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Chapter 8

DNA Methylation in the Pathogenesis of Head and Neck Cancer

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Additional information is available at the end of the chapter

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1. Introduction

Head and neck cancer is the sixth most common cancer worldwide and one of the most aggressive malignancies in human population. The most common histologic type among the head and neck tumors are the squamous cell carcinomas (*SCC*). Despite the significant efforts committed during the last decades in its early detection, prevention and treatment, head and neck cancer prognosis remains very poor with the rising incidence in developed countries and younger population. Carcinogenesis of Head and Neck Squamous Cell Carcinoma (*HNSCC*) is a multistep process, which arises through an accumulation of genetic and epigenetic alterations. Although the impact of genetic changes in oral carcinogenesis is well-known, over the last decade it has been demonstrated that epigenetic changes, especially aberrant DNA methylation, play a significant role in *HNSCC*.

1.1. Head and neck cancer – Etiology and risk factors

Head and Neck Squamous Cell Carcinoma is the sixth most common cancer in males and tenth in females worldwide [1]. Despite the fact that significant results have been achieved during the last decades in its early detection, prevention and treatment, the survival rate has remained less than 40%, and *HNSCC* remains one of the most aggressive malignancies. Furthermore, the incidence of this carcinoma is rising in developed countries and younger population, particularly young women [2, 3]. Early stages of the disease are associated with minimal symptoms, thus small percentage of *HNSCC* has been diagnosed at an early clinical stage. Advanced stages respond poorly to current cancer therapies, with high incidence of local and regional relapse and lymph node metastasis [2, 4, 5].

Head and neck cancers include malignancies arising from different anatomical sites within the upper aero-digestive tract. Head and neck cancers are characterized by heterogeneous histology. The majority carcinomas that arise from squamous cell epithelia are head and neck squamous cell carcinomas (*HNSCC*), while other cancer types that can occur in the head and neck include thyroid cancer, malignant salivary gland tumors, lymphomas and sarcomas. *HNSCC* include cancers of the oral cavity, larynx, pharynx (oropharynx, hypopharynx, nasopharynx), and esophagus, [4, 5], Figure 1.

1.2. Risk factors for HNSCC

Both environmental and genetic factors play an important role in the etiology of head and neck cancers but the causal relationship between environmental factors, lifestyle and tumor development is not yet fully elucidated. The mucosa of the upper aerodigestive tract is exposed to number of carcinogens attributed to cause genetic and epigenetic changes that ultimately lead to head and neck cancer development. *HNSCC* incidence is influenced by age, genetic factors, geographic region and different lifestyle factors, including alcohol, smoking, betel quid use, oral hygiene, and Human Papilloma Virus (HPV) infection [5]. Emerging evidences are indicating that environmental factors, such as smoking, alcohol and diet could directly or indirectly affect epigenetic mechanisms of gene expression regulation and DNA methylation in *HNSCC*.

1.3. Tobacco and alcohol use in HNSCC

Smoking and alcohol consumption are the major risk factors for head and neck cancers [6, 7]. In addition, the combination of both alcohol and tobacco use synergically increases the risk for *HNSCC*development more than 10 times [8]. Cigarette smoking is the major cause of lung cancer and is associated with head and neck, esophagus, bladder, breast and kidney cancer [9]. Increased risk with smoking may be due to the direct effect of tobacco carcinogens or due to genetic polymorphisms in enzymes that activate or detoxify carcinogens.

Several carcinogens present in cigarette smoke are inactivated by a family of enzymes cytochrome P-450 (CYP), which convert carcinogens into reactivated intermediates. These intermediates form DNA adducts that need to be detoxified by a number of enzymes, including glutathione S-transferase [GST) [10]. Single nucleotide polymorphisms (SNPs) in these genes could be an alternative mechanism that modulates the effects of cigarette smoke. Even though alcohol and smoking are known risk factors, only a fraction of smokers and alcohol consumers develop *HNSCC,* suggesting that genetic susceptibility and interactions between genetic, epigenetic and environmental factors could play an important role in the etiology of *HNSCC* [11, 12].

Alcohol consumption has been associated with an increased risk of the head and neck, esophagus, liver, colorectal, and breast cancer [13]. Possible mechanisms by which alcohol exerts its harmful effect includes the genotoxic effect of ethanol metabolite acetaldehyde, production of reactive oxygen- and nitrogen species, changes in folate metabolism, generation of DNA adducts and inhibition of DNA repair. Also, alcohol could exert its damaging

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effect directly, either acting as a solvent of carcinogens from tobacco smoke or damaging the oral mucosa, that enhances the penetration of carcinogens from tobacco smoke [7]. Genetic polymorphisms of ethanol-metabolizing enzymes, including alcohol dehydrogenases (ADH) (which metabolizes alcohol into acetaldehyde), with a different ability to generate carcinogen acetaldehyde, may determine individual susceptibility to head and neck cancer. Acetaldehyde can form adducts with DNA, interfering with DNA synthesis and repair [7, 13]. "Fast-metabolizing" ADHs genotype was associated with the increase of *OSCC* and *HNSCC* risk [14, 15, 16]. By the contrast, in other studies "fast-metabolizing" ADHs genotype was found to be associated with decreased risk of *HNSCC* [11, 17, 18]. Therefore, the mechanism by which smoking and alcohol causes increased risk for *HNSCC* and the role of alcohol and tobacco-related polymorphisms have not been fully elucidated.

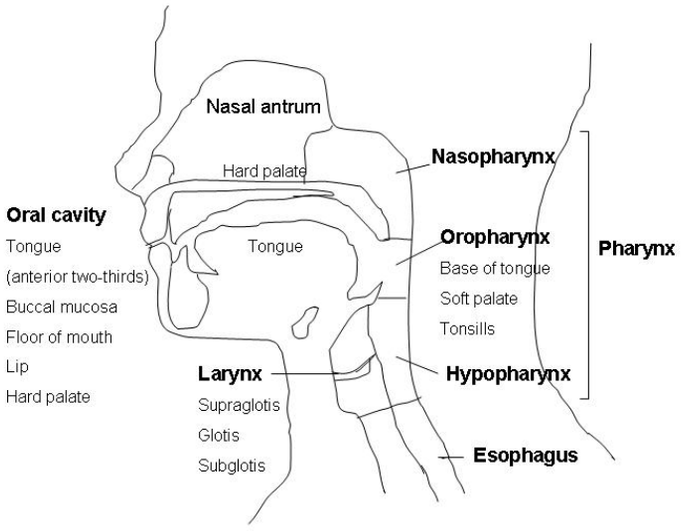

Figure 1. Diverse anatomical localization of Head and Neck Squamous Cell Carcinomas. Although head and neck cancers are characterized by heterogeneous histology, the majority carcinomas arise from squamous cell epithelia of the oral cavity (tongue, buccal mucosa, floor of the mouth), oropharynx (soft palate, base of tongue, tonsils), hypopharynx, nasopharynx, larynx (supraglotis, glottis, subglotis) and esophagus.

1.4. HPV infection in HNSCC

In addition to alcohol and tobacco exposure, human papilloma virus (HPV) infection has also a significant part in the etiology of head and neck cancer. HPV cancers predominantly arise from tongue and palatine tonsils within the oropharynx [5, 19]. Acting in synergy with tobacco use and heavy alcohol consumption, HPV infection with high-risk types is considered as an etiological factor in the development of *HSCC* and *OSCC* [5]. Reported incidence

of high-risk HPVs in oral carcinoma patients varied from 0% to 100% [20], depending on the methods used for HPV detection, tumor-host characteristics of the examined group of patients, varying numbers of included tissue samples, but it is also affected with different distribution of oncogenic HPVs in different world regions. The DNA of oncogenic HPVs is present in 20% of all *HNSSCs* and in nearly 60% of tonsillar cancers [5, 8]. There is increasing evidence that HPV-associated *HNSCC* carcinomas are distinct clinical and pathological tumor entity from alcohol and smoking-associated *HNSCC*s with regards to risk factors, tumor biology and progression [5]. Infection with oncogenic HPV types, predominantly HPV16 and HPV18 is associated with increased risk of *HNSSC* [5, 19].

HPV infection causes deregulation of cell cycle and apoptosis by inactivation of *Rb* and *p53* tumor suppressor gene protein products, involved in the maintenance of genome stability. E6 protein of high risk HPVs affects the *p53* protein function by ubiquitin-dependent degradation. Although *p53* tumor suppressor gene is the most often mutated gene in human oncology, in tumors with HPV etiology is not clear in which extent the mechanism of *p53* mutation is included in *p53* inactivation. It can be assumed that *p53* mutations occur most frequently in HPV negative than in the HPV positive head and neck cancers, but it is questionable if the presence of *p53* mutation in HPV infected tumors additionally influences prognosis of the patients. Numerous studies with conflicting results deal with the influence of oncogenic HPV types and prognosis of *HSCC*. The majority of them reported that *HSCC* patients with HPV infection have better prognosis than the patients who are HPV negative [5, 21, 22]. On the contrary, our previous results concerning *OSCC* patients in stage III of the disease showed worse overall and disease-free survival for patients infected with high-risk HPV types (HPV 16, 18 and 31) [23], indicating the presence of more aggressive disease in these patients. Our study comparing disease-free interval (DFI) and overall survival (OS) between patients with HPV infection only and the patients with HPV infection and *p53* mutation showed significantly shorter disease-free interval as well as overall survival in patients with both HPV infection and *p53* mutation. Presence of *p53* mutations in HPV infected tumors confer a higher risk of recurrence in this disease. In addition, since all patients were treated with postoperative radiotherapy, shorter DFI indicate that the response to radiotherapy may be influenced by *p53* status [24]. However, it has been recently reported that it is unlikely that HPV infection plays significant role in mobile tongue carcinogenesis in young *OSCC* patients [25].

The role of HPV infection in the etiology, as well as in prognosis of head and neck cancers varies and it depends on head and neck cancer subtypes and different anatomic site of tumors. From that point of view, it is clear that HPV typing, together with other molecular markers may help in defining a particular group of tumors in regard to prognosis and response to anti cancer therapies.

1.5. Diet and HNSCC

Recent studies provide growing evidence that some dietary components, might affect the process of carcinogenesis. There is a growing body of evidence that bioactive food components, such as isothiocyanates from cruciferous vegetables (cauliflower, cabbage, and broccoli), diallyl sulfide (an organosulphur compound from garlic), green tea flavonoids, folate, and selenium, effect on cancer might be mediated through epigenetic mechanisms. Several

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recent studies indicate that dietary habits and fruit and vegetable intake could be associated with cancer risk [26, 27]. Increased fruit and vegetable consumption was recently associated with reduced occurrence of *HNSCC* [28]. However, the findings of association of diet and cancer risk are still confounding.

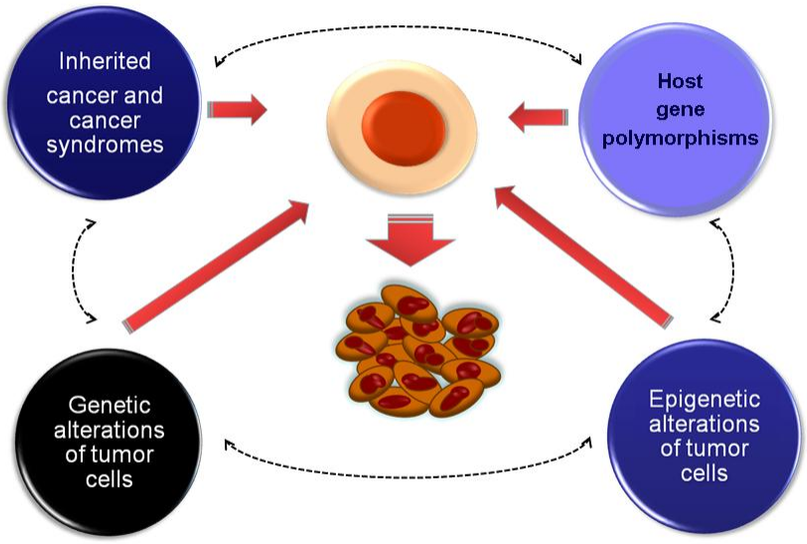

Figure 2. Schematic presentation of synergistic effect of different genetic alterations in transformation of normal to malignant cell. Gene mutations can be inherited (BRCA1, BRCA2, RET, VHL, APC) and associated with a predisposition to certain cancer or cancer syndromes. Also, mutations can be acquired leading to the strong predisposition to malignant transformation of cells (K-*ras*, p53, HER2, *bcr-abl, bcl-2*, Rb). The human genome is characterized with enormous number of genetic polymorphisms that sometimes results with impaired function of the gene product, especially when cells are exposed to environmental or host risk factors. Hypermethylation of gene promoter sequences causes gene silencing that is of great importance for tumor suppressor genes. All these genetic alterations can impair function of other genes and further contribute to increased risk of malignant transformation.

2. Molecular changes In *HNSCC*

Second half of 20th century and beginning of 21st century are marked with almost revolutionary breakthrough in our understanding of human gene structure and functions. The rapid development of biotechniques led to the sequencing of complete human genome and later of genome and transcriptome of many human tumors [29, 30]. Even before these achievements, it was well accepted that malignant tumors, on the molecular level, are disease of genes. This knowledge led to enormous number of worldwide research projects and publications concerning association of gene alterations in tumor cells with cancerogenesis and early diagnosis, tumor progression, tumor staging and response to therapy. In spite of many practical/

clinical results of these studies [Her-2/neu, *BRCA-1* and *BRCA-2* in Breast Cancer; *K-ras* and *EGFR* in Colon Cancer; *bcr-abl* in Chronic Myeloid Leukemia; *B-RAF* and *c-KIT* in Melanoma; detection of dominant clone of B- and T-lymphocytes in tracking minimal residual disease in lymphomas and leukemia's [29-33], still much more questions concerning significance of gene alterations for tumor development and treatment remains unanswered. In the last 10-15 years significant efforts are done to fulfill these gaps in our understanding of molecular pathogenesis of cancer by studying epigenetic changes of tumor tissues.

These alterations are of special interest because epigenetic changes are tumor/tissue specific, although inheritable epigenetic alterations are under strong influence of environmental risk factors and patient behavior and they are potentially reversible. Intensive research of epigenetic alterations in human tumors and their association with environmental risk factors and patient behavior put an attention to another group of gene alterations, i.e. on inherited gene polymorphisms. These researches tried to elucidate connection of certain gene polymorphisms with environmental and host risk factors and epigenetic alterations in different human tumors and in *HNSCC* [12, 16, 34-36], Figure 2.

Carcinogenesis of *HNSCC* is a multistep process, which arises through an accumulation of genetic and epigenetic alterations. These genetic alterations result in inactivation of multiple tumor suppressor genes and activation of proto-oncogenes by deletions, point mutations, promoter methylation, and gene amplification [3], Table 1.

Genetic changes	Locus / gene	Cancer type	Frequency	Ref.
LOH	9p21-22/ ***p16INK4a/p14ARF***	*HNSCC* *OSCC*	70–80%	37, 38,39, 40
	3p/ ***RASSF1A, FHIT, RARB2***	*OSCC* *HNSCC*	30–70%	37, 39, 40, 47
	17p13/***p53***	*HNSCC*	76%	39
	11q	*OSCC*	20-33%	37
	13q14/***Rb***	*HNSCC*	68%	39
	8p	*OSCC*	53-83%	37
Mutation	9p21-22/ ***p16***	*OSCC*	70%	41
	5q21-22/***APC***	*OSCC*	50%	37
	17p13/***p53***	*HNSCC*	40-79%	49
	11p15/***H-ras***	*OSCC*	35-55%	37
Amplification	11q13/(***PRAD-1/Cyclin D1/hst-1/int-2***)	*HNSCC*	30–50%	46, 47
	7p12/***EGFR***	*OSCC*	30%	53, 54

Table 1. Frequent genetic abnormalities in head and neck cancer.

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Genetic instability in regions leads to loss of chromosomal region that contains tumor suppressor genes. A high incidence of Loss of heterozygosity (LOH) observed at 9p, 8p, 3p, 9q, and 11q regions were associated with tumor stage, and poor histological differentiation in *HNSCC* [37]. Loss of heterozygosity (LOH) of 9p21 appears is an early and frequent event in head and neck carcinogenesis [37, 38-40]. The CDKN2A gene locus found in 9p21 region encodes two different transcripts, *p16* and *p14*ARF, which are responsible for G1 cell cycle regulation and MDM2 mediated degradation of *p53*. *P16* and *p14* are often inactivated in *HNSCC* through homozygous deletion, by promoter methylation, and by point mutations [41-44].

Loss of chromosome region 3p is a common early genetic event in *HNSCC* [2, 40, 45]. Genetic alterations at 3p that are common in *HNSCC* and *OSCC* contains tumor suppressor genes Fragile histidine triad (*FHIT*) gene mapped at 3*p14*, Ras association family (*RASSF1A*) gene, mapped at 3p21, and Retinoic acid receptor B2 gene (*RARB2*), mapped to chromosome region 3p24 [37, 40]. Amplification of 11q13 leads to overexpression of cyclin D1 [46], detected in 30–60% of *HNSCC* and 40% of cases of oral squamous dysplasia [47].

There has been much research on the tumor suppressor gene *p53* and its role in *HNSCC* and *OSCC* carcinogenesis. The *p53* protein blocks cell division at the G1 to S boundary, stimulates DNA repair after DNA damage, and induces apoptosis. *P53* has been shown to be functionally inactivated in oral and head and neck tumors [48]. LOH of 17p13, which contains *p53* and point mutations of the *p53* are seen in more than 50% of *HNSCC* cases [39, 49]. Cigarette smoking has been associated with the mutation of *p53* in head and neck cancers [14]. Investigations about the prognostic value of *p53* status in head and neck cancer subtypes hardly can get a clear result due to the presence of large heterogeneity in regard to patients characteristics, and methods for *p53* detection. Meta-analysis of Tandon et al [50] pointed out that evidence about the prognostic value of *p53* in head and neck cancer squamous cell carcinomas has been inconclusive. However, determination of *p53* status of *HNSCC* may be of particular interests due to its possible role in prediction of the response to cisplatin based chemotherapy. Cisplatin chemotherapy is widely used chemotherapeutical approach in the treatment of head and neck cancers and data that patients with *p53* mutations respond better to cisplatin chemotherapy need further confirmation [51]. Chemotherapy responses in *HNSCC* patients is also influenced by polymorphism at codon 72 of *p53* gene. In patients with wild type *p53*, arginine at codon 72 is associated with better response to chemotherapy. On the contrary, if *p53* is mutated, proline at position 72 is better option [52]. Better understanding of cellular processes and their actors such as *p53* network and their relation to clinical settings will help in appropriate biomarker characterization. Epidermal growth factor receptor (*EGFR*) is overexpressed in 90% of *HNSCC* [53], often due to amplification [54]. Thus, frequent molecular abnormalities in head and neck squamous cell carcinoma include alterations in tumor suppressor genes *p16*INK4A, *p53*, *p14*ARF, *FHIT*, *RASSF1A*, *Rb*, cyclin D1, and activation of oncogenes, such as members of the *ras* gene family, *c-myc*, and *EGFR* [2-4], Table 1.

A large number of previous studies have also suggested the correlation between polymorphisms of genes involved in cell cycle control, angiogenesis and metabolism of alcohol and carcinogens and susceptibility to head and neck cancer [10, 16, 34-36].

Although the impact of genetic changes in oral carcinogenesis is well-known, over the last decade it has been demonstrated that epigenetic changes, especially aberrant DNA methylation, play a significant role in *HNSCC* [55]. Epigenetic modifications are heritable changes in gene expression that are not coded in the DNA sequence [56]. These changes are mitoticaly (clonally) heritable and potentially reversible, which provides large possibilities of epigenetic therapy. Additionally, epigenetic changes could be modulated by the nutrition, environmental and genetic factors and/or gene-by-environment interactions. Main mechanisms of epigenetic control in mammals include DNA methylation, histone modifications and RNA interference (RNA silencing).

3. DNA methylation

DNA methylation of cytosine in a CpG dinucleotide is the key epigenetic modification in mammals. The covalent addition of a methyl group to the 5-carbon (C5) position of cytosine that are located 5' to a guanosine base in a CpG dinucleotide are catalyzed by a family of enzymes DNA methyltransferases (*DNMTs*). CpG dinucleotides are asymmetrically distributed in the genome. Throughout the genome rare solitary CpGs are heavily methylated. In contrast, CpG clustered in small stretches of DNA (0.5-4kb regions), with greater than 50% GC content, are termed 'CpG islands'. Of approximately 50% the genes in the genome CpG islands are associated with promoter regions. In normal cells, most CpG sites outside of CpG islands are methylated, whereas CpG islands in gene promoters are usually unmethylated, independently of the gene transcription status [56]. During the process of cancerogenesis paradoxical changes in DNA methylation patterns occurs, with simultaneous global hypomethylation and regional hypermethylation changes. Global DNA hypomethylation may activate, 'unlock' repetitive elements that could affect genome stability or could lead to transcriptional activation of latent viruses or oncogenes. Simultaneously, regional hypermethylation leads to transcriptional silencing of tumor suppressor genes. Cytosine methylation of CpG islands in the promoters of tumor suppressor genes causes their inactivation, transcriptional silencing and consequently malignant transformation. Investigations showed that the number of cancer-related genes that are inactivated by epigenetic modifications equals or even exceeds the number of genes inactivated by mutation. Furthermore, genetic and epigenetic changes have almost identical biological effect and pattern of gene expression [56]. Pattern of tumor suppressor genes hypermethylation shows tumor-type specificity.

While *DNMT3A* and *DNMT3B* are mostly involved in *de novo* methylation, *DNMT1* is involved in the maintenance of DNA methylation after replication [56]. Several studies have shown *DNMT* overexpression (mainly *DNMT1* and *DNMT3B*) in cancer, including *HNSCC* [57]. The analysis of *DNMT* knockout cells revealed that individual enzymes also interact between each other, and interact with other chromatin modifying enzymes, histone deacetylases (*HDAC*) and methyl-CpG binding proteins (MBPs). Methylation of cytosine within CpG islands is associated with binding of MBPs, which recruit *HDAC* to methylated DNA in regions of transcriptional silencing. Histone deacetylation, catalyzed by *HDACs*, leads to the

chromatin condensation and suppression of DNA transcription. Thus, DNA methylation and histone modifications are not isolated events, but highly coordinated [56].

3.1. DNA methylation in *HNSCC*

Frequent hypermethylation of tumor-related genes were observed in cancer tissue of *OSCC* and *HNSCC*, in the normal adjacent mucous in the *OSCC* [58], dysplastic tissue [59], and leukoplakia [60]. Promoter hypermethylation can also be detected in buccal swabs samples of tobacco and alcohol users [58], Thus, DNA methylation has been considered as an early event in head and neck carcinogenesis.

The list of genes that are found to be inactivated by DNA methylation events in *HNSCC* is growing rapidly and includes genes involved in the cell cycle control (*p14*, p15, *p16* and p53), DNA damage repair (*MGMT*, *hMLH1*, and ATM), apoptosis (*DAPK*, *RASSF1A*, and RARβ), *Wnt* signalling (*APC*, *RUNX3*, *WIF1*, *E-cad* and *DCC*) *SFRP* family genes, *TCF21*, etc. [42, 44, 55, 58-62], Table 2.

FUNCTION	*Gene*	Gene name	Locus	Gene Action	*HNSCC methylation*	Ref.
Cell Cycle Control	***p16***	Cyclin-dependent kinase inhibitor 2A (CDKN2A)	9p21	Regulation of the *Rb* pathway	10-70%	42, 96
	p15	Cyclin-dependent kinase inhibitor 2B	9p21	TGF beta-mediated cell cycle arrest	23-80%	151
Apoptosis	***p14***	Alternative open reading frame (ARF) of INK4a locus	9p21	Pro-apoptosis	18-46%	75-77, 96
	DAPK1	Death-associated protein kinase 1	19q34	p53-dependent apoptosis	18-77%	42, 44, 61, 68, 72, 78
Wnt signaling pathway	***APC***	Adenomatous polyposis coli	5q21-22	*Wnt* signaling and adhesion	20-68%	44, 92-95
	CDH1	*E-cad*herin	16q22	Cell–cell adhesion	2-66%	44, 72, 81-88
	SFRP	Soluble frizzled receptor protein genes family	10q24	Antagonists of the Wnt pathway	30-94%	97-99

FUNCTION	*Gene*	Gene name	Locus	Gene Action	*HNSCC methylation*	Ref.
	WIF1	Wnt inhibitory factor 1	12q14	Secreted Wnt antagonist	25-90%	62, 97, 102
	RUNX3	Runt-related transcription factor 3	1p36	*Wnt* signaling inhibitor, TGF-β-induced tumor suppression	18-36%	62, 99-101
DNA damage repair	***hMLH1***	human mutL homolog 1	3p21	DNA mismatch repair	8-76%	96, 106, 107
	MGMT	O^6-mehylguanine DNA methyltransferase	10q26	DNA repair for alkylated guanine	10-57%	59, 44, 72, 103-105
Tumor suppressor	***FHIT***	Fragile Histidine Triad	3*p14*	Control of cell cycle	33-84%	96, 110
	RASSF1A	Ras association (RalGDS/AF-6) domain family member 1A	3p21	RAS pathway regulation and tumor suppression	10-76%	45, 72, 78, 79, 96
	DCC	Deleted in Colorectal Cancer	18q21	Cell–cell adhesion	17-75%	108, 109
	RARβ	Retinoic acid receptor beta	3p24	Regulatory protein and apoptosis	53-88%	59, 78

Table 2. Genes commonly methylated in *HNSCC*.

3.2. DNA methylation association with progression and prognosis in *HNSCC*

A number of tumor suppressor genes hypermethylation has been associated with worse outcome in various cancer types. Hypermethylation of the *p16* and *WIF1* genes [63] and *RASSF1A* and *RUNX3* methylation status [64] were found to be independent prognostic factors non-small cell lung cancers. *DAPK* promoter hypermethylation has been associated with tumor aggressiveness and poor prognosis in lung cancer [65]. *E-cad*herin promoter hypermethylation was found to be the prognostic factor of worse prognosis in diffuse gastric cancer [66], and in non-small cell lung carcinoma [67]. Epigenetic inactivation of several genes by DNA methylation has been found to associate with *HNSCC* progression [44, 58, 61, 68].

$p16^{INK4a}$ is cyclin-dependent kinase inhibitor which regulates the *Rb* pathway, leading to inhibition of cell cycle progression. An alternate spliced product of the same INK4a locus is another tumor suppressor gene *p14ARF* (Alternative open reading frame). Loss of *p16* in head and neck and oral tumors has been frequently reported [69, 70]. *p16* methylation was correlated with malignant transformation of oral epithelial dysplasia and is a potential biomarker for prediction of prognosis of mild or moderate oral epithelial dysplasia, with the overall sensitivity and specificity of >60% [71]. Previously, *p16* methylation status has been correlated with tumor stage, lymph node metastasis and tumor size in *HNSCC* [72], and

poorly differentiated *HNSCC* [73]. *p16* hypermethylation has been associated with poor prognosis in *HNSCC* [55, 74] and *OSCC* [75]. Interestingly, *p16*I*NK4A* and *p14ARF* genes, transcribed from the same locus INK4A by alternate splicing, could have a diametrically opposite clinical effect in oral cancer patients when methylated. Promoter methylation of *p16* was associated with increased disease recurrences and worse prognosis, whereas *p14* methylation was strongly associated with lower disease recurrence and was found to be a good prognostic predictor for oral carcinoma [75]. However, other studies associated *p14* methylation with tumor stage and lymph node involvement of *OSCC* [76] and poor prognosis [77]. These findings indicate that DNA methylation status of *INK4A*/*ARF* locus could be used as a prognostic biomarker for assessing the aggressiveness of disease in oral and head and neck carcinoma patients.

Death associated protein-kinase (DAPK), plays a critical role in apoptosis regulation in tumor development, and commonly is hypermethylated in *HNSCC* [42] and *OSCC* [68]. *DAPK* promoter methylation showed the positive correlation with lymph node involvement [42, 72, 78] and advanced disease stage in *HNSCC* [42, 72]. The presence of *DAPK* promoter hypermethylation detected in surgical margins was associated with the decreased overall survival and was shown to be an independent prognostic factor for overall survival in *OSCC* patients [61].

RASSF1A Ras association (RalGDS/AF-6) domain family member 1A, involved in RAS pathway regulation and tumor suppression, is frequently inactivated by promoter hypermethylation in *HNSCC* [45, 72]. *RASSF1A* methylation correlates with head and neck tumor stage [72], poor disease-free survival of *OSCC* [79] and lymph node metastasis in nasophageal carcinomas [78].

E-cadherin (*E-cad*) has a dual role in a mammalian cell, as a Ca^{+2}-dependent cell adhesion molecule, and as a part of the complex signaling Wnt pathway interacting with β- and α-catenine [80]. Hypermethylation of *E-cad* is a common event in *HNSCC* [44, 72, 81, 82]. Reduced *E-cad* expression as a consequence of promoter hypermethylation leads to the development of the invasive phenotype in the *OSCC* [83], and is associated with lymph node metastasis in the *OSCC* [84] and *HNSCC* [85]. Hypermethylation of *E-cad* has previously been associated with tumor stage of *HNSCC* [72] and nasopharyngeal carcinomas [86] and lymph node metastasis of oral [82] and nasopharyngeal carcinomas [87]. Down-regulation of CDH1 due to hypermethylation contributed to the progression of esophageal cancer [88], led to poor differentiation and the development of the invasive phenotype in the *OSCC* [83], and salivary gland carcinomas [89]. Furthermore, *E-cad* hypermethylation was associated with poor survival in *the OSCC* [84] and *HNSCC* [85]. Recently published investigations showed that *E-cad* gene hypermethylation was associated with the decreased survival in *HNSCC* patients [90]. In addition, advanced *OSCC* patients with *E-cad* promoter methylation had significantly worse 3- and 5-year survival rates [44], and *E-cad* hypermethylation was found to be an independent prognostic factor in oral tongue carcinoma [81]. However, *HNSCC* patients with *E-cad* promoter methylation had lower rates of local recurrences and better disease-specific survival and outcome [91], which indicates that the role of *E-cad* gene methylation in head and neck carcinomas has not been fully elucidated.

APC gene plays an integral role in *the Wnt* signaling pathway in binding and degrading of β-catenine. Hypermethylation of *the APC* gene promoter is frequent in *HNSCC* [92-94], and *OSCC* [44]. *APC* methylation status has previously been associated with worse prognosis of esophageal carcinoma [95]. However, in another study on esophageal cancer, hypermethylation of the *APC* gene was related to a lower number of metastatic lymph nodes and better recurrence rates [96].

SFRP (soluble frizzled receptor protein) family genes are involved in the inhibition of *Wnt* signaling. Promoters of SFRP-2, SFRP-4, SFRP-5 genes showed methylation in *OSCC*, whereas SFRP-1 was demethylated in oral cancer [97]. SFRP-1 has been associated with tumor grade in *OSCC* [98]. Hypermethylation of SFRP-1 was associated with an increased risk of esophageal cancer recurrence [99].

RUNX3 Runt-related transcription factor 3 gene plays a role in the transforming growth factor-beta (TGF-β) -induced tumor suppression pathway. *RUNX3* may have an oncogenic role in *HNSCC* and its expression may predict malignant behavior [100]. In contrast to *HNSCC*, in *OSCC* the *RUNX3* gene is downregulated due to promoter hypermethylation [101] and associated with lymph node involvement and tumor stage in tongue carcinomas [62]. Hypermethylation of *RUNX3* detected in the plasma of esophageal cancer patients was significantly associated with an increased risk of cancer recurrence [99].

WIF1 Wnt inhibitory factor 1, gene involved in the inhibition of *Wnt* signaling, is commonly methylated in the *HNSCC* [102] and *OSCC* [62, 97]. Its methylation status was correlated with lymph node metastasis in nasopharyngeal carcinoma [102].

MGMT O(6)-methylguanine-DNAmethyltransferase a DNA repair gene that removes mutagenic O^6-guanine adducts from DNA, is inactivated by hypermethylation in *HNSCC* [59] and *OSCC* [44]. *MGMT* promoter hypermethylation was associated with tumor stage of *HNSCC* [72] and with lymph node involvement in laryngeal carcinomas [103]. In addition, the methylation of *MGMT* was associated with poor survival and reduced disease-free survival in *OSCC* [104] and increased recurrences rate and poor prognosis of *HNSCC* [105].

hMLH1 mutL homolog 1 is involved in the DNA repair process. The *hMLH1* promoter methylation occurred in high frequency of the majority of the early stage *OSCC* and in about half of the late stage carcinomas [106], indicating its potential role in the tumor progression. Promoter methylation of *hMLH1* gene is also a common event in *HNSCC*, and was correlated with poor survival in *HNSCC* [107].

RARß Retinoic acid receptor beta is commonly methylated in *HNSCC* [59] and associated with highly differentiated tumors, advanced tumor stage and the presence of lymph node metastasis of nasopharyngeal carcinomas [78].

TIMP3 Tissue inhibitor of metalloproteinases 3 gene is involved in the inhibition of angiogenesis and tumor growth. Promoter methylation of TIMP3 predicts better outcome in *HNSCC* treated by radiotherapy [91].

DCC Deleted in colorectal cancer, tumor suppressor gene highly methylated in *HNSCC* [108] was correlated with mandibular invasion and poor survival in oral cancer [109].

FHIT Fragile Histidine Triad gene is associated highly methylated in *HNSCC* [96], and associated with poor prognosis in early stage esophageal squamous cell carcinoma [110].

MINT1 and *MINT 31,* are the members of Methylated in tumor gene family, which methylation was previously associated with invasiveness and poor survival in the *OSCC* [109].

A number of recent data suggests that promoter hypermethylation of specific genes does not occur independently or randomly, but concurrently, which indicate that during the tumor progression progressive accumulation of epigenetic alterations could occur. High degree of methylation of multiple genes CpG island regions, associated with microsatellite instability and *hMLH1* gene hypermethylation, has been defined as a CpG island methylator phenotype (CIMP) in colorectal cancer, and is characterized by a poor outcome [111]. It has been suggested that a form of the CpG island methylator phenotype (CIMP) exists in other solid tumors, including *HNSCC* [112]. As opposed of CIMP positive colorectal cancers In oral cancer CIMP is characterized by less aggressive tumor biology [113],. The panel of tumor-related genes used to classify multiple methylation and CIMP differs substantially between studies [12, 112, 113], which could likely affect the results of association with clinical parameters and prognosis. Future extensive investigations are needed to establish a reliable set of reference cancer-related genes whose methylation status should be examined in the specific types of tumors. Further investigations are needed to better characterize the etiology of this methylation phenotype as well as to determine if this phenotype has important prognostic or clinical use.

3.3. DNA methylation in early detection of *HNSCC*

The detection of aberrant DNA hypermethylation emerged as a potential biomarker strategy for early detection of various carcinomas. Since DNA methylation is an early event in tumorigenesis of *HNSCC*, identification of methylation markers could provide great promise for early detection and treatment in *HNSCC* [68]. As opposed to advanced *HNSCC* diagnosis, early *HNSCC* detection increases survival to 80%. Therefore, early detection markers may potentially serve as a predictive tool for diagnosis and recurrence of *HNSCC*. However, even though such markers provide great promise for early detection, epigenetic biomarkers have not yet been clinically implemented [114, 115].

Routine oral visual screening could significantly reduce oral cavity mortality [116]. However, oral cavity screening will not identify cancers deep in the pharynx or larynx which requires special instrumentation and examination. In addition, screening of *HNSCC* should involve noninvasive detection of *OSCC* and *HNSCC*, such as detection in saliva and oral rinses, or minimally invasive detection, such as blood and serum analysis. Saliva presents an ideal means for *HNSCC* biomarker detection because of its proximity to the primary tumor site, availability of exfoliated cancer cells, and ease of sampling [117, 118].

Soluble CD44 promoter gene hypermethylation detected in saliva samples has been indicated as an early detection marker in *HNSCC* screening [119]. Recently, *NID2* and *HOXA9* promoter hypermethylation have been identified as biomarkers for prevention and early detection in oral carcinoma tissues and saliva [120]. Hypermethylation of multiple tumor-

related genes (*RAR-β*, *DAPK*, *CDH1*, *p16* and *RASSF1A*) analyzed in combination could serve as a biomarker for early diagnosis of esophageal squamous cell carcinoma [57]. Moreover, these multiple tumor-related gene hypermethylation were associated with the increase of *DNMT3b* expression in early stages of esophageal cancer [57].

The development methylation-specific polymerase chain reaction (PCR) techniques has resulted in the identification of methylated genes specific to *HNSCC* detected in tumor samples [68], including *DAPK* [42], and *RASSF1A* [72].

Saliva and oral rinses could be ideal diagnostic and predictive biofluids for head and neck cancer since they samples cells from the entire lesion and the entire oral cavity. The detection of hypermethylated marker genes from oral rinse and saliva samples has a great potential for the noninvasive detection of *OSCC* and *HNSCC* [118, 68, 121, 122, 123, 124]. DNA hypermethylation of *p16*, *MGMT*, and *DAPK* in saliva showed aberrant methylation in 56% of samples from *HNSCC* patients, and only in one of the 30 control subjects [68]. The aberrant methylation of a combination of marker genes *E-cadherin*, transmembrane protein with epidermal growth factor-like and 2 follistatin-like domains 2 (*TMEFF2*), and *MGMT*, present in oral rinses was used to detect *OSCC* with >90% sensitivity and specificity [124].

Using the approach of gene selection according to previous studies on promoter methylation in *HNSCC* Carvalho et al. analyzed both saliva and serum in 211 *HNSCC* patients and 527 normal controls, and showed high specificity of promoter hypermethylation in *HNSCC* patients compared with normal subjects (>90%); however, the sensitivity of this panel was 31.4% [118]. In another study with different approaches, genome-wide methylation array analysis of 807 cancer-associated genes, was conducted on the matched preoperative saliva, postoperative saliva, and oral cancer tissue, and compared to saliva of normal subjects. Multiple potential diagnostic gene panels that consisted of 4 to 7 genes ranged in their sensitivity from 62% to 77% and in their specificity from 83% to 100% [123], significantly higher than previous studies of aberrant methylation detected in saliva in head and neck cancer patients [121, 122, 118]. Using this approach, new genes could be discovered that can be used as a reliable biomarker for the early detection of oral and head and neck cancer.

The *KIF1A* (Kinesin family member 1A) gene, that encodes a microtubule-dependent molecular motor protein involved in organelle transport and cell division, has recently been associated with aberrant DNA methylation in *HNSCC* [125]. *EDNRB* (Endothelin receptor type B) is a G protein coupled receptor, which activates a phosphatidylinositol calcium second messenger system, has previously been associated with nasopharyngeal carcinomas [126] and *HNSCC* [125]. Promoter hypermethylation of *KIF1A* and *EDNRB* is a frequent event in primary *HNSCC*, and these genes are preferentially methylated in salivary rinses from *HNSCC* patients. *KIF1A* (97.8% specificity and 36.6% sensitivity) and *EDNRB* (93.2% specificity and 67.6% sensitivity) are highly sensitive markers that could potentially be used as biomarkers for *HNSCC* detection. In addition, combining the markers improves sensitivity while maintains good specificity (93.1% specificity and 77.4% sensitivity) [125].

3.4. Predictive significance of DNA methylation

Epigenetic alterations such as DNA methylation could be a marker of response to radio and/or chemotherapy in the treatment of cancer. DNA methylation could play an important role in the regulation of gene expression for genes involved in cell cycle and apoptosis, thus affecting the chemosensitivity of cancers. Treatment of cancer cells with demethylating agent could lead to re-expression of gene involved in the activation of the apoptotic process and restore the sensitivity to the chemotherapy. Targeting the epigenetic mechanisms of apoptosis related genes inactivation may increase the efficacy of chemotherapy in various cancer types. Studies in vitro and in model systems certainly suggest that treatment with epipgenetic agents can reverse drug resistance.

The best known example of DNA promoter hypermethylation and response to chemotherapy is *MGMT* promoter methylation in glioma patients treated with alkylating agents [127]. The expression of DNA repair gene *MGMT*, which removes alkyl groups added to guanine in DNA, is controlled by its promoter methylation. A cell that expresses a low amount of *MGMT* is known to be more sensitive to the antiproliferative effects of alkylating agents. It has been shown that hypermethylation of *MGMT* is the predictor of good response to temozolomide therapy in gliomas [128], to carmustine therapy in gliomas [127], and to cyclophosphamide therapy in diffuse B cell lymphoma [129]. Acquired hypermethylation of DNA mismatch repair gene *hMLH1* (detectable in peripheral blood) during carboplatinum/taxane therapy of ovarian cancer predicts poorer outcome [130]. In addition, *WRN* gene promoter hypermethylation was associated with hypersensitivity to topoisomerase inhibitor irinotecan in primary colon cancer [131].

It has been shown that DNA methylation could have the predictive significance in *OSCC* and *HNSCC*. Hypermethylation of *MGMT* was the predictor of good response to temozolomide therapy in oral squamous cell carcinoma [105]. GPx3 promoter hypermethylation was associated with tumorigenesis and chemotherapy response in *HNSCC* [132]. A correlation between methylation of mitotic checkpoint gene CHFR (checkpoint with ring finger) and sensitivity to microtubule inhibitors (docetaxel or paclitaxel) was observed in *OSCC* cells [133]. Downregulation of *SMG-1* (suppressor with morphogenetic effect on genitalia) due to promoter hypermethylation in HPV-Positive *HNSCC* resulted in increased radiation sensitivity and correlated with improved survival, whereas *SMG-1* overexpression protected HPV-positive tumor cells from irradiation [134]. DNA methylation could be a regulatory mechanism for chemosensitivity to 5-fluorouracil and cisplatin by zebularine, a novel DNA methyltransferase inhibitor, in oral squamous cell carcinomas [135]. It has been shown that zebularine suppresses the apoptotic potential of 5-fluorouracil via cAMP/PKA/CREB pathway in human oral carcinoma cells [136].

Radiotherapy is the standard adjuvant treatment for *OSCC* and the Ras/PI3K/AKT pathway plays an important role in *OSCC* radioresistance. A combination of *RASSF1A*, *RASSF2A*, and *HIN-1* methylation was found to be significantly associated with poor disease-free survival in patients treated with radiotherapy after surgery but not in patients treated with surgery alone [79].

TGF-β signaling has been found to be disrupted in *HNSCC* progression [137], thus this pathway has been targeted for therapy [138]. Downregulation of disabled homolog 2 (*DAB2*) gene expression via promoter DNA methylation frequently occurs in *HNSCC* and acts as an independent predictor of metastasis and poor prognosis [139]. Epigenetic downregulation of *DAB2* switches *TGF-β* from a tumor suppressor to a tumor promoter, suggest a way to stratify patients with advanced SCC who may benefit from anti-TGF-β therapies [139].

Although methylation of certain genes appears to influence the sensitivity to chemotherapeutic drugs, the majority of studies were performed on cell line models, or a small number of subjects. In addition, combination therapies of epigenetic agents and standard chemotherapy/radiotherapy have to be carefully investigated due to potential harmful effects in the clinical application of *DNMT* inhibitors. Epigenetic profile in predicting the chemosensitivity of individual cancers would contribute to personalized therapy [140]. Studies of pharmacoepigenomics will require large-scale analyses and genome-wide methylation analyses using microarrays and next-generation sequencers, necessary to confirm the usage of epigenetic changes in predicting responses to chemotherapeutic drugs.

3.5. Epigenetic therapy of *HNSCC*

Unlike mutations, epigenetic changes are reversible, which provides large possibilities of epigenetic therapy [141]. Demethylating agents that inhibit DNA methyltransferases *DNMTs*, could have a utility to effectively activate expression of previously epigenetically silenced genes. 5-azanucleosides, nucleoside analogs that inhibit *DNMTs*, with consequent hypomethylation of DNA, have long been known to have DNA demethylating activities, but are too toxic for clinical use [141]. However, recent studies have shown that therapeutic efficacy could be achieved at low drug doses [142 -4]. 5-Azacytidine, a cytosine analog treatment has been tested in multiple cancer cell lines and are shown to reexpress methylated genes in myelodysplastic syndromes (MDS) [142, 143]. Low doses were used in a large trial in patients with MDS, and showed an increase in the time of conversion of MDS to leukemia, and increased overall survival [144].

5-Azacytidine application resulted in partial demethylation of the *MGMT* and *RASSF1A* tumor suppressor genes and reduced proliferation of the tumor cells suggesting further investigation of 5-azacytidine for *HNSCC* treatment [145]. Methylation status and expression of *HIC1*, a potential tumor suppressor gene, was restored after demethylation treatment of *HNSCC* cell lines with 5-azacytidine [146].

Epigenetic therapy in monotherapy could reactivate tumor suppressor genes or in combination therapy may enhance the anti-proliferative effect of standard chemotherapy, such as cisplatin, 5-fluorouracil, etc. The low dose of a novel DNA methyltransferase inhibitor, zebularine may sensitize oral cancer cells to cisplatin, an important characteristic of solid cancer treatment. DNA methylation could be a regulatory mechanism for dihydropyrimidine dehydrogenase (*DPD)*, known to be a principal factor in 5-fluorouracil (5-FU) resistance and that DPD activated by zebularine in *OSCC* could be an inhibiting factor in the response to apoptosis induced by 5-FU [135].

The epidermal growth factor receptor (*EGFR*) has been extensively investigated and validated as a therapeutic target in lung, colorectal, pancreatic, and head and neck cancers. However, patients with wild type *EGFR* obtain little sustained benefit from anti-*EGFR* monotherapy Broad restoration of tumor suppressor function by demethylation could enhance the anti-proliferative and pro-apoptotic effect of *EGFR* blockade in solid malignancy. Re-expression of *p15*, *p21*, or *p27*, cell cycle inhibitors downstream of *EGFR*, or *PTEN*, a PI3K/Akt inhibitory protein, may have particular synergy with anti-*EGFR* therapy. Recent phase I study evaluated the combination of anti-*EGFR* erlotinib and 5-azacytidine in solid carcinomas showed the beneficial clinical effect in lung and head and neck cancers [147]. Efficacy of erlotinib could be enhanced by concurrent hypomethylating therapy with 5-azacytidine, secondary to re-expression of tumor suppressors interacting with the *EGFR* signaling cascade. In recent study of resistance to anti-*EGFR* therapy agents, promoter methylation of commonly methylated genes was investigated in two parental non-small cell lung cancer (*NSCLC*) and *HNSCC* cell lines and their resistant derivatives to either erlotinib or cetuximab [148]. It was found that *DAPK* gene promoter was hypermethylated in drug-resistant derivatives generated from both parental cell lines. Restoration of *DAPK* into the resistant *NSCLC* cells by stable transfection re-sensitized the cells to both erlotinib and cetuximab [148], thus indicating that *DAPK* promoter methylation could be a potential biomarker of drug response.

These results demonstrate that DNA methylation could play an important role in both chemotherapy and radiotherapy resistance, and that gene silencing through promoter methylation is one of the key mechanisms of developed resistance to anti-*EGFR* therapeutic agents. Epigenetic therapy could be a novel treatment to overcome chemo- and/or radiotherapy resistance and to improve the benefits of current therapies.

3.6. DNA methylation in *HNSCC* and etiological factors

Epidemiological studies have reported the association of DNA methylation status in *HNSCC* with exposure to environmental factors, including tobacco smoke, alcohol intake, genotoxic betel quid consumption, HPV infection, diet and environmental pollutants.

Recent studies have shown a correlation between tobacco and/or alcohol use and hypermethylation of tumor related genes. DNA global hypomethylation was associated with alcohol consumption [149]. *p16* methylation has been correlated with alcohol use and smoking [72]. Promoter methylation of *p16* was previously correlated with alcohol intake [150], while *RASSF1A* methylation was associated with tobacco use in *HNSCC* [72]. Smoking and drinking was associated with *p15* promoter methylation in the upper aerodigestive tract of healthy individuals and *HNSCC* patients [151]. Promoter methylation of *SFRP1* occurred more often in both heavy and light drinkers compared to nondrinkers in head and neck squamous cell carcinoma [152]. Significant association of tumor-associated genes hypermethylation with alcohol use was observed in *HNSCC* [153].

Recent studies are indicating that increased fruit and vegetable consumption are associated with reduced head and neck cancer risk [28]. There is a growing body of evidence that bioactive food components, such as isothiocyanates from cruciferous vegetables (cauliflower,

cabbage, and broccoli), diallyl sulfide, an organosulphur compound from garlic, isoflavone, phytosterole, folate, selenium, vitamin E, flavonoids might reduce cancer risk through epigenetic mechanisms. The chemoprevention of cancer by natural compounds could be a promising approach with less side effects and toxicity. Main polyphenols with the properties of *DNMT* inhibition are tea polyphenols, soy isoflavones, organosulphur compounds from garlic, and isothiocyanates from cruciferous vegetables. Dietary folate intake was associated with *p16* promoter methylation in head and neck squamous cell carcinoma [154]. Epigallocatechin-3-gallate (*EGCG*), the major polyphenol in green tea, is believed to be a key active ingredient with anti-cancer properties. *EGCG* is methylated by catechol-O-methyltransferase and inhibits DNA methyltransferase (*DNMT*), thus reversing the hypermethylation and inducing the re-expression of the silenced genes [141].

EGCG has been reported to reverse hypermethylation and reactivate several tumor suppressor genes in human esophageal squamous cell carcinoma cell lines [155]. The reversion-inducing cysteine-rich protein with Kazal motifs (*RECK*), a novel matrix metalloproteinases (*MMP*) inhibitor, is involved in the inhibition of tumor angiogenesis, invasion, and metastasis. *EGCG* treatment enhanced *RECK* expression by reversal of hypermethylation of *RECK* promoter and inhibiting *MMP* activities and invasion in *OSCC* cell lines [156]. Genistein, soy dietary isoflavone, reversed DNA hypermethylation and reactivated *RARβ*, *p16*, and *MGMT* in esophageal cancer cells [157]. However, the findings of association of diet and epigenetic modifications in the head and cancer and other carcinomas are still confounding.

4. Conclusions

Head and neck squamous cell carcinoma (*HNSCC*) is one of the most common and highly aggressive malignancies worldwide, despite the significant efforts committed in the last decades in its detection, prevention and treatment. Therefore, early detection and better disease prediction via genetic and epigenetic biomarkers is crucial. However, very few reliable markers are currently known. Recent studies provide strong evidence that DNA methylation could have an important role in head and neck cancer. The detection of promoter methylation status may be a useful molecular marker for early detection of *HNSCC* from tissue, saliva, and serum samples and in real time analysis of margins during surgery. In addition, the creation of methylation gene panels could be useful for *HNSCC* screening in the timely inclusion of treatment and thorough surveillance during follow-up period. Unlike genetic changes, epigenetic modifications are heritable and potentially reversible, thus providing the potential for therapy. Frequent DNA methylation detected in *HNSCC* and association with tumor progression and survival indicates that DNA methylation plays an important role in head and neck carcinogenesis and may be a useful diagnostic marker and a potential therapeutic target for *HNSCC*.

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