SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

3210.

RÉVÉSZ MÓNIKA

Klinikai onkológia és sugárterápia

című program

Programvezető: Dr. Polgár Csaba, egyetemi tanár

Témavezető: Dr. Takácsi-Nagy Zoltán, c. egyetemi tanár

The role of etiological factors in carcinogenesis in young head and neck cancer patients

PhD thesis

Mónika Révész MD

Semmelweis University Doctoral School

Pathological and Oncological Division

Supervisor: Zoltán Takácsi-Nagy MD, Ph.D.

Official reviewers: Szabolcs Bellyei, MD, Ph.D.
Sándor Bogdán, MD, Ph.D.

Head of the Complex Examination Committee:
Prof. Gábor Répássy MD, Ph.D.

Members of the Complex Examination Committee:
Judit Halász MD, Ph.D.
Balázs Szabó MD, Ph.D.

Budapest
2025

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List of Abbreviations

ALDH2: ALDH2 aldehyde dehydrogenase 2

APC: Adenomatous polyposis coli

ATR: ataxia telangiectasia and Rad3

AYA: Adolescent and Young Adult

BRCA: BReast CAncer gene

CDKN2A: cyclin-dependent kinase inhibitor 2A

CI: confidence interval

CS: Chi-square test

DFS: disease-free-survival

EED: embryonic ectoderm development

EZH2: enhancer of zeste homolog2

ESMO: European Society for Medical Oncology

FA: Fanconi anemia

FAMMM: familial atypical multiple mole melanoma

FE: Fisher's exact test

FOXM1: forkhead box M1

GCO: Global Cancer Observatory

GWAS: genome-wide association study

HIF-1α: hypoxia-inducible factor-1α

HNSCC: head and neck squamous cell carcinoma

HPV: human papillomavirus

HR: hazard ratio

K-W test: Kruskal–Wallis test

MDM2: murine double minute 2

MDMX: murine double minute X

MHC: major histocompatibility complex

MMP: matrix-metalloproteinase

MWU: Mann–Whitney U test

NCCN: National Comprehensive Cancer Network

OR: odds ratio

OS: overall survival

PcGs: polycomb group genes

PD-L1: programmed death ligand 1

PRC2: polycomb repressive complex 2

RBBP: Retinoblastoma-Binding Protein

RR: relative risk

SIR: standardized incidence ratio

SNP: single nucleotid polymorphism

SUZ12: suppressor of zeste 12

TME: tumor microenvironmental

vHL: von Hippel-Lindau

1. Introduction

1.1. Epidemiology of head and neck squamous cell carcinoma (HNSCC) in young patients

Head and neck cancer (including tumors of the lip, oral cavity, naso-oro-hypopharynx, larynx, paranasal sinuses and salivary glands) was estimated to be the sixth most common cancer with approximately 800,000 new cases annually worldwide based on the Global Cancer Observatory (GCO) database (1). The incidence and mortality rates of head and neck squamous cell carcinoma (HNSCC) vary widely by geographic regions. The highest rates were observed in South and Southeast Asia, followed by Europe and South America. The annual incidence of 17.2/100.000 in Hungary is the highest in Europe (2). The HNSCC is a generic term, it includes any malignancies arising from the squamous cells of the head and neck region, such as the lip and the oral cavity, mesopharynx, hypopharynx, larynx, nasopharynx, paranasal sinuses, and salivary glands. However in a strict sense the term HNSCC involves only the first four localizations in the above list due to their common mucosal origin. The gender distribution ratio of HNSCC generally varies around 2:1 for males to females, respectively and it usually occurs in patients over 50 years (3), however, in the past half century the incidence also increased in young adults. The proportion of HNSCC patients accepted as young patients depends on the age limit chosen by various authors in the literature, which is necessarily arbitrary. The most accepted definition for young HNSCC patient is currently an age of ≤39 years defined by the National Comprehensive Cancer Network (NCCN) Adolescent and Young Adult (AYA) Oncology's guideline (4). The European Society for Medical Oncology (ESMO) guidelines on AYAs with cancer use the aforementioned age limit according to the World Health Organization (WHO) age classification (5). Estimates for oral cancer incidence varies between 0.4-3.6% of all cancers in patients under 40 years according to the literature, and 6.7% under the age of 45 (6,7). The results of different studies are not fully coherent regarding the behavior and prognosis of HNSCC in young patients when compared with the general HNSCC patient population, which might also be related to the uncertain age limit to define the young patient group (8). Certain authors even suggested that this patient group may form a distinct disease entity (8). Whereas the survival data of

the general HNSCC patients population are well-known from multicenter researches (9), only few reports compare the prognosis and the overall survival in the young and the older HNSCC groups and the results are conflicting (10–12).

1.2. Etiological factors of the carcinogenesis

The causes of the early carcinogenesis are yet unclear. Various extrinsic and intrinsic factors might play a role in the development of HNSCC. Of note, when considering etiological factors, it is necessary to highlight that extrinsic and intrinsic risks might mutually enhance each others impact and this network of interacting risks is largely yet to be explored. Its importance might also be relevant in young HNSCC patients, in the majority of whom the early onset of their disease yet cannot be linked to any dominant etiological entity (13).

1.2.1. Extrinsic factors - environmental carcinogens

Although previous chemotherapy or irradiation obviously have their direct destructive effect on mucosal tissue, the primary risk factors are tobacco and alcohol consumption. Biotransformation, detoxification and elimination of carcinogens, DNA repair mechanisms and apoptotic pathways are the most important mechanisms of defence against carcinogenesis (14). Genetic alterations in the genes encoding for enzymes involved in biotransformation, such as cytochrome P-450s, glutathione S-transferases, UDP glucoronyltransferases, aldehyde dehydrogenase, and alcohol dehydrogenase, and also polymorphisms of the DNA repair mechanisms and apoptosis have been associated with HNSCC risk (14). Tobacco contains numerous (>70) carcinogenic agents which are released during combustion. Most carcinogens bind to the DNA, form DNA adducts, and disrupt the double-helical DNA structure. If the damage of the DNA integrity is not corrected, DNA adducts can cause mutations via activating oncogenic pathways or inactivate tumor suppressor genes such as P53 (14). Heavy smokers have an elevated risk for HNSCC in comparison with non-smokers (odds ratio: OR: 3.46) (15). In Asia betel nut chewing is also popular, which is also associated with an increased risk for HNSCC

(OR: 1.7) and for oral cancer specifically (OR:3.0) (15). The risk of HNSCC also increases dose-dependently with the amount and frequency of alcohol consumption. The alcohol might play a role in the development of cancer in two well-known ways: ethanol is oxidated into acetaldehyde, which is mutagenic by inducing point mutations, single- and double-strand DNA breaks, forming adducts, and inducing inter- and intrastrand crosslinks (16). It also has a direct local effect on cell membranes, by which it enhances the penetration of carcinogenic chemicals into the mucosa. Heavy drinkers (>50g/day) have a 2.5-fold relative risk to develop HNSCC (3). Tobacco and alcohol have a synergistic effect (with a cumulative OR: 5.73) regarding carcinogenesis (17).

Various alimentary factors as well as the oral microbiom might also have significant impact on the mucosa and potentially play a role in the development of HNSCC. Although this field is yet largely elusive, the role of poor oral hygiene in the development of oral cavity lesions is well established (18). Furthermore certain oral bacteria were recently identified to be linked to an increased risk of oral cavity HNSCC (19). In addition, a more established ethiological infective agent has emerged, namely the human papillomavirus (HPV) which has a strong and direct etiological role in the development of squamous cell carcinoma of the oropharynx. A steeply rising incidence was observed in the recent decades. In the United States the population-level incidence of HPV-positive oropharyngeal cancers increased by 225% from 1988 to 2004, and incidence for HPV-negative cancers declined by 50% (20). According to the literature currently over 20% of oropharyngeal squamous cell cancers might be attributed to HPV (21). A list of potential environmental risk factors is outlined in Table 1.

1.2.2. Intrinsic factors - genetic susceptibility to HNSCC

Certain types of rare genetic mutations with high penetrance are strongly linked to a high susceptibility for certain types of malignancies (eg. breast cancer - BRCA1, BRCA2, colorectal cancer - APC), but no such specific alleles are known in relation with HNSCC. However, certain germline mutations, which promote the formation and/or survival of tumor cells do pose a significant risk also for HNSCC, along with other types of cancer and are related to specific syndromes. There are currently 3 known genes the mutation of

which lead to a highly elevated risk of HNSCC, such as *CDKN2A*, *ATR* (ataxia telangiectasia and Rad3) - related gene and the Fanconi anemia (*FA*) gene family. The *CDKN2A* gene plays an important role in cell cycle regulation and its loss-of-function mutation leads to FAMMM (familial atypical multiple mole melanoma) syndrome, which involves melanoma, pancreatic cancer, and HNSCC. The germline mutations of the *ATR* gene can cause a combination of skin, breast, cervical and oropharyngeal cancers. More than 13 different *FA* (Fanconi anemia) genes have been described, which are linked to high risk for HNSCC (standardized incidence ratio: SIR: 500-800), congenital abnormalities and bone marrow failure (aplastic anemia) (22,23). In addition to germline mutations, somatic variants are also associated with HNSCC. Recent studies confirmed that mutations of the *TP53* tumor suppressor gene were the most frequent of all somatic genomic alterations in HNSCCs with a mutation frequency ranging from 75% to 85% in non-HPV-associated HNSCC cases (24). While some P53 mutations lead to a loss of function, many P53 mutations result in gain-of-function mutations that (in contrast to physiological P53 function) promote tumour invasion, metastasis, genomic instability, inflammation and cancer cell proliferation (24). *TP53* mutations are significantly associated with short survival time and tumor resistance to radiotherapy and chemotherapy, which makes the *TP53* mutation status a potentially useful biomarker for prognosis and a predictor of therapy response (24). The prevalence of germline *TP53* pathogenic variants in the general population is estimated to be between 1:3.000 and 1:10.000 (25). Nearly three-quarters of families with Li-Fraumeni syndrome and about one-quarter with Li-Fraumeni-like syndrome have germline mutations in the *TP53* gene (26). Individuals with Li- Fraumeni syndrome have a nearly 24-times higher incidence of any cancer compared with the general population (SIR: 23.9) (27).

A positive family history of HNSCC increases the individual risk with an OR (odds ratio) of 1.7 when a first degree relative is affected (17). Recently the International Head and Neck Cancer Epidemiology (INHANCE) study found that a family history of HNSCC in a first-degree relative conferred an increased risk of HNSCC in the patients examined with an OR: 2.2 for siblings and 1.5 for parents (28). This pattern is typical for most polygenic diseases, the risk for which is established by numerous genetic polymorphisms, which one by one only slightly increase the risk, but might also have additive or synergistic effects with each other and also certain environmental risks. According to the

National Human Genome Research Institute- European Bioinformatics Institute (NHGRI-EBI) Catalog database only fourteen associated single nucleotid polymorphisms (SNPs) were identified in five GWAS (genome-wide association study), when searching for the trait „head and neck squamous cell carcinoma" (29). The odds ratio attributed to certain SNPs varies between 1.14-3.95 (30–34). These are summarized in Table 2. Of note, affected genes are involved in immune regulation (*HLA-DQB1, NCR3, SCIMP*), cell adhesion (*VCAN*), and the breakdown of aldehids (*ALDH2, AKR1C1*), all of which processes might closely affect damage control on the tissue level.

Table 1. Extrinsic risk factors for head and neck cancers

Risk for HNSCC	Type of exposure	Odds ratio	References
Tobacco	Cigarettes	3.46	(15)
Alcohol	Three or more drinks per day vs never (30g)	2.04	(35)
	>50g/day	2.5	(3)
Tobacco + Alcohol joint effect		5.73	(17)
Betel nut chewing		1.7-3.0	(15)
Marijuana		0.88	(17)
Human papilloma virus infection	oropharynx/tonsil - oral cavity - larynx site	15.1 – 2.0 – 2.0	(17)
Epstein Barr virus infection	nasopharyngeal site/non- keratinizing oral cavity site	15.1 5.03 (pooled OR)	(36) (37)
Human immunodeficiency virus		1.7-4.0	(38)
Poor oral hygiene	lower frequency of tooth brushing	2.08	(39)
	periodontal diseases	2.63	(39)
Dietary components	high fruit vs low	0.52	(17)
	high red meat vs low	1.4	(17)
Previous chemotherapy	bone marrow transplantation	6.635	(40)
Occupational risks	asbestos cement dyes/paints/solvents polycyclic aromates	8.7 12.9 2.3 (larynx) 3.6 (oral cavity) 5.2	(41)

Table 2. SNPs associated with head and neck squamous cell carcinoma (29–34)

Affected SNP	Mapped Genes	Chromosomal location	Trait	p	Allelel frequency	Estimated risk (OR and 95% CI)	Function of gene product
rs3135001	MTCO3P1, HLA-DQB1	6:32702359	HNSCC	1.00E-16	0.79	1.3 [1.22-1.37]	MTCO3P1: pseudogene, HLA-DQB1: part of MHC II
rs1265081	CCHCR1	6:31143898	HNSCC	4.00E-10	0.52	1.18 [1.11-1.23]	Probably associated with P-bodies which have pivotal role in RNA decay
rs259919	POLR1HASP	6:30057726	HNSCC	3.00E-09	0.28	1.17 [1.11-1.24]	pseudogene
rs16879870	HTR1E, RN7SL643P	6:86822028	HNSCC	4.00E-06	NR	2.02 [1.50-2.73]	HTR1E: 5-hydroxytryptamine receptor 1E, RN7SL643P: pseudogene
rs2641256	ZNF594-DT, SCIMP	17:5223379	HNSCC	8.00E-06	NR	NR	ZNF594-DT: regulation of transcription by RNA polymerase II, SCIMP: Part of receptor complex, involved in pattern recognition receptor signaling, positive regulation of cytokine production
rs671	ALDH2	12:111803962	HNSCC	1.00E-25	NR	2.08 [1.69–2.55]	aldehyde dehydrogenase
rs2857595	NCR3, UQCRHP1	6:31600692	Laryngeal SCC	2.00E-15	0.50	1.3 [1.22-1.39]	NCR3: a natural cytotoxicity receptor (NCR) that may aid NK cells in the lysis of tumor cells, UQCRHP1: pseudogene
rs174549	FADS2, FADS1	11:61803910	Laryngeal SCC	1.00E-20	0.59	1.37 [1.28-1.47]	FADS2, FADS1: fatty acid desaturases
rs9445023	U3, ATF1P1	6:92812416	Laryngeal SCC	7.00E-07	0.88	1.32 [1.18-1.47]	U3: SnoRNA , ATF1P1: pseudogene
rs310518	VCAN	5:83523300	Laryngeal SCC	7.00E-08	0.79	1.27 [1.16-1.37]	Large chondroitin sulfate proteoglycan, major component of extracellular matrix, involved in cell adhesion, proliferation, migration and angiogenesis.
rs40129	LINC02177	16:9344835	Laryngeal SCC	4.00E-07	0.25	1.21 [1.12-1.30]	long intergenic non-protein coding RNA
rs10492336	GLULP5,LINC02459	12:114147775	Laryngeal SCC	4.00E-14	0.82	1.41 [1.30-1.54]	GLULP5: pseudogene, LINC02459: long intergenic non-protein coding RNA
rs142021700	RTTN	18:70034347	Hypopharyngeal or laryngeal cancer	3.00E-09	0.01	3.95 [2.51-6.21]	Rotatin, crucial role in fetal development
rs77045180	AKR1C1	10:4981859	Hypopharyngeal or laryngeal cancer	1.00E-07	0.02	2.51 [3.52-1.79]	Aldo-Keto Reductase Family 1 Member C1

1.2.3. Epigenetic modifications and the central role of enhancer of zeste homolog 2 (EZH2)

The reasons of the increasing number of young HNSCC patients are not yet clear, but the number of the above factors make it unlikely that any single cause could be responsible for the early onset (10). In addition to the aforementioned potential etiological factors, several mechanisms may influence carcinogenesis, of which epigenetic mechanisms play a role in modulating cell proliferation, apoptosis and tumor progression (42). Epigenetic information can be described as stable, in certain part even heritable information, and can be transmitted during cell division, but is not encoded by the DNA sequence. Therefore, in contrast to the irreversible nature of genetic changes, epigenetic mechanisms reversibly regulate gene expression and are considered a link between genotype and phenotype. Epigenetic modifications include DNA methylation, covalent histone modifications, chromatin remodeling, and the effects of non- coding RNAs and polycomb proteins on gene expression. The most frequently studied epigenetic mechanisms regulating the expression of genes and their role in carcinogenesis are DNA methylation and, more recently, the posttranscriptional regulation of gene expression by microRNAs. Proteins from the polycomb group (PcG) are involved in the epigenetic regulation of gene expression, and play a fundamental role in the maintenance and differentiation of stem cells (22,43).

The enhancer of zeste homolog 2 (EZH2) is a member of the family of polycomb group genes, which are important epigenetic regulators by repressing transcription. EZH2 is an enzimatic subunit of the Polycomb repressive complex 2 (PRC2), which can silence gene expression through the inhibition of transcription by the trimethylation of lysine-27 in histone 3 (H3K27me3), which results in the down-regulation of CXC chemokin expression and major histocompatibility complex (MHC) Class I antigen presentation, hence the immunological visibility (44). The PRC2-linked effects of EZH2 also support cellular proliferation and might hinder differentiation. The tumor suppressor protein P53 acts against the expression of PRC2 subunits (embryonic ectoderm development - EED and EZH2) through promoting the maintenance of the pRB-mediated inhibition of E2F (45,46).

EZH2 also exerts certain functions PRC2-independently through interactions with other mediators, such as hypoxia-inducible factor-1α (HIF-1α) and forkhead box M1 (FoxM1). Hypoxia, a common phenomenon in the tumor microenvironment, might up-regulate the PRC2-independent EZH2 functions both by down-regulating the expression of PRC2 subunits (EED and suppressor of zeste 12: SUZ12) and by facilitating the binding of free EZH2 to FoxM1 instead of other PRC2 members, thus modulating the expression of matrix-metalloproteinases (MMPs), hence the invasive capacity of tumor cells. EZH2 itself might facilitate the dissociation of EAF2 from VHL, thus activating HIF-1α - linked transcriptional alterations, including the up-regulation of glycolitic capacity, hypoxia-tolerance, and the expression of free EZH2 (45,47–53). The EZH1, as a subunit of PRC2, also has catalytic abilty to methylate H3K27, but it is known to be inferior in comparison with EZH2. While the function of EZH2 has been broadly studied, the function of EZH1 still needs to be investigated (54). The functions of EZH2 are summarized in Figure1.

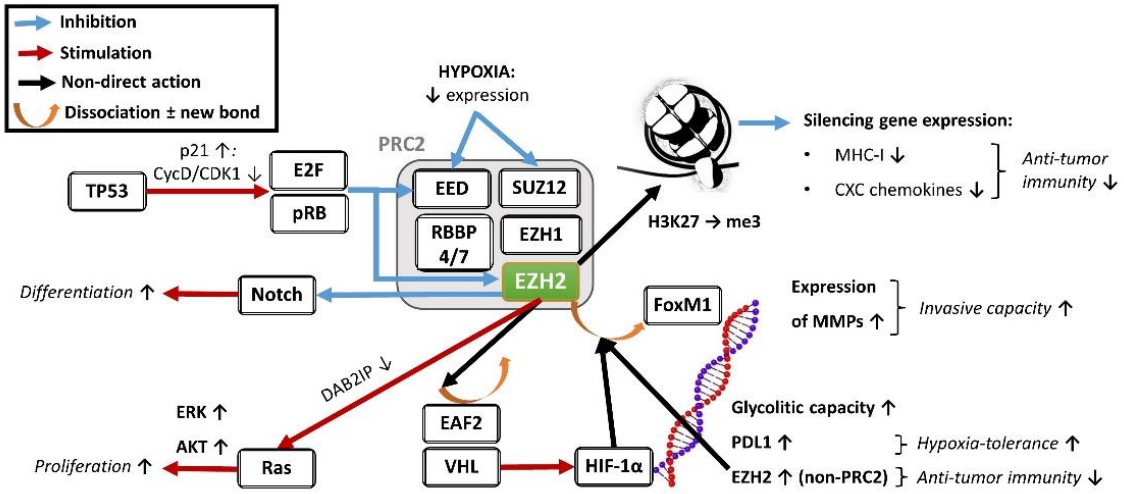

Figure 1. Summary of the main molecular interactions of EZH2 (with functional outcomes italicized) (44–53)

EZH2 has a well-defined oncogenic role and is frequently upregulated in different cancer types (43,55–60). The EZH2 overexpression of tumor cells is associated with poor

prognosis also in head and neck cancer (61). EZH2 exerts its impact on tumor viability through the aforementioned mechanisms, and also modulates the effect of the cisplatin treatment. The two-thirds of head and neck cancer patients present with locoregionally advanced stage disease (stage III-IV), which requires aggressive multimodality therapy (62) with cisplatin- containing chemotherapy (63). A recent study shows that high EZH2 expression decreases sensitivity to cisplatin-based chemotherapy in HNSCC (61). EZH2 might influence cisplatin resistance by promoting DNA repair through the upregulation of the expression of DNA damage repair genes (64).

The anti- tumor immunity in cancers can also be modulated by EZH2 through MHC I down- and PD-1 up-regulation. Immune checkpoint inhibitors were shown to improve the overall survival (OS) in metastatic or recurrent (stage IV) HNSCC (9).

1.2.4. The interplay between traditional carcinogens and patient's genotype

It is well-known that tobacco and alcohol exposure play a key role in the initiation of HNSCC. However, even among highly exposed individuals, HNSCC develops in only a small proportion of them. The most important processes against carcinogens are biotransformation, detoxification, DNA repair mechanisms and apoptotic pathways. Recent studies described that various genetic polymorphisms and epigenetic variations may alter the activity enzymes involved in the aforementioned mechanisms, which might result in an elevated risk for cancer development. This might mean that interactions between carcinogens and the patient's genotype could affect the individual susceptibility to HNSCC. The most common genetic polymorphisms affecting the above pathways are single nucleotide polymorphism (SNP) of cytochrome P-450 family enzymes and glutathione S-transferases (GSTs), UDP-glucuronosyltransferase (UGTs), aldehyde dehydrogenase (ALDH), and alcohol dehydrogenase (ADH) enzymes. Epigenetic modifications such as DNA methylation, histone modifications, chromatin remodeling, and the effect of non- coding RNAs and polycomb proteins in gene expression could also regulate the expression of genes and potentially their role in carcinogenesis (22).

1.3. Novel therapeutic strategies in HNSCC with epigenetic drugs

There are three main types of standard cancer treatments in HNSCC: surgery, radiation therapy and chemotherapy according to the traditional treatment modell. However, over the past decade, it has become increasingly clear that each individual cancer has its own specific molecular phenotype and therefore responds differently to common treatments. This paradigm-shift leads to the concept of precision and personalized medicine, in which therapy selection is tailored to each individual (65). Nowadays the algorithms of oncological therapies include tyrosine kinase inhibitors, immunotherapy (especially immune checkpoint inhibitors) as well as the combination of the latter with chemotherapy if the faster tumor shrinkage is the aim and the potentially enhanced toxicity is tolerable (9). A better understanding of the epigenetic modifications might also lead to the introduction of new therapeutic options with epigenetic targets also in HNSCC. Currently, such therapies are classified into five groups: DNA methyltransferase inhibitors, histone methyltransferase inhibitors, histone demethylase inhibitors, histone acetyltransferase inhibitors, histone deacetylase inhibitors and microRNAs. Of the five categories, DNA methyltransferase inhibitors and histone deacetylase inhibitors are broad re-programmers that lead to general changes in the epigenome. It is important to note that both of them directly also modulate non-histone proteins such as P53. Studies have shown that treatment with these agents causes growth inhibition by cell cycle arrest and reduction in clonogenic survival on human tumor cell lines. Both of them have been successfully used in the treatment of hematological malignancies in monotherapy. Co-administration of histone acetyltransferase inhibitor and cisplatin displays synergistic effects in inducing greater cytotoxicity and apoptosis induction, compared to cisplatin alone in HNSCC (43,66–68). Other inhibitors are used to treat the changes in epigenetic pathways, such as the EZH2 inhibitors. Recently, numerous EZH2 inhibitors have been introduced. Tazemetostat acts as a histone methyltransferase inhibitor, which selectively blocks the activity of EZH2 methyltransferase. It was the first oral EZH2 inhibitor approved by the U.S. Food and Drug Administration (FDA) in 2020 for the treatment of adults and adolescents (≥16 years) with advanced or metastatic epithelioid sarcomas, and for relapsed/refractory follicular lymphoma (69). Administration of tazemetostat is well tolerated, causing mostly grade 1- grade 2 gastrointestinal and hematologic adverse

events (70). It has been examined in several clinical trials to evaluate the anti-tumor activity and safety in relapsed or refractory malignant pleural mesothelioma, synovial sarcoma, and recurrent ovarian or endometrial cancers (71).

Of note, epigenetic "errors" rate are more frequent than clear genetic drivers of diseases and the epigenetic regulators play an important role in the development of new cancers as well as tumor heterogeneity. These mechanisms provide another barrier to the effective treatment with developing drug resistance. The combination of epigenetic modifiers with standard therapies (chemotherapy, radiotherapy, angiogenesis inhibition, epidermal growth factor receptor inhibition, and hormone therapy) are of interest, as well as the co-administration with immunotherapy (71,72).

Combination therapies consisting of EZH2 inhibitors and cisplatin could potentially be beneficial for the treatment of lung, ovarian, and breast cancer. Synergistic effects were reported in two in vitro models; however, another study suggested there was an antagonistic effect in HNSCC cell lines (64). The combination of EZH2 and checkpoint inhibitor therapies might improve the therapeutic response due to the immunomodulatory effects of EZH2 through MHC I down- and PD-1 up-regulation (44). In a phase 1/2 ongoing study (NCT04624113) tazemetostat has been administered with pembrolizumab in patients with pembrolizumab- or nivolumab-resistant, recurrent or metastatic HNSCC. The initial results were discussed at ASCO Annual Meeting 2023 (73). A new clinical trial (NCT05879484) has been designed with front-line administration of valemetostat (oral EZH1/EZH2 dual inhibitor) and pembrolizumab (PD-1 inhibitor) in PD-L1 positive, HPV-negative recurrent/metastatic HNSCC (The PANTHERAS). This Phase 1 trial is expected to determine the recommended dose of the two drugs and evaluate how effective and safe the combination is. The participants will receive treatment for 2 years, the results will expected to be publicated after July of 2027 (71,74).

Due to its outstanding role in modulating both tumor cell survival and tumor immunity EZH2 seemed to make a suitable target to further characterize the tumors of young patients.

2. Objectives

It is still a matter of debate whether young HNSCC patients might form a distinct disease entity or sub-category. Our aim was to clarify the relation of this group with the general patient population.

The study design consisted of two sequential parts.

1. In the first one we aimed to characterize this group epidemiologically. Three main aspects seemed to have outstanding relevance. Firstly, we hypothesized that an etiologically distinct group has to have its own age distribution. In contrast, the distortion of the age distribution towards the older age groups is against a group-specific causative factor. Secondly, we aimed to compare the distribution of the primary tumor localizations in the young and the non-preselected general HNSCC patient population. Finally, we also aimed to assess the prevalence of classical risks (namely smoking and alcohol consumption) in both study groups in comparison with the local general population. In addition we also intended to compare the tumor variables (grade, stage, TNM status) and the prognosis (including 5-year survival rates) in the young and the general HNSCC patient population.

2. In the second part of the study we aimed to characterize the molecular phenotype of the tumors of young patients in comparison with the general HNSCC patient population. This might be indicative of whether tumor characteristics (i.e. aggressivity) or permissive patient factors might rather characterize the increased risk in this patient subpopulation. Having a well-defined oncogenic role in cancer initiation, progression, metastasis, metabolism, and drug resistance, and in the modulation of antitumor immunity, EZH2 has been defined as an effective marker of the tumor aggressivity and tumorigenic potential. Thus, we determined the EZH2 expression in combination with P53 of our examined groups' tumors.

3. Methods

3.1. Study design

In the first part of the study we collected and analyzed the medical records of 85 consecutive young patients with histologically verified HNSCC between 2000 and 2018 based on the institutional medical database of the National Institute of Oncology. The definition of young patients were accepted as individuals age under 40 years (≤39) at the time of the diagnosis according to the National Comprehensive Cancer Network (NCCN) Adolescent and Young Adult (AYA) Oncology's guideline (4). We involved patients, who suffered from lip, oral cavity, meso-hypopharynx or laryngeal cancer. We excluded patients with metastases of unknown primary tumors, nasopharyngeal and salivary gland tumors, sinonasal carcinomas, thyroid tumors and lymphomas. Patient data (age, gender, drug and clinical history, tobacco and alcohol consumption, tumor site, stage, grade, and nodal status at the time of the diagnosis) were extracted from our institutional database and were compared with an institutional control group of 140 consecutive general HNSCC patients from year 2014. Only those who had never smoked were considered non-smokers. Drinkers were defined as at or above World Health Organization (WHO) medium risk category (>40 g alcohol for men and >20 g for women). The tumors were classified according to the 7^{th} edition of TNM classification system.

In the second part of the study we involved formalin fixed, paraffin embedded tissue blocks of 68 random young HNSCC patients (≤39 years, between 2000-2018) in comparison with the samples of 58 gender and tumor localization matched general HNSCC subjects (all diagnosed in the year 2014). We also collected the clinicopathologic data of the examined groups. The proportion of certain tumor localizations in the control group were also matched to the young study group to avoid bias.

3.2. Immunohistochemical staining

Paraffin-embedded tissue sections were used for the confirmation of the diagnosis and the immunohistochemical staining for $p16^{INK4a}$ antigen with Roche CINtec® p16 ($E6H4^{TM}$) antibody (Roche, Basle, Switzerland).

EZH2 expressions of the tumors were also detected by immunohistochemical staining (mouse monoclonal anti-EZH2 antibody, clone 11, BD Biosciences, Franklin Lakes, NJ, USA). Two independent examiners evaluated the reactions, scored the staining intensity and the proportion of positive cells. The proportion of the positive tumor cells (%) and the intensity of the nuclear staining (0, 1+, 2+, 3+) were recorded for each slide (such as in Figure 2). The P53 protein levels were also examined by immunohistochemistry (mouse monoclonal anti- P53 antibody, clone DO7, DAKO, Glostrup, Denmark). For P53 staining intensity, the positive control was a monoclonal, P53-expressing, high-grade serosus ovarian cancer cell line. Negative nuclear staining corresponded to a score of 0, focal or heterogenous staining patterns corresponded to a score of 1, and diffuse intensive nuclear staining in ≥80% of the tumor cells was classified as a score of 2.

3.3. Statistical analysis

Statistical analyses were conducted using KyPlot 5.0 (KyensLab Inc., Tokyo, Japan) and Statistica 14.0.1.25 (TIBCO Software Inc., Palo Alto, CA) softwares. Kolmogorov–Smirnov test was used to assess the distributions of numeric variables. T test or Mann–Whitney U test (MWU) were used to compare independent variables according to distribution normality. Kruskal–Wallis (K-W) test was used for non- parametric multiple comparisons, using Dunn's test for post hoc analysis. Chi-square test (CS) and Fisher's exact test (FE) were used to compare the compositions of groups. Overall survival intervals were determined as the time period from discovery date to the time of death in months. Survival data were processed using the Kaplan–Meier method, while the survival of subgroups was compared using the Log-Rank (Cox–Mantel–Haenszel) test. Univariate and multivariate analyses of prognostic factors were performed using the Cox's regression model. Statistical significance was determined when p values were under 0.05.

3.4. Ethical approval

The study was conducted under the ethical permission of the Scientific and Research Ethics Committee of the Medical Research Council (approval number: BMEÜ/3719- 1 2022/EKU) in accordance with the Helsinki Declaration of 1975, as revised in 2008.

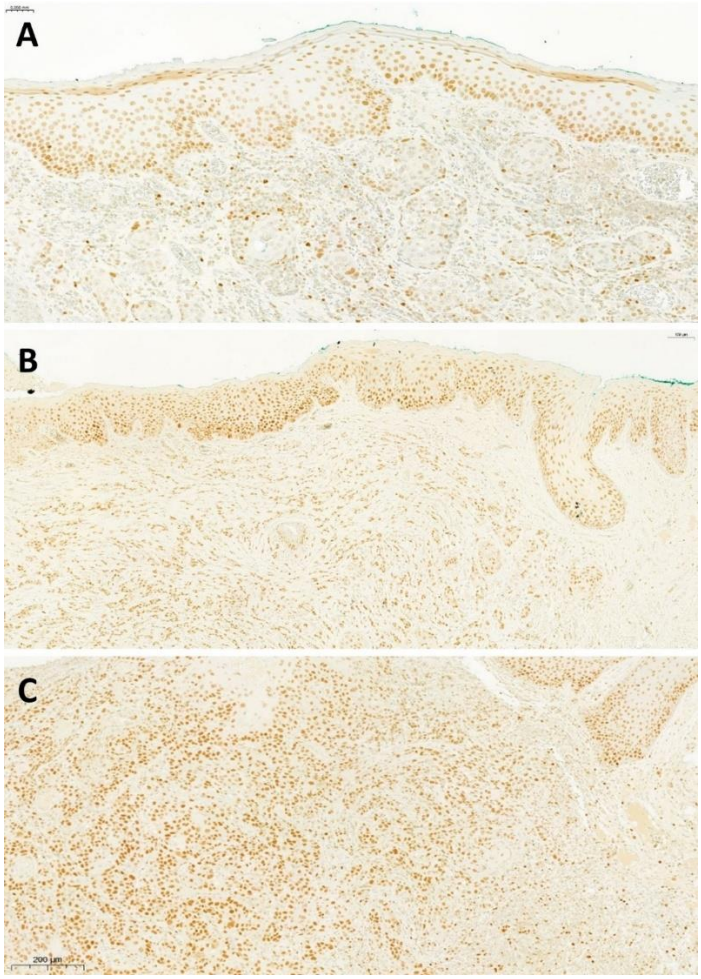

Figure 2. Three different degrees of EZH2 expression (proportions of positive nuclei: (A) 20%; (B) 80%; (C) 100%). Tumor tissue can be seen in the submucosa; the dark tone in the nucleus indicates EZH2 expression. Positivity in the basal layer of physiological mucosal epithelium is normal. In the pictures, EZH2 expression extends to the full length of the epithelium due to the in situ carcinoma or epithelial dysplasia in the tumor environment. Scale bars represent 50 µm, on picture (A), 100 µm, on picture (B), 200 µm, on picture (C).

4. Results

4.1. First part of the research

4.1.1. Tumor characteristics of young patients

We found male predominance (78.9%, male/female: 67/18) among the young patients. The median age at the time of the diagnosis was 37 years (range 21-39) and two-third of the patients were 35-39 years old. For comparison we involved an institutional general HNSCC group (male/female: 104/36; median age: 61.5 years, range 34-88). The most common primary tumor locations were the lip and the oral cavity in the young group in contrast to the general patient population (48/85 [56.47%] vs. 27/140 [19.28%]; $p<0.001$, CS). The most common subsite was the tongue in contrast to the oral cavity tumors of the general HNSCC patients (26/48, [54.1%] vs. 6/27, [22.2%], $p<0.007$, CS). In the oral cavity subgroup 6 of 48 young patients (12.5%) and 3 of 27 general HNSCC patients (11.1%) presented with lip cancer, the difference was not significant ($p=0.85$, Chi-square test). More than half of the young patients (57/85) had early (T1-T2) primary tumor status at the time of the diagnosis, which is significantly different from the control group (57/85 [67%] vs. 66/140 [47.1%], respectively, $p<0.037$). Fifty-four (63.5%) patients had cervical lymph node involvement (N+) according to the physical examination and radiological imaging (ultrasound, CT scan, MRI). Lymph nodes were seldom involved in laryngeal cancer (N0: 83.3%, 10 of 12). None of the patients had distant metastasis (M) at the time of the diagnosis. Well-differentiated tumors (grade 1-2) are significantly more common among young patients than in the control group (55/73 [75.3%] vs. 73/119 [61.3%], respectively, $p=0.043$). No differences were found in other histological characteristics, such as perineural invasion, lymphovascular and vascular spreading. Detailed characteristics of all study groups are reported in Table 3.

Table 3. Clinical characteristics and treatment methods of the young patients and the institutional control group

	Young patients (n=85)	Control group (n=140)	p (Chi-square)
Sex			
male	67 (78.8%)	104 (74.3%)	0.44
female	18 (21.2%)	36 (25.7%)	
Median age (yr)	37	61.5	**0.001**
Interquartile range (yr)	33-39	56.75-67	
Smoking history			
pos	52 (65.8%)	123 (87.9%)	**<0.001**
neg	27 (34.1%)	17 (12.1%)	
NA	6	0	
Alcohol consumption			
pos	38 (48.1%)	79 (56.4%)	0.235
neg	41 (51.8%)	61 (43.6%)	
NA	6	0	
Localization			
Lip, oral cavity	48 (56.5%)	27 (19.3%)	
Mesopharynx	15 (17.6%)	35 (25%)	**<0.001**
Hypopharynx	10 (11.8%)	34 (24.3%)	
Larynx	12 (14.1%)	44 (31.4%)	
Primary tumor size			
T1	24 (28.2%)	28 (20%)	
T2	33 (38.8%)	38 (27.1%)	**<0.037**
T3	12 (14.1%)	30 (21.4%)	
T4	16 (18.8%)	44 (31.4%)	
Nodal status			
N0	31 (36.5%)	49 (35%)	
N1	16 (18.8%)	24 (17.1%)	0.83
N2	33 (38.8%)	54 (38.6%)	
N3	5 (5.9%)	13 (9.3%)	

Stage			
I	16 (18.8%)	16 (11.4%)	
II	8 (9.4%)	10 (7.1%)	0.375
III	18 (21.2%)	31 (22.1%)	
IV	43 (50.5%)	83 (59.3%)	
Grade			
1-2	55 (75.3%)	73 (61.3%)	**0.043**
3-4	18 (24.7%)	46 (38.6%)	
NA	12	21	
Perineural spread			
pos	13 (38.2%)	17 (29.3%)	
neg	21 (61.8%)	41 (70.7%)	0.37
NA	51	82	
Vascular invasion			
pos	10 (28.6%)	24 (40.7%)	
neg	25 (71.4%)	35 (59.3%)	0.23
NA	50	81	
Primary treatment			
only surgery	15 (17.6%)	12 (8.6%)	
only non-surgery	28 (32.9%)	46 (32.8%)	0.11
combined (surgery+ non-surgery)	42 (49.4%)	82 (58.5%)	

NA: not available

4.1.2. Prevalence of potential risk factors in young patients

We analyzed the potential intrinsic factors of the enrolled patients. Two of 85 patients had Fanconi syndrome, which was associated with aplastic anemia. Both of them had bone marrow transplantation before the development of HNSCC. The extrinsic (smoking and alcohol consumption) etiological factors were also collected from the medical records of 79 young patients. Fifty-two (65.8%) of them were (current or previous) smokers, 38 (48.1%) consumed alcohol and 33 (41.7%) enjoyed both risk factors. However, smoking

was significantly less prevalent in the young group than in the general HNSCC patient population (65.8% vs. 87.9% respectively, p<0.001, CS). In contrast, the alcohol consumption was similar in the young and the general HNSCC group (48.1% vs. 56.4%, respectively, p<0.001 CS) (Table 4.). The p16 status was assessed in 65 young and 36 control patients. The positivity rate was 20% (n=13) and 11.1% (n=4), with no significant difference between the two groups (p=0.25, Chi-square test).

Table 4. Prevalence of risk factors in the examined group and the regional data from Hungary (75–77)

	Cases (n)	**Age at diagnosis** (median; years)	**Gender** (male/female)	**Smokers (%)** former or present	**Alcohol consumers (%)**	**p16 positive status**
Young HNSCC group	85	37	67/18	**65.8** (52/79)	**48.1** (38/79)	**20** (13/65)
Control HNSCC group	140	61.5	104/36	**87.9** (123/140)	**56.4** (79/140)	**11.1** (4/36)
Prevalence in regional population (%)				**30.6**	**32.3**	**18**
p (Chi-square)				**<0.001**	**<0.001**	0.25

4.1.3. Front-line therapy and histological among the young patients

Fifty-seven of the 85 patients (67%) underwent surgical therapy. 85.4% of lip and oral cavity (n=41), 75% of laryngeal (n=9), 40% of hypopharyngeal (n=4) and 20% of mesopharyngeal (n=3) cancer patients underwent surgery. Forty of the 57 patients (70.2%) underwent R0 resection, among them 10 patients with close R0 resection (the free margin is <5mm). In 29.8% (n=17) of patients R1 resection was performed.

Two of the patients operated with close R0 resection (20%) and six of the patients operated with R1 resection (35.2%) underwent re-excision surgery. The indications for postoperative irradiation were pT3 or pT4 stage, perineural spreading, vascular invasion, extranodal extension and close or positive surgical resection margins. Altogether 42 patients (73.6%; n=31 patients with R0; n=11 with R1 resection) received adjuvant therapy after the surgical therapy (Table 3.)

4.1.4. Overall survival (OS) of young patients

The median follow-up time since the diagnosis of cancer was 28.5 months (range 3-228). Cumulative five-year OS for all head and neck sites was 44.2% (Figure 3A), 44.9% for lip and oral cavity (n=47), 58.3% for oropharynx (n=15), 0% for hypopharynx (n=10) and 62.8% for larynx (n=12) carcinoma. Only the subgroup with hypopharynx carcinoma differed significantly from all others (Figure 3B). The cumulative 5-year survival for all tumor sites was significantly better in the young than in the control group (44.2% vs. 32% respectively, p=0.005). The 5-year OS was analysed by risk factors. Significantly better OS was observed in the alcohol abstinent group (59.6% vs. 32.7%; p=0.0297). The 5-year OS was significantly better in patients with early, than in advanced T status (T1-2: 52.6% vs. T3-4: 26.7%; p=0.0058) (Figure 3C) and in patients with N0 vs. N+ nodal status (65.2% vs. 32.3% respectively; p=0.0013) (Figure 3D).

The 5-year OS was better among young patients with p16 positive status (n=13) in comparison the p16 negative young patient group (n=51), however the difference did not reach the level of significance (66.6% vs.43.3%; p=0.23).

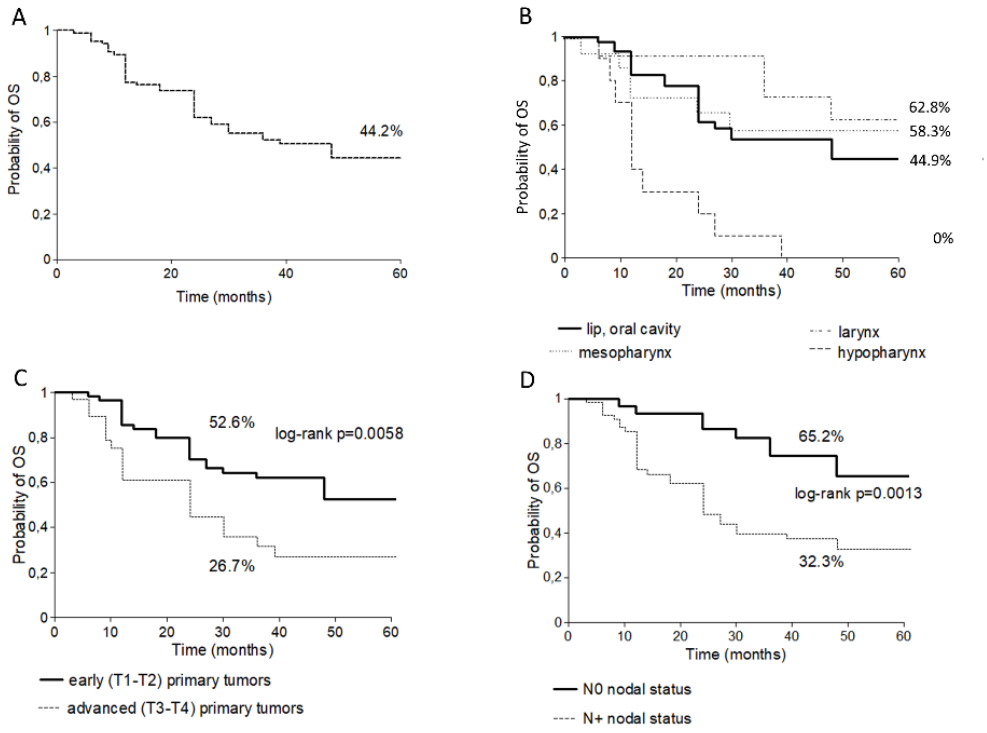

Figure 3. Five-year overall survival curve of all sites of head and neck cancers in young patients (A); Five-year overall survival curve of subtypes of head and neck cancers in young patients (B); Five-year overall survival curve stratified by T stage in young patients (C); Five-year overall survival curve stratified by N stage in young patients (D)

4.1.5. Results of the regression model analysis

Univariate Cox regression model for the entire study population revealed that young age, a non-smoker or abstinent status, as well as laryngeal tumor site might predict a significantly better prognosis. Data adjustment for multivariate Cox regression model proved that from the aforementioned parameters young age, abstinence and earlier stage remained independent prognostic parameters, which were associated with more favorable outcome (Table 5.). Contrarily, alcohol consumption, older age and advanced stage almost doubled the relative risk. When analyzing the young group separately, an alcohol-abstinent status, as well as advanced stage proved significant predictors of the prognosis. All the significancies and trends in the young age group were in line with those in the entire study group (Table 6.).

4.1.6. Locoregional control

Thirty-six of 85 patients (42.3%) had residual disease. Positive margins were reported in 17 cases according to the histological analysis of the specimens. Residual disease was also detected in 15 cases after the completion of other treatment modalities (e.g. definitive radio(chemo)therapy, induction chemotherapy+radio(chemo)therapy) by radiological imaging. We also considered four further cases of locoregional recurrences to constitute residual disease, as recurrence occured within 3 months (average of 10 weeks, range:8-12) after surgical treatment with tumor-free margin (R0) status.

Recurrent disease was observed in 24.7% (n=21) of the 85 patients (12 local, 4 regional, 3 distant metastasis and 2 locoregional recurrence) after that the patient was defined as disease free, and occurred within an average of 24 months after the diagnosis (local: 15.14 months /range 4-48/; regional: 9 months /range 4-20/; distant: 20 months /range 14-24/). The incidence of second primary cancers (lung, oropharynx and tongue) was 4.7% (n=4).

Table 5. Univariate and multivariate Cox regression model for the assessment of prognostic factors in the entire (young patients and control group) study population (RR= relative risk)

	Univariate model		**Multivariate model**	
	RR (95% CI)	**p**	**RR (95% CI)**	**p**
Young group (vs. control)	0.497 (0.309-0.797)	0.004	0.617 (0.426-0.894)	0.011
Male sex (vs. female)	0.837 (0.525-1.334)	0.454	0.708 (0.474-1.057)	0.091
Smokers (vs. non-smokers)	1.799 (1.037-3.121)	0.037	1.577 (0.871-2.855)	0.133
Alcohol (vs. abstinent)	1.641 (1.073-2.508)	0.022	2.007 (1.394-2.888)	0.0001
Localization (vs. oral cavity)				
mesopharynx	0.877 (0.448-1.714)	0.308		
hypopharynx	0.814 (0.456-1.453)	0.164		
larynx	0.398 (0.221-0.718)	0.005		
Grade 3/4 (vs. 1/2)	0.854 (0.581-1.255)	0.421	0.746 (0.482-1.155)	0.189
Stage IV (vs. I-III)	2.454 (1.605-3.752)	0.00003	1.883 (1.343-2.64)	0.0002

RR: relative risk; CI: confidence interval

Table 6. Univariate and multivariate Cox regression model for the assessment of prognostic factors among young head and neck squamous cancer patients

	Univariate model		**Multivariate model**	
	RR (95% CI)	**p**	**RR (95% CI)**	**p**
Male sex (vs. female)	1.146 (0.531-2.472)	0.728	0.592 (0.208-1.688)	0.327
Smokers (vs. non-smokers)	1.796 (0.85-3.797)	0.125	1.746 (0.664-4.589)	0.258
Alcohol (vs. abstinent)	1.973 (1.029-3.783)	0.041	1.433 (0.61-3.369)	0.409
Tumor site (vs. oral cavity)				
mesopharynx	1.683 (0.464-6.113)	0.282	0.434 (0.05-3.79)	0.043
hypopharynx	4.08 (1.898-8.769)	0.00003	3.602 (1.404-9.244)	0.00003
larynx	0.546 (0.188-1.582)	0.063	0.325 (0.071-1.491)	0.189
Grade 3/4 (vs. 1/2)	0.734 (0.333-1.618)	0.444	0.939 (0.396-2.227)	0.887
Stage IV (vs. I-III)	1.904 (1.026-3.535)	0.041	1.948 (0.908-4.179)	0.087

RR: relative risk; CI: confidence interval

4.2. Second part of the research

4.2.1. Epidemiological characteristics of the matched groups

The median age at diagnosis was 36 years [total range 21-39] in the young and 62 years [total range 45-88] in the general HNSCC group. Young patients with laryngeal cancer had the best five years disease-free survival (DFS) with 83.3%, followed by the oropharyngeal, lip and oral cavity, and hypopharyngeal tumors (61.5%, 43.6% and 0% respectively). Of note, the general HNSCC (control) group is not epidemiologically representative, as its members were selected to match the young group for gender distribution and tumor localizations (lip, oral cavity, mesopharynx, hypopharynx or larynx). Group characteristics are shown in detail in Table 7. The survival data of the young HNSCC group is also presented on Figure 4.

Table 7. Clinical characteristics of the young patients and control group

	Young HNSCC	General HNSCC	p
Case number (n)	68	58	
Sex (female/male)	16/52	10/48	0.39 (χ2)
Age (median; min-max)	36 (21-39)	62 (45-88)	**0.001** (MWU)
Smoking history			
pos	43 (63.2%)	46 (79.3%)	0.10 (χ2)
neg	22 (32.3%)	12 (20.7%)	
NA	3 (4.4%)	0 (0%)	
Alcohol consumption (n; % of group)			
pos	29 (42.6%)	37 (63.8%)	**0.03** (χ2)
neg	36 (52.9%)	21 (36.2%)	
NA	3 (4.4%)	0 (0%)	
Localization (n; % of group)			
Lip, oral cavity	41 (60.2%)	26 (44.8%)	0.19 (χ2)
Mesopharynx	13 (19.1%)	10 (17.2%)	
Hypopharynx	7 (10.3%)	11 (18.9%)	
Larynx	7 (10.3%)	11 (18.9%)	
5 years DFS (yes/known)			
Total	46,2% (30/65)	34,5% (20/58)*	0.18 (χ2)*
Lip, oral cavity	43,6% (17/39)	30,8% (8/26)*	0.29 (χ2)*
Mesopharynx	61,5% (8/13)	50% (5/10)*	0.58 (χ2)*
Hypopharynx	0% (0/7)	18,2%(2/11)*	0.67 (χ2)*
Larynx	83,3% (5/6)	45,5% (5/11)*	0.11 (χ2)*
Primary tumor size (n)			
T1	19	12	0.71 (χ2)
T2	28	24	
T3	8	10	
T4	13	12	
Nodal status (n)			
N0	27	32	0.83 (χ2)
N1	11	6	
N2	27	17	
N3	3	3	
Stage (n)			
I	12	10	0.67 (χ2)
II	8	11	
III	14	9	
IV	34	28	
Grade (n)			
1-2	49	35	0.13 (χ2)
3-4	15	21	
NA	4	2	

NA: not available; DFS: disease-free-survival

*Survival data is presented to characterize the study groups. Due to group matching for localization and gender the data are not suitable for epidemiological and survival conclusions

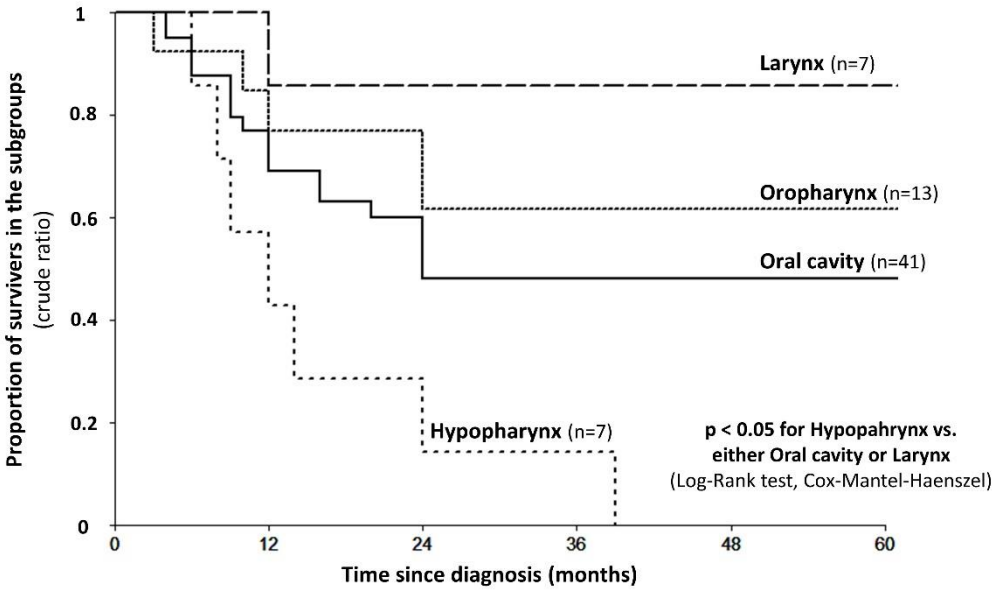

Figure 4. Five-year overall survival curves of subtypes of head-and-neck cancers in young patients.

4.2.2. Expression of EZH2 in squamous cell carcinoma of the head and neck region

EZH2 staining was predominantly nuclear. The median proportion of EZH2 expressing cells among all malignant cells was 60% [IQR: 30-80] in the general and 40% [IQR: 3.75-72.5] in the young HNSCC group (p=0.003; MWU, Figure 5/A). We also found a significant difference in the median staining intensity of EZH2 (young and control group medians 1 [IQR: 0.75-2] vs. 1.5 [IQR: 1-2] respectively, p=0.0001, MWU, Figure 5/B). In the subgroup analysis a lower EZH2 expression both regarding the proportion of expressing cells and the intensity of staining was characteristic for young patients in all tumor localizations, although the difference only reached statistical signficance for the oral cavity and hypopharynx when regarding proportions (Figure 6). All EZH2 and P53 expression data are presented in Table 8. EZH2 expression was not correlated with histological grade, primary tumor status, and presence of nodal metastasis. Higher EZH2 expression seems to characterize hypopharyngeal tumors when compared to either oral cavity or laryngeal malignancies (percent of positive cells, control group; oral cavity 60% [IQR: 20-90] or larynx 40% [IQR: 10-80] vs. hypopharynx 80% [60-90] Dunn post-hoc

p<0.05). Although a similar trend exists in the young group, it did not reach statistical significance.

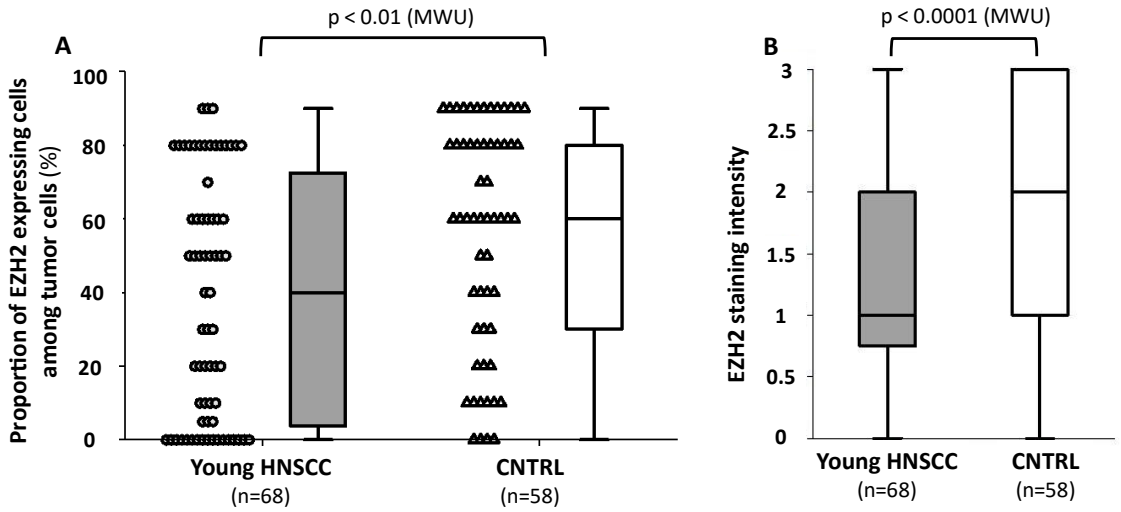

Figure 5. (A) Comparison of the median proportion of EZH2-expressing cells in the two examined groups, dots represent individual data points. (B) comparison of the median staining intensity of EZH2 (young and control groups). Medians, interquartile ranges and total ranges are indicated.

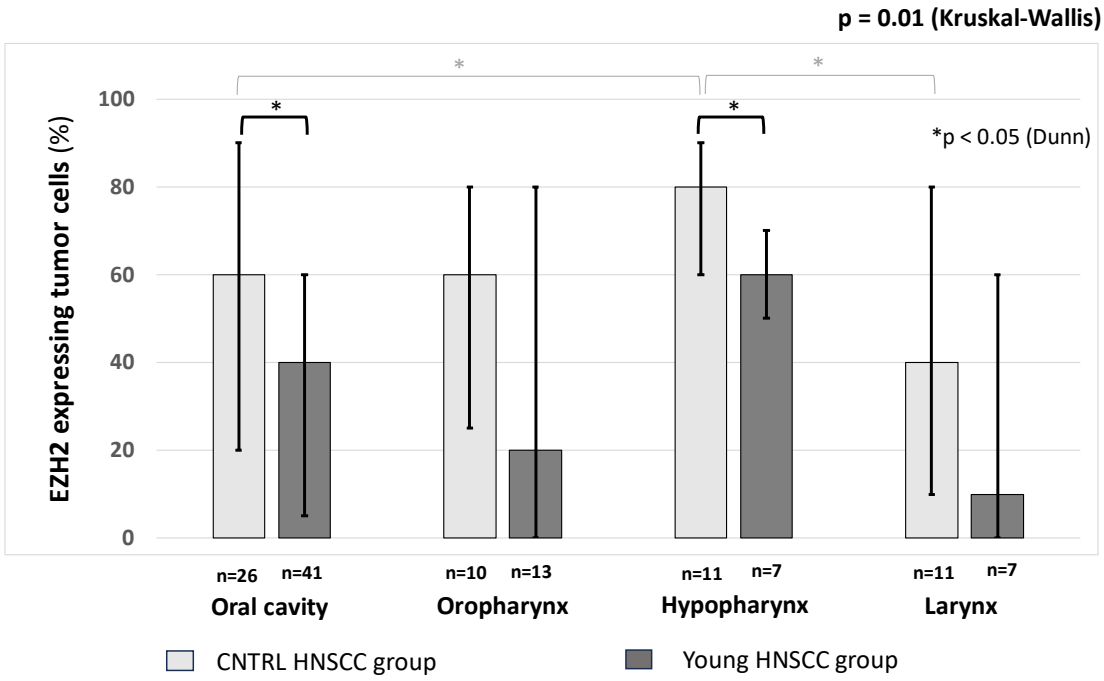

Figure 6. Proportions of EZH2-expressing tumor cells in certain locations in the young and control HNSCC groups. Medians and interquartile ranges are indicated.

Table 8. Staining characteristics of tumor cells

	Young patients	Control	p (stat. method)
EZH2 expression (% of malignant cells)	**median [IQR]**	**median [IQR]**	
Total	40 [3.75-72.5]	60 [30-80]	0.003 (MWU)
(multiple comparison)	-	-	0.01 (K-W)
Lip, oral cavity	40 [5-60]	60 [20-90]	**0.02 (Dunn)**
Oropharynx	20 [0-80]	60 [25-80]	0.20 (Dunn)
Hypopharynx	60 [50-70]	80 [60-90]	**0.04 (Dunn)**
Larynx	10 [0-60]	40 [10-80]	0.08 (Dunn)
EZH2 expression (staining intensity)	**median [IQR]**	**median [IQR]**	
Total	1 [0.75-2]	1.5 [1-2]	0.0001 (MWU)
(multiple comparison)	-	-	<0.001 (K-W)
Lip, oral cavity	1 [1-2]	2 [1-3]	**0.02** (Dunn)
Oropharynx	2 [0-2]	2 [1-2]	0.16 (Dunn)
Hypopharynx	2 [1-2]	3 [2-3]	**0.006** (Dunn)
Larynx	1 [0-1]	1 [1-2]	**0.02** (Dunn)
P53 expression (staining intensity)	**median [IQR]**	**median [IQR]**	
Total	1 [0-2]	1 [0-2]	0.26 (MWU)
(multiple comparison)	-	-	0.70 (K-W)
Lip, oral cavity	1 [0-2]1	1.5 [0-2]	N/A
Oropharynx	1 [0-1]	1 [0.5-2]	N/A
Hypopharynx	1.5 [0.75-2]	2 [0.5-2]	N/A
Larynx	1 [0-1.25]	1.5 [0.5-2]	N/A

N/A: not applicable

4.2.3. Survival characteristics and potential associations

Five-year overall survival (OS) for all head and neck sites was 46.1% in young patients, and 34.5% in the control group, ($p>0.05$, $\chi 2$). The 5-year OS was significantly better in patients with N0 vs. N+ nodal status in both groups (young group: 69% vs. 31.8%, $p=0.004$, $\chi 2$; control group: 46.8% vs. 19.2%, $p=0.0074$, $\chi 2$). A linear regression analysis suggested that there was only a marginal effect of the proportion of EZH2-expressing tumor cells on the crude disease-free survival of young patients ($F(1,66) = 3.95$, $p=0.05$, with an $R2=0.06$ and a regression coefficient of -0.4); thus, the one percent increase in the proportion of EZH2-expressing cells might be related to a 0.4 month decrease in the predicted DFS with great scatter in the data.

Cox regression analysis was performed to test the true predictive values of tumoral EZH2 and P53 expression, alcohol and tobacco consumption, and T and N statuses (Table 9). Univariate Cox analysis identified a more advanced T status (T3-4 vs. T1-2), a positive

nodal status, and alcohol consumption above the WHO medium risk category as significant predictors of mortality. In multivariate Cox analysis, the nodal status was verified as the single highly significant predictor of mortality, whereas alcohol consumption did not reach the level of significance. Neither a higher proportion of EZH2-expressing tumor cells (defined as exceeding the group median of 40%) nor intratumoral P53 expression were identified as significant predictors of an unfavorable outcome among young HNSCC patients (although the trend of the HR was coherent with the known biological nature of both mediators).

Table 9. Cox analysis for the predictors of patient survival among young HNSCC-afflicted individuals

	Univariate		**Multivariate**	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**
EZH2 expressing tumor cells (%, above vs. at or below median)	1.2 (0.63-2.29)	0.59	1.17 (0.54-2.51)	0.69
P53 expression (expressing vs. non-expressing)	0.9 (0.47-1.78)	0.80	0.8 (0.35-1.63)	0.47
T status (T3-4 vs. T1-2)	**2.34 (1.23-4.46)**	**0.01**	1.64 (0.78-3.47)	0.19
N status (N+ vs. N0)	**2.92 (1.38-6.2)**	**< 0.01**	**3.04 (1.38-6.68)**	**< 0.01**
Alcohol consumption (vs. below WHO medium risk)	**2.26 (1.16-4.4)**	**0.02**	1.96 (0.98-4.08)	0.06
Smoking (vs. non-smoker)	2.02 (0.92-4.44)	0.08	1.59 (0.65-3.94)	0.31

HR: hazard ratio; CI: confidence interval

5. Discussion

The incidence of HNSCC in young people is relatively low however there has been a trend for an increasing percentage of younger patients in the US, various European countries and China. In India, where the overall HNSCC burden is much higher than in other countries, this increasing proportion of young patients seems to be similar (8). However, the percentage of young HNSCC patients depends greatly on the chosen age cut off, which is not consistent in the literature. To the best of our knowledge, our study was the first in Europe to investigate the clinical characteristics of young HNSCC patients under 39 years of age, as defined by the NCCN Adolescent and Young Adult oncology guidelines, and involving patients with tumor localizations of HNSCC in the strict sense (oral cavity, pharynx, larynx). Due to the low number of cases and the heterogeneous patient groups with different age ranges in the literature, the question of whether the young patient group constitutes a distinct etiological entity is still unanswered. Our research strategy was characterized by the funnel approach. As a great bulk of knowledge is already known regarding the etiological factors of HNSCC in the general patient population, we first aimed to map the epidemiological points in which the young population might differ. Although uncountable pathways are involved, the formation of tumors eventually involves four major phase of events, namely the effects of certain harmful agents, incomplete DNA damage control, incomplete immunological control of tumor cells and finally the viability and aggressivity of the established tumor tissue.

In our study populations (both the young and the general) we found male predominance, which is in line with the literature. It can be assumed that estrogen exposure in women may protect against the development of these tumours. The results of recent in vitro studies render the beneficial effect of estrogen in reducing the migration ability of tumor cells controversial. However, the beneficial role of antiestrogens in the treatment of HNSCC is plausible (78).

The age distribution of our young patients was found to be heavily distorted towards the cut-off value of 39 years, which might indicate that the majority of them might represent the “lower or left tail” of the age distribution’s Gaussian curve of the general patient population.

Previous studies confirmed that smoking, alcohol consumption and HPV are the most important risk factors of HNSCC (10,79). It is known that these carcinogens have a delayed effect, and it is under debate whether the shorter duration of the exposure in younger patients might be sufficient to significantly increase the risk (10). Di Credico et al. investigated the risk for HNSCC in terms of the joint effect of the duration and intensity of the alcohol consumption. Their study indicated that the cancer risk of oral cavity, hypopharynx and larynx cancer increases with drinking intensity, however the duration did not substantially modify cancer risk (80). The prevalence of tobacco and alcohol consumption in our young study population was in-between of the regional general population over 15 years old (30.6% (75); 32.3% (76)) respectively and the general HNSCC population.

The proportions of smokers and alcohol consumers, as well as the gender distribution in our general HNSCC study group were practically identical with large-scale international hypothesis-free epidemiological HNSCC studies (81,82). We found that, in contrast to the general HNSCC population, the oral cavity proved to be the most common tumor site in young patients. The oral cavity is the most exposed area to carcinogens, which mix with saliva and tend to pool on the non-keratinized mucosa (83). The majority of young patients were diagnosed in early T status, however two-thirds of them had cervical lymph node metastases. Advanced T and N statuses were associated with significantly worse 5-year survival also in our young study population (in line with previous reports in general HNSCC patients (84)). Several studies demonstrated that HPV associated head and neck cancer presents with a very high percentage of node positivity. However, in our study we found low prevalence of p16 positivity, which is in line with a recently published Hungarian article with similar data regarding the prevalence of p16 positivity in HNSCC (77). It is known that not only the tumor characteristics but also the prognosis differs in patients with HPV-related cancers. Next-generation DNA sequencing indicates that HPV-positive tumors have less intratumor heterogeneity than HPV-negative tumors, and thus may be more likely to respond to therapy without recurrence and offer a favorable prognosis (85). This altered therapeutic response might originate from the extensive cell cycle arrest in G2/M phase causing higher cellular radiosensitivity (20). After irradiation the larger number of residual DNA double-strand breaks are attributed to diminished DNA repair capacity, which also contribute to the better efficacy of therapetic regimens

and the favorable outcome (20). In our study population we also found better survival in the HPV-positive group, with a non-significant trend, likely due to the small subgroups size.

Tumor stage is a well-known predictor of the prognosis and survival. However on its own the histologic grade was not found to be clearly correlated with the oncological outcome in HNSCC (86). Of note, poorly differentiated tumors have a higher risk of nodal metastasis (86). In our study we found that the tumors of young patients were significantly more differentiated in comparison with the general HNSCC patients' tumors, but the proportion of positive cervical nodal metastases did not differ between the two examined groups. This raises the possibility of either the contribution of additional factors to early metastasis formation or the possibility of a relatively late recognition of malignancies in young patients.

Several studies aimed to compare the prognosis of young and older patients, but the results are inconsistent (10–12), which could be explained by the different definition of young patients' age and the paucity of comprehensive studies on more than one tumor site. In our study the young age was associated with a relatively better outcome in comparison with the general HNSCC group. The 5-year survival analysis showed significantly worse survival in the hypopharynx subgroup than in the other subgroups in young patients.

According to our results, the age distribution (two third of the patients were 35-39 years old) and the high prevalence of traditional risk factors among the young patients as well as the predominance of oral cavity tumor localization suggest that young HNSCC patients might have an exceeded vulnerability to various environmental carcinogens which might originate from a diminished damage control on the cellular level or a weaker capacity to eliminate tumor cells by the immune system.

It also seemed to be possible that the early-onset might originate from the behaviour of the tumor cells. There is no consensus whether the HNSCC in young patients has a more aggressive behaviour than tumors in older people (87). Several studies found neither significant differences in the clinical parameters of tumors nor differences in the histological grading of tumors between young and older patients (87). A recently published article reported that young patients with non-HPV related HNSCCs are thought

to have inherently more aggressive forms of the disease when compared with older patients, however their conclusion is based only on survival data. The disease free survival was similar between the two groups, and they suggested that the better overall survival of young patients was only due to their fewer comorbidities hence better overall health. The adverse features of the tumors (extracapsular extension, lymphovascular invasion, perineural invasion) did not differ significantly in the two examined groups, corroborating with our findings (88).

To examine and compare the molecular profile of the HNSCC tumors in the young and the older group, our choice fell on EZH2 (in combination with P53), which can modulate central components of pathways involved in the regulation of cell proliferation, apoptosis inhibition and tumor progression chiefly through epigenetic regulatory mechanisms. EZH2 overexpression is identified as a negative prognostic marker and it has been associated with poor prognosis from various types of human cancers, including lung cancer, melanoma, ovarian cancer as well as hematological malignancies (55,89,90) and in HNSCC (91). Different types of EZH2 inhibitors have been developed, and there are a number of ongoing clinical trials of drugs targeting EZH2 in different cancer types (60). Combining EZH2 inhibitors with other therapy methods such as immune therapy, conventional chemotherapy, and target therapy might improve the treatment efficacy by helping to overcome resistence and boost anti-tumor immunity (60). Therefore, tazemetostat is being tested in combination with other therapies, such as chemotherapy or immunotherapy in HNSCC (73). It represents a promising class of targeted therapies for head and neck cancer, but further investigations are needed.

The role of the EZH2 expression has been investigated in few studies in HNSCC. To our best knowledge, this work is the first to compare the tumoral EZH2 expressions in the young and the general HNSCC patient groups. Lower EZH2 expression was detected in the young patients' tumors in comparison with the tumors of the general HNSCC group. This finding discourages those speculations, which suggest that more viable tumors (either through more successful immune evasion or a more malignant cellular phenotype) might be in the background of the early-onset tumor manifestation in young patients. It is likely that it is not a question of increased biological aggressivity, but rather of diminished function of tumor recognition or elimination, which may result in early carcinogenesis in young HNSCC patients. Shan et al. suggested that young HNSCC patients may present

with unique biological and tumor microenvironmental (TME) characteristics, which includes various immune cells, fibroblasts, endothelial cells, and various cytokines. These cellular components show different characteristics in terms of inflammatory responses, immune evasion and microenvironmental regulation. Recent studies reported that the TME in younger patients may contain elevated levels and activity of immunosuppressive cells, such as regulatory T cells and myeloid-derived suppressor cells, or a higher proportion of programmed death ligand 1 (PD-L1) expressing antigen presenting cells (92).

In our study, the proportion of EZH2-expressing cells was the highest in the subpopulation with the worst prognosis (hypopharyngeal carcinoma patients) and the lowest in those with the best DFS rates (laryngeal carcinoma patients). For our young patients, linear regression analysis indicated only a possible marginal effect of EZH2 expression on patient survival. Although numerous authors agree that high EZH2 expression indicates poor prognosis in regard to HNSCC (61,93,94), in our study Cox regression analysis only identified advanced N and T statuses, as well as alcohol consumption, as significant negative prognostic factors for survival. This discrepancy is in line with the results of Nienstedt et al, who found that EZH2 expression had no impact on patient survival, but did affect the development of lymph node metastases (91). The synthesis of the seemingly contradicting results could be that EZH2 is not a direct effector but a multitasking regulator of tumor cell characteristics. In vitro, the effect of EZH2 on cell proliferation and survival could be clearly demonstrated (95). However, in the living organism, multiple factors may interfere, including the capacity of the organism to control cellular DNA damage or to contain tissue proliferation of malignant phenotype cells through tumor immunity.

Many studies have shown that expression of P53 affects the prognosis of oral squamous cell carcinoma, oropharyngeal squamous cell carcinoma, and laryngeal carcinoma (96). According to our results, young HNSCC patients might be characterized by similar P53 expression in comparison with general HNSCC patient population. Both P53 and EZH2 play critical roles in regulating cell growth, apoptosis, and differentiation, and their dysregulation can contribute to cancer development and progression. The correlation between EZH2 and P53 was further confirmed at both mRNA and protein levels (97). Kuser-Abali et al. reported that P53-regulated MDM2 (murine double minute 2) that,

together with MDMX (murine double minute x), controls EZH2 turnover to determine tissue sensitivity to DNA-damaging agents (98). Zhao et al. found that EZH2 overexpression and P53 mutations frequently occur in late-stage cancers, which is in line with previous studies (97). In vitro studies have shown that EZH2 enhances P53 protein translation and amplify P53 gain-of-function mutant-mediated cancer growth and metastasis formation, therefore EZH2 is a viable therapeutic target in P53-mutated cancer (97). Our results suggest that the balance of this complex system may be different in young patients compared to the general HNSCC patient population, which warrants further, targeted studies.

6. Conclusions

In conclusion our results reveal that early- onset head and neck carcinomas occur mostly between 35-39 years, and their distribution is heavily distorted toward the older age group, which might suggests that the majority of young patients are unlikely to carry independent etiologies such as typical tumor predisposition syndromes, as these rather manifest at a younger age and are expect to carry their own age distribution. In the young group the traditional risk factors (tobacco and alcohol consumption) might play a significant role in the development of cancer, however the exposition time of the harmful agents are necessarily shorter. Based on these findings we concluded that young patients with HNSCC might represent an extreme value within the spectrum of the general HNSCC patient population, and we suggest that this group does not form a distinct entity. Histological examination indicated that young patients suffered from significantly more differentiated tumors in comparison with general HNSCC patients group, however there were no other histological markers that differed between the two groups.

We hypothesized increased vulnerability in the background of the early carcinogenesis, which might be either tumor or host (patient factors) induced. For investigating the biological aggressivity of the young patients' tumors our choice fell on EZH2, which has an outstanding role in modulating tumor cell survival, proliferation and tumor immunity. We found lower EZH2 expression in the young group, which discourages the assumptions that we are dealing with biologically more aggressive cancer phenotypes in young patients. Of note, the P53 immunohistochemical staining intensitiy was similar in the two study groups. Further studies should probably put more focus on patient characteristics, including a potentially altered tumor immunity and also the cellular capacity to restore DNA damages. Understanding the EZH2 mechanisms of action and interplay with other regulator proteins can guide the development of targeted therapies for improved cancer treatment outcomes. Based on our results, pharmacological trials might need to be supplemented with appropriate subgroup studies to investigate potential differences in the efficacy of EZH2 inhibitor therapies in different age groups.

7. Summary

Head and neck squamous cell carcinoma (HNSCC) was estimated to be the sixth most common cancer with approximately 800,000 new cases annually worldwide. Although HNSCC usually occurs in patients over 60 years, in the past half century the incidence also increased in young adults. Our aim was to characterize the clinical features, the age distribution and the prevalence of classical risk factors in the young patient group in comparison with a non- preselected institutional general HNSCC population.

The study design consisted of two sequential parts. In both studies we involved patients who were diagnosed with primary head and neck squamous cell carcinoma which originated from lip, oral cavity, meso- hypopharynx and larynx localizations.

In the first part of the study we analyzed the data of 85 young patients in comparison with 140 institutional general HNSCC patients. The age distribution (two third of the young patients were 35-39 years old) and the high prevalence of traditional risk factors among the young patients as well as the predominance of the oral cavity tumor localization suggest that young HNSCC patients might have an exceeded vulnerability to various carcinogens, however, a more aggressive tumor phenotype could not be exluded solely on an epidemiological basis. To investigate the latter possibility, in the second part of the study, we chose the enhancer of zeste homolog 2 (EZH2) in combination with the tumor supressor protein P53 to characterize the HNSCC tumor cells of young patients. EZH2 is a marker of tumor cell viability, and aggressivity, which has a well-defined oncogenic role also in tumor progression, metastasis, drug resistance, and in the modulation of anti-tumor immunity in various cancers. We determined the expression of EZH2 and P53 in the HNSCCs of 68 young patients in comparison with 58 gender and localization matched general HNSCC patients. Lower EZH2 and similar P53 expressions were found to characterize tumors of all localizations in young HNSCC patients.

The lower EZH2 expression of young HNSCC patients' tumors discourages speculations toward a more malignant phenotype of early onset tumors and suggests the dominant role of host factors (including cell cycle control and tumor immunity). Furthermore our results might also raise the possibility of an altered efficacy of the novel anti-EZH2 therapies in this patient subgroup.

8. References

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9. Bibliography of the candidate's publications

9.1. Publications related to the thesis

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IF: -

10. Acknowledgements

I would like to express my profound gratitude towards all who contributed to this work, namely:

I am especially grateful to my supervisor, Zoltán Takácsi-Nagy MD for his inspiration, guidance, and for his continuous support.

I am grateful to Professor Ferenc Oberna MD, gave me the opportunity to spend time writing scientific articles in addition to my clinical work.

I am grateful to István Kenessey MD for his comprehensive introduction to statistical analysis and for reviewing the manuscripts.

I would like to thank Erika Tóth MD and András Slezák MD pathologists for evaluating the immunohistochemical stainings.

I would like to thank the support for Professor Csaba Polgár MD DSc and Professor András Matolcsy MD DSc, the head of the Doctoral School of Pathological Sciences, who allowed me to write this dissertation.

I would like to thank all members of my family, especially my husband, András, and my son, Dani, for being my safe place and supporting my studies.