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Original Research

DNA promoter methylation of cancer-associated genes in patients with head and neck squamous cell carcinoma

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ABSTRACT

Introduction: Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world. Abnormal methylation can be one reason for this cancer. This study aimed to investigate the DNA promotor methylation status of cancer-associated genes (*ATM, APC, CDO1, RB1, TP53,* and *WIF1*) in patients with HNSCC. **Methods**: Bisulfite conversion and methylation-sensitive high-resolution melting were used to analyze the DNA methylation levels in normal and tumor tissues in 44 patients. **Results**: Significant differences in DNA methylation were observed between tumor and normal tissues for *CDO1* and *WIF1* genes in all subjects and subgroups ($p < 0.05$). In the T_3 subgroup, a significant correlation was found between CDO1 gene methylation and age in normal tissue. The same correlation was detected for the WIF1 gene methylation in tumor tissue samples in the subgroup with T_3 and normal tissue samples in the subgroup with T_4 ($p < 0.05$). In all genes, no significant differences were found between the patient subgroups (T_2, T_3, T_4 stage, primary/recurrent lesion, non-keratinizing/keratinizing SCC, age before/after 50, and smokers/non-smokers). **Conclusion**: Changes in the expressions of *CDO1* and *WIF1* genes can affect the mechanisms of the occurrence and development of HNSCC. Methylation in the ATM, APC, RB1, and TP53 genes is not specific to HNSCC.

Key words: DNA methylation, cancer associated genes, head and neck squamous cell carcinoma

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INTRODUCTION

Epigenetic gene regulation changes (such as DNA methylation, histone modification, chromatin remodeling, and non-coding RNA) and DNA mutation are the basis of the molecular mechanisms of cancerogenesis and tumorigenesis. Therefore, studying epigenetic markers for diagnosis, prognosis, and cancer prevention is particularly interesting. Detection of DNA methylation levels has recently been used as biomarkers for early diagnosis and prognosis to identify target genes for drug therapy.

The hypomethylation of single CpG dinucleotides has been one of the first changes found in tumor cells[1]. A large part of the investigation has been carried out during DNA methylation in the promoter region of various genes[2,3]. There are changes in the methylation levels of general and tissue-specific cell genes in the ontogenesis *in utero* and post-natal periods.

The non-mutagenic etiology of cancer, such as disturbance of DNA methylation, is influenced by age, diet, transplacental, environmental, and occupational exposure. Moreover, its dual ability to be reversible and transgenerational demands a specific approach to interpreting results. Age-related DNA methylation is associated with different diseases[4] and sex hormone status in some cancer types[5]. There is a significant gap in understanding the association between the age-expected DNA methylation level and cancer[6]. In particular, no data exist regarding pre- and post-menopausal differences in DNA methylation levels and their effects on cancer development.

The most intensive processes are in the genome of active proliferating cells, such as epithelial cells. Therefore, epithelial cells in various cancers are convenient models for studying epigenetic malformation. Head and neck squamous cell carcinoma (HNSCC) is an example of oncopathology with epithelial tissue damage.

HNSCC corresponds to the majority of cases (~90%) of head and neck cancers (HNCs)[7]. HNCs are rated 6–7 in global prevalence among cancers, with 700,000 newly registered cases and 470,000 deaths a year[8]. The therapeutic options for HNCs include surgical treatment, radiotherapy, and chemotherapy. The management tactics should be personalized by a multidisciplinary board comprising an oncological surgeon, a chemotherapist, a radiotherapist, a psychologist, a rehabilitation specialist, and a dentist[9,10].

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Despite the advanced diagnostics, cancer screening, and HPV vaccination programs, 60–70% of the cases are diagnosed as late as during stage III–IV, associated with low life expectancy and high risks of recurrence [11]. Therefore, finding new potential targets for therapy is crucial.

The main risk factor for HNSCC is smoking. In some publications, it has been shown that smokers have a 10-fold higher risk of developing the disease than non-smokers. Smoking, in combination with frequent alcohol consumption, increases the risk more than 35-fold [12–14]. Other risk factors include ultraviolet and ionizing radiation, various toxic compounds, a weakened immune system, a diet low in vitamins A and B, age over 40, male, and some viral infections [15].

DNA methylation also varies due to several factors, including age and disease status. Silenced genes are often methylated, while active genes remain largely unmethylated [16,17]. DNA methylation is the most studied epigenetic mechanism in HNSCC. It allows us to observe changes in methylation patterns in a whole genome and individual genes that can be used to identify new disease biomarkers [18]. Various biomaterials can be used for studies, including liquid biopsy samples, which provide a noninvasive alternative for early cancer detection. In particular, Zhou C. studied 27 aberrantly methylated genes with altered expression and showed that FAM135B methylation is a favorable independent prognostic marker for overall survival in patients with HNSCC [19].

We previously investigated the DNA methylation status of some tumor-associated genes (*CDO1, MEST, RASSF1A, RASSF2, RASSF5*, and *WIF1*) in patients with HNSCC [20]. We observed significant differences in DNA methylation levels between tumor and normal tissues in the *CDO1* and *WIF1* genes in all patient groups and subgroups (larynx and other cancers, SCC keratinizing and non-keratinizing, primary and recurrent tumor, and smokers and non-smokers) [21]. The methylation level in the *CDO1* gene in tumor tissue was significantly increased in the T_4 and T_3 stage subgroups over the T_2 stage [22].

For further research, we investigated another group of tumors and normal tissue from patients with HNSCC. We chose other cancer-associated genes: *ATM, APC, RB1,* and *TP53*. We also used these earlier studied genes (*CDO1* and *WIF1*), as they had shown notable results in other patients (**Table 1**). The selected genes play a considerable role in the regulation of cell proliferation, differentiation, and apoptosis, the disruption of which can lead to oncopathology.

This study aimed to investigate the DNA promotor methylation status of cancer-associated genes (*ATM, APC, CDO1, RB1, TP53,* and *WIF1*) in patients with HNSCC.

Table 1: Characteristics of studied genes

Symbol	Gene name	Location	Exon count	Gene ID	Transcripts	MIM	Gene type	Gene function	References
APC GS; DP2; DP3; BTPS2; DESMD; DP2.5; PPP1R46	Adenomatous Polyposis Coli	5q22.2	20	5624	NM_000038.6	611731	protein-coding	tumor suppressor	37; 39
ATM AT1; ATA; ATC; ATD; ATE; ATDC; TEL1; TELO1	Ataxia-telangiectasia-mutated	11q22.3	67	472	NM_000051.4	607585	protein-coding	cell cycle regulation.	42-44
CDO1 (CDO-I)	Cysteine dioxygenase type 1	5q22.3	9	1036	NM_001323565.2	603943	protein-coding	tumor suppressor	26-28
TP53 (P53; BCC7; LFS1; BMFS5; TRP53)	Tumor Protein P53	17p13.1	11	7157	NM_000546.6.	191170	protein-coding	tumor suppressor	45-50
RB1 (RB; pRb; OSRC; pp110; p105-Rb; PPP1R130; p110-RB1)	Retinoblastoma 1	13q14.2	27	5925	NM_000321.3...	614041	protein-coding	cell cycle regulator, tumor suppressor	51-53
WIF1 (WIF-1)	WNT inhibitory factor 1	12q14.3	10	11197	NM_007191.5	605186	protein-coding	tumor suppressor	19; 30-36

Table 2: Characteristics of patient

Patient ID	Tumor origin	ICD-10	TNM	Type of lesion	Histological glade	Gender	Age	Smoker
1	Tongue n=9	C02.0	T1N0M0	Prim	SCC non-keratinizing	M	50	y
2		C02.1	T2N0M0	Prim	SCC non-keratinizing	F	59	n
3		C02.1	T3N0M0	Rec	SCC keratinizing	F	66	y
4		C02.1	T3N0M0	Rec	SCC non-keratinizing	F	47	n
5		C02.1	T3N0M0	Prim	SCC keratinizing	M	63	n
6		C02.1	T3N0M0	Prim	SCC keratinizing	M	40	n
7		C02.1	T3N0M0	Prim	SCC keratinizing	M	46	n
8		C02.1	T3N1M0	Prim	SCC non-keratinizing	M	52	n
9		C02.1	T4N2M0	Prim	SCC keratinizing	M	36	n
10	Oral cavity n=6	C03.0	T2N0M0	Prim	SCC non-keratinizing	F	59	n
11		C03.0	T3N0M0	Prim	SCC keratinizing	M	69	y
12		C03.0	T4N0M0	Prim	SCC keratinizing	M	37	y
13		C03.1	T4N0M0	Prim	SCC non-keratinizing	M	30	n
14		C03.1	T4N0M0	Prim	SCC non-keratinizing	F	63	n
15		C03.1	T4N0M0	Prim	SCC non-keratinizing	F	48	n
16	Floor of mouth n=3	C04.1	T2N0M0	Prim	SCC keratinizing	F	67	y
17		C04.1	T3N1M0	Prim	SCC non-keratinizing	F	66	y
18		C04.1	T4N0M0	Prim	SCC keratinizing	F	64	y
19	Maxillary sinus n=6	C31.0	T3N0M0	Prim	SCC non-keratinizing	M	58	y
20		C31.1	T3N0M0	Prim	SCC non-keratinizing	M	55	y
21		C31.8	T3N0M0	Rec	SCC non-keratinizing	M	64	y
22		C31.0	T4N0M0	Rec	SCC non-keratinizing	M	41	n
23		C31.8	T4aN0M0	Prim	SCC non-keratinizing	M	61	n
24		C31.0	T4aN0M0	Prim	SCC non-keratinizing	M	67	n
25	Larynx n=20	C32.1	T1N2M0	Prim	SCC non-keratinizing	M	48	n
26		C32.0	T2N0M0	Prim	SCC non-keratinizing	F	55	n
27		C32.8	T2N0M0	Prim	SCC non-keratinizing	M	70	n

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Table 2 continued

Patient ID	Tumor origin	ICD-10	TNM	Type of lesion	Histological glade	Gender	Age	Smoker
28		C32.0	T2N0M0	Prim	SCC non-keratinizing	M	69	y
29		C32.0	T2N0M0	Prim	SCC non-keratinizing	M	58	y
30		C32.0	T3N0M0	Prim	SCC keratinizing	M	64	y
31		C32.0	T3N0M0	Prim	SCC non-keratinizing	M	62	n
32		C32.0	T3N0M0	Prim	SCC keratinizing	M	62	n
33		C32.8	T3N0M0	Rec	SCC non-keratinizing	M	64	y
34		C32.8	T3N0M0	Prim	SCC non-keratinizing	M	69	y
35		C32.8	T3N0M0	Prim	SCC non-keratinizing	M	58	y
36		C32.9	T3N0M0	Prim	SCC keratinizing	M	61	n
37		C32.8	T3N1M0	Prim	SCC non-keratinizing	M	49	y
38		C32.8	T3N0M0	Rec	SCC non-keratinizing	M	69	n
39		C32.0	T4N2M0	Prim	SCC non-keratinizing	M	64	n
40		C32.0	T4N0M0	Prim	SCC keratinizing	M	71	y
41		C32.8	T4aN0M0	Prim	SCC keratinizing	M	72	y
42		C32.8	T4aN0M0	Prim	SCC non-keratinizing	M	58	n
43		C32.8	T4aN2aM0	Prim	SCC non-keratinizing	M	70	y
44		C32.8	T4aN2bM0	Rec	SCC non-keratinizing	F	81	n

*M: male, F: female, SCC: squamous cell carcinoma, Prim: primary tumor, Rec: recurrent tumor, y: yes, n: no, ICD-10: International Statistical Classification of Diseases and Related Health Problems

Table 3: Primer sequences used for Methylation-Specific PCR (MSP)

Gene	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')	Product size (bp*)
ATM	GTTGGTTATTGGTGGATATGG	TAATTCCAAAACCCAAACTCTTAAC	696
APC	GTTGGTTATTGGTGGATATGG	AACCTACAAAACCAAAAACCAACTA	600
CDO1	GGGAGGATGA ATTTTATAGATTTG	TAAACTTCCATA ATAACCTACACCTC	396
RB1	GATAGGGATGAGGTTTATAGTTATTTATTAAAATCCTATCACCATTCTACAAAC		770
TP53	GGATTATTTGTTTTTATTTGTTATGG	CAAAACTCCACTCCTCTACCTAAAC	495
WIF1	GAGTGATGTT TTAGGGGT	CCTCAACCA AAACTATTCC	464

* bp: base pair

Table 4: Average level of promotor genes methylation in tumor and normal tissue in all patient and their subgroups

Patients (n)	Sample	All (n = 44)	TNM **			Type of lesion		SCC		Age		Smokers	
Genes										before 50 years (n= 10)	after 50 years (n = 34)	Yes (n = 20)	No (n = 24)
			T2 (n = 7)	T3 (n = 20)	T4 (n = 15)	Prim (n = 37)	Rec (n =7)	non-keratinizing (n = 30)	keratinizing (n = 14)				
		M ± m, Range											
APC	T	0.36±0.17 (0.01÷0.54	0.31±0.14 (0.05÷0.47)	0.32±0.18 (0.01÷0.54)	0.31±0.16 (0.01÷0.52)	0.30±0.18 (0.01÷0.54)	0.36±0.16 (0.01÷0.47)	0.36±0.14 (0.01÷0.54)	0.24±0.20 (0.01÷0.52)	0.32±0.17 (0.01÷0.50)	0.31±0.17 (0.01÷0.54)	0.31±0.16 (0.01÷0.50)	0.31±0.20 (0.01÷0.54)
	N	0.28±0.18 (0.01÷0.56	0.22±0.18 (0.07÷0.48)	0.31±0.18 (0.04÷0.56)	0.24±0.18 (0.01÷0.56)	0.27±0.19 (0.01÷0.56)	0.34±0.14 (0.01÷0.46)	0.32±0.17 (0.01÷0.56)	0.21±0.19 (0.01÷0.56)	0.27±0.18 (0.04÷0.50)	0.28±0.19 (0.01÷0.56)	0.27±0.17 (0.01÷0.50)	0.29±0.20 (0.01÷0.56)
ATM	T	0.35±0.18 (0.01÷0.54	0.31±0.20 (0.04÷0.54)	0.34±0.19 (0.01÷0.54)	0.37±0.18 (0.06÷0.54)	0.34±0.19 (0.01÷0.54)	0.38±0.19 (0.01÷0.53)	0.39±0.18 (0.01÷0.54)	0.28±0.18 (0.01÷0.53)	0.30±0.23 (0.01÷0.53)	0.37±0.17 (0.04÷0.54)	0.33±0.19 (0.01÷0.54)	0.37±0.18 (0.04÷0.54)
	N	0.30±0.19 (0.01÷0.57	0.27±0.22 (0.06÷0.57)	0.30±0.19 (0.01÷0.56)	0.29±0.17 (0.07÷0.49)	0.31±0.19 (0.02÷0.57)	0.27±0.21 (0.01÷0.49)	0.36±0.18 (0.01÷0.57)	0.20±0.18 (0.01÷0.55)	0.29±0.19 (0.02÷0.50)	0.30±0.19 (0.01÷0.57)	0.30±0.18 (0.02÷0.57)	0.30±0.20 (0.01÷0.56)
CDO1	T	0.41±0.16* (0.18÷0.76	0.30±0.11 (0.18÷0.48)	0.44±0.16* (0.18÷0.76)	0.42±0.16* (0.18÷0.71)	0.38±0.15* (0.18÷0.71)	0.51±0.18* (0.26÷0.76)	0.43±0.15* (0.18÷0.71)	0.36±0.16* (0.18÷0.66)	0.38±0.15* (0.18÷0.66)	0.41±0.16* (0.18÷0.76)	0.44*±0.16 (0.18÷0.76	0.36±0.16 (0.18÷0.71)

Continued on next page

Table 4 continued

	N	0.20±0.11 (0.01÷0.53)	0.20±0.10 (0.08÷0.34)	0.19±0.10 (0.01÷0.34)	0.19±0.11 (0.01÷0.32)	0.19±0.11 (0.01÷0.34)	0.23±0.11 (0.01÷0.34)	0.21±0.10 (0.01÷0.34)	0.17±0.10 (0.01÷0.32)	0.17±0.13 (0.01÷0.32)	0.21±0.09 (0.01÷0.34)	0.20±0.11 (0.01÷0.34)	0.20±0.10 (0.01÷0.34)
TP53	T	0.10±0.06 (0.01÷0.20)	0.08±0.05 (0.01÷0.18)	0.09±0.06 (0.01÷0.20)	0.11±0.06 (0.01÷0.20)	0.10±0.06 (0.01÷0.20)	0.10±0.08 (0.01÷0.20)	0.10±0.06 (0.01÷0.18)	0.10±0.04 (0.01÷0.17)	0.10±0.06 (0.01÷0.20)	0.10±0.06 (0.01÷0.20)	0.10±0.07 (0.01÷0.20)	0.10±0.05 (0.01÷0.18)
	N	0.09±0.07 (0.01÷0.21)	0.08±0.07 (0.01÷0.19)	0.09±0.06 (0.01÷0.20)	0.09±0.07 (0.01÷0.21)	0.09±0.07 (0.01÷0.21)	0.07±0.05 (0.01÷0.16)	0.08±0.07 (0.01÷0.21)	0.09±0.06 (0.01÷0.21)	0.15±0.04 (0.09÷0.21)	0.07±0.06 (0.01÷0.20)	0.10±0.07 (0.01÷0.20)	0.07±0.06 (0.01÷0.21)
RB1	T	0.26±0.12 (0.01÷0.40)	0.21±0.12 (0.01÷0.36)	0.27±0.13 (0.01÷0.40)	0.27±0.12 (0.01÷0.38)	0.26±0.13 (0.01÷0.40)	0.27±0.13 (0.01÷0.36)	0.28±0.11 (0.01÷0.40)	0.24±0.15 (0.01÷0.38)	0.27±0.10 (0.06÷0.39)	0.25±0.14 (0.01÷0.40)	0.26±0.12 (0.01÷0.39)	0.27±0.14 (0.01÷0.40)
	N	0.25±0.11 (0.01÷0.40)	0.26±0.11 (0.01÷0.38)	0.26±0.11 (0.01÷0.40)	0.23±0.10 (0.01÷0.36)	0.26±0.10 (0.01÷0.40)	0.23±0.14 (0.01÷0.36)	0.26±0.10 (0.01÷0.40)	0.23±0.12 (0.01÷0.36)	0.22±0.10 (0.01÷0.34)	0.24±0.12 (0.01÷0.40)	0.25±0.10 (0.01÷0.38)	0.26±0.12 (0.01÷0.40
WIF1	T	0.30±0.16* (0.08÷0.58)	0.33±0.15 (0.14÷0.58)	0.30±0.17 (0.08÷0.58)	0.29±0.15* (0.09÷0.58)	0.29±0.15* (0.08÷0.58)	0.34±0.16* (0.09÷0.56)	0.26±0.13 (0.08÷0.58)	0.37±0.18* (0.08÷0.59)	0.23±0.13* (0.09÷0.55)	0.32±0.15 (0.09÷0.58)	0.29*±0.16 (0.08÷0.58)	0.32±0.16 (0.08÷0.58)
	N	0.16±0.08 (0.01÷0.42)	0.21±0.05 (0.15÷0.30)	0.18±0.10 (0.01÷0.42)	0.11±0.05 (0.01÷0.22)	0.16±0.07 (0.01÷0.42)	0.18±0.08 (0.08÷0.26)	0.16±0.09 (0.01÷0.42)	0.15±0.08 (0.01÷0.30)	0.10±0.06 (0.01÷0.20)	0.18±0.08 (0.01÷0.20)	0.14±0.06 (0.01÷0.26)	0.18±0.11 (0.01÷0.42)

T: tumor, N: normal

*Significant differences between tumor and normal tissues ($p < 0.05$).

** Two patients had $T_1N_0M_0$ and $T_1N_2M_0$ stage with average methylation levels in ATM, APC, CDO1, RB1, TP53, WIF1genes in patient's tumor tissue equal to 0.26, 0.46, 0.33, 0.15, 0.35, 0.31 respectively, and in patient's normal tissue equal to 0.46, 0.53, 0.31, 0.10,0.30 and 0.23 respectively.

METHODS

Patient characteristics

The study involved 44 patients (34 men and 10 women) with HNSCC treated at A. Tsyb Medical Radiology Center, Obninsk, and P.A. Herzen Cancer Research Institute, Moscow. The study was approved by the A. Tsyb MRC Ethics Committee (approval № 634-17.11.2021).

For the first step of therapy, all patients underwent surgery. The absence of other treatments before surgery was the main inclusion criterion.

The sampling time duration was 6 months. All patients were sampled before starting further therapies. The characteristics of the patients are listed in Table 2. In total, 37 patients were diagnosed with primary HNSCC, and tumor recurrence after therapy was observed in seven cases. Squamous cell carcinoma of the tongue was observed in nine cases, cancer of the oral cavity was observed in six, cancer of the floor of the mouth was observed in three, cancer of the maxillary sinus was observed in six, and larynx cancer was observed in 20 patients. The non-keratinizing tumors were observed in 28 patients, with the keratinizing type observed in 16 patients [21]. Of the 44 patients, 26 were smokers. The mean age of the men was 58 (30÷72), and that of the women was 58 (47÷81) years old. Distribution by age in all patients is shown in **Figure 1**: four patients were 30–40 years of age (9%), eight patients were 41–50 years (18%), nine patients were 52–59 years (20%), 20 patients were 61–70 years (44%), and four patients were 71–81 (9%). The patients are classified according to the tumor, node, metastasis (TNM) system [22], in which T_1 - 2 (5%), T_2 - 7 (16%), T_3 - 20 (45%), and T_4 - 15 (34%).

The pre-operative contrast-enhanced CT images of three patients with various diagnoses are shown in **Figure 2**.

Sampling

Tumor and normal tissue samples from each patient were obtained during surgery and stored at −20°C.

DNA extraction

DNA isolation from biomaterials was performed on microcolumns (K-SORB, № EX-514, Syntol, Russia) following the manufacturer's instructions.

DNA methylation analysis

Bisulfite conversion was performed using the EZ DNA Methylation-Lightning Kit (ThermoFisher Epi-JET Bisulfite Conversion Kit, K1461, ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions.

Methylation of the promoter regions of the genes was performed using the methylation-sensitive high-resolution melting (MS-HRM) method using the CFX 96 Connect real-time system (BioRad, USA).

The primers for the reaction were selected using Primer Blast software (Table 3). Ready-mix (PCR-Mix, M-428, Syntol, Russia) was used for two-step PCR. The program of amplification included 95°C — 5 min; (95°C — 15 s, 60°C — 30 s, 72°C — 45 s) × 30 cycles; (95°C — 15 s, 50°C — 30 s, 72°C — 45 s) × 25 cycles [23]. Furthermore, the intercalating dye EVA Green (Syntol, Russia) was added to the obtained products. Each sample was run in duplicate. The melting curve was constructed according to the following program: first stage - 95° - 30 s; second stage - 60° - 10 min; third stage — melting analysis in the range of 60–90° with a 0.2° step. MS-HRM was performed using Precision Melt Analysis Software, version 3 (BioRad, USA). A CFX96 amplifier (BioRad, USA) was used for PCR and MS-HRM. The methylation level was detected by fluorescence expressed in relative fluorescence units (RFU) [24].

Statistical analysis

Statistical analysis of the data was carried out using R language. The method used was a chi-squared test. P values less than 0.05 were considered statistically significant.

RESULTS

The average methylation level results of the DNA promoter for all normal and tumor tissues of the patients and subgroups are shown in Table 4. Significant differences in DNA methylation levels between the tumor and normal tissues were found for *CDO1* and *WIF1* genes in all patients ($p < 0.05$). Significant differences were also observed for *CDO1* and *WIF1* genes in the tumor and normal tissues in the different subgroups ($p < 0.05$). The exception was the *CDO1* gene in a subgroup of patients with T_2 and non-smokers. Regarding the *WIF1* gene, the exceptions were found in subgroups of patients with T_2 and T_3, patients over 50, and non-smokers. The methylation levels of the DNA promoter of *ATM, APC, RB1,* and *TP53* genes were insignificant. Furthermore, no significant differences were found between the subgroups (T_2, T_3, and T_4 stage, with primary and recurrent lesions, non-keratinizing and keratinizing SCC, before and after 50 years of age, and smokers and non-smokers) of the patients.

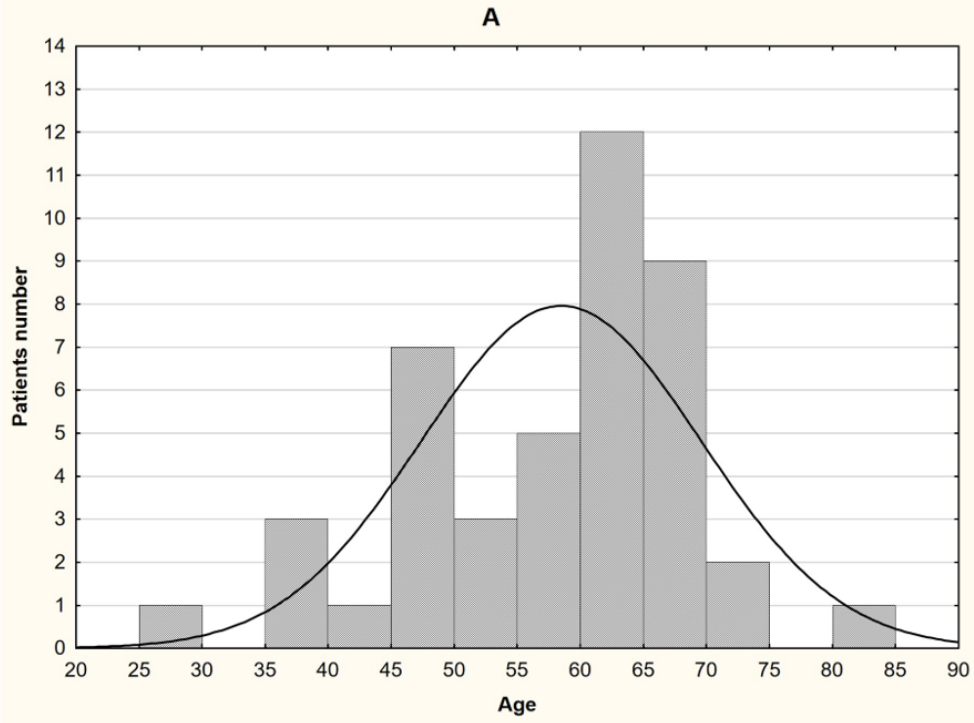

Figure 1: **Distribution of all patients according to the years (58.68 ± 11.05).**

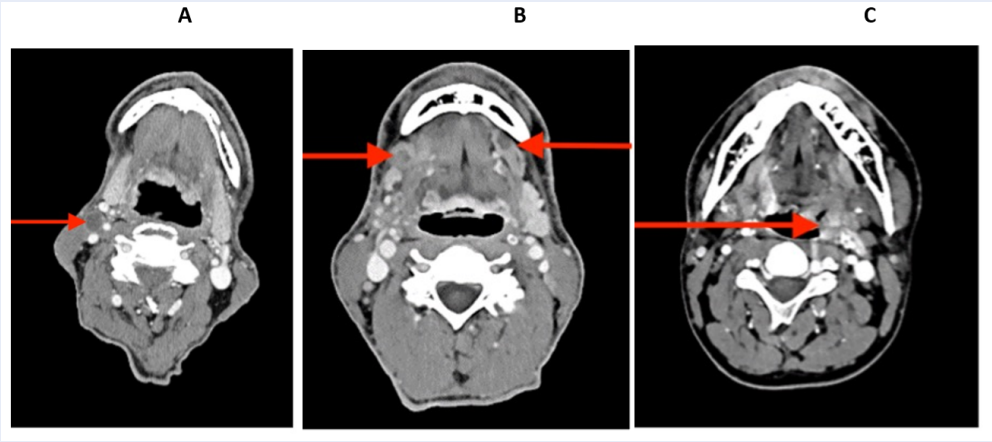

Figure 2: **Pre-operative contrast-enhanced CT images of three patients**. **A**. Patient №37 - 49-year-old male diagnosed with locally advanced (T3N1M0) stage of laryngeal squamous cell non-keratinizing cancer. **B**. Patient №17 - 66-year-old female diagnosed with locally advanced (T3N1M0) stage squamous cell non-keratinizing cancer of mouth floor. **C**. Patient №41 - 72-year-old male diagnosed with locally advanced (T4aN0M0) stage laryngeal squamous cell keratinizing cancer.

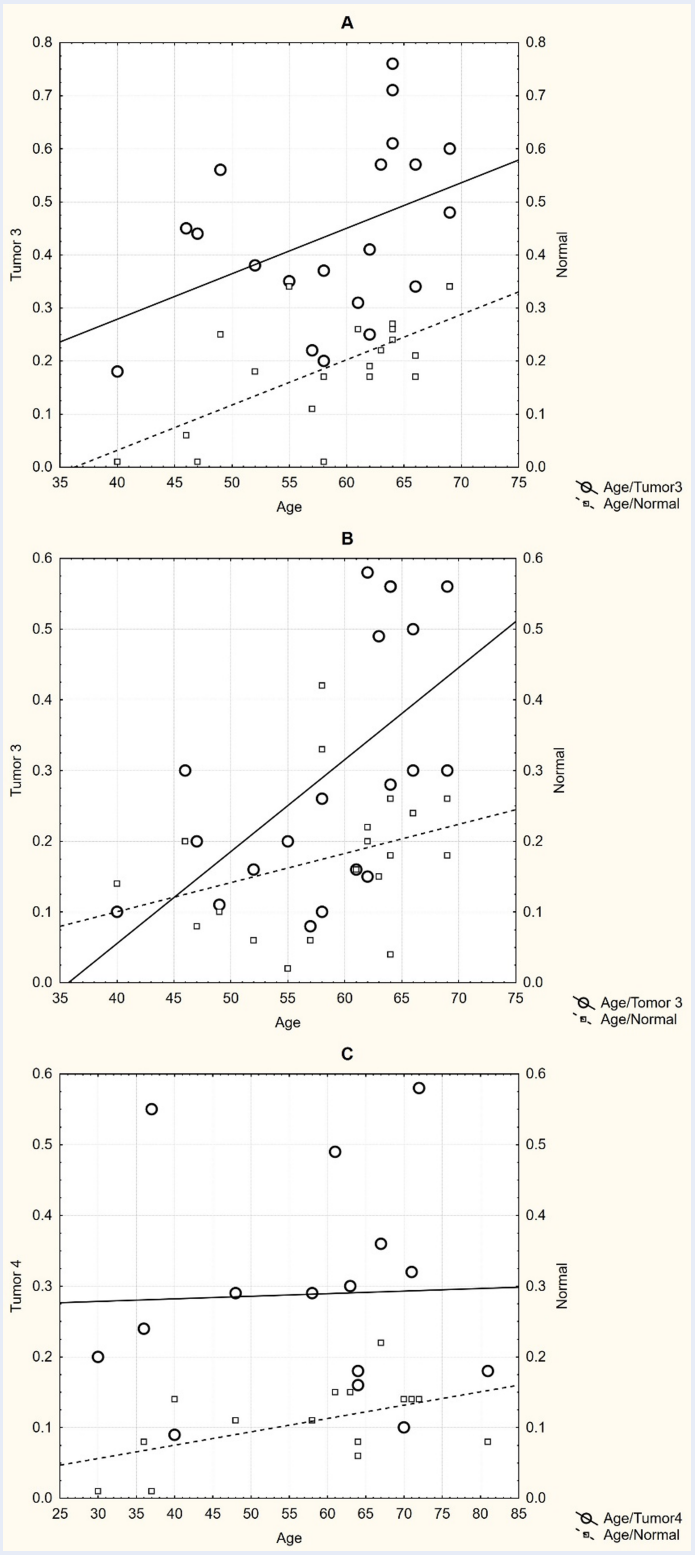

Figure 3: **The relationship between individual DNA methylation level in tumor and normal tissue in genes (CDO1, WIF1) and age in patients with different TNM classification**. **A**. CDO1 gene, patients with T3; **B**. WIF1 gene, patients with T3; **C**. WIF1 gene, patients with T4.

Afterward, significant differences were observed in the methylation level between the tumor and normal tissues only for the *CDO1* and *WIF1* genes. Using different TNM systems, we analyzed the relationship between these data and age in the patient samples (**Figure 3**). We observed a significant positive correlation in the methylation level in the *CDO1* gene with age in normal tissue from patients with T_3 (Age/Normal: $y = -0.3102 + 0.0085^*x$; $r = 0.66$; $p = 0.002$; $r^2 = 0.43$). The correlation between the methylation level in *CDO1* with age in tumor tissue from patients with T_3 was not significant but was different (Age/Tumor3: $y = -0.0641 + 0.0086^*x$; $r = 0.42$; $p = 0.07$; $r^2 = 0.17$). We observed a significant correlation between methylation level in the *WIF1* gene and age in the tumor tissue samples from the subgroup with T_3 (Age/Tumor3: $y = -0.4654 + 0.013^*x$; $r = 0.59$; $p = 0.006$; $r^2 = 0.35$) and normal tissue samples in the subgroup with T_4 (Age/Normal: $y = -0.0002 + 0.0019^*x$; $r = 0.52$; $p = 0.05$; $r^2 = 0.27$).

DISCUSSION

Epigenetic disorders in tumor tissue cells in various types of oncopathology are as important as mutations, according to abundant literature data from the last decade. Early-stage epigenetic alterations can be identified and exploited to diagnose tumors early and predict cancer prognosis[25], and DNA methylation can be used as a molecular target for cancer treatment because it is reversible via pharmacological inhibition of DNA methyltransferase.

(CDO-I) Cysteine dioxygenase type 1 protein-coding gene and tumor suppressor. *CDO1* expression may be silenced by promoter DNA methylation. *CDO1* can augment the generation of reactive oxygen species to induce apoptosis. Many studies have shown increased promoter methylation and, consequently, suppression of the *CDO1* gene in various tumor cells[26]. In primary breast cancer patients and those with prostate cancer, the methylation level of the *CDO1* gene's promoter region correlates strongly with tumor development. It can be utilized as a reliable prognostic indicator[27]. High levels of promoter *CDO1* methylation have been observed in Barrett esophageal adenocarcinoma and prostate cancer patients[28,29]. Our findings align with the evidence from the literature because the overall group of patients with HNSCC and subgroups had considerably higher *CDO1* gene promoter methylation levels in tumor tissue than in normal tissue. Moreover, significant differences were found between smokers and non-smokers.

(WIF1) WNT inhibitory factor 1 is a protein-coding gene that is a tumor suppressor gene. These genes are epigenetically silenced in various cancers. The protein encoded by this gene functions to inhibit WNT proteins, which are extracellular signaling molecules that play a role in embryonic development. Wnt signal transduction dysregulation can contribute to head and neck cancer. Inhibition of Wnt signaling induces apoptosis and inhibits tumor growth in many cancer types[30]. Mutations in the genes encoding proteins in the Wnt (Wingless/Int-1) pathway are rare in HNSCC. Thus, this pathway is not considered significant for the pathogenesis of head and neck carcinomas[31]. Few studies exist on the methylation levels of the *Wnt* gene. Our data indicated no significant differences in normal and tumor tissues from patients in terms of the DNA methylation level of the *Wnt* gene.

Methylation of the *WIF 1* gene, one of the antagonists of this pathway, is often associated with the development of this pathology[32]. *WIF1* has the highest methylation frequency in oral carcinomas. *WIF1* is methylated in 18% of patients with oral squamous cell carcinoma[33] and 35% of patients with tongue carcinoma[34], and it is significantly methylated in nasopharyngeal tumors[35]. In addition, non-small cell lung cancer frequently exhibits hypermethylation of the *WIF1* (Wnt inhibitory factor-1) promoter region, even though patient characteristics, such as age, sex, and smoking history, are unrelated to the methylation status[36].

Our study showed substantial variations in the average *WIF1* promoter methylation levels between tumor and normal tissues in the entire patient population and in the subgroups. There were also significant differences in this parameter in the tumor tissue in the subgroups of patients with keratinizing and non-keratinizing HNSCC.

It is well known that DNA methylation is associated with cancer and aging. Aberrant DNA methylation frequently occurs with aging and illnesses such as cancer. DNA methylation status can become disrupted according to age and a particular disease stage. For example, promoter-specific hypermethylation and concurrent gene silence are linked to various malignancies[37]. We found a significant correlation between methylation level for the *CDO1* and *WIF 1* genes in the normal and tumor tissues and age only in the subgroups with T_3 and T_4. These data indicate some dependence of the methylation level on the patient's age. The individual epigenetic features of the patient's genome likely play an essential role in the results obtained. Thus, the study should be repeated with a larger sample size, and/or patients should be stratified according to other criteria.

Adenomatous polyposis coli (*APC*) is a tumor suppressor gene that inhibits cell proliferation through the Wnt/β-catenin signaling pathway. *APC* interacts with DNA repair proteins, DNA replication proteins, tubulin, and other components. It is not expressed in some types of cancer, in particular prostate, breast, and colorectal cancer. Lack of expression is associated with reduced survival in cancer patients. Moreover, *APC* regulates the response to chemotherapy in cancer cells[38]. The Wnt/β-catenin signaling pathway is activated by *APC* mutations in colorectal, endometrial, and prostate malignancies[39]. Hypermethylation of this gene has been detected in oral squamous cell carcinoma samples in some studies but not in patients with head and neck cancers. Additionally, there are no differences in the methylation of the *APC* gene between HNSCC patients and healthy individuals. The *APC* promoter was methylated in 7% of DNA samples taken from the plasma of a population free of cancer. It was unrelated to several other putative risk factors, such as age, tobacco and alcohol use, family history of cancer, diet, and nutrition. Other research failed to find a connection between clinical traits and outcomes, such as survival and aberrant methylation[40,41]. Our results showed no significant differences in DNA methylation for the *APC* gene between tumor and normal tissues.

The ataxia–telangiectasia mutation (*ATM)* gene product is a well-known tumor suppressor essential for maintaining genomic stability. The mutated form of the *ATM* gene is involved in cell cycle control, apoptosis, oxidative stress, and telomere maintenance, and its role as a risk factor for cancer development is well established. In addition, several studies have been conducted to evaluate the risk of *ATM* gene mutations for breast cancer. In addition, several studies have been conducted to evaluate the screening of *ATM* gene mutations for the occurrence of breast cancer[42,43]. In HNSCC, the *ATM* gene promoter is a target for abnormal hypermethylation. Reduced *ATM* function through epigenetic silencing is a likely mechanism contributing to HNSCC and other tumor types, given its significant role in maintaining genome integrity and the causal role that genome instability plays in cancer onset and progression. It has been found that hypermethylation of the *ATM* promoter is significantly correlated with a decrease in the average survival rate of patients. Furthermore, this link is unrelated to other possible predictive elements, such as tumor size, lymph node condition, clinical stage, and history of tobacco and alcohol use. Thus, these data indicate that hypermethylation of the *ATM* promoter can be used as a prognostic factor in HNSCC[44]. However, our data revealed no significant differences in DNA methylation levels for the *ATM* gene in the tumor and normal tissues of patients.

Functional properties caused by tumor protein p53 (*TP53*) gene mutations are involved in cancer development and progression[45,46]. Somatic mutation is one of the most prevalent changes in the *TP53* gene in human tumors. Moreover, the underlying cause of Li-Fraumeni syndrome, which predisposes patients to various early-onset cancers, is germline mutation. *TP53* gene alterations are also possible prognostic and predictive indicators and prospective drug targets[47]. According to a thorough integrative genomic study, most somatic genomic changes in HNSCC are mutations in the *TP53* gene. Invasion, metastasis, genomic instability, and cancer cell proliferation are all facilitated by *TP53* mutations, which can result in a loss of wild-type *p53* activity or an increase in those activities. Interestingly, aggressiveness and worse survival following surgical treatment of HNSCC are related to disruptive *TP53* mutations in tumor DNA[48]. Harris C.C. proposed that genetic analysis of the *p53* gene in exfoliated cells detected in either body fluids or tissue biopsies might identify people at elevated cancer risk because mutations in the *p53* gene can arise in precancerous lesions in the lung, breast, esophagus, and colon[49]. Hypermethylation of the promoter region of *TP53* is often associated with a decrease in its activity and can even lead to its silence and, thus, to the loss of its function. This, in turn, contributes to malignant transformation[50]. Unfortunately, our research showed no significant differences in DNA methylation for the *TP53* gene between tumor and normal tissues in the patients.

The tumor suppressor retinoblastoma gene (*RB1*) is essential for controlling the cell cycle by increasing G1/S arrest and limiting growth by blocking the E2F transcription factor. Even aneuploidy can result from *RB1* loss, dramatically increasing the chance of developing cancer. The *RB1* gene is part of a large gene family that includes RBL1 and RBL2, each encoding the structurally related proteins pRb, p107, and p130[51]. One frameshift and seven non-synonymous missense mutations were discovered in *RB1* (pocket domain and spacer region) sequencing analysis. In HNC tumor samples, an *RB1* promoter methylation study showed that 16% of its cytosines (3% in CpG) were methylated[52]. Sabir M. and colleagues indicated that genetic and epigenetic *RB1* changes might contribute to the pathogenesis of HNSCC in the Pakistani population[53]. However, our study showed no significant differences in DNA methylation forthe *RB1* gene

between tumor and normal tissues in our examined patients.

Even though in our patients with HNSCC, in the tumor-associated genes *APC*, *ATM*, *TP53*, and *RB1*, no significant changes were observed in the methylation level between normal and tumor tissue, these genes are not specific for epigenetic analysis.

We also did not find significant differences in any genes (T_2, T_3, and T_4 stages, with primary and recurrent lesion, non-keratinizing and keratinizing SCC, age before and after 50 years, and smokers, and non-smokers) of the patients. This may be due to the small number of patients examined.

CONCLUSIONS

Thus, we can conclude that changes in the expressions of the *CDO1* and *WIF1* genes can affect the occurrence and development of HNSCC and can be considered prognostic and diagnostic markers for this pathology. Future application of molecular–genetic and epigenetic studies on HNSCC can serve as the basis for target therapy and contribute to personalized medicine.

ABBREVIATIONS

None.

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None.

AUTHOR'S CONTRIBUTIONS

Kurevlev S., molecular-genetic investigation, development of research design;

Aghajanyan A. data analysis and interpretation, article writing, review of publications on the article topic, statistical analysis investigation;

Tskhovrebova L. data analysis and interpretation, article writing, review of publications on the article topic;

Gordon K. collection of biomaterials, clinical data analysis;

Polyakov A. collection of biomaterials, clinical data analysis;

Ratushny M. collection of biomaterials, clinical data analysis;

Fatkhutdinov T., scientific editing supervision;

All authors have read and agreed to the published version of the manuscript.

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AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by the Biomedical Ethics Committee of the A.F. Tsyb Medical Radiology Research Center Branch of the Federal State Institution "National Medical Research Center of Radiology" of the Ministry of Health of the Russian Federation (protocol № 634 from 17.11.2021).

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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