Role of DNA methylation in head and neck cancer

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Abstract Head and neck cancer (HNC) is a heterogenous and complex entity including diverse anatomical sites and a variety of tumor types displaying unique characteristics and different etiologies. Both environmental and genetic factors play a role in the development of the disease, but the underlying mechanism is still far from clear. Previous studies suggest that alterations in the genes acting in cellular signal pathways may contribute to head and neck carcinogenesis. In cancer, DNA methylation patterns display specific aberrations even in the early and precancerous stages and may confer susceptibility to further genetic or epigenetic changes. Silencing of the genes by hypermethylation or induction of oncogenes by promoter hypomethylation are frequent mechanisms in different types of cancer and achieve increasing diagnostic and therapeutic importance since the changes are reversible. Therefore, methylation analysis may provide promising clinical applications, including the development of new biomarkers and prediction of the therapeutic response or prognosis. In this review, we aimed to analyze the available information indicating a role for the epigenetic changes in HNC.

Keywords Methylation · Epigenetics · Head and neck cancer

Introduction

Head and neck cancer (HNC) is a highly heterogenous group of malignant diseases and the sixth most frequently observed cancer type in developing countries (Crowe et al. 2002; Ohshima et al. 2005). It reveals different combinations of various sites and disease types which result from sequential genetic changes in multiple intracellular pathways and inherent viral infections. HNC displays serial dysplastic alterations before acquiring invasive characteristics. Tobacco and/or alcohol use are the main etiological factors and play an important role in oral cavity, pharynx, and larynx cancer (Ohshima et al. 2005). Acute laryngitis which can be caused by chronic irritation, inhalation of irritants, viral infections, or allergic reactions may also increase the risk of cancer development (Kumar et al. 2000). The prognosis of the disease varies according to tumor size, local invasion, histology, and grade as well as ethnic origin. These diverse varieties reflect the versatile pathogenesis of the disease. Identification of novel therapeutic targets and new and specific biomarkers for the early detection of HNC could greatly increase the survival rate and might also help as prognostic indicators.

Head and neck carcinogenesis is a multistep and multifactorial complex mechanism containing a variety of genetic and epigenetic abnormalities in DNA repair, signal transduction, apoptosis, angiogenesis, proliferation, differentiation, and cell cycle regulation (Scully et al. 2000). In recent years, the role of epigenetic alterations have been increasingly recognized. Changes in the methylation patterns are one of the most frequent events in human tumors (Jones...
and Baylin 2002). Two types of methylation changes are usually observed: hypermethylation of the CpG islands and global hypomethylation in a variety of tumors. DNA hypomethylation has been associated with the activation of oncogenes and chromosomal instability leading to over-expression of the oncogenes, while DNA hypermethylation is associated with the repression of tumor suppressor genes (TSG) and genomic instability. DNA methylation also plays an important role in tumor initiation and progression (Jones and Baylin 2002; Momparler and Bovenzi 2000). Thus, gene silencing by hypermethylation is an important mechanism that has great promise for therapy and for the discovery of new biomarkers. However, the information on the frequency and specificity of methylation changes is still insufficient. It presents a challenge to identify crucial genes that are susceptible to methylation-induced silencing and are directly associated with the development of HNC. Since HNC is a heterogenous disease, the methylation status may vary according to clinical characteristics and environmental and genetic factors such as anatomic involvement of tumor (Dikshit et al. 2007; Azarschab et al. 2003), human papilloma virus (HPV) positivity (Bennett et al. 2010; Richards et al. 2009), smoking (Sharma et al. 2010), microsatellite instability (MSI) (Zuo et al. 2009; Demokan et al. 2006; Sengupta et al. 2007; Liu et al. 2003a, b, 2002), and geographic location (Li et al. 2003a, b; Ayadi et al. 2002). Therefore, analysis of the epigenetic changes in genes playing important roles in these vital molecular mechanisms is crucial in order to understand the molecular biology of head and neck carcinogenesis.

Genetic evidence indicates that the majority of squamous HNC originate from hyperplastic fields characterized by higher numbers of proliferating cells and clonal selection (Rubin 2011; Tabor et al. 2002). Almost two thirds of the tumor recurrences following resection occur in these regions (Tabor et al. 2004; Ha and Califano 2003). Clonal selection, divergence, and expression are the basic characteristics of the field development. Molecular evidence including cells harboring mutations of the TSG and recent data on p53 mutations support the field cancerization theory. The presence of cells with p53 gene mutations in these fields have also been shown in HNC (Tabor et al. 2001). The genetic changes characterizing these regions can be used to identify individuals at risk of developing cancer (Dakubo et al. 2007). Furthermore, methylation in noncancerous tissues is now considered as a marker for potential cancer risk and previous exposure to certain carcinogenic factors (Nakajima et al. 2008). Knowledge of the methylation status in these regions may enable intervention by using demethylating agents as chemopreventive means (Dakubo et al. 2007). An epigenetic field effect has been first described by increased aberrant methylation in normal tissue from patients with liver cancer (Kondo et al. 2000). Later studies have unequivocally shown the presence of an epigenetic field for cancerization (Ushijima 2007). Recently, the presence of an epigenetic field for cancerization has also been shown in colon (Shen et al. 2005), breast (Yan et al. 2006), and renal (Arai et al. 2006) cancers.

Recent advances in technology make it possible to analyze DNA methylation by highly sensitive and quantitative methods. For DNA methylation analysis, a variety of different methodologies have been used, almost all of which rely on three different approaches for treating DNA: the action of methylation-specific endonucleases, bisulfite modification of unmethylated cytosines, or immunoprecipitation (IP) of the methylated DNA fragments. Combination of these initial steps with different detection techniques for analysis have resulted in various analytical methods to investigate candidate genes or to study global DNA methylation. The main methodologies used for methylation analysis are summarized in Table 1. Several techniques initially confined to smaller regions of the genome have now been extended to perform analysis of the whole genome. New high-throughput methodologies provide information to characterize methylated sequences at single-base resolution on a genome-wide scale.

Recent studies have shown that hypermethylation of some TSG may be a valuable biomarker (Kim et al. 2006; Demokan et al. 2010; Kaur et al. 2010; Pattani et al. 2010) in different cancer types. Furthermore, results of clinical

| Table 1: The main methodologies used in DNA methylation analysis |
|---------------------------------------------|
| **Sequence-specific methylation**              |
| Quantitative methylation-specific PCR (Q MSP) |
| Methylation-sensitive restriction enzyme analysis |
| Sensitive restriction-multiplex PCR |
| Combined bisulfite restriction analysis (COBRA) |
| Bisulfite sequencing |
| Pyrosequencing |
| Multiplex ligation-dependent probe amplification (MLPA) |
| Mass array analysis |
| **Global methylation**                          |
| Restriction landmark genomic scanning (RGLS)   |
| Arbitrarily primed methylation-sensitive PCR (AP-MSP) |
| Methylated CpG island recovery assay (MIRA) |
| Differential methylation hybridization (DMH)   |
| Analysis of intermethylated sites (AIMS)       |
| Methylated DNA immunoprecipitation (MeDIP)    |
| Whole-genome shotgun bisulfite sequencing (WGSBS) |
studies using DNA methyltransferase and histone deacetylase inhibitors indicate the potential of epigenetic therapeutics in clinical oncology (Ren et al. 2011; Wagner et al. 2010). The epigenetic changes may help to predict the prognosis and identify individuals who may benefit from the therapy with demethylating agents (Issa et al. 2004).

In this review, we aimed to summarize the present state of knowledge in head and neck carcinogenesis by analyzing the studies investigating the relationship between methylation and HNC. The publications in the literature were retrieved by literature and keyword search using the ISI, PubMed, and Scopus databases to identify the studies relevant to this review. Citations in these publications were also evaluated for their relevance.

**Data from analysis of tumor suppressor gene panels**

In recent years, most studies have focused on the analysis of promoter methylation of TSG panels playing a role in vital cellular mechanisms via the candidate gene strategy approach (Table 2). In a study among the North American population (Demokan et al. 2010), KIF1A and EDNRB genes were highly methylated (97% and 98%, respectively) in the primary tumor tissue and salivary rinse samples from patients with squamous cell carcinoma, while normal salivary and normal mucosal samples were minimally methylated. A significant association between KIF1A methylation and tumor site was reported. Kaur et al. (2010) found high methylation levels of the KIF1A, EDNRB, DCC, and p16 genes in Indian patients with oral squamous cell carcinoma (OSCC) and Pattani et al. (2010) have shown that, in salivary samples from patients with pre-malignant or malignant oral cavity lesions, promoter hypermethylation of the KIF1A and EDNRB genes was associated with malignancy. The endothelin receptor type B (EDNRB) is a G protein-coupled receptor which activates a phosphatidylinositol-calcium second messenger system (Smollich and Wülfing 2008). The kinesin family member 1A (KIF1A) gene encodes a protein that is a microtubule-dependent molecular motor involved in important intracellular functions such as organelle transport and cell division (Okada et al. 1995).

We have evaluated the epigenetic changes specific to head and neck squamous cell carcinoma (HNSCC) by investigating promoter hypermethylation of a panel of 24 TSG via candidate gene approach in a recent study (Yalniz et al. 2011). CHFR, RAR3, DAPK1, and RASSF1A genes were found to be the most frequently methylated genes in HNC tumor tissue by methylation-specific multiplex ligation-dependent probe amplification. A further collaborative study analyzing a panel of 22 genes confirmed these findings and found that the RAR3, APC, and CHFR genes were frequently hypermethylated in HNC (Chen et al. 2007).

In another study, six genes were analyzed in patients with HNSCC treated by radiotherapy (de Schutter et al. 2009). The MGMT and TIMP3 genes displayed higher methylation rates. Promoter hypermethylation of the TIMP3 and CDH1 genes were significantly associated with better locoregional control (LRC) and overall survival (OS) or disease-free survival (DFS). TIMP3 methylation highly correlated with DAPK methylation, indicating a very strong functional association between these two genes (Nayak et al. 2007). In other gene panel studies, methylation of the TIMP3, CDH1, p16, MGMT, DAPK, and RASSF1 genes were observed in HNSCC tumors and paired saliva samples (Righini et al. 2007; Hasegawa et al. 2002; Rosas et al. 2001; Sanchez-Cespedes et al. 2000). A study analyzing promoter methylation of 15 candidate genes (Steinmann et al. 2009) in tumors and matched normal tissue from patients with HNSCC has shown that methylation of the p16, MGMT, DAPK, RAR3, hMLH1, CDH1, RASSF2, RASSF5, and MST1 genes was significantly more frequent in the tumors than the normal tissue (Steinmann et al. 2008). The hMLH1 (Puri et al. 2005), RAR3 (Maruya et al. 2004), p16, and MGMT (Puri et al. 2005; Maruya et al. 2004) genes have also been found to be frequently methylated in HNSCC. The higher increased methylation of the TSG is usually associated with advanced tumor stages and undifferentiated HNSCC. This has been primarily shown for the p16 and RASSF5 genes (Steinmann et al. 2009). In the same study, RASSF4 gene methylation was more frequent in patients with advanced tumor stage and recurrent HNSCC than patients without relapse. Higher methylation of the p16, RAR3, and RASSF1 genes was also reported in a study in which matched normal samples were not available (Okami et al. 2005).

In a study investigating three TSG functioning in carcinogen metabolism (CYP1A1, CYP2A13, and GSTM1), the genes were found to be moderately (27.4–58.1%) methylated in the tumors, while methylation levels in normal tissue were much lower. A significant correlation was reported between smoking and the methylation status of the CYP1A1 and CYP2A13 genes (Sharma et al. 2010).

Hypermethylation of the hMLH1 gene, which plays an important role in DNA mismatch repair, has been found to be significantly associated with decreased hMLH1 protein expression, MSI, and decreased cause-specific survival for HNSCC patients in various studies (Zuo et al. 2009; Demokan et al. 2006; Sengupta et al. 2007; Liu et al. 2003a, b, 2002). Methylation of both hMLH1 and hMSH2
### Table 2 Candidate genes frequently methylated in HNC

| Gene | Gene name | Gene function | Tumor type$^a$ | Method$^b$ | Clinical association$^c$ | Samples$^d$ | Methylation range (%) |
|------|-----------|---------------|---------------|-----------|----------------------|------------|----------------------|
| **APC** | Adenomatous polyposis coli | Tumor suppression | HNSCC (Chen et al. 2007), SGC (Durr et al. 2010; Lee et al. 2010), NPC (Loyo et al. 2011) | MS-MLPA (Chen et al. 2007) QMSP (Durr et al. 2010; Loyo et al. 2011), MSP (Lee et al. 2010) | (HG,St,H) | (Chen et al. 2007; Durr et al. 2010; Lee et al. 2008; Loyo et al. 2011), MN (Durr et al. 2010; Lee et al. 2010) | 21–34 |
| **BLU** | MYND-type containing 10 | Tumor suppression | NPC (Ayadi et al. 2008; Liu et al. 2003b; Loyo et al. 2011; Qiu et al. 2004) | QMSP (Loyo et al. 2011), MSP (Ayadi et al. 2008; Liu et al. 2003b; Qiu et al. 2004) | H (Ayadi et al. 2008) | (Ayadi et al. 2008; Liu et al. 2003b; Loyo et al. 2011; Qiu et al. 2004), NE (Liu et al. 2003b; Qiu et al. 2004) | 34–74 |
| **CCNA1** | Cyclin A1 | Cell cycle | OSCC (Shaw et al. 2006), HNC (Tan et al. 2008; Tokumaru et al. 2004), NPC (Yanatatsaneejit et al. 2008) | PS (Shaw et al. 2006), BS (Tokumaru et al. 2004), QMSP (Tan et al. 2008) MSP (Yanatatsaneejit et al. 2008), COBRA (Yanatatsaneejit et al. 2008) | HG (Shaw et al. 2006) | (Shaw et al. 2006; Tan et al. 2008; Tokumaru et al. 2004; Yanatatsaneejit et al. 2008) NE (Tokumaru et al. 2008; Yanatatsaneejit et al. 2008) MN (Shaw et al. 2006), SM (Tan et al. 2008), L (Yanatatsaneejit et al. 2008) | 45–53 |
| **CDH1** | E-cadherin | Cell adhesion | HNSCC (De Schutter et al. 2009; Hasegawa et al. 2002; Righini et al. 2007; Steinmann et al. 2009), HPC (Dikshit et al. 2007) SGC (Zhang et al. 2007a), OSCC (Chang et al. 2002; De Moraes et al. 2008; Maeda et al. 2007a; Viswanathan et al. 2003; Yeh et al. 2002) LC (Azarschab et al. 2003; Dikshit et al. 2007) NPC (Ayadi et al. 2008; Chang et al. 2003b; Kao et al. 2002; Krishna et al. 2005) | MSP (Ayadi et al. 2008; Azarschab et al. 2003; Chang et al. 2003b; Chang et al. 2002; De Moraes et al. 2008; De Schutter et al. 2009; Dikshit et al. 2007; Hasegawa et al. 2002; Krishna et al. 2005; Li et al. 2003a, b) MSP (Maeda et al. 2007a; Niemhom et al. 2008; Righini et al. 2007; Steinmann et al. 2009; Tsaö et al. 2003) NPC (Ayadi et al. 2008; Chang et al. 2003b; Kao et al. 2002; Krishna et al. 2005) | (B-LRC, B-S) (De Schutter et al. 2009) (B-DFS, B-DSS, B-O) (De Schutter et al. 2009), (TU,TS,TS) (Hasegawa et al. 2002) MET (Azarschab et al. 2003; Li et al. 2003a), (PD,PF) (Zhang et al. 2007a) LN (Chang et al. 2008; De Moraes et al. 2008; Li et al. 2003b), I (Li et al. 2003a) EBV (Kao et al. 2002; M-SA (Hasegawa et al. 2002; Righini et al. 2007), NE (Chang et al. 2003b; Niemhom et al. 2008; Tsaö et al. 2003) MN (Righini et al. 2007; Steinmann et al. 2009; Viswanathan et al. 2003; Yeh et al. 2002) SA-H (Righini et al. 2007) LN (Chang et al. 2002; Li et al. 2003a), T (Ayadi et al. 2008; Azarschab et al. 2003; Chang et al. 2002, 2003b;
| Gene  | Gene name | Gene function | Tumor type\(^a\) | Method\(^b\) | Clinical association\(^c\) | Samples\(^d\) | Methylation range (%) |
|-------|-----------|---------------|------------------|-------------|-----------------------------|---------------|----------------------|
| Li et al. 2003a, b; Niemhom et al. 2008; Tsao et al. 2003; Wong et al. 2003b) | CHFR | Checkpoint with forkhead and ring finger domains | Early G2/M checkpoint | Zhang et al. 2007a), SRM-PCR (Viswanathan et al. 2003) BS (Kao et al. 2002) | Krishna et al. 2005; Li et al. 2003a; Niemhom et al. 2008; Tsao et al. 2003) St (Niemhom et al. 2008) | De Moraes et al. 2008; De Schutter et al. 2009; Dikshit et al. 2007; Hasegawa et al. 2002; Kao et al. 2002; Krishna et al. 2005; Li et al. 2003a) T (Li et al. 2003b; Maeda et al. 2007a; Niemhom et al. 2008; Righini et al. 2007; Steinmann et al. 2009; Tsao et al. 2003; Viswanathan et al. 2003) T (Wong et al. 2003b; Yeh et al. 2002; Zhang et al. 2007a) | n.a. T (Baba et al. 2009; Cheung et al. 2005; Toyoda et al. 2003; Yahniz et al. 2011), MN (Baba et al. 2009; Toyoda et al. 2003) NE (Baba et al. 2009; Cheung et al. 2005; Toyoda et al. 2003) |
| DAPK1 | Death-associated protein kinase 1 | Apoptosis | HNSCC (Chen et al. 2007; Hasegawa et al. 2002; Nayak et al. 2007; Righini et al. 2007; Rosas et al. 2001; Sanchez-Cespedes et al. 2000; Steinmann et al. 2009) OSCC (Ogi et al. 2002), SGC (Li et al. 2005), LC (Zhang and Kong 2004) | MS-MLPA (Chen et al. 2007; Yahniz et al. 2011) MSP (Baba et al. 2009; Cheung et al. 2005), MSRE (Toyota et al. 2003) | (St,TS,TU) (Hasegawa et al. 2002) LNM (Calmon et al. 2007; Fendri et al. 2009; Hasegawa et al. 2002; Sanchez-Cespedes et al. 2000) St (Sanchez-Cespedes et al. 2000) | M-SA (Chang et al. 2003b; Hasegawa et al. 2002; Righini et al. 2007; Rosas et al. 2001) NE (Chang et al. 2003b; Kwong et al. 2002; Wong et al. 2002), M-SE (Sanchez-Cespedes et al. 2000) SA-H (Righini et al. 2007), CINT (Kong et al. 2006) MN (Fendri et al. 2009; Righini et al. 2007; Steinmann et al. 2009; Zhang and Kong 2004), (L) (Wong et al. 2002) T (Calmon et al. 2007; Chang et al. 2003b) | 19–61 |

\(^{a}\) Tumor type: HNSCC = Head and Neck Squamous Cell Carcinoma, OSCC = Oral Squamous Cell Carcinoma, NPC = Nasopharyngeal Carcinoma.
\(^{b}\) Method: MS-MLPA = Multiplex Ligation-Dependent Probe Amplification, QMSP = Quantitative Methylation-Specific PCR, MSRE = Methylation-Specific Restriction Enzyme, BS = Bisulfite Sequencing, BS = Bisulfite Sequencing.
\(^{c}\) Clinical association: St = Staining, LNM = Lymph Node Metastasis.
\(^{d}\) Samples: T = Tissue, NE = Nucleus Extract, MN = Methylation, M-SA = Methylation Specific Amplification.
### Table 2 (continued)

| Gene  | Gene name                      | Gene function               | Tumor type | Method       | Clinical association | Samples | Methylation range (%) |
|-------|--------------------------------|-----------------------------|------------|--------------|----------------------|---------|------------------------|
| **DCC** | Deleted in colorectal cancer | Tumor suppression           | OSCC (Kaur et al. 2010; Ogi et al. 2002), HNSCC (Carvalho et al. 2006; Tan et al. 2008), NPC (Loyo et al. 2011) | QMSP (Carvalho et al. 2006; Kaur et al. 2010; Loyo et al. 2011; Tan et al. 2008), BS (Ogi et al. 2002) | (I, P-S) (Ogi et al. 2002) | T (Carvalho et al. 2006; Kaur et al. 2010; Loyo et al. 2011; Ogi et al. 2002; Tan et al. 2008), SA-H (Carvalho et al. 2006; Kaur et al. 2010), SE-H (Kaur et al. 2010), NE (Kaur et al. 2010), SM (Carvalho et al. 2006; Tan et al. 2008) | 50–75 |
| **DLCI** | Deleted in liver cancer 1 | Tumor suppression           | NPC (Loyo et al. 2011; Seng et al. 2007) | QMSP (Loyo et al. 2011; Seng et al. 2007) | n.a. | T (Loyo et al. 2011; Seng et al. 2007), (MN,NE, SM) (Seng et al. 2007) | 43–89 |
| **DLEC1** | Deleted in lung and esophageal cancer | Tumor suppression           | NPC (Ayadi et al. 2008; Loyo et al. 2011) | QMSP (Loyo et al. 2011), MSP (Ayadi et al. 2008) | H (Ayadi et al. 2008) | T (Ayadi et al. 2008; Loyo et al. 2011) | 60–86 |
| **EDNRB** | Endothelin receptor type B | Signaling mechanism         | HNSCC (Demokan et al. 2010), OSCC (Kaur et al. 2010; Pattani et al. 2010), PM-OCL (Pattani et al. 2010), NPC (Lo et al. 2002; Zhou et al. 2007) | QMSP (Demokan et al. 2010; Kaur et al. 2010; Pattani et al. 2010), MSP (Lo et al. 2002; Zhou et al. 2007) | H (Pattani et al. 2010) | T (Demokan et al. 2010; Kaur et al. 2010; Lo et al. 2002; Pattani et al. 2010; Zhou et al. 2007), SA-H (Demokan et al. 2010; Kaur et al. 2010), SE-H (Kaur et al. 2010), NE (Kaur et al. 2010; Lo et al. 2002) | 60–97 |
| Gene       | Gene name                  | Gene function                                                                 | Tumor type | Method                      | Clinical association               | Samples         | Methylation range (%) |
|------------|----------------------------|-------------------------------------------------------------------------------|------------|-----------------------------|-----------------------------------|----------------|-----------------------|
| HIN-1      | High-in-normal 1           | Tumor suppression                                                             | OSCC (Huang et al. 2009), NPC (Wong et al. 2003a) | PCR-DHPLC (Huang et al. 2009), MSP (Wong et al. 2009) | T (Huang et al. 2009; Wong et al. 2003a) | 73-77          | 32.5-50               |
| hMLH1      | mutL homolog 1             | DNA repair                                                                    | HNSCC (Demokan et al. 2002, 2006; Liu et al. 2003a; Steinmann et al. 2006; Sengupta et al. 2006; Tan et al. 2006), MSP (Czerninski et al. 2009), OSRE (Demokan et al. 2006; Puri et al. 2005; Steinmann et al. 2009; Tan et al. 2006) | H-MSI (Demokan et al. 2006; Sengupta et al. 2007; Zuo et al. 2009), P-S (Zuo et al. 2009) | n.a.             | 32.5-50               |
| hMSH2      | mutS homolog 2             | DNA repair                                                                    | HNSCC (Demokan et al. 2010; Hoque et al. 2008), OSCC (Kaur et al. 2010; Pattani et al. 2010), PM-OCL (Pattani et al. 2010), NPC (Loyo et al. 2011) | QMSP (Demokan et al. 2010; Hoque et al. 2008; Kaur et al. 2010; Loyo et al. 2011; Pattani et al. 2010) | T (Demokan et al. 2010; Hoque et al. 2008; Kaur et al. 2010; Loyo et al. 2011; Pattani et al. 2010) | 35-98           | 30-50                 |
| KIF1A      | Kinesin family member 1A   | Axonal transport of synaptic vesicles and cell division                      | HNSCC (De Schutter et al. 2009; Hasegawa et al. 2002; Maruyama et al. 2004; Puri et al. 2005; Righini et al. 2007; Rosas et al. 2001) | MSP (De Schutter et al. 2009; Dikshit et al. 2007; Hasegawa et al. 2002; Katue et al. 2006; Maruyama et al. 2004; Puri et al. 2005; Righini et al. 2007; Rosas et al. 2001) | M-SA (Hasegawa et al. 2002), AU (Puri et al. 2005) (P-S, P-DFS) (Fanfani et al. 2009) | 6-56            | 6-56                  |
| MGMT       | O(6)-methylguanine-DNA methyltransferase | DNA repair                                                                    | HNSCC (De Schutter et al. 2009; Hasegawa et al. 2002, 2006; Maruyama et al. 2004; Puri et al. 2005; Righini et al. 2007; Rosas et al. 2001) | MSP (De Schutter et al. 2009; Dikshit et al. 2007; Hasegawa et al. 2002; Katue et al. 2006; Maruyama et al. 2004; Puri et al. 2005; Righini et al. 2007; Rosas et al. 2001) | M-SA (Hasegawa et al. 2002), AU (Puri et al. 2005) (P-S, P-DFS) (Fanfani et al. 2009) | 6-56            | 6-56                  |
| Gene     | Gene name                  | Function                                      | Tumor type          | Method       | Clinical association                  | Samples              | Methylation range (%) |
|----------|----------------------------|-----------------------------------------------|--------------------|--------------|---------------------------------------|----------------------|-----------------------|
|          |                            |                                               | (OSCC)             | Rosas et al. | MN (Kato et al. 2006;                       |                      | 23–48                 |
| MINT1    | Methylated in tumor-1      | Synaptic vesicle exocytosis                   | (OSCC)             | Rosas et al. | MN (Kato et al. 2006;                       |                      | 23–48                 |
|          |                            |                                               |                    | (Durr et al. 2010) | (Ogi et al. 2002;                       | (Durr et al. 2010)   |                      |
| MINT31   | Methylated in tumor-31     | Tumor suppression                             | (OSCC)             | MSRE (Ogi et al. 2002), QMSP (Durr et al. 2010) | (St, P-P) (Ogi et al. 2002) | (Carvalho et al. 2008; Ogi et al. 2002, NE (Carvalho et al. 2008) | 15–64                 |
| p14      | Alternative open reading frame (ARF) of INK4a locus | Tumor suppression                             | (OSCC)             | MSRE (Ogi et al. 2002), QMSP (Carvalho et al. 2008) | G-P (Ogi et al. 2002; Sailsree et al. 2008), R-RC (Sailsree et al. 2008) (LNM,TS,St) (Ishida et al. 2005) | (Ishida et al. 2005) | 14–34                 |
|          |                            |                                               |                    |              | (Ishida et al. 2005; Kordi-Tamandani et al. 2010; Ogi et al. 2002; Sailsree et al. 2008) | (Ishida et al. 2005) |                      |

Notes:
- Method: MSRE (Mutationally Stable Reagent Extraction), QMSP (Quantitative Methylated DNA Sequencing), MSP (Methylation Specific PCR)
- Clinical association: (ANS, P-P) (Ogi et al. 2002), R-RC (Sailsree et al. 2008)
| Gene   | Gene name                                      | Gene function     | Tumor type\(^a\)                                                                 | Method\(^b\)                                      | Clinical association\(^c\)                              | Samples\(^d\)                        | Methylation range (%) |
|--------|-----------------------------------------------|-------------------|---------------------------------------------------------------------------------|--------------------------------------------------|--------------------------------------------------------|--------------------------------------|------------------------|
| p15    | Cyclin-dependent kinase inhibitor 2B          | Tumor suppression | OSCC (Ogi et al. 2002; Shintani et al. 2001; Viswanathan et al. 2003; Yeh et al. 2002), HNC (Chang et al. 2004) PM-OCL (Takeshima et al. 2008), NPC (Chang et al. 2003b; Wong et al. 2003b) | SRM-PCR (Viswanathan et al. 2003), MSRE (Ogi et al. 2002) QMSP (Chang et al. 2004) MSP (Chang et al. 2003b; Shintani et al. 2001; Takeshima et al. 2008; Wong et al. 2003b; Yeh et al. 2003) | ANS (Ogi et al. 2002), (TU, AU) (Chang et al. 2004) QMSP (Chang et al. 2003b; Shintani et al. 2001; Takeshima et al. 2008; Wong et al. 2003b; Yeh et al. 2003) | MN (Viswanathan et al. 2003), M-SA (Chang et al. 2003b; Chang et al. 2004; Takeshima et al. 2008) NE (Chang et al. 2003b; Takeshima et al. 2008) T (Chang et al. 2003b; Chang et al. 2004; Ogi et al. 2002; Shintani et al. 2001; Takeshima et al. 2008; Viswanathan et al. 2003; Wong et al. 2003b; Yeh et al. 2003) | 22–65                  |
| p16    | Cyclin-dependent kinase inhibitor 2A (CDKN2A) | Tumor suppression | OSCC (El-Naggar et al. 1997; González et al. 1997; Hall et al. 2008; Huang et al. 2002; Ishida et al. 2005; Kato et al. 2006; Kaur et al. 2010; Miracca et al. 1999; Nakahara et al. 2005) OSCC (Nakahara et al. 2001; Ogi et al. 2002; Ohta et al. 2009; Reed et al. 1996; Riese et al. 1999; Ruesga et al. 2007; Sailasree et al. 2008) OSCC (Shaw et al. 2006; Shintani et al. 2001; Sinha et al. 2009; Tao et al. 1997; Viswanathan et al. 2003; Yáñez-Jiménez et al. 2003; Yáñez et al. 2003) HNSCC (Ai et al. 2003; Carvalho et al. 2008; Haségawa et al. 2002; Kocsicenyi et al. 2007; Manyu et al. 2004; Okami et al. 2005; Puri et al. 2005) HNSCC (Righini et al. 2007; Rosas et al. 2001; Sanchez-Cespedes et al. 2003; Carvalho et al. 2008; Haségawa et al. 2002; Kocsicenyi et al. 2007; Manyu et al. 2004; Okami et al. 2005; Puri et al. 2005) | QMSP (Carvalho et al. 2008; Kaur et al. 2010; Tan et al. 2008) SRM-PCR (Viswanathan et al. 2003), PS (Hall et al. 2008; Shaw et al. 2006) MSRE (El-Naggar et al. 1997; González et al. 1997; Miracca et al. 1999; Ogi et al. 2002; Tao et al. 1997) MSP (Agnese et al. 2006; Ai et al. 2003; Cao et al. 2009; Chang et al. 2003b; Dikshit et al. 2007; El-Naggar et al. 1997; González et al. 1997; Guo et al. 2007; Ha and Califano 2003; Kato et al. 2006; Pocock et al. 2007; Kwong et al. 2002; Li et al. 2005; Lo et al. 1996; Manyu et al. 2004; Miracca et al. 1999; Nakahara et al. 2005; T (Nakahara et al. 2005) QMSP (Chang et al. 2003b; Shintani et al. 2001; Takeshima et al. 2008; Wong et al. 2003b; Yeh et al. 2003) | (A-TU,TU) (Haségawa et al. 2002) (St, LNM, TS) (Haségawa et al. 2002; Ishida et al. 2005) ANS (Kresty et al. 2002), PD (Steinmamm et al. 2009) (I-RC, P-P) (Sailasree et al. 2008) | T (Agnese et al. 2006; Ai et al. 2003; Cao et al. 2009; Carvalho et al. 2008; Chang et al. 2003b; Chang et al. 2004; El-Naggar et al. 1997; González et al. 1997; Guo et al. 2007; Ha and Califano 2003; Kato et al. 2006; Kauer et al. 2007; Kresty et al. 2002; Kwong et al. 2002; Li et al. 2005; Lo et al. 1996; Manyu et al. 2004; Miracca et al. 1999; Nakahara et al. 2005; T (Nakahara et al. 2005) QMSP (Chang et al. 2003b; Shintani et al. 2001; Takeshima et al. 2008; Wong et al. 2003b; Yeh et al. 2003) | 5–68
| Gene        | Gene name | Gene function | Tumor type | Method | Clinical association | Samples | Methylation range (%) |
|-------------|-----------|---------------|------------|--------|----------------------|---------|-----------------------|
|            |           |               | PM-HNL     | 2003   | MSP (Ohta et al.     | 2003)   | T (Riese et al.       |
|            |           |               |            |        | 2009; Okami et al.   |         | 1999; Righini et al.  |
|            |           |               |            |        | 2005; Puri et al. 2005; Riese et al. 1999; Righini et al. 2007; Rosas et al. 2001; Rubin et al. 2011; Ruesga et al. 2007; Sailasree et al. 2008; Sanchez-Cespedes et al. 2000; Shaw et al. 2006; Shintani et al. 2001; Sinha et al. 2009; Smigiel et al. 2004; Steinmann et al. 2009) T (Takeshima et al. 2008; Tan et al. 2006, 2008; Tao et al. 1997; Viswanathan et al. 2003; Weber et al. 2002; Wong et al. 2003b; Yakushiji et al. 2001; Yeh et al. 2003) |         |                       |
|            |           |               |            |        | 2000) MSP (Shintani et al. 2001; Sinha et al. 2009; Smigiel et al. 2004; Steinmann et al. 2009; Takeshima et al. 2008; Tan et al. 2006) MSP (Weber et al. 2002; Wong et al. 2003b; Yakushiji et al. 2001; Yeh et al. 2003) |         |                       |
|            |           |               |            |        |                      |         |                       |
|            |           |               | SGC  (Agnese et al. 2006; Dikshit et al. 2007; Smigiel et al. 2004) HPC (Dikshit et al. 2007), NPC (Chang et al. 2003b; Kwong et al. 2002; Lo et al. 1996; Tan et al. 2006; Wong et al. 2003b) |         |                          |         |                           |
|            |           |               |            |        |                      |         |                           |
Table 2 (continued)

| Gene       | Gene name                  | Gene function                                                                 | Tumor type\( ^e \)                                                                 | Method\( ^d \)                  | Clinical association\( ^e \)                      | Samples\( ^d \)                | Methylation range (%) |
|------------|----------------------------|-------------------------------------------------------------------------------|---------------------------------------------------------------------------------|---------------------------------|-----------------------------------------------|---------------------------|----------------------|
| **PGP9.5** (UCHL1) | Ubiquitin carboxy-terminal esterase L1 (ubiquitin thiolesterase) | Processing of ubiquitin precursors and of ubiquitinated proteins               | SGC (Durr et al. 2010), HNC (Tokumaru et al. 2004, 2008) OSCC (Tokumaru et al. 2008), NPC (Li et al. 2010; Loyo et al. 2011) | BS (Tokumaru et al. 2004), MSP (Li et al. 2010) QMSP (Durr et al. 2010; Loyo et al. 2011; Tokumaru et al. 2008) | n.a.                                    | T (Durr et al. 2010; Li et al. 2010; Loyo et al. 2011; Tokumaru et al. 2004, 2008) MN (Durr et al. 2010, NE (Tokumaru et al. 2004) | 60–82 |
| **RARB**   | Retinoic acid receptor beta | Tumor suppression                                                              | HNSCC (Chen et al. 2007; Maruya et al. 2004; Okami et al. 2005; Steinmann et al. 2009; Yahniz et al. 2011; Youssef et al. 2004) PM-HNL (Maruya et al. 2004), PM-OCL (Youssef et al. 2004) SGC (Durr et al. 2010), NPC (Fendri et al. 2009; Kwong et al. 2002; Kwong et al. 2005b) | MS-MLPA (Chen et al. 2007; Yahniz et al. 2011) MSP (Fendri et al. 2009; Kwong et al. 2005b; Maruya et al. 2004; Okami et al. 2005) MSP (Steinmann et al. 2009; Youssef et al. 2004), QMSP (Durr et al. 2010) | (St, H) (Fendri et al. 2009) | MN (Durr et al. 2010; Fendri et al. 2009; Maruya et al. 2004; Steinmann et al. 2009) NE (Kwong et al. 2002; Youssef et al. 2004) T (Chen et al. 2007; Durr et al. 2010; Fendri et al. 2009; Kwong et al. 2002, 2005b; Maruya et al. 2004; Okami et al. 2005; Steinmann et al. 2009; Yahniz et al. 2011; Youssef et al. 2004) | 15–80 |
| **RASSF1A** | Ras association (RalGDS/AF-6) domain family member 1A | Tumor suppression                                                              | HNSCC (Carvalho et al. 2008; Chen et al. 2007; Dong et al. 2003; Hasegawa et al. 2002; Okami et al. 2005; Righini et al. 2007; Steinmann et al. 2009), OSCC (Huang et al. 2009), SGC (Li et al. 2005), NPC (Chang et al. 2003b; Fendri et al. 2009; Kwong et al. 2002; Lo et al. 2001; Steinmann et al. 2009) | MS-MLPA (Chen et al. 2007), QMSP (Carvalho et al. 2008) PCR-DHPLC (Huang et al. 2009) MSP (Chang et al. 2003b; Dong et al. 2003; Fendri et al. 2009; Hasegawa et al. 2002; Kwong et al. 2002; Li et al. 2005) MSP (Lo et al. 2001; Okami et al. 2005) (Qu et al. 2004; Righini et al. 2007; Steinmann et al. 2009), HPV (Dong et al. 2003) (S,TS,TU) (Hasegawa et al. 2002) (P-DFS) (Huang et al. 2009) (HGS,MET) (Li et al. 2005) (LN,M,A) (Fendri et al. 2009), EBV (Zhou et al. 2005) | M-SA (Chang et al. 2003b; Hasegawa et al. 2002; Righini et al. 2007) MN (Righini et al. 2007) NE (Carvalho et al. 2008; Chang et al. 2003b; Dong et al. 2003; Fendri et al. 2009; Kwong et al. 2005b; Lo et al. 2001; Qu et al. 2004; Wang et al. 2005) | 2.4–84 |
| Gene   | Gene name                  | Gene function                          | Tumor typea                                      | Methodb                                      | Clinical associationc                                      | Samplesd                              | Methylation range (%) |
|--------|----------------------------|----------------------------------------|-------------------------------------------------|----------------------------------------------|----------------------------------------------------------|----------------------------------------|-----------------------|
| RASSF2 | Ras association (RAKIDS/AF-6) domain family member 2 | Tumor suppression                      | HNSCC (Steinmann et al. 2009) OSCC (Huang et al. 2009; Imai et al. 2008), NPC (Zhang et al. 2007b) | COBRA (Imai et al. 2008; Steinmann et al. 2009) PCR-DHPLC (Huang et al. 2009), MSP (Zhang et al. 2007b) | P-DFS (Huang et al. 2009), LNM (Zhang et al. 2007b) | T (Huang et al. 2009; Imai et al. 2008; Steinmann et al. 2009; Zhang et al. 2007b), MN (Imai et al. 2008; Steinmann et al. 2009) NE (Zhang et al. 2007b) | 22–51 |
| SFRP1  | Secreted protein 1 Inhibition of Wnt signaling | Response to retinoic acid receptor | SGC (Lee et al. 2010), OSCC (Sogabe et al. 2008) | MSP (Lee et al. 2010; Sogabe et al. 2008) | (HG,ST,H) (Lee et al. 2010), G (Sogabe et al. 2008) | T (Kwong et al. 2005a; Tokumaru et al. 2005; Yanatatsaneejit et al. 2008), NE (Kwong et al. 2005a; Tokumaru et al. 2005; Yanatatsaneejit et al. 2008) L (Yanatatsaneejit et al. 2008) | 24–58.6 |
| TIG1   | Tazarotene-induced gene-1 Tissue inhibitor of metalloproteinases 3 Inhibition of angiogenesis and tumor growth | HNSCC (Tokumaru et al. 2005), NPC (Kwong et al. 2005a; Yanatatsaneejit et al. 2008) | MSP (Kwong et al. 2005a; Tokumaru et al. 2005) MSP-COBRA (Yanatatsaneejit et al. 2008) | n.a. | T (Kwong et al. 2005a; Tokumaru et al. 2005; Yanatatsaneejit et al. 2008), NE (Kwong et al. 2005a; Tokumaru et al. 2005; Yanatatsaneejit et al. 2008) L (Yanatatsaneejit et al. 2008) | 51–91 |
| TIMP3  | Tissue inhibitor of metalloproteinases 3 | Inhibition of angiogenesis and tumor growth | HNSCC (De Schutter et al. 2009; Nayak et al. 2007; Righini et al. 2007), SGC (Durr et al. 2010) | MSP (De Schutter et al. 2009; Righini et al. 2007), QMSP (Durr et al. 2010; Nayak et al. 2007) | (B-LRC, B-S) (De Schutter et al. 2009) (B-DFS, B-DSS, B-O) (De Schutter et al. 2009) | T (De Schutter et al. 2009; Durr et al. 2010; Nayak et al. 2007; Righini et al. 2007), MN (Durr et al. 2010; Righini et al. 2007) | 40.5–71.8 |
| Gene | Gene name | Gene function | Tumor type<sup>a</sup> | Method<sup>b</sup> | Clinical association<sup>c</sup> | Samples<sup>d</sup> | Methylation range (%) |
|------|-----------|---------------|------------------------|-------------------|-------------------------------|-------------------|----------------------|
| WIF1 | Wnt inhibitory factor 1 | Inhibition of Wnt signaling | NPC (Chan et al. 2007; Fendri et al. 2010) | MSP (Chan et al. 2007; Fendri et al. 2010) | (TS, LNM) (Chan et al. 2007; Fendri et al. 2010) (MET, A) (Chan et al. 2007; Fendri et al. 2010) | M-SA (Righini et al. 2007), SA-H (Righini et al. 2007) (T, NE) (Chan et al. 2007; Fendri et al. 2010) | 85–89.7 |
| 14-3-3 sigma | Stratifin | Signaling mechanism | SGC (Gasco et al. 2002; Uchida et al. 2004), OSCC (Gasco et al. 2002) PM-OCL (Gasco et al. 2002), NPC (Yi et al. 2009) | MSP (Gasco et al. 2002; Uchida et al. 2004; Yi et al. 2009) | H (Uchida et al. 2004) (LNM, MET, S) (Yi et al. 2009) | T (Gasco et al. 2002; Uchida et al. 2004; Yi et al. 2009), MN (Gasco et al. 2002; Yi et al. 2009) | 35–84 |

<sup>a</sup>Tumor types: OSCC oral squamous cell carcinoma, PM-OCL premalignant oral cavity lesions, PM-HNL premalignant head and neck lesions, SGC salivary gland carcinomas, LC larynx cancer, HPC hypopharyngeal cancer, PC pharyngeal cancer, NPC nasopharyngeal carcinoma

<sup>b</sup>Methods: PCR polymerase chain reaction, MSP methylation-specific PCR, QMSP quantitative methylation-specific PCR, QRT-PCR quantitative reverse transcriptase PCR, MSRE methylation-specific restriction enzyme analysis, SRM-PCR sensitive restriction-multiplex PCR, MS-MLPA methylation-specific multiplex ligation-dependent probe amplification, COBRA combined bisulfite restriction analysis, PS pyrosequencing, SB Southern blot, BS bisulfite sequencing, PCR-DHPLC PCR-denaturing high-performance liquid chromatography

<sup>c</sup>Clinical: B-LRC better local recurrence, B/P-S better/poor survival, B/P-DFS better/poor disease-free survival, B/P-DSS better/poor disease-specific survival, B/P-O better/poor outcome, ANS anatomic site, I invasion, A-S age of smoking, S stage, LNM lymph node metastasis, TS tumor size, TU tobacco usage, I/R-RC increased/reduced recurrence, P/G-P poor/good prognosis, AU alcohol usage, H histology, HG high grade, MET distant metastasis, H-MSI high microsatellite instability, PD poor differentiation, PI perineural invasion, G gender, A age

<sup>d</sup>Samples: T tumor, MN matched normal tissue, NE normal epithelium, M-SA matched salivary rinse, M-SE matched serum, SA-H salivary rinses from healthy people, SM surgical margin, SE-H serum from healthy people, LN lymph node, L lymphocytes, CINT chronic inflammatory nasopharyngeal tissues
genes may have an important role in oral carcinogenesis and have been associated with the susceptibility for oral malignancies (Czerninski et al. 2009).

Biomarkers predicting clinical response, tumor recurrence, or patient survival are not available for many cancer types, particularly for oral and pharyngeal cancer. Taioli et al. (2009) have studied the methylation of a panel of TSG in order to identify a possible correlation with survival and recurrence rates in patients with oral or pharyngeal cancer. MGMT promoter methylation was inversely associated with poor OS and DFS, indicating that MGMT promoter methylation may act as a possible prognostic biomarker for oral and pharyngeal cancer (Taioli et al. 2009). In oral epithelial dysplasia that transforms to OSCC, p16 methylation may act as a candidate biomarker of malignant transformation, whereas methylation of the MGMT, CYGB, and CCNA1 genes are not associated with malignancy (Hall et al. 2008). In oral carcinomas, methylation of the p16 and MGMT genes are frequently observed as an early event (Kato et al. 2006).

Promoter hypermethylation of the p16, p15, hMLH1, MGMT, and CDH1 genes has been reported in OSCC (Viswanathan et al. 2003). The Ras/Pi3K/AKT pathway is a major mechanism associated with radioresistance in OSCC. A study investigating four genes (RASSF1A, RASSF2A, PTEN, and HIN-1) in this pathway revealed that RASSF1A and RASSF2A methylation were more frequent in the tumors and significantly associated with poor DFS (Huang et al. 2009). Methylation of CCNA1, CYGB, and p16 genes has also been correlated with the clinicopathological parameters in oral cancer (Shaw et al. 2006). In OSCC, different levels of methylation have been reported for the p16, p15, p14, DCC, DAPK, MINT1, MINT2, MINT27, and MINT31 gene promoters and DCC methylation was associated with bone invasion of gingival tumors, invasiveness, and reduced survival (Ogi et al. 2002). Aberrant methylation of the p14, p15, and p16 gene promoters has been reported in HNSCC (Weber et al. 2002), oral precancerous lesions (Takekawa et al. 2008), oral carcinomas (Sailasree et al. 2008; Yeh et al. 2003; Shintani et al. 2001), and salivary gland carcinomas (Nishimine et al. 2003). When normal tissue and benign and malignant salivary gland tumors were compared, significantly higher methylation of the APC, RARβ, MINT1, PGP9.5, and TIMP3 genes were observed in salivary duct carcinoma (Durr et al. 2010). In adenoid cystic carcinomas of the salivary gland, p16, RASSF1A, and DAPK gene methylation is also a common event (Li et al. 2005).

In patients with laryngeal and hypopharyngeal cancers, promoter methylation of the p16, MGMT, DAPK, and CDH1 genes are observed frequently (Dikshit et al. 2007; Azarschab et al. 2003). For tumors in the oropharynx, a statistically significant association between hypermethylation of the DAPK1 gene and risk of lymph node metastases has been reported, and significant evidence indicate an association between hypermethylation of the ADAM23 gene and advanced tumor stage in larynx cancer (Calmon et al. 2007).

In a study investigating the methylation status of candidate TSG in surgical margins as a predictor of local recurrence in HNSCC, it has been shown that analysis of the CDKN2A, CCNA1, and DCC genes in the surgical margins by quantitative methylation-specific PCR (Q MSP) can correctly predict local recurrences in HNSCC (Tan et al. 2008). Using candidate gene and discovery approaches, 21 genes were investigated in HNSCC and normal tissue samples. p16, MINT31, and RASSF1A gene methylation were detected only in HNSCC but not in the controls (Carvalho et al. 2008).

Data from mRNA expression array studies

Recently, studies investigating novel methylated TSG specific to the tumor types have made use of mRNA expression arrays to analyze more than 40,000 genetic regions on a single platform via pharmacological unmasking and discovery approaches (Yamashita et al. 2002; Tokumaru et al. 2004).

After bisulfite sequencing, rapid subtractive hybridization, and microarray analysis in order to determine genes that are induced to reexpression by the demethylating agent 5-aza-2′-deoxycytidine in HNSCC cell lines, 35 out of 78 genes were selected and only 3 of these (CRABP2, MX1, and SLC15A3) were verified by QMSP (Calmon et al. 2009). After methylation-specific PCR (MSP) analysis, CRABP2 and MX1 genes were highly methylated in primary HNSCC compared with lymphocytes from a healthy cohort. In addition, lack of the CRABP2 protein was associated with poor survival rates, indicating that CRABP2 expression may be a potential prognostic biomarker for patients with HNSCC.

In a study, PGP9.5, CCNA1, bone morphogenetic protein 2A (BMP2A), metallothionein 1G (MT1G), and neuromedin U (NmU) genes were highly methylated and CCNA1 hypermethylation displayed an inverse correlation with p53 mutations (Tokumaru et al. 2004).

Methylation of the Nischarin (NISCH), p21-activated protein kinase 3 (Bak3), KIF1A, and OGDH1 genes has been reported in 8–52% of the patients with HNC, whereas no methylation was observed in the normal cohort (Hoque et al. 2008).

Global methylation

A recent study indicated the involvement of global DNA hypermethylation in the pathogenesis of HNSCC (Worsham
et al. 2010). Using DNA IP and Affymetrix whole-genome tiling arrays, 231 new and previously unreported genes out of 1,143 cancer genes on the array were identified via the whole-genome methylation approach.

In a study aiming to characterize early molecular changes in premalignant lesions of the oral cavity and the role of tobacco and alcohol consumption or HPV infections, the global methylation index was found to be 4.28 (95%CI, 4.1, 4.4) in the oral cancer case series. Methylation was inversely associated with tobacco use (Guerrero-Preston et al. 2009).

In cancer, abnormal demethylation leads to the loss of silencing in repetitive elements which are located on approximately 50% of the human genome, whereas in normal cells, these repetitive sequences are regulated by epigenetic silencing. DNA methylation alterations in HNC and adjacent nontumor tissues was investigated via a genome-wide microarray approach (Szpakowski et al. 2009). Of the more than 250,000 repetitive elements probed, between 5% and 8% displayed disease-related DNA methylation changes. Among the SVA, HERV, LINE-1P, AluY and MalR families, LINE-1 (Richards et al. 2009; Subbalekha et al. 2009; Smith et al. 2007), SINE (Alu) (Richards et al. 2009) repetitive elements, and LRE1 (Furniss et al. 2008; Hsiung et al. 2007) showed loss of DNA methylation in the tumors when compared to matched normal adjacent tissue. LINE and LRE1 hypomethylation were more frequently observed in HPV-negative than in HPV-positive tumors (Richards et al. 2009).

The Runt-related transcription factor 3 (RUNX3) gene plays a role in the transforming growth factor-beta (TGF-β)-induced tumor suppression pathway. Although RUNX3 has been considered as a TSG in some studies (Bae et al. 1995; Li et al. 2002), it has been shown that the expression level of RUNX3 in HNSCC tissues are higher than that in normal oral epithelial tissues due to demethylation (Ginos et al. 2004; Salto-Tellez et al. 2006). Therefore, it has been suggested that RUNX3 may have an oncogenic role in HNSCC and its expression may predict malignant behavior and the effect of chemotherapeutic drugs in HNSCC as a potential biomarker (Tsunematsu et al. 2009). In contrast to HNSCC, the RUNX3 gene is underexpressed in OSCC due to promoter hypermethylation, indicating that RUNX3 plays an important role in oral carcinogenesis and may be a useful diagnostic marker and a potential therapeutic target for OSCC (Gao et al. 2009).

A recent report has shown that the SEPT9, SLC5A8, FUSSEL18, EBF3, and IRX1 genes which act in the TGF-β signaling pathway are commonly methylated and down-regulated in HNC (Bennett et al. 2009). In IP studies, all these candidate genes were observed to interact with the components of the TGF-β pathway (Bennett et al. 2008). It was reported that decreased mitotic activity and increased apoptosis rates were observed when the SLC5A8, EBF3, and IRX1 genes are overexpressed.

Recently, HPV-infected HNSCC tumors have been shown to display higher levels of global DNA methylation. These tumors were universally methylated irrespective of the clinical factors and methylation of the FUSSEL18, IRX1, and EBF3 genes were likely correlated with recurrences. Promoter methylation of the FUSSEL18 and SEPT9 genes was significantly associated with alcohol and tobacco consumption. A trend between HPV16 positivity and methylation of the IRX1, EBF3, SLC5A8, and SEPT9 genes was noted and it has been suggested that this gene panel may be used for the selection of treatment modality (Bennett et al. 2010).

The Transketolase-like 1 (TKTL1) gene is a novel candidate oncogene, which is hypomethylated in human HNSCC tumor samples and contributes to HNSCC carcinogenesis via aerobic glycolysis and HIF1-α stabilization (Sun et al. 2010).

Studies of individual genes

Tumor suppressor genes

Significantly higher methylation of the p14ARF gene has been reported in OSCC compared to normal control tissues, implying that the methylation status of p14ARF may be an important determinant in the early diagnosis and treatment of OSCC (Kordi-Tamandani et al. 2010; Ishida et al. 2005).

Data on the methylation of the p16 gene promoter is not consistent. The gene has been analyzed individually or in gene panels but with discordant results. In a study, p16 methylation has been associated with malignant transformation of oral epithelial dysplasia and was considered a potential biomarker for the prediction of prognosis of mild or moderate oral epithelial dysplasia (Cao et al. 2009; Kresty et al. 2002). High methylation of the p16 promoter region has also been reported in carcinomas of the tongue and methylation in the surgical margins were found to increase the risk of local recurrences 6.3-fold when compared with patients with negative margins (Sinha et al. 2009). Varying degrees of p16 methylation has been reported in OSCC (Ohta et al. 2009; Ruesga et al. 2007; Nakahara et al. 2006; Huang et al. 2002; Yakashiiji et al. 2001; Nakahara et al. 2001; Miracca et al. 1999; Riese et al. 1999; El-Naggar et al. 1997; Tao et al. 1997; González et al. 1997; Reed et al. 1996), in mucoepidermoid carcinoma (MEC) of the salivary glands (Guo et al. 2007; Agnese et al. 2006), in HNC (Yalniz et al. 2011; Koscielny et al. 2007; Ai et al. 2003), and in larynx cancer (Smigiel et al. 2004). Conversely, there is a also a report of low p16 methylation in larynx cancer (Agnese et al. 2006).

DCC is a candidate TSG located on chromosome 18q21. Hypermethylation of DCC as a mechanism for inactivation
in HNSCC has been investigated (Carvalho et al. 2006). The \( DCC \) promoter was highly methylated in the tumors and there was a significant correlation between \( DCC \) promoter region hypermethylation and lack of \( DCC \) expression.

In a study investigating the methylation and expression levels of the \( APC \) and \( SFRP \) genes, both genes were highly methylated in MEC but not in adjacent normal tissue. There was significant correlation between methylation and low \( SFRP1 \) expression. Methylation of the \( SFRP1 \) gene was the main cause of decreased \( SFRP1 \) expression. Beta-catenin expression was also associated with reduced \( SFRP1 \) expression. In addition, both \( SFRP1 \) and beta-catenin expression were associated with tumor grade and stage. Survival was particularly poor in patients with reduced \( SFRP1 \) and cytoplasmic/nuclear beta-catenin expression. It has been suggested that detection of \( SFRP1 \) expression and aberrant beta-catenin expression in the cell may be useful biomarkers of tumor progression and prognosis in patients with MEC (Lee et al. 2010).

Alterations in \( TGF-\beta \) signaling are common in HNSCC. Hypermethylation of \( TGF-\beta \) type I receptor (\( TGFBR-I \)) gene was evaluated via MSP and restriction enzyme-mediated PCR (MSRE). \( TGFBR-I \) expression was lost in 83% of the HNSCC tumors and was linked to DNA hypermethylation of the CpG-rich promoter region in 62% of the samples (Muñoz-Antonia et al. 2009).

\( NDRG2 \) is a candidate TSG involved in oral squamous cell cancers via the Akt signaling pathway. Reduced \( NDRG2 \) mRNA levels, caused by promoter methylation, have been reported in most of the OSCC patients and in several cases of precancerous leukoplakia with dysplasia (Furuta et al. 2010).

Hypermethylation of the \( FancB \) gene has been observed in sporadic HNSCC tumors (Smith et al. 2010), while methylation of the \( SYK \) gene has been frequently observed in OSCC; the downregulation of \( SYK \) expression due to promoter methylation was associated with metastasis (Ogane et al. 2009). \( MGMT \) gene methylation is observed in laryngeal cancer (Zhang et al. 2006, 2004) and HNSCC (Zuo et al. 2004), but not in normal larynx tissue.

The \( MAL T1 \) gene, responsible for activating nuclear factor-kappaB in lymphocyte lineages, is located in a genomic region encoding putative TSG and is expressed in the nucleus of oral epithelial cells. Absence of expression due to epigenetic inactivation during tumor progression has been associated with tumor recurrence and poor patient survival, suggesting that analysis of \( MAL T1 \) expression may be a useful predictive and prognostic marker for OSCC (Chiba et al. 2009).

In primary OSCC, aberrant methylation of the \( R A S S F 2 \) gene and a high frequency of \( ROBO1 \) methylation have been reported in early dysplastic lesions of the head and neck (Imai et al. 2008; Ghosh et al. 2009). A significant inverse correlation between \( R A S S F 1 A \) promoter methylation and HPV infection in HNSCC has been shown (Dong et al. 2003).

\( GAL R1 \) methylation is observed in primary HNSCC tumors and correlates with decreased \( GAL R1 \) expression, as well as increased tumor size, lymph node status, tumor stage, \( CC N D 1 \) expression, \( p l 6 \) methylation, and survival (Misawa et al. 2008).

Methylation of the \( C D H 1 \) gene promoter has been reported in tongue cancer (Chang et al. 2002), salivary gland adenoid cystic carcinoma (Zhang et al. 2007a), and nonmetastatic oral cancer as an early event (De Moraes et al. 2008; Maeda et al. 2007a; Yeh et al. 2002).

Expression of the \( R A R \beta \) gene has been studied in primary tissue specimens of different anatomical sites from patients with HNSCC and a strong correlation was found between hypermethylation and reduced expression of \( R A R \beta 2 \) (Youssef et al. 2004). In particular, significantly lower hypermethylation and higher \( R A R \beta 2 \) mRNA expression levels when compared to the tumors located at other sites of the head and neck were observed in tumors from the hypopharynx (Olasz et al. 2007) and salivary duct or acinic cell carcinomas displaying \( R A S S F 1 \) methylation (Williams et al. 2006).

Genes with different functions

\( C H F R \) is a putative early mitotic checkpoint gene which causes a delay in chromosome condensation in response to mitotic stress. In a study, aberrant promoter methylation of the \( C H F R \) gene was reported in patients with OSCC, while the gene was methylated minimally in the surrounding normal mucosa and no methylation was observed in the \( C H F R \) promoter in a healthy cohort (Baba et al. 2009; Toyota et al. 2003).

The \( H I C 1 \) gene plays a role in the regulation of transcription. A study using MSP has reported that \( H I C 1 \) is highly (95%) methylated in HNSCC and reexpression of the gene was associated with decreased aggressiveness (Brieger et al. 2010). On the other hand, in a recent study from our group, no significant methylation of the \( H I C 1 \) gene was observed in patients with HNC using a more sensitive technique (Yañiz et al. 2011).

The \( C Y G B \) gene was first described as an intracellular globin of unknown function (Burmester et al. 2002). \( C Y G B \) downregulation is a key event in the familial cancer syndrome of the upper aerodigestive tract. Increased expression of the \( C Y G B \) gene displays an inverse correlation with promoter methylation and a strong correlation with tumor hypoxia. It has been consistently associated with aggressive tumors in oral and oropharyngeal squamous cell carcinoma when compared with histologically tumor-free surgical margins (Shaw et al. 2009).
In individual studies, aberrant methylation of RBB1 (Kishi et al. 2005) and 14-3-3 sigma (Uchida et al. 2004; Gasco et al. 2002) in salivary gland cancer, Apaf-1 (Huang et al. 2004) and DAPK (Zhang and Kong 2004) in laryngeal squamous cell carcinoma, TSC2 (Chakraborty et al. 2008), SFRP1, SFRP2, SFRP5 (Sogabe et al. 2008), RECK (Long et al. 2008), EpCAM (Shiah et al. 2009), MTNR1A (Nakamura et al. 2008), IKKalpha (Shiah et al. 2009), and PGP9.5 (Tokumaru et al. 2008) have also been reported. miR-137 plays an important role in the cell cycle control. It has been shown that miR-137 and miR-193a are epigenetically silenced during oral carcinogenesis (Kozaki et al. 2008). Methylation of miR-137 has been reported in squamous cell carcinoma tissue and oral rinse samples and was associated with gender and inversely associated with body mass index (Langevin et al. 2010).

Promoter methylation of the RASSF1A, DAPK, and RAR/32 genes was analyzed by MSP in primary NPC tumors and normal nasopharyngeal epithelia. All genes were highly methylated in tumor tissue, whereas methylation was not observed in the normal nasopharyngeal tissue. Methylation of the three genes was significantly associated with lymph node involvement. RASSF1A and RAR/32 methylation also correlated with age at diagnosis, T stage, and histological type (Fendri et al. 2009). Epigenetic silencing of cellular retinol-binding proteins, CRBP1, CRBP1K, and RAR/32 are also commonly observed in NPC tumor samples (Kwong et al. 2005a, 2002).

High methylation frequencies for the DAPK, RASSF1A, CDH1, and p16 genes are frequently observed in NPC tumors (Kwong et al. 2002; Chang et al. 2003a). In contrast to HNSCC (Dikshit et al. 2007), in NPC, no change has been reported in the RUNX3 promoter region, whereas the p16, RASSF1A, CDH1, and hMLH1 gene promoters were frequently methylated (Tan et al. 2006).

In the patients with NPC from Southeast Asia but not from North Africa, methylation of p16, DLEC1, BLU, and CDH1 genes were found to be associated with the juvenile and adult forms of the disease. Strong correlations were observed between aberrant promoter methylation of the CDH1 and BLU genes and lymph node invasion (Li et al. 2003a, b) or undifferentiated tumors, respectively (Ayadi et al. 2008).

**Nasopharyngeal carcinoma and studies of TSG gene panels**

Nasopharyngeal carcinoma (NPC) is a rare malignancy with unique genetic, viral, and environmental characteristics that distinguish it from other types of head and neck carcinoma. It has a different etiology, epidemiology, prognosis, and therapy. The clinical management of NPC remains challenging largely due to the lack of early detection strategies for this tumor (Loyo et al. 2011; Razak et al. 2010).

A recent study investigating a panel of 18 marker genes in nasopharyngeal tumors has shown that the methylation status of the AIMP1, APC, CALCA, DCC, DLEC, DLC1, ESR, FHIT, KIF1A, and PGP9.5 genes were significantly associated with NPC when compared with other tumors or the benign nasopharyngeal biopsy samples (Loyo et al. 2011).

In an 11-gene panel, promoter methylation levels of CDH1, p15, THBS1, RASSF1A, MLH1, MGMT, p16, and TP73 genes were significantly higher in the tumor samples from patients with NPC when compared with the lymphocytes from the same individuals (Wong et al. 2003a).

Using the discovery approach after expression profiling among eight potential candidate TSG, promoter methylation of only three genes (CCNA1, RARRES1, and HRASLS) have been significantly associated with NPC (Yanatasaneejit et al. 2008).

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**Single-gene studies in NPC**

**Tumor suppressor genes**

WIFI1 is a highly conserved gene on chromosome 12 and encodes a protein of the sFRP family, which inhibits the Wnt signaling pathway (Kawano and Kypta 2003). Inhibition of Wnt signaling induces apoptosis and inhibits tumor growth in many cancer types (He et al. 2004). Silencing of the WIFI1 gene by hypermethylation may result in the activation of some tumors (Suzuki et al. 2004; Caldwell et al. 2004; Ai et al. 2006; Taniguchi et al. 2005). The WIFI1 promoter region is highly methylated in nasopharyngeal tumors, whereas no methylation is observed in the normal mucosa (Fendri et al. 2010; Chan et al. 2007). WIFI1 methylation has been found to be associated with tumor size, node involvement and metastasis, and age (Fendri et al. 2010).
Expression of the **MIPOL1** gene is also downregulated in some NPC tumors via promoter hypermethylation and allelic loss (Cheung et al. 2009). Likewise, **DAB2** is frequently methylated in NPC, which correlates with the loss of expression in NPC tumors (Tong et al. 2010).

Two studies investigating the **RASSF1A** gene have shown that the gene is highly methylated in primary NPC but not in normal nasopharyngeal epithelia (Wang et al. 2009; Lo et al. 2001). It has been suggested that aberrant hypermethylation of **RASSF1A** and high Epstein–Barr virus (EBV) load may play an important role in NPC carcinogenesis (Zhou et al. 2005).

Methylation of **CDH1** is more frequently observed in advanced stages of NPC (Niemhom et al. 2008). Hypermethylation of **CDH1** promoter and presence of EBV are predominantly detected in undifferentiated and nonkeratinizing NPC compared to squamous cell NPC. Most of the NPC samples demonstrating **CDH1** hypermethylation were EBV-positive, whereas the EBV genome and hypermethylation were not detected in normal nasopharyngeal tissue when **CDH1** methylation was absent, indicating a correlation between **CDH1** hypermethylation and EBV infection (Niemhom et al. 2008; Krishna et al. 2005; Li et al. 2003a, b; Tsao et al. 2003; Kao et al. 2002).

Genes with various functions

**14-3-3 sigma**, the downstream target of p53, is a negative regulator of cell cycle G2–M phase checkpoint in response to DNA damage. By MSP, 100% methylation of **14-3-3 sigma** was shown in tumor tissue but not in any of adjacent normal nasopharyngeal epithelial tissue (Yi et al. 2009). Analysis by real-time PCR, Western blotting, and immunohistochemistry have revealed that **14-3-3 sigma** expression is downregulated or absent in NPC samples displaying high methylation. In addition, hypermethylation of **14-3-3 sigma** has been associated with lymph node and distant metastasis.

Methylation of the **PGP9.5** gene is frequently detected in primary NPC but only minimally observed in normal nasopharyngeal tissue, indicating that the methylation-mediated silencing of **PGP9.5** may be important in nasopharyngeal carcinogenesis (Li et al. 2010). The **EDNRB** gene, located on chromosome 13q22, was highly methylated in primary NPC tumor samples, while no methylation was observed in normal nasopharyngeal epithelia (Zhou et al. 2007; Lo et al. 2002). In patients with NPC, high methylation of the **LARS2** gene, which is located at the chromosome 3 common eliminated region-1 (**C3CER1**) on 3p21.3, has been reported (Zhou et al. 2009).

Several genes which have been studied in gene panels were also evaluated in single-gene studies. **RASSF2** (Zhang et al. 2007b), **DLC1** (Seng et al. 2007), **DAPK** (Kong et al. 2006; Wong et al. 2002), **CHFR** (Cheung et al. 2005), **TIG1** (Kwong et al. 2005b), **BLU** (Qiu et al. 2004; Liu et al. 2003a, b), and **p16** (Lo et al. 1996) are among those. The corresponding data are given in Table 3. Other newly identified TSG are **IRF8** (Lee et al. 2008), **ADAMTS18**, which is a novel gene located on 16q23 (Jin et al. 2007), **LTF** (Yi et al. 2006), **CDH13** (Sun et al. 2007), **PCH10** (Ying et al. 2006), **TSLC1** (Hui et al. 2003), **HIN-1** (Wong et al. 2003b), and **RIZ1** (Chang et al. 2003b). The **GNAT1** gene has been shown to be methylated not only in all primary NPC tissue samples but also in 80% of tissue samples with chronic nasopharyngitis (Yi et al. 2007).

**Conclusions**

Biomarkers predicting clinical response, tumor recurrence, or patient survival are not available for HNC. Further studies are needed to identify new biomarkers for early detection and prediction of the therapeutic response or prognosis. Follow-up studies using quantitative MSP analysis, global methylation profiling, and detailed analysis of downstream DNA repair genes regulated by promoter methylation may provide new insight into the issue. Considering the great heterogeneity of HNC, a combination of multiple genes for analysis may provide a higher coverage for diverse tumors than the analysis of a single gene (Carvalho et al. 2008). Recent data indicate that promoter hypermethylation of the **KIF1A** and **EDNRB** genes is a frequent event in primary HNSCC and combining only the **KIF1A** and **EDNRB** genes provides a higher specificity and sensitivity than using a panel of 10 different genes (Demokan et al. 2010). Preferential methylation of these genes in salivary rinses from HNSCC patients may provide a promising potential biomarker for the disease. Other genes investigated frequently and found to display significant differential methylation are **RASSF1A**, **DAPK1**, **MGMT**, **RARβ**, **CDH1**, **hMLH1**, and **CHFR**, although a wide range of different methylation ratios have been reported. These variations most probably result from the differences in the sensitivity of the methods, variations in the processing of the samples, and composition of the patient cohorts.

Methylation analysis has the additional advantage that methylation patterns are not affected by external factors or temporary physiological changes. They persist and are usually increased during disease progression. The higher specificity and sensitivity provides a suitable tool to obtain predictive or prognostic information. So far, the information about DNA methylation has not translated into useful and reliable markers for HNC in the clinical practice. However, given the sensitive and high-throughput quantitative methodologies for methylation analysis, specific markers for HNC will certainly
| Gene | Name | Function | Tumor type | Method | Samples | Methylation range (%) |
|------|------|----------|------------|--------|---------|-----------------------|
| ADAM23 | ADAM metallopeptidase domain 23 | Cell–cell and cell–matrix interactions | NPC (Calmon et al. 2007) | T (Calmon et al. 2007) | 42 |
| ADAMTS18 | A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 18 | Tumor suppression | NPC (Jin et al. 2007) | T (Jin et al. 2007) | 70 |
| AIM1 | Absent in melanoma 1 | Tumor suppression | NPC (Loyo et al. 2011) | T (Loyo et al. 2011) | 30 |
| APOF1 | Apoptotic peptidase activating factor 1 | Apoptosis | LC (Huang et al. 2004) | T (Huang et al. 2004) | 40 |
| ATM | Ataxia telangiectasia mutated | Regulating DNA damage response mechanism | HNC (Ai et al. 2004) | T (Ai et al. 2004) | 25 |
| BMP2A | Bone morphogenetic protein 2A | Induction of cartilage and bone formation | HNSCC (Tokumaru et al. 2004) | T (Tokumaru et al. 2004) | 25 |
| C/EBPalpha | Tumor suppressor CCAAT/enhancer binding protein alpha | Transcriptional regulation | HNC (Bennett et al. 2007) | T (Bennett et al. 2007) | 68 |
| CALCA | Calcitonin-related polypeptide alpha | Calcium regulation and phosphorus metabolism | NPC (Loyo et al. 2011) | T (Loyo et al. 2011) | 44 |
| CHD13 | Cadherin 13 | Calcium-dependent cell adhesion | NPC (Sun et al. 2007) | (TNE) (Sun et al. 2007) | 89.7 |
| CRABP2 | Cellular retinoic acid binding protein 2 | Retinoic acid-mediated regulation of human skin growth and differentiation | HNSCC (Calmon et al. 2009) | (TL) (Calmon et al. 2009) | 58 |
| CRBP1 | Cellular retinol-binding protein type I | Intracellular transport of retinol | NPC (Kwong et al. 2005b) | T (Kwong et al. 2005b) | 87.8 |
| CRBPIV | Cellular retinol-binding protein type IV | Intracellular transport of retinol | NPC (Kwong et al. 2005b) | T (Kwong et al. 2005b) | 54 |
| CYGB | Cytoglobin | Transfer of oxygen from arterial blood to the brain | OSCC (Shaw et al. 2006) | PS (Shaw et al. 2006) | 65 |
| CYPIA1 | Cytochrome P450 family 1A | Carcinogen metabolism | HNC (Sharma et al. 2010) | (TNE) (Sharma et al. 2010) | 39.7 |
| CYPIA13 | Cytochrome P450, family 2, subfamily A, polypeptide 13 | Carcinogen metabolism | HNC (Sharma et al. 2010) | (TNE) (Sharma et al. 2010) | 27.4 |
| DAB2 | Human disabled-2 | Signal transduction | NPC (Tong et al. 2010) | T (Tong et al. 2010) | 65 |
| DBCCRI | Deleted in bladder cancer 1 | Inhibition of cell proliferation, involvement in cell death, and regulation of plasminogen pathway | OSCC, PM-OCL (Gao et al. 2004) | (TLMN) (Gao et al. 2004) | 44 |
| EpCAM | Epithelial cell adhesion molecule | Homotypic calcium-independent cell adhesion | OSCC (Shiah et al. 2009) | T (Shiah et al. 2009) | 51 |
| ESR | Estrogen receptor alpha | Hormone binding, DNA binding and activation of transcription | NPC (Loyo et al. 2011) | T (Loyo et al. 2011) | 26 |
| FancB | Fanconi anemia, complementation group B | DNA repair | HNSCC (Smith et al. 2010) | (TNE) (Smith et al. 2010) | 31 |
| GALR1 | Galanin receptor 1 | Tumor suppression | HNSCC (Misawa et al. 2008) | T (Misawa et al. 2008) | 38 |
| GNA11 | Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1 | Signal transduction | NPC (Yi et al. 2007) | (TCINT) (Yi et al. 2007) | 100 |
| Gene | Name | Function | Tumor type | Method | Samples | Methylation range (%) |
|------|------|----------|------------|--------|---------|-----------------------|
| GSTM1 | Glutathione S-transferase mu 1 | Carcinogen metabolism | HNC (Sharma et al. 2010) | MSP (Sharma et al. 2010) | (T,NE) (Sharma et al. 2010) | 58.1 |
| HpC | Hypermethylated in cancer 1 | Transcriptional regulation | HNSCC (El-Naggar et al. 1997) | MSP (El-Naggar et al. 1997) | T (El-Naggar et al. 1997) | 9 |
| HRASLS | HRAS-like suppressor | Formation of peripheral nerve trajectories | NPC (Yanatsaneejit et al. 2008) | MSP-COBRA (Yanatsaneejit et al. 2008) | (T,NE,L) (Yanatsaneejit et al. 2008) | 17 |
| Ikkalpha | IkappaB kinase-alpha | Interferon-gamma signaling pathway | OSCC (Maeda et al. 2007b) | BS (Maeda et al. 2007b) | (T,NE) (Maeda et al. 2007b) | 63 |
| IRF8 | Interferon regulatory factor 8 | Transcriptional regulation | NPC (Lee et al. 2008) | MSP (Lee et al. 2008) | T (Lee et al. 2008) | 78 |
| LARS2 | Leucyl-tRNA synthetase 2, mitochondrial | The action of a catalyst | NPC (Zhou et al. 2009) | PCR-SSCP (Zhou et al. 2009) | T (Zhou et al. 2009) | 64 |
| LHX6 | LIM homeobox 6 | The specification of cortical interneuron subtypes and in the migration of GABAergic interneuron precursors | HNC (Estécio et al. 2006) (MS/AP-PCR) (Estécio et al. 2006), COBRA (Estécio et al. 2006) | (Estécio et al. 2006) | (T,NE) (Estécio et al. 2006) | 65.6 |
| LRP1B | Low-density lipoprotein receptor-related protein 1B | Process of receptor-mediated endocytosis | OSCC (Nakagawa et al. 2006) | COBRA (Nakagawa et al. 2006) | T (Nakagawa et al. 2006) | 43 |
| LTF | Lactotransferrin | Antimicrobial activity | NPC (Yi et al. 2006) | MSP (Yi et al. 2006) | T (Yi et al. 2006) | 43 |
| LTP1 | LTP1 | MALT1 | OSCC (Chiba et al. 2009) | MSP (Chiba et al. 2009) | T (Chiba et al. 2009) | 43 |
| LTP2 | LTP2 | MALT1 | OSCC (Chiba et al. 2009) | MSP (Chiba et al. 2009) | T (Chiba et al. 2009) | 43 |
| LTP27 | LTP27 | MALT1 | OSCC (Chiba et al. 2009) | MSP (Chiba et al. 2009) | T (Chiba et al. 2009) | 43 |
| MALT1 | MALT1 | Maltose-binding protein 1 | Reproductive and circadian actions of melatonin | OSCC (Takahama et al. 2008) | T (Takahama et al. 2008) | 43 |
| MAP4K1 | MAP4K1 | N-myc downstream-regulated gene 2 | Reproductive and circadian actions of melatonin | OSCC (Takahama et al. 2008) | T (Takahama et al. 2008) | 43 |
| Gene | Name                        | Function                                                                 | Tumor type      | Method       | Samples           | Methylation range (%) |
|------|-----------------------------|---------------------------------------------------------------------------|-----------------|--------------|-------------------|-----------------------|
| NmU  | Neuromedin U                | Stimulation of contractions of the ileum and urinary bladder              | HNSCC (Tokumaru et al. 2004) | BS (Tokumaru et al. 2004) | T (Tokumaru et al. 2004) | 20                    |
| OGDHL| Otoglutarate dehydrogenase-like | Citric acid cycle                                                          | HNSCC (Hoque et al. 2008) | QMSP (Hoque et al. 2008) | T (Hoque et al. 2008) ) (SA-H,NE) (Hoque et al. 2008) | 8                     |
| PAK3 | p21-activated protein kinase 3 | Cytoskeleton reorganization and nuclear signaling                         | HNSCC (Hoque et al. 2008) | QMSP (Hoque et al. 2008) | T (Hoque et al. 2008) ) (SA-H,NE) (Hoque et al. 2008) | 52                    |
| PCDH10| Protocadherin 10            | Calcium-dependent cell adhesion                                            | NPC (Ying et al. 2006) | MSP (Ying et al. 2006) | T (Ying et al. 2006) ) (SA-H,NE) (Hoque et al. 2008) | 8                     |
| PRTFDC1| Phosphoribosyl transferase domain containing 1 | Tumor suppression                                                        | OSCC (Suzuki et al. 2007) | COBRA (Suzuki et al. 2007) | (T,MN) (Steinmann et al. 2009) | 13                    |
| RASSF4| Ras association (RatGDS/AF-6) domain family member 4 | Tumor suppression                                                        | HNSCC (Steinmann et al. 2009) | COBRA (Steinmann et al. 2009) | (T,MN) (Steinmann et al. 2009) | 58                    |
| RASSF5| Ras association (RatGDS/AF-6) domain family member 5 | Tumor suppression                                                        | HNSCC (Steinmann et al. 2009) | COBRA (Steinmann et al. 2009) | (T,MN) (Steinmann et al. 2009) | 58                    |
| RBI  | Retinoblastoma 1            | Tumor suppression                                                         | SGC (Kishi et al. 2005) | MSP (Kishi et al. 2005) | T (Kishi et al. 2005) | 41.7                  |
| RECK | Reversion-inducing cysteine-rich protein with kazal motifs               | Suppression of tumor invasion and metastasis                              | OSCC (Long et al. 2008) | MSP (Long et al. 2008) | (T,MN,NE) (Long et al. 2008) | 52                    |
| RIZI | Retinoblastoma protein-interacting zinc finger protein                    | Transcriptional activation                                                | NPC (Chang et al. 2003a) | MSP (Chang et al. 2003a) | (T,L,SA-P) (Chang et al. 2003a) | 60                    |
| ROBO1| Roundabout, axon guidance receptor, homolog 1 (Drosophila)              | Tumor suppression                                                         | (HNSCC) (Ghosh et al. 2009) (PM-HNL) (Ghosh et al. 2009) | MSRE (Ghosh et al. 2009) | T (Ghosh et al. 2009) | 21                    |
| SFRP2| Secreted protein 2          | Inhibition of Wnt signaling                                               | OSCC (Sogabe et al. 2008) | MSP (Sogabe et al. 2008) | (T, MN) (Sogabe et al. 2008) | 36                    |
| SFRP5| Secreted protein 5          | Inhibition of Wnt signaling                                               | OSCC (Sogabe et al. 2008) | MSP (Sogabe et al. 2008) | (T, MN) (Sogabe et al. 2008) | 16                    |
| SOCS3| Suppressor of cytokine signaling 3                                      | Signal transduction                                                      | (HNC, PM-HNC) (Weber et al. 2005) | MSP (Weber et al. 2005) | (TMN,LN) (Weber et al. 2005) | 90                    |
| STAT1| Signal transducer and activator of transcription 1                      | Signal transduction                                                      | HNC (Xi et al. 2006) | MSP (Xi et al. 2006) | (T,L) (Xi et al. 2006) | 75                    |
| SYK  | Spleen tyrosine kinase      | Signal transduction                                                      | OSCC (Ogane et al. 2009) | QMSP (Ogane et al. 2009) | (TMN) (Ogane et al. 2009) | 63                    |
| TGFBR1| Transforming growth factor, beta receptor 1                             | Signal transduction                                                      | HNSCC (Muñoz-Antonia et al. 2009) | MSP (Muñoz-Antonia et al. 2009) | T (Muñoz-Antonia et al. 2009) | 62                    |
| TCF21| Transcription factor 21                                              | Epithelial–mesenchymal interactions                                      | HNC (Smith et al. 2006) | COBRA (Smith et al. 2006) | (TMN) (Smith et al. 2006) | 76                    |
| TSC2 | Tuberous sclerosis 2        | Tumor suppression                                                         | OSCC (Chakraborty et al. 2008) | COBRA (Chakraborty et al. 2008) | (TMN) (Chakraborty et al. 2008) | 100                   |
| TSCL1| Tumor suppressor in lung cancer                                      | Tumor suppression                                                         | NPC (Hui et al. 2003) | MSP (Hui et al. 2003) | (T,NE) (Hui et al. 2003) | 34.2                  |
emerge by careful evaluation and combination of different marker panels to achieve a high sensitivity for the disease. Over the next years, clinical tests based on diagnostic and therapeutic methylation markers will certainly be available and will be used for the assessment of prognosis, treatment planning, and to predict the response.

Future studies aiming to determine the causative role and significance of these epigenetic alterations may provide important clues into the mechanism and contribution of the specific events and help to establish a sequence of methylation events during tumor development associated with different stages of head and neck carcinogenesis. Since tumor-specific DNA can be easily detected in blood, serum, and saliva, methylation analysis can be used as a noninvasive method for the early detection using a panel of the genes specific for the disease. Furthermore, epigenetic silencing of the genes offers new therapeutic approaches using demethylating agents. Analysis of genome-wide methylation profiles by new high-throughput technologies which enable simultaneous analysis of thousands of genetic loci will certainly help to identify highly specific novel methylation biomarkers.

Conflict of interest The authors declare that they have no conflicts of interest.

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