Global DNA methylation status in laryngeal cancer.

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Abstract

Laryngeal squamous cell carcinoma (LSCC) belongs to the heterogeneous group of head and neck cancers. In the etiology of LSCC, beside important environmental factors, genetic and epigenetic factors play a role in defining an individual's susceptibility to LSCC. One such epigenetic factor is DNA hypomethylation, which due to the large heterogeneity of the tissue, and invasiveness of the methods used, is often evaluated in easily available tissue, such as peripheral blood, for cancer screening.

In this study we evaluated global DNA methylation status in laryngeal cancer tissues compared to normal laryngeal tissues and peripheral blood leukocytes in a homogeneous group of 72 patients with LSCC using a UPLC-based method (ultra performance liquid chromatography) to assess the total content of 5'-methylcytosine.

Among the 72 patients, aged from 43 to 86 (mean 59.19, standard deviation 7.99), were 64 men and 8 women. A survey of the patients was carried out regarding their age at onset, exposure to environmental carcinogens (alcohol, cigarettes, etc.), the type of treatment, duration of treatment from the time of diagnosis and family history. Each tumor was characterized in terms of clinical and pathological features.

We found DNA hypomethylation both in tumor tissue and normal tissue (about 56% and 49% of tumor and normal tissues, respectively, were substantially hypomethylated. There was a highly significant correlation between the levels of 5'-methylcytosine in these two types of tissue (p = 0.001, Spearman's test for correlation). The level of 5-methylcytosine in blood leukocytes was higher than in cancerous and normal tissues. A negative correlation was found between tumor grade and blood levels of 5-methylcytosine.

The level of leukocyte DNA methylation measured using total 5-methylcytosine content cannot be used as a surrogate marker for genome methylation status in cancer tissues. Further studies are necessary to determine the correlation between the tumor grade and blood levels of 5-methylcytosine.

Keywords: epigenetic, DNA hypomethylation, laryngeal cancer, head and neck cancer
Abstract 2 (150 words)

Background
Global DNA hypomethylation is often determined in easily available tissue, such as peripheral blood.

Methods
We evaluated global DNA methylation status in cancer tissues compared to adjacent normal tissues and peripheral blood leukocytes in a homogeneous group of 72 patients with laryngeal cancer using a UPLC-based method (ultra performance liquid chromatography) to assess the total content of 5′-methylcytosine.

Results
DNA hypomethylation was found in tumor tissue and normal tissue (56% and 49% of tumor and normal tissues, respectively, were substantially hypomethylated). There was a significant correlation between the levels of 5′-methylcytosine in these two types of tissue (p = 0.001, Spearman’s test for correlation). There was no significant DNA hypomethylation in blood leukocytes. A negative correlation between tumor grade and blood levels of 5-methylcytosine was found.

Conclusion
The level of leukocyte DNA methylation measured using total 5-methylcytosine content cannot be used as a surrogate marker for genome methylation status in laryngeal cancer tissues.
Introduction

Head and neck squamous cell carcinomas (HNSCCs) are a highly heterogeneous group of cancers at the morphological, genetic and epigenetic levels [Crowe 2002, Demokan 2011, Martinez 2012]. One type of head and neck cancers is laryngeal cancer. The majority of such cases (95%) are diagnosed as laryngeal squamous cell carcinoma (LSCC) [Wang 2011]. According to the European Cancer Observatory’s 2008 data for the European Union, laryngeal cancer ranks as the 13th most common malignancy in men and 21th most common malignancy in women [http://eu-cancer.iarc.fr; Marioni 2012].

In Poland laryngeal cancer was in 9th place among all malignancies reported in 2009. In the years 1999-2009, from about 2100 to about 2700 new cases of laryngeal cancer were diagnosed annually [http://onkologia.org.pl]. Carcinogenesis in LSCC is a multifactorial process, in which both exposure to environmental factors and genetic/epigenetic changes play a role. Some interactions between two or more factors may have a multiplicative effect [Al-Kayed 2006, Pelucchi 2011, Sinha 2012].

One of the common epigenetic events observed in various types of human tumors is global DNA hypomethylation, which affects a variety of DNA sequences, including repetitive sequences (transposable elements interspersed throughout the genome, large repeat sequences and simple repeat ones), as well as single-copy genes (e.g. growth regulatory genes, tissue specific genes, developmentally critical genes) [Daura-Oller 2009, Das 2004, De Smet 2010, Ehrlich 2002, Hoffmann 2005, Poage 2011]. DNA hypomethylation may lead to chromosomal instability, loss of the imprinting (LOI) of specific genes, retrotransposition and oncogene activation [Das 2004, Daura-Oller 2009, De Smet 2010, Jintaridith 2010, McCabe 2005, Pogribny 2010, Steinhoff 2002].

To estimate levels of global DNA methylation, various methods determining the methylation of cytosine in the genome, such as assessment of the content of 5-methylcytosine or the methylation level in repetitive DNA sequences, can be used [Stach 2003, Weisenberger 2005, Smith 2007, Yang 2004]. These repetitive elements, constituting about half of the human genome, are relatively rich in CpG dinucleotides (LINE - long interspersed nuclear element, mainly LINE-1, SINE - short interpresed nuclear element and Alu), and are used as surrogate markers for estimating genomic DNA methylation levels [Smith 2007, Yang 2004]. It is generally accepted that there is an association between global hypomethylation (the 5-methylcytosine content) and hypomethylation of repetitive sequences [Kaneda 2004, Weisenberger 2005], although some studies have found no such relation [Choi 2009].

DNA hypomethylation in different cancers varies according to the type and stage of
cancer [Furniss 2008, Hoffmann 2005]. In view of the positive association between tumor progression and metastasis, DNA hypomethylation may be a useful marker in classifying a cancer and predicting its clinical course [Hoffmann 2005].

DNA hypomethylation is namely a reduction (usually by an average of 10%) in the level of methylation (5-methylcytosine content) in malignant tumors usually compared to adjacent normal tissue or analogous normal tissue from healthy controls [De Smet 2012, Ehrlich 2002, Taby 2010, Yang 2004, Zhu 2010]. However, due to the large heterogeneity of tissue, and invasiveness of the methods used, global DNA methylation is often evaluated in easily available tissue, such as peripheral blood, for epidemiological purposes and cancer screening [Choi 2009, Woo 2012, Zhu 2010]. According to the meta-analysis done by Woo and Kim [Woo 2012], global DNA hypomethylation in peripheral leukocytes may be considered as a biomarker for cancer risk. However, the authors emphasize that this is dependent on the experimental methods and targeted region of DNA used to measure the global DNA hypomethylation level and cancer type. Several independent studies have found that global DNA hypomethylation in peripheral blood leukocytes may be a risk factor in cancer of the colon, breast, liver, bladder and some head and neck tumors [Cho 2010, Choi 2009, Hsiung 2007, Moore 2008, Pufulete 2003, Wu 2012].

Therefore, we evaluated global DNA methylation status in laryngeal cancer tissues compared to normal laryngeal tissues and peripheral blood leukocytes in a homogeneous group of patients with LSCC using a UPLC-based method (UPLC, ultra performance liquid chromatography) to assess the total content of 5′-methylcytosine.
Material

Tissue samples used to analyze 5′-methylcytosine levels were obtained from 72 patients with LSCC (Table 1, 2). All the patients were Caucasians from the same region of Poland. Histopathologically confirmed laryngeal cancer tissue, adjacent normal laryngeal tissue (1.5-2 cm from the margin of the neoplastic lesion), and peripheral blood were collected from each patient. All tissue samples from the study group were taken during laryngectomy in the Department of Otolaryngology, Wroclaw Medical University, Poland and in the Department of Clinical Otolaryngology, 4th Military Hospital, Wroclaw, Poland. The clinical data were collected in these centers and in the Lower Silesian Oncology Center, Wroclaw, Poland.

The study received approval from the Bioethics Committee of Wroclaw Medical University (KB-332/2006).

The mean age of the patients was 59.19 years with standard deviation 7.99 (range 43-86 yrs). The median follow-up time was 29.4 months with standard deviation 27.7 months (range, 5 to 172 months). None of the patients had distant metastases at the moment of diagnosis. During follow-up, 7 patients were observed with distant metastases (9.7%), 19 patients (26.4%) with recurrences of LSCC and 4 patients (2.8%) with secondary tumors. A family history of cancer was found in 36% of LSCC patients (26 of 72). However, only 3 of these 26 cases related to head and neck tumors (2 carcinomas of the larynx, 1 cancer of the lower lip).

Table 1. Clinical characteristics of the study group.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Number of cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laryngeal squamous cell carcinoma</td>
<td></td>
</tr>
<tr>
<td>keratinizing</td>
<td>55 (76.39)</td>
</tr>
<tr>
<td>nonkeratinizing</td>
<td>17 (23.61)</td>
</tr>
<tr>
<td>Localisation</td>
<td></td>
</tr>
<tr>
<td>glottis</td>
<td>24 (33.33)</td>
</tr>
<tr>
<td>epiglottis</td>
<td>8 (11.11)</td>
</tr>
<tr>
<td>subglottis</td>
<td>0 (0.00)</td>
</tr>
<tr>
<td>transglottis</td>
<td>40 (55.56)</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>16 (22.22)</td>
</tr>
<tr>
<td>G2</td>
<td>45 (62.50)</td>
</tr>
<tr>
<td>G3</td>
<td>11 (15.28)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>3 (4.17)</td>
</tr>
</tbody>
</table>
Table 2. Demographic and lifestyle characteristics of the patients with laryngeal cancer.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking</td>
<td></td>
</tr>
<tr>
<td>smoking pack yrs.</td>
<td></td>
</tr>
<tr>
<td>(\leq 20)</td>
<td>9 (12.68%)</td>
</tr>
<tr>
<td>(&gt;20)</td>
<td>62 (87.32%)</td>
</tr>
<tr>
<td>time of smoking (yrs.)</td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>9 (12.68%)</td>
</tr>
<tr>
<td>21-30</td>
<td>32 (45.07%)</td>
</tr>
<tr>
<td>31-40</td>
<td>20 (28.17%)</td>
</tr>
<tr>
<td>41-50</td>
<td>10 (14.08%)</td>
</tr>
<tr>
<td>(&gt;51)</td>
<td>1 (1.41%)</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>4 (5.56%)</td>
</tr>
<tr>
<td>occasionally</td>
<td>9 (12.50%)</td>
</tr>
<tr>
<td>often</td>
<td>32 (44.44%)</td>
</tr>
<tr>
<td>abuse</td>
<td>27 (37.50%)</td>
</tr>
<tr>
<td>Place of residence</td>
<td></td>
</tr>
<tr>
<td>village</td>
<td>21 (29.17%)</td>
</tr>
<tr>
<td>small city</td>
<td>34 (47.22%)</td>
</tr>
<tr>
<td>big city</td>
<td>17 (23.61%)</td>
</tr>
<tr>
<td>Education</td>
<td></td>
</tr>
<tr>
<td>primary</td>
<td>22 (30.56%)</td>
</tr>
<tr>
<td>vocational</td>
<td>34 (47.22%)</td>
</tr>
<tr>
<td>secondary</td>
<td>12 (16.67%)</td>
</tr>
<tr>
<td>higher</td>
<td>4 (5.56%)</td>
</tr>
<tr>
<td>Negative environmental factors at work</td>
<td></td>
</tr>
<tr>
<td>yes</td>
<td>36 (50.00%)</td>
</tr>
<tr>
<td>no</td>
<td>36 (50.00%)</td>
</tr>
</tbody>
</table>
Methods

Genomic DNA was isolated from fresh-frozen solid tissues using a Gentra Puregene Tissue Kit (QIAGEN) and from peripheral blood leukocytes using a QIAamp DNA Blood Midi Kit (QIAGEN). UPLC was the method used to assess the total content of 5-methylcytosine (5mC) in the DNA of the studied tissues.

Analysis procedure:

I. DNA enzymatic hydrolysis to 2'-deoxynucleosides (dN): dG (2'-deoxyguanosine), dC (2'-deoxycytidine), dT (2'-deoxythymidine), dA (2'-deoxyadenosine), 5mdC (5-methyl-2'-deoxycytidine).

1. 2 - 5μg DNA in a volume of 20μl was denatured at 95°C for 10 minutes
2. 50μl 30mM NaOAc (pH=5.2 Chempur), 3μl 20mM Zn₂SO₄ (Chempur) and 5μl 0.3 U/μl Nuclease P1 (Sigma Aldrich) were added to denatured DNA.
3. The mixture was incubated at 45°C for 30 minutes.
4. 10 μl 1M TRIS (pH=8.0 Sigma Aldrich) and 3μl 1U/μl SAP (Shrimp Alkaline Phosphatase, Fermentas) were added to the mixture.
5. The mixture was incubated at 37°C for 2h.

II. UPLC-based chromatographic separation using the ACQUITY Ultra Performance Liquid Chromatography™ system (Waters) with Tunable UV Detector.

Separation of the five deoxynucleotides was performed using the ACQUITY UPLC BEH C18 Column, with a flow rate of 0.5ml/min. The mobile phase consisted of 50 mM KH₂PO₄ (Chempur) and 1.5% Metanol (Roche). The UV absorbance was monitored at 273 nm.

The standard solution consisted of 30mM from each dN: dA (2'-Deoxyadenosine monohydrate, Sigma-Aldrich), dG (2'-Deoxyguanosine, MP Biomedicals), dC (2'-Deoxycytidine, Sigma-Aldrich), dT (Thymidine, Sigma-Aldrich) and 10mM - 5mdC (5-Methyl-2'-deoxycytidine, TriLink BioTechnologies).

All the data were processed using EMPOWER2 Software (Waters).

The level of 5mdC (% of the dC level) present in DNA samples was calculated using the following equation [Coney 1997, Fuke 2004]:

\[
\text{%5mdC}=\frac{5\text{mdC}}{(d\text{C}+5\text{mdC})} \times 100
\]
Statistical analysis

The Mann-Whitney test and Spearman and Kendall’s test for correlation were used (using the R and SPSS statistical packages).
Results

A clearly significant positive correlation between the frequency of methylation in cancer tissues and the frequency of methylation in normal tissues (i.e. individuals with a high frequency of methylation in normal tissues tend to have a high frequency of methylation in cancer tissues) was observed (p<0.001, Spearman’s test of correlation). The mean level of 5mC in laryngeal cancer tissues and normal laryngeal tissues was similar, about 3.7% (Table 3, Figure 1). We found tumor-to-tumor and normal tissue-to-normal tissue variability in the DNA methylation level (Table 3). According to previous studies by other authors, the range of the global 5-methylcytosine content in human DNA samples from various normal tissues is from about 3.4 to 4.3% and a DNA sample is classified as substantially hypomethylated if the global DNA methylation is ≤3.2% [Ehrlich 2002, Weisenberger 2005]. We found that about 56% of cancer tissues and 49% of normal tissues were substantially hypomethylated, while Ehrlich found hypomethylation in about 35% of colon tumors and Smith et al. in about 67% of head and neck squamous cell cancers [Ehrlich 2002, Smith 2007].

The frequency of methylation in peripheral blood leukocytes in our studies was higher than in both tumor and normal tissues (p<0.001, Wilcoxon signed rank test) (Table 3). The DNA methylation levels in peripheral blood leukocytes from patients with LSCC were higher than in normal leukocytes in the study by Ehrlich et al. (mean levels 5.6% and 3.9, respectively) [Ehrlich 2002]. Only 15.2% of the blood samples in our studies were hypomethylated.

We did not observe any correlations of the level of methylation in the examined tissues with the age of the patients, despite evidence that the level of global genomic demethylation is an age dependent process and differs according to the type of tissue [De Smet 2010, Fraga 2005, Fuke 2004, Suzuki 2006].

Table 3. Levels of 5’-methylcytosine in the studied tissues.

<table>
<thead>
<tr>
<th>Methylation</th>
<th>N</th>
<th>Minimum (%</th>
<th>Maximum (%)</th>
<th>Mean (%)</th>
<th>Std. Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>72</td>
<td>0.012 (1.2)</td>
<td>0.113 (11.3)</td>
<td>0.03657</td>
<td>0.020855</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>72</td>
<td>0.017 (1.7)</td>
<td>0.099 (9.9)</td>
<td>0.03711</td>
<td>0.016755</td>
</tr>
<tr>
<td>Periferal blood leukocytes</td>
<td>65*</td>
<td>0.020 (2.0)</td>
<td>0.144 (14.4)</td>
<td>0.05568</td>
<td>0.023790</td>
</tr>
</tbody>
</table>
seven DNA samples were degraded

Figure 1. A typical image of the chromatographic separation of nucleotides after enzymatic DNA hydrolysis of cancerous tissue (authors' results).

We did not observe any significant correlations between the 5mC level and the clinical data with the exception of the following:
- a negative correlation between the tumor grade and levels of 5’-methylcytosine in peripheral blood leukocytes (p=0.018, Kendall’s correlation coefficient), and
- a positive correlation between the frequency of alcohol consumption and the level of 5mC in the blood (p=0.032, Kendall’s correlation coefficient) was found.
Discussion

Changes in the level of genomic DNA methylation play an important role in many types of human malignancies during both initiation and progression [De Smet 2010, Dong 2003, Eden 2003, Hasegawa 2002, Pogribny 2010, Smiraglia 2003]. The methylation status in such a heterogeneous group of neoplasms as head and neck cancers may vary according to many factors, such as clinical characteristics, e.g. anatomic location, environmental factors [Demokan 2011].

We presented global DNA methylation based on assessing the total 5’-methylcytosine content in a very homogenous group of HNSCC, in LSCC.

We found a significantly decreased level of DNA methylation in both types of laryngeal tissues, cancerous and normal. This is consistent with the observations of other authors, who described global DNA hypomethylation, e.g. in normal colon mucosa, colon adenomas and colon carcinomas or in other types of tumors and adjacent normal tissues [Choi 2009, Cravo 1994, Figueiredo 2009, Poage 2011, Pufulete 2003]. Global hypomethylation in tumor tissues of head and neck tumors was also observed in LINE repetitive sequences by other authors, such as Smith et al. [Smith 2007]. The DNA methylation level of LINE-1 elements in the study of Smith et al. was positively correlated with environmental factors, such as alcohol use and tobacco smoking, as well as high tumor stage [Smith 2007]. In our study we did not observe any correlation between DNA hypomethylation and the clinical data, such as disease stage, tumor size or recurrence, except for a negative correlation between the tumor grade and blood levels of 5-methylcytosine. This suggests that DNA hypomethylation in peripheral blood leukocytes may be a marker for more aggressive tumors. Further studies are necessary to establish such a correlation, because the sample used had a prominent bias towards a specific grade (over 60% of samples were in G2). However, there are several reports of progressively increasing hypomethylation with increasing malignancy grade in a variety of tumors [Costello 2001, Zhu 2010].

The study performed by Szpakowski et al. using a genome-wide microarray approach to analyze squamous carcinomas of the head and neck showed disease-related alterations in DNA methylation [Szpakowski 2009]. This analysis suggested that in tissues adjacent to the tumor there was generalized and highly variable disruption of epigenetic control across the repetitive DNA loci, while in tumor cells LINE-1 elements were preferentially demethylated. Recent studies of head and neck cancer by Poage et al. [Poage 2011] confirmed that global methylation is associated in a sequence-dependent manner. Also, Jackson et al. [Jackson 2004] on the basis of their research consider that different types of cancers may vary in the
timing and frequency of the DNA demethylation of specific repetitive sequences during tumorigenesis.

The role of hypomethylation in carcinogenesis of LSCC is also confirmed by results from our previous studies. We found high level, statistically significant chromosomal rearrangements in centromeric and telomeric regions in LSCC in a CGH study [unpublished]. It has been shown that the demethylation of repetitive sequences located in centromeres, as well as regions around the centromeres or subtelomeric regions, can induce chromosomal instability [Narayan 1998, Martinez 2012, Pogribny 2010, Stach 2003], which play a role in HNSCC carcinogenesis [De Smet 2010, Eden 2003, Hermsen 1996, Jin 2000, Stembalska 2002 - PhD thesis].

Taking into account the theory of field cancerization in HNSCC [Braakhuis 2003], we suggest that decreased DNA methylation levels in LSCC patients for cancerous and normal tissues may result from the impact of tobacco smoke [Smith 2007] and/or alcohol consumption (see Table 2) [Gabriel 2006, Pufulete 2005]. Current studies have shown that smoking and drinking alcohol are associated with low levels of vitamin B12, which is required for the synthesis of S-adenosylmethionine, SAM (a source of methyl groups in the methylation process) [Gabriel 2006, Pufulete et al. 2005, Smith 2007]. Also, microenvironmental hypoxia, which may be caused by smoking and alcohol consumption, can influence local epigenetic changes, leading to the inappropriate silencing and re-activation of genes involved in carcinogenesis [Fraga 2005, Shahrzad 2007]. Regions of solid tumors are temporarily and/or chronically exposed to hypoxia and reperfusion, which are known to contribute to the development of cancer [Shahrzad 2007]. Hypomethylation may also be related to a folate deficiency in one's diet [Figueiredo 2009, Pufulete 2003, Pufulete 2005, Sapkota 2008], or the presence of mutations or polymorphisms in genes encoding enzymes involved in folate and methionine (a precursor of SAM) metabolism, such as MTR (methionine synthase) or MTHFR (the 5,10-methylenetetrahydrofolate reductase), [Kruszyna 2010, Paz 2002, Pufulete 2005]. The present study did not consider dietary and genetic factors, which could modify the results by influencing the metabolism of folic acid. Although it may be assumed that in our study group, which consisted almost entirely of smokers (only one person did not smoke) and over 80% of whom were alcohol drinkers, there is a deficiency of various micronutrients in their diets. Piyathilake et al., who did not observe hypomethylation in oral squamous cell carcinomas in comparison to the results of earlier studies for squamous cell lung carcinomas using monoclonal antibodies specific to 5-methylcytosine, suggested that various risk factors (including nutrition) and the activity of
methyltransferases may affect the difference between hypomethylation levels in studied tumors [Piyathilake 2005].

Except for a positive association between the frequency of alcohol consumption and the level of 5-methylcytosine in peripheral blood leukocytes, we did not find any effects of demographic or lifestyle factors on DNA methylation. An negative association was reported by Choi et al. However, the frequency of alcohol consumption did not affect the association between global DNA methylation and the risk of breast cancer [Choi 2009].

The methylation levels observed in our study in all tissues are similar to the specific kind of variation in methylation in head and neck cancers found by Poage et al. [Poage 2011]. The differences in methylation levels could indicate the coexistence of cells actually presenting hypermethylation. This observation is consistent with the results of other authors, such as Smith et al. [2007] and Ehrlich et al. [2002]. Further research is required to clarify the high level of methylation in peripheral blood leukocytes of LSCC patients.

We did not find an association between DNA hypomethylation in tumor tissues and hypomethylation in peripheral blood leukocytes. Our results are similar to Furniss et al. [2008], who did not find any relationship between the level of methylation in tumor tissue and in blood for patients with laryngeal cancer. Therefore, we believe that peripheral leukocyte DNA methylation measured by 5-methylcytosine content cannot be used as a surrogate marker of genomic instability in LSCC.

Conclusion:
We found decreased global DNA methylation level in laryngeal cancer tissues and surrounding laryngeal tissue, indicating the presence of hypomethylation in both types of tissue. We suggest that hypomethylation in both tissues may be the result of field cancerization, but further studies are needed to confirm this. Subsequent work will be necessary to investigate the negative correlation between the tumor grade and blood levels of 5-methylcytosine found here.
The level of leukocyte DNA methylation measured using total content of 5-methylcytosine cannot be used as a surrogate marker for genome methylation status in cancer tissues.

Acknowledgements
This work was supported by a Polish government grant for science research projects (Contract No. 2360/B/P01/2008/34).
The study was supported by a research fellowship within “The Development Program of Wroclaw Medical University” funded from the European Social Fund, Human Capital, National Cohesion Strategy” (contract no. UDA-POKL.04.01.01-00-010/08-01)”. 
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