Predictive value of epigenetic alterations in head and neck squamous cell carcinoma

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Head and neck cancer collectively describes malignant tumors originating from the mucosal surface of the upper aerodigestive tract. These tumors pose a great threat to public health because of their high incidence and mortality. Traditional risk factors are tobacco and alcohol abuse. More recently, infection by high-risk types of human papilloma virus (HPV) has been identified as an additional risk factor, especially for oropharyngeal squamous cell carcinoma (OPSCC). Moreover, HPV-positive OPSCC is considered a distinct tumor entity with an improved clinical outcome compared to HPV-negative OPSCC. Epigenetic alterations act as key events in the pathogenesis of cancer and are of special interest for basic and translational oncology because of their reversible nature. This review provides a comprehensive summary of alterations of the epigenome in head and neck squamous cell carcinoma (HNSCC) with a focus on the methylome (hypomethylation and hypermethylation) and its predictive value in the evaluation of pathologic states and clinical outcome, or monitoring response rates to certain therapies.

Introduction

Head and neck cancer is one of the most prevalent human malignancies, affecting different anatomic sites of the upper aero-digestive tract such as the oral cavity, larynx, and naso-, oro-, and hypopharynx.[1] Despite improvements in early detection, surgical techniques, and radiation and chemotherapeutic regimens and the implementation of multimodal therapy, appropriate treatment of advanced head and neck cancer remains a major challenge. Poor survival mainly results from its aggressive growth behavior and resistance to treatment, which lead to high rates of therapy failure.[2-3] The majority of head and neck cancers present as squamous cell carcinoma (HNSCC) and traditional risk factors are tobacco and alcohol abuse. However, the escalating incidence of oropharyngeal squamous cell carcinoma (OPSCC) in Western countries in the absence of a parallel rise in smoking and alcohol consumption suggests the involvement of additional non-traditional behavioral and environmental factors.[1,4] Indeed, infection by high-risk types of human papilloma virus (HPV), predominantly type 16, has been found to be etiologically associated with OPSCC in an increasing number of patients.[5-7] Numerous studies have confirmed that patients with advanced stage HPV-positive tumors have a significantly better clinical outcome than their HPV-negative counterparts.[8-10] As a consequence, ongoing clinical trials aim to exploit the favorable prognosis for HPV-positive tumors by a focused approach involving de-escalating strategies in order to decrease unnecessary treatment-related toxicities.[11] However, additional molecular biomarkers are needed to improve stratification of OPSCC patients suitable for treatment by de-escalation.[8,12] Furthermore, a better understanding of the molecular principles underlying the differential clinical outcome of HPV-positive and HPV-negative OPSCCs could drive the development of novel targeted therapies for advanced-stage HPV-negative tumors that currently have a dismal prognosis.

Similar to other human malignancies, the development of HNSCC is a multistep process involving the accumulation of genetic and epigenetic alterations that affect the expression and function of numerous oncogenes and tumor suppressor genes.[1,3,13] Recent studies demonstrated characteristic differences in global gene expression profiles between HPV-positive and HPV-negative tumors indicating that a favorable clinical outcome is at least partially due to HPV-related changes in the cancer genome and epigenome.[14-18] In accordance with this assumption, quantitative and qualitative differences in genomic aberrations and somatic mutations[19-24] and epigenomic patterns,[25,26] especially DNA methylation, have been found between HPV-positive and HPV-negative tumors.

Regulation and Function of Epigenetics in Cancer

Epigenetic alterations refer to reversible changes that are reproduced during DNA replication and affect the spatial

conformation of DNA and its transcriptional activity without changing the underlying genetic code. Appropriate cell-type specific epigenetic patterning is required to ensure cell identity during development and tissue homeostasis. Consequently, deregulation of such programming can contribute to human diseases such as cancer.[27,28] There are several known epigenetic mechanisms, including DNA methylation, changes in chromatin conformation, and histone modifications.[29]

DNA methylation involves an enzymatic reaction catalyzed by a family of enzymes named DNA methyltransferases (DNMTs). Three subtypes of DNMT have been identified in humans: DNMT1, DNMT3A, and DNMT3B.[30] DNMT1 is responsible for maintaining the DNA methylation pattern in the daughter cells during mitosis or meiosis, whereas DNMT3A and DNMT3B are involved in the *de novo* methylation process, for example during genomic imprinting. Possible mechanisms involved in the initiation of *de novo* methylation are DNA target sequences, interference RNA, and chromatin changes induced by histones and protein interactions.

The primary targets of DNA methylation are CpG islands, regions rich in CpG dinucleotides that are predominantly present in the promoter region of certain genes. The methylome is profoundly altered in human cancers, with a characteristic loss of global DNA methylation in repetitive regions and concomitant accumulation of gene promoter methylation. Promoter hypermethylation of tumor suppressor genes involved in cellular processes of DNA damage repair, detoxification, cell cycle regulation, and apoptosis is one of the best-characterized epigenetic changes and often leads to transcriptional silencing.[31] In cancer, gene silencing by promoter methylation may occur even more frequently than structural inactivation of genes by deletion or somatic mutations, and represents an attractive target for new therapeutic strategies because of its reversible nature.[31,32] The effect of global DNA hypomethylation is less clear, but is thought to contribute to chromosomal instability and activate the expression of proto-oncogenes.[33-35] In support of this notion, hypomethylation of repetitive elements such as LINE-1 is frequently seen in various types of human cancer and correlates with chromosomal instability. Global DNA hypomethylation was demonstrated for HNSCC compared to normal mucosa,[36] and 3 studies showed a distinct difference in hypomethylation of LINE-1 elements depending on the HPV status.[37-39] These findings indicate a more efficient maintenance of global DNA methylation accompanied by reduced genomic instability in HPV-positive tumors, which is in line with recent studies addressing the quality and quantity of genomic aberrations.[19,20,23,24] Although the exact mechanism of global DNA hypomethylation in cancer cells remains elusive, differences in expression or function of DNMTs may explain HPV-related differences among OPSCCs.[39-41]

Gene Promoter Methylation in HNSCC

Aberrant gene promoter methylation is a hallmark in the pathogenesis of HNSCC, and is partially influenced by tobacco use, alcohol consumption, and virus infection.[42-44] As these aberrant
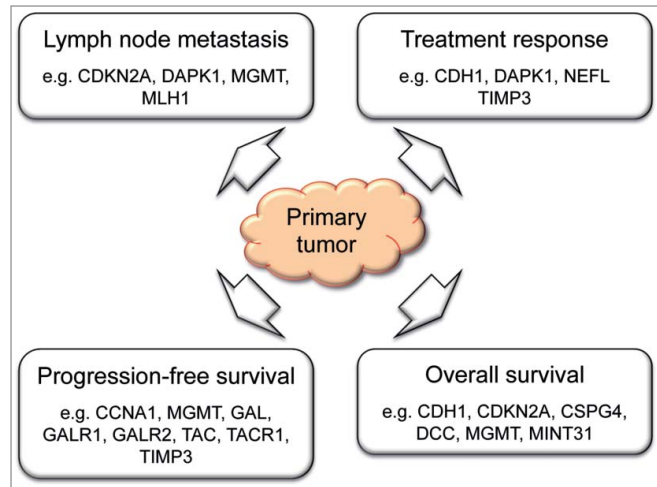

Figure 1. Predictive value of promoter hypermethylation in head and neck squamous cell carcinoma (HNSCC). Aberrant promoter methylation of distinct genes is a hallmark in the pathogenesis of HNSCC. DNA methylation profiling represents an attractive tool to obtain diagnostic or prognostic information regarding lymph node metastasis, treatment response, and progression-free or overall survival.

methylation patterns persist, and usually increase, during disease progression, assessment of methylation profiles is a suitable tool for obtaining diagnostic or prognostic information (**Fig. 1**). Most published studies on promoter methylation in HNSCC evaluated only a limited number of candidate genes, which were selected on the basis of their functional relevance during carcinogenesis. Many genes, such as *CCNA1, CDKN2A, CYGB, DAPK1, DCC, FHIT, GAL, GALR1, GALR2 MGMT, MINT31, MLH1, MST1, NEIL1, NEFL, RARB, RASSF1, RASSF5, SFRP1, TAC1, TACR1, TCF21,* and *TIMP3,* show promoter hypermethylation during neoplastic transformation or malignant progression.[43,45-62] In contrast, there only a few reported examples of genes that show promoter hypomethylation in tumors compared to normal tissue, such as *CSPG4* and *MAGEB2.*[63,64] Aberrant promoter methylation status of many genes has been associated with clinical and pathologic features, such as lymph node metastasis, locoregional control, and overall or disease-free survival.[43,50-52,55-57,59,62,63,65-67] These findings are in part related to a significant correlation between changes in promoter methylation and resistance to treatment with radiation or chemotherapy. For example, promoter methylation of *TIMP3* and *CDH1* in advanced HNSCC treated by radiotherapy predicts a better clinical outcome[68] whereas *NEFL* promoter hypermethylation is associated with resistance to cisplatin-based chemotherapy and predicts decreased overall and disease-free survival for patients treated with cisplatin-based chemotherapy.[61] Finally, promoter methylation of *DAPK1* is associated with resistance to both cetuximab and erlotinib.[69]

HPV-Related Gene Promoter Methylation and Affected Signaling Pathways

Several studies have explored differences in gene promoter methylation profiles between HPV-positive and HPV-negative

HNSCC. However, as above, most reports evaluated only single genes or a limited number of selected genes and did not focus solely on OPSCC, which is the most common site for HPV-related tumors in the upper aerodigestive tract.[25,26] HPV-related promoter hypermethylation was demonstrated for genes involved in cell cycle progression, DNA damage response, mRNA decay, and cell fate decision, such as *CCNA1, RASSF1A, SMG1,* and *TCF21* (**Table 1**).[45,58,70,71] However, few studies provide experimental evidence for the possible involvement of genes affected by HPV-related promoter methylation in determining clinical outcome. Gubanova and colleagues demonstrated HPV-related hypermethylation of *SMG1*, which encodes a PI3K-related kinase involved in the maintenance of genome integrity via genotoxic stress response pathways.[70] *SMG1* gene silencing in HPV-negative HNSCC cells results in increased radiation sensitivity, whereas *SMG1* overexpression protects HPV-positive tumor cells from irradiation.[70] Unquestionably, further studies are needed to confirm the prognostic value of *SMG1* methylation and/or expression in HNSCC in the setting of primary or adjuvant radiation therapy and to test the concept that pharmacologic targeting of SMG1-regulated signaling pathways would improve sensitivity to radiotherapy in HPV-negative HNSCC.

Recent studies focused on comprehensive analysis of gene promoter methylation in HPV-positive versus HPV-negative HNSCC with the aim of gaining a detailed global view of clinically relevant alterations and unraveling associated signaling and gene regulatory networks (**Table 1**). Worsham and colleagues subjected DNA from 4 HPV-positive and 4 HPV-negative primary HNSCCs to genome-wide methylation profiling, revealing a large list of genes that are differentially methylated depending on the HPV status.[72] Although the small number of tumor samples in both subgroups limited data interpretation, functional annotation of genes with a HPV-related methylation pattern suggested differential activity of several signaling pathways, including WNT/β-catenin signaling, epidermal growth factor receptor (EGFR) signaling, retinoic acid signaling, and cell–cell or cell–matrix adhesion.

Lleras and colleagues conducted a genome-wide DNA methylation profiling of primary tumor samples and corresponding adjacent mucosa from 118 HNSCC patients. When these datasets were individually analyzed according to anatomic site of the primary tumor, 460 differentially methylated CpG loci in OPSCC could be identified. Moreover, stratification by HPV status revealed a significantly higher number of differentially methylated CpG loci in HPV-positive OPSCC, suggesting that silencing of genes was less affected in HPV-negative tumors.[73] A trend toward a widespread gain of promoter hypermethylation in HPV-positive tumors was also reported in 2 independent global studies.[41,74] Colacino and colleagues quantified the methylation status at 1,505 CpG sites across 807 genes in a panel of 68 well-annotated HNSCC tumor samples.[74] Unsupervised hierarchical clustering based on methylation patterns identified 6 distinct tumor clusters, which significantly differed with respect to age, HPV status, and 3-year survival. As expected, the cluster enriched for HPV-positive tumors had the best 3-year survival. Thirteen individual CpG sites were found to be significantly associated with HPV status; these sites predominantly affected the expression of genes involved in cell cycle regulation and JAK-STAT signaling. Promoter hypermethylation of *CCNA1, CDH11, GRB7, RUNX1T1, SYBL1,* and *TUSC3* was found in HPV-positive tumors, whereas HPV-related hypomethylation was detected in *ESR2, HSD17B12, JAK3, MGMT, RASSF1, SPDEF,* and *STAT5A*.[74] In a similar study, Lechner and colleagues used samples derived from laser-capture micro-dissection of 42 HNSCCs to generate DNA methylation profiles of HPV-positive and HPV-negative tumors.[41] Methylation data were validated in 2 independent sets of HPV-related HNSCC samples (fresh-frozen samples and cell lines). Gene-set enrichment analysis identified several targets of polycomb repressive complex 2 (PRC2) that were affected by HPV-related methylation, including multiple members of the cadherin superfamily (e.g., *CDH8, CDH15, PCDH8, PCDH9, PCDH10,* and *PCDHB3*) that are implicated in many cancers and cancer-specific processes.

Fertig and colleagues applied integrative data analysis based on DNA methylation and gene expression patterns to infer biologically significant molecular pathways that may be exploited as therapeutic targets.[75] This approach revealed specific epigenetic changes that regulate gene expression in HPV-negative HNSCC and distinguish it from HPV-positive HNSCC. Notably, analysis of these differentially regulated genes indicated that activation of

Table 1. Molecular mechanisms affected by HPV-related promoter methylation in head and neck squamous cell carcinoma (HNSCC)

Cellular mechanism	HPV-related gene promoter methylation	References
Cell adhesion	CDH1, CDH8, CDH11, CDH15, PCDH8, PCDH9, PCDH10, PCDHB3	41, 71, 74
Cell cycle progression	CCNA1, RASSF1A	45, 71, 74
DNA damage response	MGMT	74
mRNA decay	SMG1	70
Cell fate decision	TCF21	58
Cellular signaling		
Hedgehog signaling	GLI1, SMO	75
EGFR signaling	EGFR, ERBB2, ERBB4, EREG, GRB7	72, 74
Estrogen receptor signaling	ESR2, HSD17B12	74
JAK-STAT signaling	JAK3, STAT3, STAT5A	72, 74
Retinoic acid signaling	ALDH1A2 PPARA, PPARG, RXRG, THRB	72, 76
WNT/β-catenin signaling	APC, AXIN1, CD44, DDK3, FZD2, FZD3, GPC1, GSK3B, KREMEN2, SDC2, SFRP2, SFRP4, TCF7, WIF1, WNT3, WNT3A, WNT5A, WNT7A, WNT9B, WNT10A, WNT11, WNT16	72, 75

the Hedgehog pathway was specific for HPV-negative HNSCC, which was confirmed by increased levels of GLI1, the primary Hedgehog target, in HNSCC compared to normal mucosa with the highest GLI1 expression in HPV-negative tumors.[75]

Finally, our group applied an array-based approach to monitor global changes in CpG island hypermethylation between HPV-negative and HPV-positive OPSCCs and identified a specific pattern of differentially methylated regions that critically depends on the presence of viral transcripts.[76] A combined promoter methylation profile of low methylation levels in *ALDH1A2* and *OSR2* genes and high methylation levels in *GATA4, GRIA4,* and *IRX4* genes not only correlated with HPV-related tumors, but also served as a reliable methylation signature for improved progression-free and overall survival in 3 independent patient cohorts.[76] A common feature of all 5 genes (*ALDH1A2, OSR2, GATA4, GRIA4,* and *IRX4*) that constitute the methylation signature of our study is their involvement in tissue development and regeneration, and 4 of these genes (*ALDH1A2, OSR2, GATA4,* and *IRX4*) are related to retinoid metabolism and signaling. Since retinoic acids exert potent effects on cell growth, differentiation, and apoptosis, pharmaceutical modulation of retinoid metabolism and signaling pathways may offer a novel approach to targeted treatment of HPV-negative HNSCC.

Alternative Resources for Assessment of DNA Methylation Profiles

Local recurrence develops in a considerable number of patients with HNSCC despite an apparent complete excision and histopathologic tumor-free surgical margins. This can be at least partly explained by the existence of cancer cells in negative surgical margins that are not detectable by conventional microscopic inspection. Assessment of DNA methylation profiles of the surgical margins could serve as an additional and more sensitive strategy for determining appropriate treatment and minimizing morbidity. Indeed, quantitative profiling of candidate genes, including *CCNA1, CDH1, CDKN2A, DAPK1, DCC, MGMT,* and *RASSF1A,* revealed that hypermethylation patterns in surgical margin specimens from HNSCC patients predicted local recurrence and clinical outcome.[77,78]

The ability to study solid cancers through noninvasive sampling of body fluids is one of the most exciting and rapidly advancing developments in cancer diagnostics and monitoring treatment response.[79] This is now possible as a result of major technologic advances including the isolation of intact cancer cells and assessment of cancer cell-derived DNA from body fluid. Accordingly, analysis of DNA methylation profiles using blood samples or salivary rinses of HNSCC patients is a promising method that offers diagnostic as well as prognostic value. Moreover, its minimally invasive nature enables easy access to samples for longitudinal monitoring of patients for assessment of therapy response and tumor relapse. In one study, *DAPK1* hypermethylation was analyzed in tissue and blood samples of 77 patients with oral precancerous lesions and 32 patients with oral SCC.[80] The frequency of promoter hypermethylation was significantly higher in patients with oral SCC compared to those with precancer lesions and there was a strong correlation between hypermethylation frequencies in tumor tissue and blood samples. Corresponding DNA methylation patterns between paired tumor and serum samples were also demonstrated for *CDKN2A, DAPK1,* and *MGMT* in a panel of 50 HNSCC patients.[46] Notably, 5 patients with hypermethylated serum DNA developed distant metastasis, whereas metastasis occurred in only one patient who was negative for serum promoter hypermethylation. Frequent hypermethylation of several candidate genes, including *CDH1, CDKN2A, CDKN2B,* and *DAKP1,* was also found in plasma samples of patients with nasopharyngeal carcinoma (NPC) before treatment. Post-treatment, hypermethylation of at least 1 of 3 genes (*CDH1, CDKN2A,* and *DAPK1*) was detectable in plasma samples of 38% of NPC patients with tumor recurrence, but not in patients without recurrence.[81] In summary, these findings demonstrate that DNA hypermethylation in blood samples could be used as a serologic tumor biomarker for early detection of malignant progression as well as locoregional recurrence and distant metastasis (**Fig. 2**).

Promoter hypermethylation of selected genes, including *CDH1, CDKN2A, DAPK1, EDNRB, MGMT, RASSF1A,* and *TIMP3,* was also assessed in salivary rinses of patients with HNSCC in order to
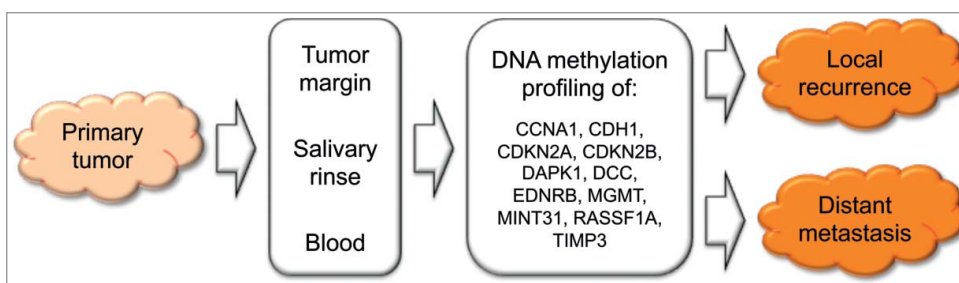

Figure 2. Assessment of promoter methylation profiles using DNA samples from tumor margin or body fluids. DNA obtained from tissue samples during surgical resection (tumor margin) or from body fluids obtained using minimally invasive procedures (blood sample or salivary rinse with or without exfoliating brush) are additional resources with predictive value for diagnosis, prognosis, and treatment decision making for patients with head and neck squamous cell carcinoma (HNSCC). Promoter methylation profiling of DNA derived from tumor margins has been shown to predict patients at high risk for local recurrence and poor clinical outcome and could serve as an additional strategy for the stratification of patients for appropriate treatment, which might in turn minimize morbidity. Promoter methylation profiling of DNA derived from salivary rinses or blood samples not only has considerable diagnostic and prognostic value, but also enables easy access to samples for longitudinal monitoring of patients for assessment of therapeutic response and tumor relapse.

(i) verify whether changes found in tumors could be detected in body fluid containing cells from the oral mucosa and pharynx, and (ii) investigate whether these changes could be used as reliable biomarkers for HNSCC surveillance and early detection of locoregional tumor recurrence.[82-86] As an example, Sun and colleagues assessed the methylation status of a candidate gene panel (*CDKN2A, CCNA1, DAPK1, DCC, MGMT, MINT31,* and *TIMP3*) in salivary rinses of 197 HNSCC patients before treatment.[87] Univariate analysis revealed significant associations of *CCNA1, MGMT,* and *MINT31* hypermethylation with poor overall survival, *TIMP3* hypermethylation with local recurrence-free survival, and *MINT31* hypermethylation with poor disease-free survival. In multivariate analysis, only hypermethylation of *TIMP3* in salivary rinse had an independent and significant association with local recurrence-free survival. A significant association between *TIMP3* methylation in salivary rinse and local recurrence-free survival was also evident in another independent study using samples collected 6 months after the last curative treatment.[88] In this study, multivariate analysis confirmed for the first time that *TIMP3* promoter hypermethylation in post-treatment salivary rinse represents an independent prognostic marker for local recurrence-free survival in patients with HNSCC. These findings justify detection of DNA hypermethylation in saliva as a reliable tool for identifying and monitoring subgroups of HNSCC patients with a high risk of treatment failure (**Fig. 2**).

Use of an exfoliating brush as an alternative method to obtain clinically relevant material might improve DNA methylation profiling for the diagnosis and monitoring of HNSCC. An exfoliating brush allows sampling of the full thickness of stratified squamous epithelium of the oral and oropharyngeal mucosa with the advantage of being minimally invasive and not requiring local anesthetic. Longo and colleagues demonstrated frequent hypermethylation of *CCNA1, DCC,* and *TIMP3* in exfoliated tumor cells collected from a cohort of 96 HNSCC patients and reported that hypermethylation of *DCC* might have value as a prognostic indicator for local recurrence-free survival.[89] A recent study evaluated the concordance of promoter hypermethylation of candidate genes, including *CDKN2A, DAPK1, DCC, MGMT, MINT31,* and *TIMP3*, between salivary rinses collected with or without an exfoliating brush from identical HNSCC patients and revealed a highly significant positive correlation between data obtained using the 2 methods.[90]

heterogeneity hampers accurate prognosis, treatment planning, and identification of causative genes that may serve as molecular drug targets. The rapid development and implementation of next-generation sequencing platforms has revolutionized the identification of complex patterns of genomic aberrations and somatic mutations in cancer, including HNSCC.[19-21,91,92] Clinical and experimental studies provide compelling evidence that extensive global reprogramming of epigenetic patterns is as important in neoplastic transformation and the manifestation of cancer as alterations in the genomic coding information itself. Changes in DNA methylation profiles have been extensively investigated in HNSCC as a reflection of aberrant epigenetic regulation of tumor suppressor genes and oncogenes.[25,26,93,94] However, many studies to date have focused on either single genes or a limited number of selected candidate genes to demonstrate the predictive value of these epigenetic events in tumor diagnostic and prognosis, or in monitoring treatment response and therapy failure. New high-throughput technologies to specifically identify and quantitatively assess global epigenetic alterations are now available, enabling an integrative approach and a better understanding of the molecular principles underlying the complex interconnections among the cancer genome, epigenome, and transcriptome.[95] Head and neck oncology is entering an exciting new era of comprehensive studies that will unravel new molecular principles of HNSCC development and progression, and yield novel concepts for individualized and targeted therapy.[96,97]

Moreover, the large number of mutations found in epigenetic pathways point to a mechanistic link between a gene defect and establishment and maintenance of epigenetic patterns.[95] This makes epigenetic deregulation an attractive target for pharmaceutical intervention strategies[98-101]. Nonetheless, pleiotropic effects are a major concern when using epigenetically active drugs because general targeting of epigenetic mechanisms such as DNMT inhibition may lead to broad global effects on gene expression. A major challenge for the future will be the development of more specific intervention strategies to target epigenetic alterations at certain specific genomic loci rather than to restore global epigenetic patterns.

Conclusion and Future Perspectives

Epidemiologic and clinical studies have highlighted an unexpected heterogeneity in HNSCC regarding its etiology as well as environmental, cellular, and molecular features. This

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