DENTAL MEDICINE

Epigenetic methylation changes: implication as biomarkers in oral and maxillofacial area cancers

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Abstract

Background/Aim. Squamous cell carcinoma (SCC) is the most frequent cancer of the head and neck area in the oral cavity. Epigenetic alterations in oral and maxillofacial area cancers are urgently needed to be investigated, as the observed changes might have crucial diagnostic value for personalized medicine.

Methods. Our study aimed to identify the most frequently hypermethylated tumor suppressor gene promoters in OSCC, followed by correlation analysis with the patients' survival. We evaluated the methylation status of the promoters in a panel of 22 tumor suppressor genes in Romanian (n=9) and Bulgarian (n=12) patient groups suffering from oral and maxillofacial area cancers. The extracted DNA was further digested through EpiTect Methyl II PCR Array System containing methylation-sensitive and methylation-dependent restriction enzymes, followed by specific amplification of the products obtained by qPCR and data analysis using the online platform provided by the producer.

Results. Different methylation patterns were observed in the tumor suppressor genes' promoters. Among them, the methylation profile of Cccnd2, Chd1, Cdh13, Cdkn1c, Neurog1, Gstp1, and Runx3 genes further correlated with overall survival rates.

Conclusions. Our data emphasize that epigenetic alterations are responsible for the clinical heterogeneity of oral and maxillofacial area cancers and significantly impact on patient survival. Additional investigation on a larger patient cohort should validate these potential biomarkers.

Keywords: oral cancer, promoter methylation status, tumor suppressor genes

Introduction

Oral cancer is the most frequent cancer of the head and neck area, accounting for about 90% of all malignancies of the oral cavity [1,2]. Due to its significant mortality and persistently low cure rates, OSCC represents a major public health problem with a substantial individual and socioeconomic impact. Despite the many advancements in oral cancer prevention and multimodality treatment, five-year survival rates for this type of malignancy remain at a disappointingly low level, almost unchanged over the past 20 years [3].

There is no doubt that epigenetics and genetic and transcriptomic alterations represent essential hallmarks of the complex cancer roadmap [4,5]. These mechanisms may take place in varying order [4], but their complementarity is involved in every step of cancer evolution, from responses to environmental exposures to progression into malignancy [6-10]. The increased methylation pattern has essential effects

on cellular mechanisms, altering the transcriptional control that leads to inappropriate inhibition of suppressor genes or activation of oncogenes [6,11,12].

The reversible events represented by epigenetic alterations are related to modifications in gene expression that do not include changes in the DNA sequence [13]. The aberrant epigenetic alterations, including DNA methylation or histone modification, can interfere with the binding of transcription factors to the DNA of tumor suppressor genes, resulting in transcriptional silencing of key protein-coding and non-coding transcripts, involved in the restriction of uncontrolled cell growth. In the last decade, epigenetics has become the primary focus of numerous investigations, as it provides previously unrecognized mechanistic insights into the etiology of carcinogenesis [14,15]. Unlike genetic events, epigenetic changes do not result in DNA sequence modification but have extensive effects on gene activity, mainly by modifying their promoters. The main epigenetic changes revealed in cancer are related to DNA methylation of the "CpG islands" within promoters in tumor suppressor genes or post-translational modifications of chromatin proteins.

Consequently, epigenetic silencing of tumor suppressor genes is observed [16]. Our study aimed to identify the most frequently hypermethylated tumor suppressor gene promoters in oral and maxillofacial area cancers. Further, a correlation with clinical characteristics such as the patients' overall survival (OS) has been found.

Methods

Tissue samples. Tissue samples of both tumors and adjacent normal tissues were collected from patients with oral cavity and maxillofacial area tumors that underwent surgical treatment. The samples were collected from the Oral Maxillofacial Surgery Clinic II in Cluj-Napoca (coded: R1-9), Romania (protocol No. 147/06.05.2019), and from the Maxillofacial Surgery Clinic in Plovdiv (code: B1-12), Bulgaria, and are displayed in table I. The Institutional Ethics Committee approved the study of Medical University - Plovdiv (Protocol No1/25.02.2016). All patients included in the study signed an informed consent for this prospective study.

Analysis of DNA Methylation patterns in cancer tissues and their normal adjacent tissues. Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen). This procedure comprises a step of removal of RNA using RNase. Genomic DNA isolated from normal and tumor OSCC samples was used to evaluate the methylation status of 22 tumor suppressor gene promoters (Human Tumor Suppressor Genes EpiTectMethyl qPCR Array system, EAHS-551ZQiagen), frequently described in the literature as hypermethylated [17]. The EpiTect Methyl II PCR Array System contains methylation-sensitive and methylation-dependent restriction enzymes. This quantification system uses two endonucleases for distinct cleavage of target sequences that require the presence or absence of methylated cytosine. Then the digested products were assessed by qRT-PCR.

Table I. Clinical characteristics of patients with oral and maxillofacial area cancer.

Patient cohort	No	Age	Sex	Diagnosis	Localization	Stage
UMPh	R1	72	M	OSCC	left tonsillar space	T3N2Mx
	R2	78	M	SCC	left parotid gland	T4aNxMx
	R3	56	M	OSCC	retromolar trigone	T4aN2bMx
	R4	49	F	SCC	left parotid gland	T2N0Mx
	R5	47	F	SCC	right parotid gland	T2N0Mx
	R6	35	F	OSCC	left parapharyngeal region	T3N0Mx
	R7	44	M	OSCC	tongue	T1N0Mx
	R8	78	F	OSCC	inferior lip	T1N0Mx
	R9	67	M	OSCC	mandible	T1N0Mx
PlU	B1	66	M	OSCC	floor of mouth	T2N1Mx
	B2	47	M	OSCC	floor of mouth and tongue	T2N0Mx
	B3	52	M	OSCC	floor of mouth	T2N1Mx
	B4	65	M	OSCC	floor of mouth	T1N0Mx
	B5	76	M	OSCC	mandibular gingiva	T2N1Mx
	B6	76	F	OSCC	tongue	T2N0Mx
	B7	64	M	OSCC	tongue	T2N0Mx
	B8	60	M	OSCC	floor of mouth	T2N0Mx
	B9	56	F	OSCC	right retromolar triangle	T1N0Mx
	B10	66	M	OSCC	floor of mouth	T2N0Mx
	B11	57	M	OSCC	tongue	T1N0Mx
	B12	67	M	OSCC	tongue	T2N0Mx

DNA Methylation data analysis. Data analysis was done using the analysis templates provided by the producer, which furnish the gene promoter methylation status as a fraction of unmethylated (FUM), intermediary methylated (FIM), methylated (FM), or hypermethylated (FHM) of the fraction of DNA input, using the online platform provided by Qiagen (www.sabiosciences.com/dna_methylation_array.php).

Survival analyses. Kaplan–Meier analysis was performed to generate data concerning the effect of promoter methylation of the selected tumor suppressor genes on OS. The Kaplan-Meier survival curve showed overall survival outcomes for relative high-risk and low-risk patients considering the methylation status of selected genes based on the average methylation patterns. A log-rank test assessed the differences between the curves. Hazard ratio (HR) and 95% confidence intervals (CI) were evaluated. Kaplan–Meier curves were generated by GraphPad Prism (version 8).

Gene network. The interconnection among the methylated genes was used String Network online tool (https://string-db.org).

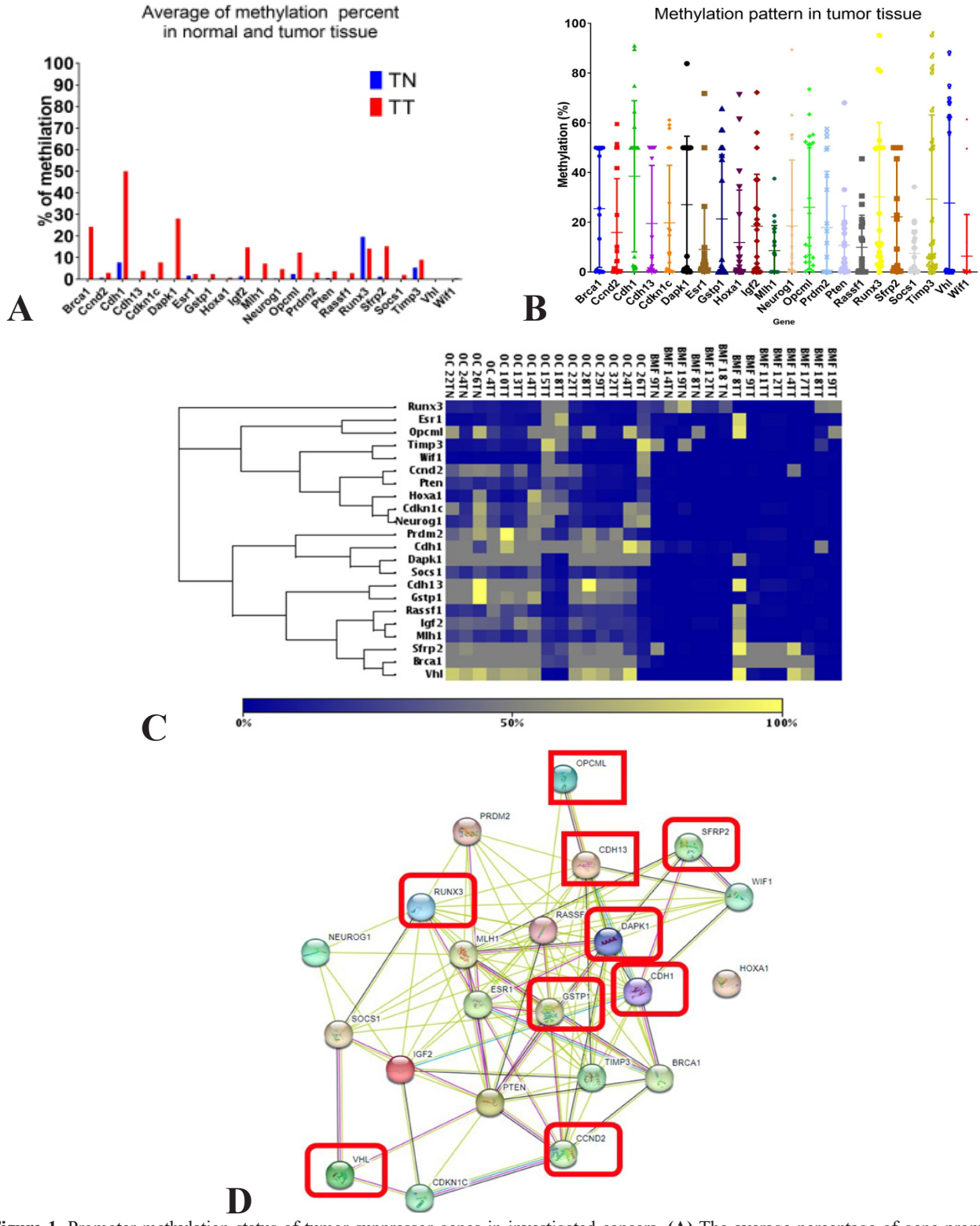

Figure 1. Promoter methylation status of tumor suppressor genes in investigated cancers. **(A)** The average percentage of gene promoter methylation pattern in normal and tumor tissues; **(B)** Individual representation of gene promoters' methylation in tumor tissue; **(C)** Representative heatmap of promoter methylation pattern of human tumor suppressor genes in normal and tumoral tissue from oral cancer patients. Blue color corresponds to a reduced methylation status, whereas yellow indicates an increased methylation pattern; **(D)** Predicted protein interaction network encoded by the methylated tumor suppressor genes. The network was generated using String9.1. The network is interconnected by different lines, indicating: deep-blue colour: co-occurrence; black colour: co-expression; pink colour: based on experimental data; light blue colour: data retrieved from the String database. Red boxes represent proteins produced by genes hypermethylated in oral cancer.

Results

The implication of promoter methylation of tumor suppressor genes in oral cavity and maxillofacial area cancers

To find the cancer-specific methylation profile, we analyzed the promoter region of 22 tumor suppressor genes through the Human Tumor Suppressor Genes EpiTectMethylqPCR Array system. Clustering data from patients with oral cancer demonstrated increased methylation pattern modifications in tumor tissues compared to normal tissues at the level of the analyzed tumor suppressor genes (Figure 1A). Our results showed that the following tumor suppressor genes had a high level of promoter methylation (an average of over 20% methylation) in tumor tissues: Brca1, Chd1, Cdh13, Cdkn1c, Dapk1, Gstp1 Opcml, Runx3, Sfrp2, Timp3 and Vhl (Figure 1B). The heatmap summarizing the methylation profile of the selected genes in two different patient cohorts (total N=21) is presented in figure 1C.

A STRING9.1 network was generated using the evaluated tumor suppressor genes list to identify the relevant methylated promoter genes with implications in oral carcinogenesis. We identified some relevant genes with methylated promoters based on these results, which correlated with survival rates (Figure 1A-C). The genes retrieved as frequently methylated are displayed in the red box, interconnected as a network (Figure D).

The methylation of crucial tumor suppressor genes is associated with shorter OS in the investigated cancers

Kaplan-Meier survival curves were analyzed using an average promoter methylation pattern (in both standard and tumor tissue) as a cut-off value for the separation in low and high methylation patterns for each analyzed sample. The OS rate correlated significantly with the methylation pattern for the following genes: Cccnd2, Chd1, Cdkn1c, Igf2, Cdh13, Neurog1, and Runx3 (Figure 2). On the contrary, no statistically significant correlation with the OS rate was observed for Brca1, Dapk1, Opcml, Prdm2, Vhl, Timp3, and Sfrp2 for the high methylated group.

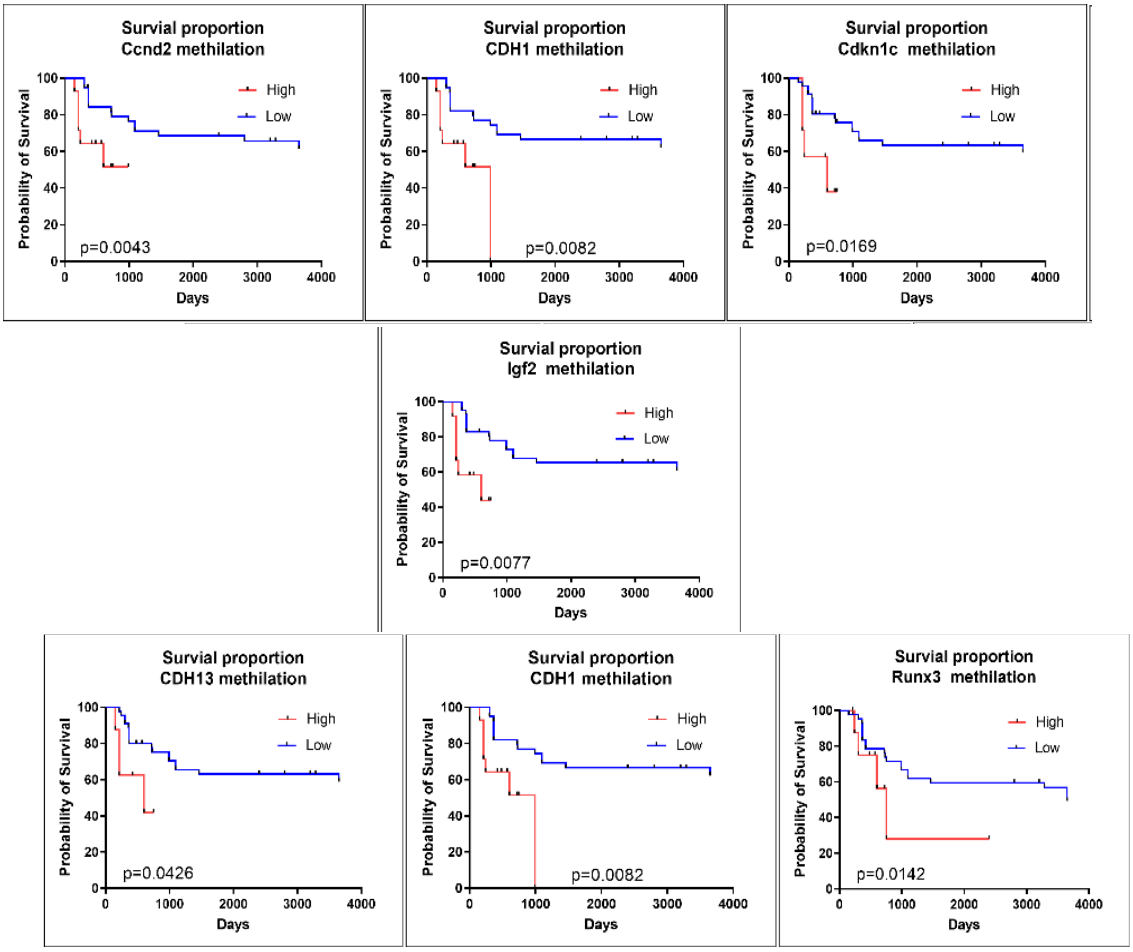

Figure 2. Kaplan-Meier survival analysis estimated OS in patients with oral cancer related to the methylation status of Cccnd2, Chd1, Cdkn1c, Igf2, Cdh13, Neurog1, and Runx3 genes. Samples were separated into low and respectively high methylated groups based on average methylation status; $p \leq 0.05$ was considered statistically significant.

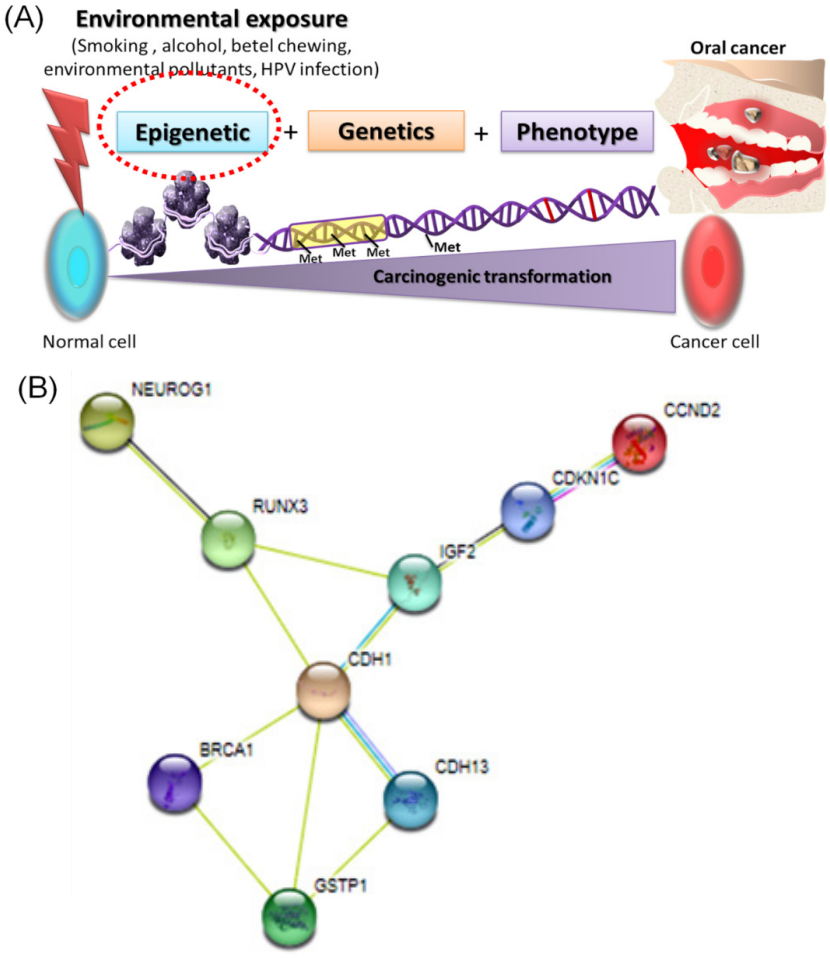

Figure 3. Epigenetic alteration in OSCC. **(A)** Schematic representation of the interconnectivity between environmental exposure, genetic and epigenetic variations, and specific phenotypes in OSCC carcinogenesis; **(B)** The network was generated using String 9.1 for the genes correlating with OS rates.

Discussion

The loss of expression of tumor suppressor genes is one of the earliest events that precede the process of oncogenesis. Our investigation provides evidence of the impact of epigenetic alterations on the cancer risk [18], including oral and maxillofacial area cancers. Therefore, our results seem applicable to precision diagnostics and patient stratification.

Based on these results, we proposed a schematic representation of the interactions between environmental exposure, genetic and epigenetic variation, and specific phenotype in OSCC carcinogenesis (Figure 3A). The presented network was generated using String 9.1 for the genes that correlate with OS rates (Figure 3B).

Based on survival data, we can assume that epigenetic alterations can have a higher impact on cancer risk than genetic alterations [6,19-23]. Oral cancer shares similar epigenetic characteristics with other cancer types related to CpG island hypermethylation of many tumor suppressor gene promoters, followed by transcriptional inactivation [24]. These events appear within genes' promoter regions and could be considered early events in the cancer initiation [25].

We observed some specifically activated pathways, showing the transition from benign to malignant tumors. This suggests that the analyzed genes might act as possible biomarkers in the early stages of malignancy, where the core of the network is represented by Chd1, and Cdh13**,** two important epithelial cell adhesion molecules (Figure 1D). As indicated by our results, there is a wide range of cellular processes regulated by genes' promoter hypermethylation, such as apoptosis (Rassf1A), Wnt signaling (Wif1, Runx3), or DNA-repair genes (Brca1 and Mlh1) [26-28].

It has been shown that the expression of homeobox genes is usually controlled by an epigenetic mechanism, such as the methylation of CpG islands in the promoter region

which further impacts patients' prognosis [29]. An increased percentage of methylation is shown in the samples of OSCC patients, particularly at the advanced stage [25].

Rassf1A (Ras association domain family one isoform A) promoter hypermethylation was proven to have negative prognostic value for patients with salivary adenoid cystic carcinoma [30]. The Ras/PI3K/AKT pathway regulates critical cellular processes, including apoptosis, cell growth, and proliferation. This pathway sustains tumorigenesis and contributes to oncogene activation in the PIK3CA/AKT pathway in OSCC [31]. In addition, it was demonstrated that epigenetic silencing of tumor suppressor genes related to this pathway promoted the radioresistance [32].

The human runt-related transcription factor 3 (Runx3) is an essential component of the TGFβ1 (transforming growth factor-beta 1) pathway shown to be hypermethylated in the gastrointestinal cancer [33]. Runx3 expression level correlates with the methylation status in the head and neck cancer [34] or oesophagal squamous cell carcinoma [33]. Moreover, Runx3 expression was low due to the methylation of its promoter in average oral epithelial cells [34]. Also, Runx3 methylation corresponded to worse patient outcomes [22].

Cdh1 (Cadherin 1, type 1, E-cadherin) is another hypermethylated gene, and this scenario determined shorter OS. It regulates the intercellular adhesion between epithelial cells [19]. Cdh1 is a tumor suppressor gene with a hypermethylated promoter in oral cancer. Promoter hypermethylation of MGMT (O-6-Methylguanine-DNA Methyltransferase) and CDH1 genes in oral cavity cancer was considered a possible molecular marker for the unfavourable prognosis of advanced cancers [35].

Mlh1 (MutL homolog 1, colon cancer, nonpolyposis type 2) promoter methylation was proven to be an early event in the oral cancer [36]. Another study observed an increased incidence of promoter hypermethylation in 23% of the cases for Mlh1 and 35% for Cdh1 in the OSCC [37]. In our study, the hypermethylation pattern was more intensive for the Mlh1 gene and relatively similar for Cdh1. Noorlag et al. revealed a higher level of methylation pattern for *RARB* (31% of cases), *CHFR* (20%), *Cdh13* (13%), *Dapk1* (12%), and *Apc* (10%) for head and neck cancer [23]. The methylation pattern was slightly different for our patient cohort, wherein the methylation percentage for *Cdh13* was 35%, and in the Noorlag et al. study, it was about 13%.

Timp3 (TIMP metallopeptidase inhibitor 3) is a critical factor that regulates epithelial-to-mesenchymal transition, a process involved in the invasion and metastasis [13,19]. This gene was frequently found methylated in our tested samples. Another study presented Dapk1, DCC, and Timp3 as hypermethylated in over 90% of oral cancers stages T1 and T2 [38]. In addition, Dapk1 and MGMT methylation correlated with lymph node metastasis [21]. Timp3 was shown to have prognostic significance in post-treatment saliva from head and neck cancer patients [39].

A meta-analysis study revealed that the DNA hypermethylation of the Dapk1 gene promoter was a promising prognostic and diagnostic biomarker [21,40]. Dapk and MGMT show potential as epigenetic markers in HPV-negative oral and oropharyngeal squamous cell carcinoma [11].

The current study provides evidence of specific promoter gene methylation signatures in oral cancer with the potential clinical application, as the DNA methylation status might be a possible biomarker correlated with overall survival for oral cancer patients. A more significant number of patients and confounding factors analysis are needed to confirm our results, not only from tissue but also from serum or saliva [41]. Additional studies should be considered based on HPV status [11].

The methylations of the tumor suppressor gene promoters in our study were considered to reflect the general epigenetic damage in individual tissues. To support this idea, the interconnectivity among the evaluated biomarkers was evaluated. Moreover, these genes were interconnected with the most frequently mutated genes, suggesting the efficacy of combining the correlation of methylation pattern with the mutational one as a single entity with crucial prognostic significance in the OSCC [4,42,43].

The interdependence between genetics and epigenetics is studied and might reveal intricate roads in cancer development (Figure 3). Epigenetic interventions using epigenetic modulation can enhance the response to therapy in oral cancer, particularly for counteracting the therapeutic resistance of this aggressive disease [16].

Conclusion

Our data argued that molecular modifications at the genetic and epigenetic levels could be merged into classical diagnostic systems to stream out more precise personal diagnoses and prognoses. The interplay between genetic and epigenetic alterations is responsible for the clinical heterogeneity of this tumor type, with another significant impact on patient prognosis.

Based on our data, we suggest that DNA methylation status can be used as a possible biomarker and can be associated with the survival rate. Furthermore, the promoter methylation analysis of a large cohort of early oral and maxillofacial area cancers with frequently hypermethylated genes can be used in a therapeutic strategy based on reversible epigenetic alterations in specific conditions.

The methylated tumor suppressor gene promoters are interdependent within a network with the most frequently mutated genes. The network exploited only those cases related to the survival rate; consequently, the epigenetic imbalances can be used for a personalized tumor profile. The current study provides evidence of specific methylation signatures in oral and maxillofacial area cancer potential clinical application.

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