; R: reverse primer; S: sequencing primer.

The profiles were screened in search for tags fulfilling two criteria: 1. "Present" call of particular tag in all three control samples. 2. "Decrease" call of the tag in the analyzed LSCC sample. To consider the gene as downregulated, the "Decrease" call must be observed in LSCC sample for all tags assigned to its sequence against a minimum 2 out of 3 controls.

Reverse transcription and quantitative PCR (RT-qPCR)

Total RNA from twenty-five cell lines and three non tumor control samples was reverse transcribed to cDNA using the Enhanced Avian RT First Strand Synthesis Kit (Sigma-Aldrich), according to the manufacturer's protocol.

Primers for qPCR were designed using Beacon Designer™ 7.5 software (PRIMER Biosoft International). The amplified sequence overlapped at least one intron/exon junction. A specificity of each primer pair was verified by Primer-BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Two reference genes, *ARNT* and *UBC* were used, in line with our previous reports [1, 12].

The qPCR was performed using iCycler iQ5 (Bio-Rad) and the gene expression level was calculated with the use of Gene Expresssion Analysis for iCycler iQ5® Real-time PCR Detection System software (Bio-Rad Laboratories). PCR amplification was facilitated by 5 × HOT FIREPol®EvaGreen® qPCR Supermix (Solis BioDyne, Estonia), following the manufacturer's protocol, with the final concentration of 250 nM for both primers. For each sample, 0.4 µl of cDNA was used (undiluted reverse transcription product derived from 8 µg of RNA in 40 µl of final reaction volume). The primer sequences, annealing temperatures, PCR efficiencies and

qPCR reaction conditions are described in **Table 2**. The melting curve, PCR efficiency, PCR data analysis and the statistics were completed as described before [12]. Each gene was amplified in triplicate and non-specific PCR products were not observed. All LSCC samples showing *CEACAM6* expression below the lowest *CEACAM6* expression observed in control samples were described as downregulated.

DNA copy number analysis-array-based comparative genomic hybridization (a-CGH)

The microarray-based DNA copy number data obtained and reported previously [1, 13] was used and was based on Human Genome CGH 244K Microarrays (ten LSCC cell lines) and Human Genome CGH 44K Microarrays (three LSCC cell lines) (Agilent Technologies, Waldbronn, Germany). The gene position was determined according to UCSC Genome Browser database (NCBI36/hg18). To identify potential DNA copy number alterations the mean log2ratio for chromosomal regions harboring *CEACAM6* was evaluated. The range of the mean log2ratio between +0.5 and -0.5 was considered as normal. Log2ratio values below -0.5 were recognized as a potential deletion.

Datamining-alterations in DNA sequence and gene copy number

The set of samples including 279 HNSCC cases from cBioPortal database was used to identify inactivating mutations in *CEACAM6* sequence and changes in gene copy number (http://www.cbioportal.org) [14-16]. Moreover the additional data was obtained from the COSMIC database (Catalogue of Somatic Mutations in Cancer http://cancer.sanger.ac.uk/cosmic [17]). The screened set of samples included 908 HNSCC cases, of which 26 were derived from larynx.

DNA methylation analysis by bisulfite pyrosequencing

Purified DNA (500 ng) from each sample were bisulfite converted using the EpiTect DNA Modification Kit (Qiagen, Germany), according to the manufacturer's protocol. The primers were designed using the PyroMark Assay Design Software 2.0. (Qiagen) as described elsewhere [10]. Amplified region: chr19:42260536-42260626 (91 bp, GRCh37/hg19) was located in the second exon of *CEACAM6*, 1168 bp downstream of the transcription start site. Two CG dinucleotides at genomic positions: CG1-chr19: 42260566-42260567; CG2-chr19: 42260568-42260569 were analyzed for methylation level. The mean DNA methylation calculated for these two CG dinucleotides is referred to as gene DNA methylation. PCR reaction was performed using PyroMark PCR kit according to the protocol supplied (Qiagen, Germany). Primer sequences, annealing temperatures and PCR reaction conditions are shown in **Table 2**. Pyrosequencing was performed using PyroMark Q24 sequencer (Qiagen) and the raw data were analyzed with PyroMarkQ24 software (ver. 2.0.6, Qiagen). Fully methylated and unmethylated controls were used in each run. The reaction mix (in total volume 25 µl) included as follows: 1 × PyroMark Master Mix (with HotStarTaq DNA Polymerase, 1 × PyroMark PCR Buffer and dNTPs), 10 pmol of each primer, 1 × CoralLoad Concentrate and 1 µl of converted DNA.

To identify hypermethylated LSCC samples the cut off value (based on results collected for oral epithelium control samples) was established by multiplying two times the mean standard deviation value of control samples methylation and adding the result to the highest value of DNA methylation observed among these controls. Additionally, dilution series of commercially available fully methylated DNA template (MET) in unmethylated DNA template (UM) from 0% to 100%, in 10% steps, were used to estimate the assay sensitivity.

DAC-induced inhibition of DNA methylation and validation by RT-qPCR and pyrosequencing

In this part of a study, DNA and RNA extracted from two LSCC cell lines (UT-SCC-29 and UT-SCC-11) with induced inhibition of DNA methylation obtained in our other study was used [18, 19]. Cell lines were incubated with the DNA methyltransfrerase inhibitor: DAC (Sigma) added to culture medium in final concentrations 0.1 µM and 0.3 µM. The control samples were cultured under the same conditions, but DAC solution was replaced by the equal volume of DAC solvent (50% acetic acid) as described recently [18, 19]. Additionally, the "mock" control (i.e. the cell line cultured without any treatment was applied). Each culture was continued until reached 80% confluence. The experiment was run in duplicate.

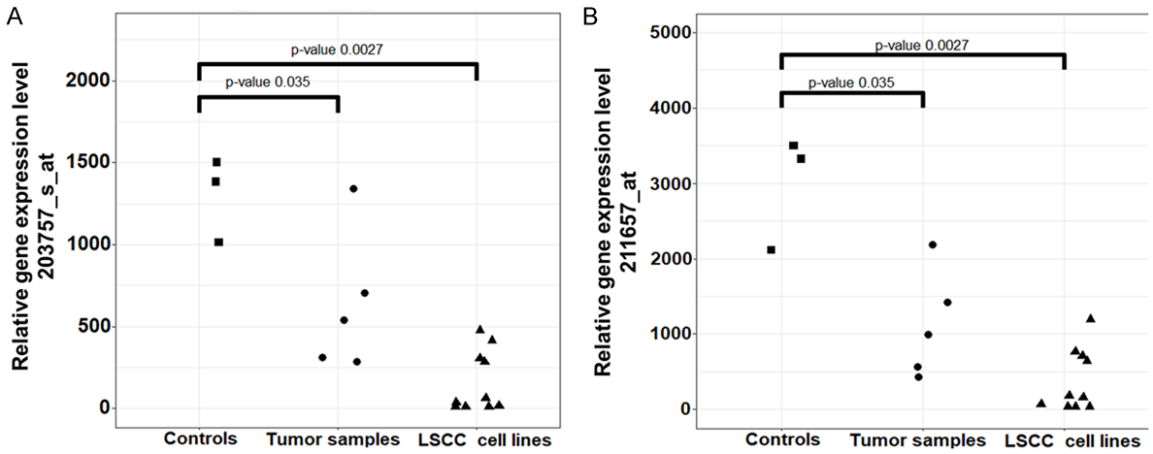

Figure 1. The relative expression of *CEACAM6* gene. Two tags assigned to gene sequence: 203757_s_at (A) and 211657_at (B) are presented. Significantly downregulated expression of *CEACAM6* in LSCC primary tumors and cell lines is shown (P<0.05; Mann Whitney U-test).

Thereafter DNA methylation level of *CEACAM6* promotor was evaluated by bisulfite pyrosequencing analysis. DNA from both, control and DAC-induced LSCC cell lines, was purified and bisulfite converted with EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, USA), according to the manufacturer's protocol. Moreover, the global DNA methylation level was estimated by the analysis of *LINE-1* (Long interspersed nucleotide element 1) DNA methylation. Primers were designed in the same manner as described for *CEACAM6* and their sequences are presented in **Table 2**. All the samples were run in triplicates.

To estimate the changes in *CEACAM6* expression in cell lines with DAC-induced inhibition of DNA methylation, total RNA was reverse transcribed using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, USA), according to manufacturer's protocol. The qPCR was performed and *GAPDH* gene was used as a reference gene. Sequences of primers are listed in **Table 2**. Total Human RNA served as a positive control of RT-qPCR reaction. The fold change of expression level was calculated from the ratio between *CEACAM6* expression observed in DAC-treated cells *versus* controls (cells incubated with acetic acid).

Nucleic acids isolation, bisulfite pyrosequencing and RT-qPCR reactions performed in this part of the study were conducted using the same techniques as described earlier for untreated LSCC cell lines.

Statistical analysis

CEACAM6 expression status of all studied cell lines was correlated with the clinical characteristics: tumor stage (T), nodal involvement (N) and tumor differentiation status (G) of the tumors from which these cell lines were originated. All cell lines were derived from the M-negative (M0) tumors; hence this parameter was not considered. To assess the statistical significance of the observed changes in DNA methylation level and gene expression the Mann-Whitney U-test was used. The changes were considered significant if P<0.05.

For analysis of correlation of *CEACAM6* expression with DNA methylation level the Pearson's test has been used. The changes were considered significant if P<0.05.

Results

CEACAM6 gene is downregulated on mRNA level in LSCC

A comparison of gene expression profiles between eleven LSCC cell lines, five primary tumor samples and three non cancer controls identified genes that are significantly downregulated in LSCC. One of the genes that fulfill the criteria established in this study was *CEACAM6*. Two tags are annotated to its sequence (203757_s_at and 211657_at). Both of them showed a "Present" call for expression in all control samples, revealing that the gene is expressed in all of these samples, whereas

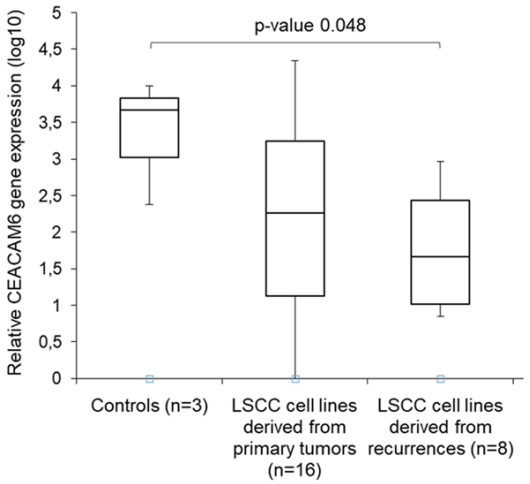

Figure 2. The RT-qPCR results. Box plots show the relative gene expression level in LSCC cell lines derived from primary and recurrent tumors and non cancer controls. One cell line: UT-SCC-6B was excluded from this analysis due to its metastatic origin. The Mann-Whitney U-test was performed for statistical analysis. The logarithmic function (log_{10}) was used for gene expression data normalization.

a "Decrease" call from both tags in all 11 cell lines indicate transcriptional loss of the gene in LSCC. In tumor samples the 211657_at tag signposted a decrease in 5/5 and the 203757_s_at tag in 4/5 cases, respectively. Thus, *CEACAM6* was shown to be downregulated on mRNA level in all 11 (100%) LSCC cell lines and in 4/5 (80%) of primary tumor samples (**Figure 1**).

Quantitative PCR confirms downregulation of CEACAM6 gene

To confirm the microarray-based expression results, the reverse transcription and qPCR were performed using RNA derived from the set of twenty-five LSCC cell lines and three non cancer controls. According to the criteria established in this study, mRNA expression level of *CEACAM6* was downregulated in 14/25 (56%) cell lines compared to controls. Moreover, the expression level was significantly downregulated in cell lines derived from recurrent tumors (U-test, P=0.048), but not from primary tumors (U-test, P=0.138), as compared to controls (**Figure 2**).

CEACAM6 copy number and DNA sequence are not altered in LSCC

To estimate the copy number of the gene we have analyzed a-CGH profiles performed on thirteen LSCC cell lines. Based on the aforedescribed criteria, we found that only in one cell line (UT-SCC-22) mean log2ratio value (-0.52) indicates a potential *CEACAM6* heterozygous deletion. Therefore, we have extended our search and analyzed the additional set of data available on the cBioPortal. In set of 279 HNSCC samples in the database no gene deletion was reported. In line with these findings neither change of *CEACAM6* copy number was reported in set of HNSCC, nor in LSCC samples in COSMIC database. These results drove us to a conclusion, that gene copy number loss is not the main molecular mechanism leading to *CEACAM6* downregulation in LSCC.

We have, moreover, screened the data from cBioPortal and COSMIC databases for potential inactivating point mutations in the *CEACAM6* sequence. Therein, missense mutations of the analyzed gene are rare with only one detected in cBioPortal database. In light of these findings we have resigned from searching for inactivating mutations in our group of samples.

CEACAM6 gene is hypermethylated in LSCC cell lines

Using bisulfite pyrosequencing we have found significant differences (U-test P=0.0001) in gene promoter DNA methylation in LSCC cell lines [n=25] and control samples [n=10]. The mean DNA methylation of *CEACAM6* promoter in LSCC cell lines ranged between 31-97% (mean for all LSCC samples: 70%, SD=21) as compared to epithelial control samples, where it ranged between 18-55% (mean for all control samples: 36%, SD=12). Based on the control samples the cut-off methylation level was determined to be at 78% and indicated hypermethylated samples. According to the outlined criteria the gene was hypermethylated in 9/25 (36%) cell lines (**Figure 3A**).

The dilution series of methylated and unmethylated standard have demonstrated nearly linear representation of DNA methylation levels (**Figure 3B**), therefore this assay was not biased towards any of DNA template (methylated or unmethylated).

Inhibition of CEACAM6 gene DNA methylation with demethylating agent results in restoration of its expression in LSCC cell lines

Two LSCC cell lines: UT-SCC-11 and -29 were used to prove a direct correlation between

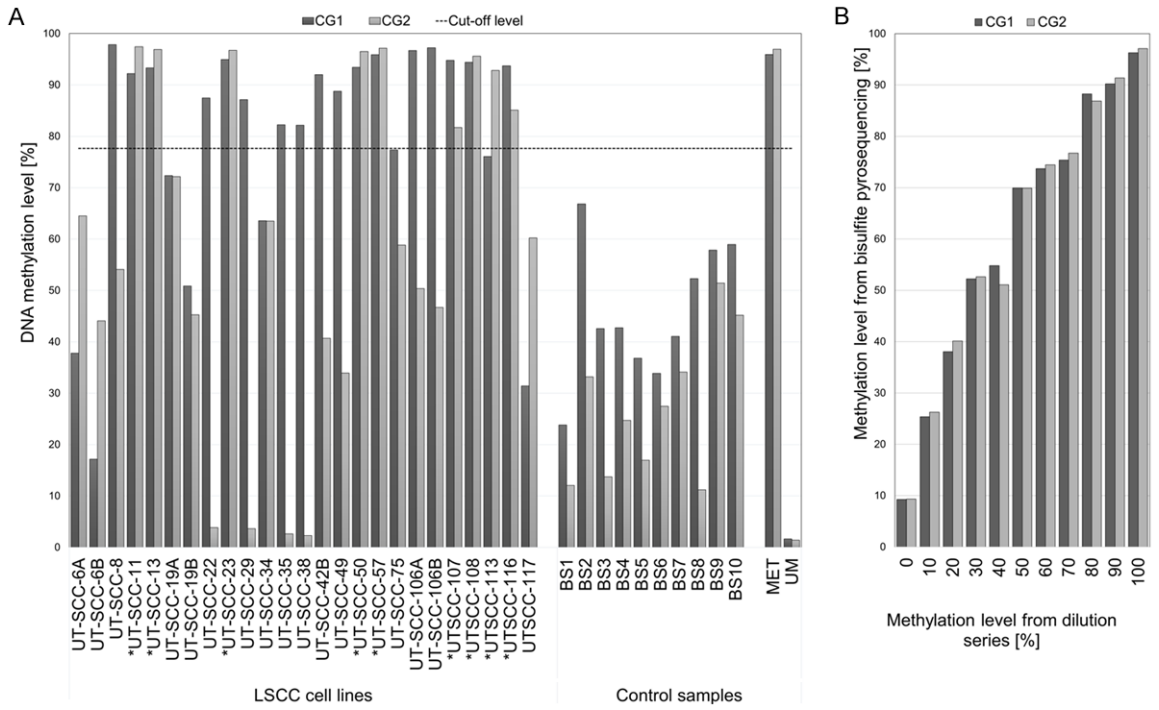

Figure 3. Analysis of DNA methylation level based on bisulfite pyrosequencing. A: DNA methylation level of *CEACAM6* gene promoter region. Dotted horizontal line indicates the cut-off level (78%) above which samples are regarded as hypermethylated and marked with an asterix. BS: control DNA from buccal swabs samples, MET: fully-methylated standard; UM: unmethylated standard. B: DNA methylation analysis of serial dilution of fully methylated (MET) control for *CEACAM6* gene. Each bar represents one CG dinucleotide.

CEACAM6 promoter methylation and expression.

First, we analyzed the global DNA methylation level in the cell lines incubated with the DNA demethylating agent-DAC. Two DAC concentrations: 0.1 μM and 0.3 μM were used. After the incubation the average *LINE-1* DNA methylation level was decreased by 21% and 19% in UT-SCC-11 cell line and by 12% and 8% for UT-SCC-29 cell line (0.1 μM and 0.3 μM DAC respectively, both cell lines) (**Figure 4A**), showing a global decrease of DNA methylation level in the analyzed cell lines.

Next, DNA methylation level of the promoter region of *CEACAM6* was analyzed. Similarly, we observed reduction of DNA methylation by 60% in the UT-SCC-11 cell line (treated with both DAC concentrations), as well as in the UT-SCC-29 cell line, that showed a reduction by 23% and 18% (0.1 μM and 0.3 μM DAC respectively). Noteworthy, one out of two analyzed CG dinucleotides was found unmethylated in UT-SCC-29 cell line (see **Figure 3A**). DNA methylation of the remaining CG was decreased by 44% and 38% in the 0.1 μM and 0.3 μM DAC-induced cells respectively (**Figure 4B**).

To verify the gene expression after inhibition of DNA methylation, the RNA extracted from the same cell lines were reverse transcribed and used for qPCR. We observed 3.18- and 3.71-fold increase in *CEACAM6* expression in the UT-SCC-11 cell line (0.1 μM DAC, 0.3 μM DAC, respectively) and 1.71- and 3.33-fold in the UT-SCC-29 cell line: (0.1 μM DAC, 0.3 μM DAC, respectively) (**Figure 4C**).

Consequently, we have shown that DAC-induced decrease of *CEACAM6* promoter methylation results in restoration of gene expression. We observed strong negative correlation between *CEACAM6* expression and methylation level for the UT-SCC-11 cell line (Pearson's test, R=-0.9654, P=0.008) and moderate negative correlation in the UT-SCC-29 cell line (Pearson's test, R=-0.6354, P=0.25). Together, these data indicate that high DNA methylation

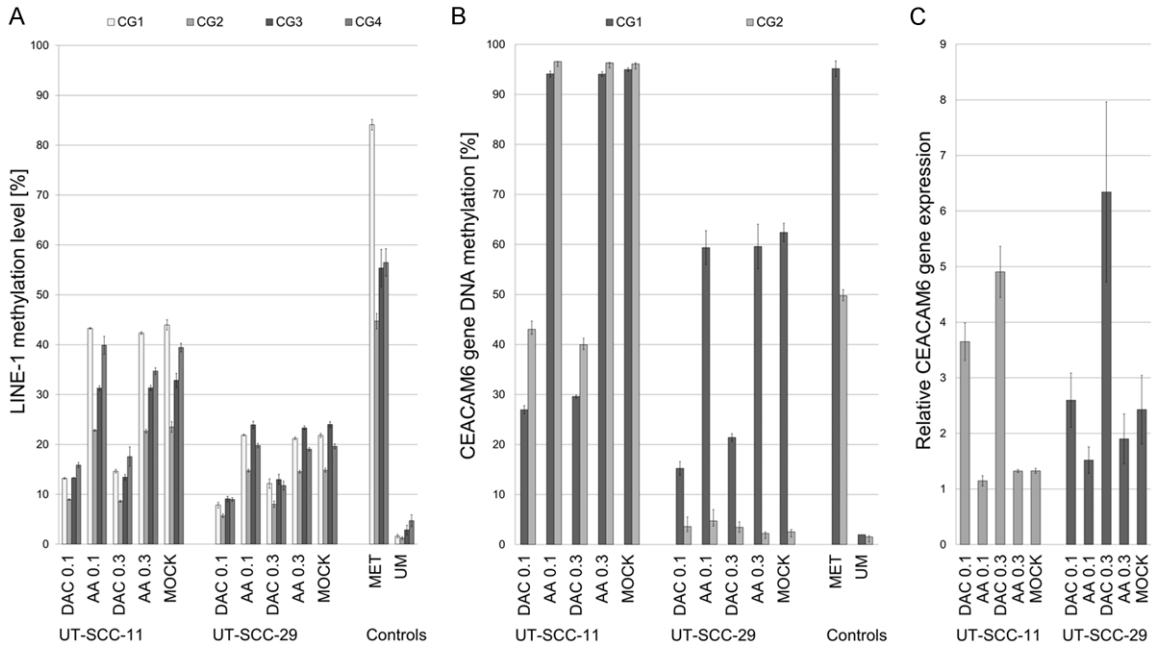

Figure 4. DAC-induced inhibition of DNA methylation in LSCC cell lines. A: Global DNA methylation level in LSCC cell lines based on a *LINE-1* sequence bisulfite pyrosequencing; B: *CEACAM6* gene promoter DNA methylation level in LSCC cell lines treated with DAC; C: The changes in *CEACAM6* gene expression level before and after DAC treatment, analyzed by RT-qPCR. DAC 0.1, DAC 0.3: 5-Aza-2'-deoxycytidine concentrations 0.1 μM and 0.3 μM respectively, AA 0.1, AA 0.3: acetic acid controls for DAC 0.1 μM and 0.3 μM concentrations, respectively; MOCK: non-treated control, MET: fully-methylated standard; UM: unmethylated standard.

level impacts mRNA gene expression and confirms, that *CEACAM6* downregulation observed in LSCC cell lines is the consequence of hypermethylation of its promoter region.

CEACAM6 gene expression in LSCC cell lines correlates with tumors/patient's clinico-histological data

Expression of *CEACAM6* was lower in the cell lines derived from recurrences (P=0.048), but not in cell lines derived from primary tumors (P=0.138) as compared to controls. The gene expression was significantly downregulated in highly advanced, less differentiated tumors (stages T3/T4 and G3) as compared to control samples (U-test, P=0.049, P=0.036 respectively), but no significant difference between N0 and N+ cancer-derived cell lines was found. The correlation between gene expression level and patient survival time was performed, however no significant association was observed.

Discussion

Understanding of genetic background of laryngeal cancer is crucial for improvement of diagnostic and prognostic tools. Searching for genes significantly deregulated in LSCC we have screened data obtained in our previous microarray-based expression analysis. We have focused on *CEACAM6*, downregulated in all 11 LSCC cell lines and in 4/5 primary tumor samples analyzed with the use of microarrays. Under physiological conditions, this gene is involved in cell adhesion and its expression occurs in granulocytes and epithelial cells [20]. Most of authors report *CEACAM6* upregulation in cancers [21], i.e. focal overexpression of *CEACAM6* in head and neck cancer was shown by Cameron et al. [6, 7]. On the contrary, *CEACAM6* expression was found significantly decreased in squamous cell lung cancer as compared to adenocarcinoma [22, 23]. The discrepancies between our results and other published data encouraged us to investigate *CEACAM6* expression in laryngeal cancer in more detail.

The aims of this study were to analyze the expression of *CEACAM6* in LSCC and determine its correlation with cancer stage and grade. Moreover, we have made an attempt to

identify the molecular mechanism leading to the observed *CEACAM6* downregulation in LSCC.

Importantly, we have confirmed the microarray results with RT-qPCR and observed a significant decrease of gene expression in 14/25 LSCC cell lines as compared to non cancer controls. The gene expression was lower in recurrent LSCC cell lines as compared to control samples (U test p=0.048). These results are in contrary to the findings by Cameron et al. [7]. However, the cell lines and tumor samples used for the study cited above were derived mainly from tongue, lip and pharynx, but not from larynx. Moreover, authors reported, that *CEACAM6* expression varies in head and neck tumors, even in tumors of the same origin. In fact, we have also observed such heterogeneity of expression level in our study. Although, we have determined the average *CEACAM6* downregulation in LSCC cell lines, we have also found that in one cell line (UT-SCC-106A) the expression level surpassed the highest level observed in control samples. These findings may elucidate a partial disagreement of our results compared to those published by Cameron et al. [7]. As was previously shown, CEACAM6 protein is strongly expressed in bronchial epithelium and alveolar epithelium of lungs, but not in adventitia submucosa or blood vessel endothelium [24]. In our opinion, the expression level of *CEACAM6* observed in epithelial non cancer controls in our study is strongly reduced in the tumor samples as a result of the malignant transformation.

The literature data and our results encouraged us to investigate for possible mechanisms of the observed gene expression changes. With the application of array-CGH we have shown heterozygous deletion in only 1/25 cell lines. In line, neither the cBioPortal, nor the COSMIC data indicate recurrent *CEACAM6* copy number alterations. The same holds true for missense mutations, observed only in 1 of total 1187 cases collected in both databases. Thus, we have eliminated these mechanisms as potentially responsible for *CEACAM6* downregulation.

Therefore, in a further attempt to find the underlying silencing mechanism we have analyzed the gene promoter DNA methylation level using bisulfite pyrosequencing. DNA hypermethylation leading to gene inactivation is a mechanism that commonly occurs during carcinogenesis [18, 25, 26]. The DNA hypermethylation (mean DNA methylation > 78%) found in *CEACAM6* promoter in 9/25 (36%) LSCC samples allowed us to assume, that this mechanism may play a pivotal role in decreasing its expression. In line with our data, *CEACAM6* was indicated as an epigenetic biomarker downregulated as a consequence of recurrent hypermethylation in basal - like subtype of triple-negative breast cancer [27, 28]. To prove the assumption, that the gene is indeed epigenetically silenced, we have inhibited DNA methylation in two LSCC cell lines, differing in their initial methylation status, with the use of DAC. In both cell lines, we have observed decrease in DNA methylation level of *CEACAM6* promoter, resulting in restoration of its expression.

These findings let us postulate, that DNA hypermethylation is the main cause of *CEACAM6* silencing in LSCC.

As aforementioned, in the current study the significant downregulation of *CEACAM6* was observed in LSCC cell lines derived from recurrent tumors. It occurs predominantly in advanced LSCC tumors (U test: T3/4 P=0.049, and G3 P=0.036), although, it is not significantly correlated with either lymph node metastases or patient's survival. This finding is contradictory to previously published data. In pancreatic, colon or colorectal cancers, *CEACAM6* is considered as an oncogene, based on its association with advanced tumor stage, deregulated cell polarization and differentiation, higher risk of recurrence, patients' lower overall and recurrence-free survival [4, 29-31]. Moreover, the gene is involved in promotion of EMT (Epithelial to Mesenchymal Transition), *in vitro* migration and invasion and *in vivo* metastasis [32].

On the other hand, *CEACAM6* was shown to be downregulated in basal breast cancer and basal-like cancer cell lines [33]. Therein, cancer cells with observed CEACAM6 protein expression were less proliferative and less tumorigenic in nude mice as compared to cells without CEACAM6 expression. Likewise it was shown, that RNAi-based suppression of *CEACAM6* decreased invasiveness of colon

cancer cell lines [30] and increased susceptibility to caspase-mediated anoikis in pancreatic adenocarcinoma cell lines [34]. Thus, the lack of metastasis, observed in our study may be due to sensitivity to anoikis after detaching from tumor. Therefore, we speculate that in LSCC cell lines *CEACAM6* downregulation may facilitate cell proliferation and tumor growth and inhibit cell differentiation and metastasis formation.

In summary, the altered gene expression level suggests the potential contribution of *CEACAM6* in LSCC development. We have demonstrated, that changes in DNA methylation lead to gene silencing in LSCC cell lines. As a consequence, *CEACAM6* downregulation might promote tumor growth, and to some extent, limit cell differentiation and migration.

Acknowledgements

This work was supported by the National Science Centre (Krakow, Poland), grant number N N403 153140. KB received a "Supportive scholarship for PhD candidates specializing in the fields considered as a strategic for the development of the Greater Poland region", Human Capital Operational Programme for year 2011/2012, Sub-measure 8.2.2 Human Capital Operational Programme, co-financed by European Union under the European Social Fund. For this work no funding from any type of organization concerning for health or medicine was received.

Disclosure of conflict of interest

None.

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