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Molecular and clinical aspects of HNSCC in the Republic of Moldova

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ABSTRACT

Introduction: Complex molecular characterization and integrated approaches in the basic research of HNSCC provide new insights into the understanding and treatment of these tumors. Mutations in the TP53 gene, HPV infection, aberrant DNA methylation are just a few factors that have a direct link with the clinical and psychological condition of patients with this type of cancer. In the Republic of Moldova, these aspects are insufficiently studied.

Methods: The study included 128 patients with HNSCC from whom the following samples were collected: fresh tumor tissue, NAT, blood, and saliva. All samples, except saliva, were tested for 3 mutations in the TP53 gene, while DNA isolated from tumor tissue was also tested for global DNA methylation assessment. HPV genotypes were tested from saliva. HPV positive samples were retested from tumor tissue.

Results: Of the total analyzed samples for TP53 pathogenic variants, in 30 (23.44%) samples there were detected one or two mutations, and in 9 samples (7.03%) – it was detected the presence of two mutations simultaneously. HPV infection was detected in 17 samples (13.28%). Regarding global DNA methylation, in patients with a high degree of exposure to stress, a 44% lower level was observed (median 13.5 ng/ml) compared to those with moderate and low exposure (median 20.5 ng/ml).

Conclusion: The most frequent mutation identified in the TP53 gene was the 524G>A substitution and the frequency of high-risk HPV infection in HNSCC patients from Moldova was 13.28%. The high degree of stress exposure showed a lower level of global methylation.

Keywords: psychological stress, HNSCC, TP53, high risk HPV, global methylation

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is an aggressive, genetically complex neoplasm with a high mortality rate. HNSCC involves several anatomical sites of the head-neck region (the oral cavity, larynx and pharynx are the most frequently affected) and it is caused by various etiological factors such as smoking, alcohol consumption, human papillomavirus (HPV) infection, etc. Worldwide, in 2020, there were registered 931,931 new cases of head and neck carcinoma (HNC) and 467,125 (50.12%) deaths, 90% of these numbers being attributed to HNSCC (1). In the Republic of Moldova, according to the same data, in 2020, 989 cases were diagnosed with HNC and there were registered 651 deaths, which is 65.82%. Previous research suggests that men are more likely than women to develop head and neck carcinoma (2), and the determination of HPV status and *TP53* mutations may help clinicians make better prognostic assessment and streamline treatment decisions (3–5). The global increase in HPV infection, especially in the pharynx, leads to the accumulation of various aberrations in the genome of normal squamous cells, especially very frequent alterations of the *TP53* gene, which are a trigger for the appearance of neoplastic cells. The incidence of HNSCC caused by HPV varies depending on anatomical region and geographic location. In the Republic of Moldova, this factor has not been monitored and there were no data until 2023 about cases of HNSCC caused by HPV infection or associated with this virus (6).

Another trigger of tumorigenesis is aberrant DNA methylation, and monitorization of such changes may guide treatment strategies. Unlike mutations and other

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non-epigenetic alterations, DNA methylation is a reversible modification, which makes it extremely interesting in terms of therapeutic approaches. In contrast to the specific methylation of certain genes, global DNA methylation refers to the total 5-methylcytosine (5mC) content in a sample compared to the total cytosine content and reflects the global epigenetic status of the entire genome. Global hypomethylation may contribute to tumour development and progression by stimulating molecular DNA rearrangement mechanisms. It is known that cancer cells show a low level of global methylation in CpG regions (4,6) and certain studies suggest that psychological stress estimated on the basis of cortisol levels is negatively associated with the methylation of certain loci in the genome (7). In this context, epigenetic changes in HNSCC under the action of psychological stress are of increased interest and one of the present research objectives was to estimate the global DNA methylation (MG) of head-neck tumours in relation to the psychological stress of the patients.

The late diagnosis, the variation in the incidence of the disease between sexes, the different prognosis in different tumour subtypes, the high tumour heterogeneity, the mutational burden related to the etiological factor and the insufficient research regarding the molecular factors in head and neck cancer in the population of the Republic of Moldova are aspects that impose polyvalent characterization of these tumours. Complex molecular characterization and integrated diagnostic approaches as well as the possibility of determining prognosis will provide new insights into HNSCC. In the present study, we aimed to carry out a statistical evaluation of a cohort of 128 patients with HNSCC from the Republic of Moldova and to address several molecular and clinical aspects of HNSCC such as the testing of *TP53* mutations, HPV infection, and global DNA methylation.

METHODS

Samples and reagents

128 patients with HNSCC were under the study (Table 1). The samples of tumour tissue and normal tissue adjacent to the tumour (NAT) were collected from the head and neck tumour section by surgical method or biopsy by oncologists from the Head and Neck Tumour Department and were accumulated in the period 2020-2023 in the Cancer Biology Scientific Laboratory, Institute of Oncology, Republic of Moldova. Informed consent was obtained from all subjects and approved by Ethics Committee (Bioethics) of IMSP Institute of Oncology, number 05-18/21 from 01.02.2021. Tumor tissue, NAT, whole blood and saliva samples were stored at -86 °C immediately after collection. Tumour tissue and NAT were stored in cryotubes with 96% alcohol. Nucleic acids from tumour tissue, NAT and whole blood were isolated with the PureLink Genomic DNA Mini Kit and GeneJET Genomic DNA Purification Kit (ThermoFisherScientific, MA, USA) while total cellular DNA from saliva- with the AmpliSens DNA-sorb-AM kit (InterLabService Ltd., Moscow, Russia). TaqMan primers and probes (FAM labeled MGB Probe + 2 primers + allele-specific blocker) for the identification of *TP53* mutations c.524G>A (Hs00000888_mu), c.818G>A (Hs00001004_mu), c.817C>T (Hs00000890_mu) and reference gene (Hs00001027_rf) by castPCR method (Competitive, allele-specific TaqMan PCR) were purchased from ThermoFisherScientific, MA, USA. The reagent kit for the detection and differentiation of 12 HPV genotypes (AmpliSens HPV HCR-genotype-FRT, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) was provided by InterLabService Ltd., Moscow, Russia. Processing of DNA samples and determination of global methylation was carried out by using Nuclease P1 from Penicillium citrinum (Sigma-Aldrich, Missouri, USA), FastAP Thermosensitive Alkaline Phosphatase (ThermoFisherScientific, MA, USA), and the DNA Methylation ELISA Kit (Cayman Chemical, Michigan, USA).

Nucleic acids extraction

DNA from tumour tissue, NAT, and whole peripheral blood samples were isolated with the same reagent kits. From each sample of tumour tissue and NAT, a 20 mg sample was sectioned with a scalpel and weighed on an electronic balance. The sectioned tumour tissue was homogenized with TissueRuptor II (Qiagen, Hilden, Germany), with the following steps being performed according to the kit protocol from the manufacturer. The purity of the samples was estimated by spectrophotometric measurement, using the NanoDropLite spectrophotometer (ThermoFisherScientific, MA, USA), λ260/

Table 1. The number of subjects involved in the study, sorted by cancer type and sex

Cancer Type Detailed Site	Total	Men	Women
Oral Cavity Squamous Cell Carcinoma	72	60	12
Oropharynx Squamous Cell Carcinoma	33	30	3
Larynx Squamous Cell Carcinoma	20	20	0
Hypopharynx Squamous Cell Carcinoma	2	2	0
Head and Neck Squamous Cell Carcinoma of Unknown Primary	1	1	0
TOTAL	128	113	15

λ280 ratio being between 1.6 and 2.0. The concentration was determined by using the Qubit dsDNA BR Assay Kit (Invitrogen, Massachusetts, USA) and the Qubit 3.0 Fluorometer (Invitrogen, Massachusetts, USA). The DNA concentration ranged from 150 – 560 ng/µL. The DNA for HPV genotyping was isolated with the DNA-sorb-AM kit without measuring concentration and purity according to the manufacturer's protocol.

CAST-PCR

The reaction involved the use of probes and primers for the detection of c.524G>A, c.818G>A and c.817C>T mutations in the *TP53* gene as well as primers/probes for the reference gene/control gene (detect mutation-free regions of the *TP53* genes). CAST-PCR assays were performed in a final volume of 20 µL in 96-well plates that included 10.0 µL TaqMan Genotyping Master Mix, 2 (ThermoFisherScientific, MA, USA), 2.0 - 4.0 µL DNA solution with a concentration of 10-5 ng/µL (20 ng DNA per reaction), 2 µL TaqMan Mutation Detection Assay for each mutation, and 4-6 µL Nuclease Free Water. Amplification was performed using the 7500 Real-Time PCR System (Life Technologies, California, USA) according to the manufacturer's protocol. Amplification conditions were as follows: initial denaturation at 95 °C for 10 min; 5 cycles at 92 °C for 15 s; 58 °C for 1 min; 40 cycles at 92 °C for 15 s; 60 °C for 1 min. The results were analyzed according to the manufacturer's recommendations based on Cts.

HPV genotyping

HPV genotyping was carried out by Real-Time PCR. Saliva samples were collected in sterile tubes then aliquoted with 1:1 mucolytic solution mixture and stored at -86°C until DNA isolation. The isolated total cellular DNA was amplified by using primers for the identification of high carcinogenic risk (HCR) HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59. In order to exclude false negative results in samples without detectable HPV DNA, there were also included primers for a fragment of β-globin gene that served as a control. Reaction mixtures were obtained according to the manufacturer's protocol. Amplification of regions of interest was performed using the 7500 Real-Time PCR System (Life Technologies, California, USA) according to the manufacturer's recommendations.

Global DNA methylation

In order to evaluate global DNA methylation, 500 ng of DNA isolated from tumour tissue was heated at 100 °C for 3 min followed by transferring the samples to ice for 1 min as a way of denaturating the DNA strands. DNA was digested overnight with P1 nuclease and then treated with alkaline phosphatase at 37°C for 2 hours followed by incubation at 100°C for 10 minutes. The obtained product was stored at 4°C until use. The amount of methylated DNA was measured by the ELISA method based on 5-methyl-2-deoxy cytidine (ng/ml) according to the manufacturer's protocol. Results were read using a StatFax 4700 strip reader (Awareness Technology Inc., Florida, USA). Blood samples were analyzed to assess the degree of MG, and the HNSCC patients from whom the samples were taken were investigated based on a clinical survey in order to evaluate the psychological stress.

RESULTS

Detection of mutations in the TP53 gene and HPV genotyping

The analyzed *TP53* mutations were selected based on the frequency and on their role in structural and functional impairment of the protein (VIPUR score). From 128 samples analyzed for those three *TP53* mutations, in 89 (69.53%) no mutation was identified, in 30 (23.44%) one or two mutations were detected, and 9 samples (7.03%)) showed an inconclusive result. Also in 9 samples (7.03%) it was detected the simultaneous presence of two mutations and in 21 (16.41%) – one mutation per sample. The most frequent mutation identified was the substitution c.524G>A (23 samples, 17.97%), followed by c.818G>A (16 samples, 12.50%). The c.817C>T pathogenic variant was not determined in any of the analyzed samples. Of 39 *TP53* mutations identified, 36 (92.31%) were determined to be somatic while 3 (7.69%) had a germline origin. Two germinal mutations were represented by the substitution c.818G>A and one by c.524G>A.

Regarding the HPV genotyping of 128 people diagnosed with HNSCC, HPV infection was detected in 17 samples (13.28%) and 15 patients (88.24%) were male. The higher percentage in men is directly proportional to the higher number of male patients (113 patients) and no difference in HPV infection was observed between sexes. Of the twelve high-risk genotypes tested, two were detected: 16 and 18 with a percentage of 94.12% and 5.88%, respectively. The most common localization of the primary tumour in which human papillomavirus infection was detected is the oropharynx (10 samples) (Table 2).

The average age of diagnosis was 60.6 years, the highest probability density being in the range of 60-65 years with a normal data distribution (Figure 1)

From the obtained results, no correlation was observed between HPV infection and the presence of *TP53* mutations.

Table 2. HPV positiveness and localization of the primary tumor by sex

Cancer Type Detailed Site	Total	HPV+	HPV-	HPV+ (F)	HPV+ (M)
Oral Cavity Squamous Cell Carcinoma	72	5	67	0	5
Oropharynx Squamous Cell Carcinoma	33	10	23	2	8
Larynx Squamous Cell Carcinoma	20	2	18	0	2
Hypopharynx Squamous Cell Carcinoma	2	0	2	0	0
Head and Neck Squamous Cell Carcinoma of Unknown Primary	1	0	1	0	0
TOTAL	128	17	111	2	15

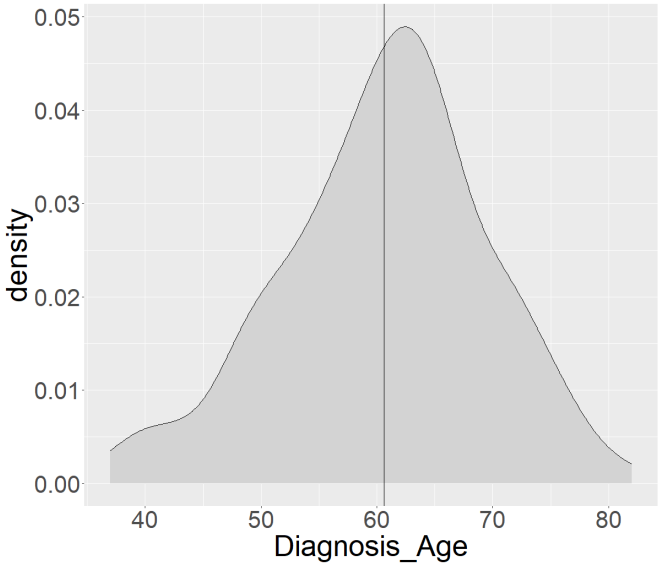

Fig.1 Average age of diagnosis

Global DNA methylation and psychological stress

The estimation of the exposure to psychological stress was determined by the doctor during the anamnesis of the patients and fell into two categories: high exposure (High) and moderate/low exposure (Moderate). Research has shown that patients with a high degree of exposure to stress had a 44% lower MG level (median 13.5 ng/ml) compared to those in the Moderate category (median 20.5 ng /ml).

DISCUSSIONS

According to the International Agency for Research on Cancer (IARC) all 3 analyzed mutations, also known as R175H, R273H and R273C, are among the most frequent, they show a high degree of pathogenicity and have a VIPUR score higher than 0.5 (8). The *TP53* pathogenic mutations, HPV infection, and DNA methylation are factors that cumulatively or separately determine the development and progression of head and neck squamous cell carcinoma. Alterations in *TP53* could create a favourable environment for HPV infection and also facilitate HPV oncogenic proteins to induce malignant transformation of cells through several mechanisms. Since the p53 protein has an important role in viral suppression by inhibiting the multiplication and persistence of HPV in host cells, mutations in *TP53*, and especially mutations that show a high score of structural and functional damage of the protein, can compromise this resistance capacity, facilitating persistent papilloma virus infection. Also, oncogenic proteins produced by HPV, such as E6 and E7, interact directly with the p53 protein and inhibit its function. E6 binds to p53 and promotes its degradation, while E7 interferes with p53's capacity to activate genes involved in tumour suppression and apoptosis (8). This interference with p53 function allows HPV-infected cells to evade tumour suppressor mechanisms and multiply uncontrollably. However, regarding the c.524G>A, c.818G>A and c.817C>T *TP53* mutations, no correlation between the presence of mutations and HPV infection was observed in the studied group.

Statistically, the value of 13.28% of cases associated with high-risk HPV in the studied group shows a lower proportion of cases in comparison to the statistics reported in other research (approx. 25%) (9), however, it is necessary to mention that only 12 high-risk papillomavirus strains were included in the present study. Also, in accordance with other studies (9) we observed that the highest incidence of infection is associated with the oropharynx. On the other hand, the average age of diagnosis of 60.6 years is lower compared to the same index observed in other European countries (63.84 years) (10), and 88.24% cases of HNSCC incidence in male patients indicate a higher value compared to the general statistics (70-80%) (11). Men are more likely to develop HNSCC, a discrepancy attributed to the fact that they are more exposed to risk factors such as smoking, excessive alcohol consumption and HPV infection.

Regarding DNA methylation, it can affect the expression of genes involved in tumour suppression and cell cycle control. In head and neck squamous cell carcinoma, global hypomethylation can lead to dysregulation of gene function, including the *TP53* gene, and loss or decrease of tumour suppressor capacity. Therefore, changes in global methylation may amplify the effects of *TP53* gene mutations and HPV infection on HNSCC development and progression. Some clinical factors such as psychological stress, anxiety and depression can also contribute to cancer development by inducing chronic

inflammation and the cellular response to stress. These biochemical changes can influence the way the DNA is methylated and can indirectly affect tumour suppression mechanisms. Previous studies suggest that psychological stress can contribute to epigenetic changes of different loci in the genome (7) and in the present study a significant change in global methylation was observed under the action of stress. However, the exact mechanisms by which psychological stress may influence global DNA methylation in HNSCC are not yet fully understood and require further research. It is known, however, that stress works through the hormonal system and neurotransmitters that can affect the way the enzymes responsible for methylation act on DNA.

In squamous cell carcinoma of the head and neck, such factors as *TP53* gene mutations, HPV infection and changes in global DNA methylation are interconnected and interact in a complex way. Understanding these interactions is essential for identifying more effective therapeutic strategies in this form of cancer, and the integrated approach of such factors will provide a more comprehensive insight into the mechanisms of HNSCC development and progression which will facilitate the development of diagnostic strategies, personalized treatment and management for patients.

CONCLUSION

The research provides insights into important HNSCC occurrence and development factors such as TP53 pathogenic mutations, HPV infection, age of diagnosis and DNA methylation under the action of psychological stress. While studied TP53 mutations and HPV infection did not reveal a correlation, the impact of stress on MG levels underscores the role of psychological factors in HNSCC epigenetic machinery. Additionally it was observed a lower percentage of high-risk HPV cases, compared to other research and also a slightly lower age of diagnosis in Moldova than in other European countries.

ABBREVIATIONS

HNSCC – Head and Neck Squamous Cell Carcinoma

NAT – Histologically normal tissues adjacent to the tumours

DNA- Deoxyribonucleic acid

HPV- Human papillomavirus

ELISA- Enzyme-Linked Immunosorbent Assay

PCR – Polymerase Chain Reaction

CastPCR- Competitive allele-specific TaqMan PCR

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AUTHORS' CONTRIBUTION

VS – Conceptualization; Project administration; Funding acquisition; Writing – review and editing; Supervision

VT – Methodology; Validation; Data Curation; Writing – original draft preparation

VS – Formal analysis; Data Curation; Writing – original draft preparation

CP – Investigation; Writing – review and editing

VB – Investigation; Writing – review and editing

VT – Investigation; Writing – review and editing

AC – Resources; Writing – review and editing

IS – Resources; Writing – review and editing

AM – Resources; Writing – review and editing

MS – Supervision; Writing – review and editing

CONFLICT OF INTEREST

None to declare.

ETHICS COMMITTEE APPROVAL

Ethics Committee (Bioethics) of IMSP Institute of Oncology, approval decision number 05-18/21 from 01.02.2021

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