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Epigenetics in Head and Neck Squamous Cell Carcinoma

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1. Introduction

In addition to the genetic information required to establish an organism, recent decades have unveiled a previously unknown type of chromatin modification, known as epigenetic, which is defined as heritable DNA changes that are not encoded in the sequence itself. Unlike genetic modifications, the epigenetic ones are reversible, and increasingly appear to serve fundamental roles in cell differentiation and development.

It is increasingly evident that genetics alone cannot explain the complexity of phenotypes in the living world. Heritable phenotypic characteristics that are not caused by DNA sequence alterations represent the object of epigenetics and include potentially reversible changes such as histone modifications, DNA methylation, and imprinting. At the interface between epigenetics and genomics, a new discipline that is emerging, epigenomics, promises to profoundly change the way we envision phenomena in the biological and medical sciences. Epigenetic modifications can provide an astronomic number of distinct signatures, with huge diagnostic and prognostic value, but it is essential to consider all the different sources of information.

Epigenomics-based diagnostic tools for early cancer detection represent an exciting development. Tumors shed their DNA into the blood, and epigenetic changes that occur early during tumorigenesis, sometimes even in premalignant lesions, can provide valuable biomarkers. Previous research at Epigenomics identified Septin 9 as a single gene in which DNA methylation changes occur very early in colorectal cancer development and are present in the vast majority of tumors of all stages. In most tissues, CpG (cytosine 5-phosphorylated guanine) islands around transcription start sites are largely unmethylated, but their methylation has been described in many tumors and can serve as potential biomarkers. One of the advantages of using epigenomic biomarkers is that, in most cases, DNA methylation changes precede clinical symptoms.

Stephen B. Baylin, M.D., professor of cancer research and deputy director of The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University and colleagues recently presented a molecular model to explain how DNA methylation causes gene silencing in mammalian cells. They used the GATA-4 gene as a model to investigate how polycomb protein complexes and DNA methylation maintain the chromatin in its silent state. They found that polycomb protein occupancy at genomic regions enriched in trimethylated

histone H3 lysine 27 marks establishes long-range interactions by chromatin looping. This finding promises to significantly improve our understanding of higher order chromatin organization and gene silencing both in stem cells and in cancer cells, which share intriguing similarities with respect to chromatin organization.

In addition to the work on acetylation and methylation in the context of cancer epigenetics another interest focuses on the involvement of heat shock proteins (Hsp) as molecular chaperones in cancer. Cancer cell metabolism creates a considerable amount of stress, and one of the main categories, known as proteotoxic stress, is mediated by the misfolded or unfolded protein response. Heat shock proteins are essential in maintaining the correctly folded conformation and activity of oncoproteins, and thus allow cancer cells to survive the stress response. The use of molecular chaperones as therapeutic targets for malignant tumors emerges as an exciting idea, and several Hsp90 inhibitors are currently being investigated as potential anticancer agents.

“Our individual life history is inscribed in our epigenome,” states Toshikazu Ushijima, Ph.D., chief of the carcinogenesis division at the National Cancer Center Research Institute, Tokyo. Dr. Ushijima and collaborators screened genes that were silenced in esophageal squamous cell carcinomas and demonstrated that methylation levels in five promoters are significantly correlated with the duration of tobacco smoking, indicating that chronic smoking induces methylation changes in many of these genes. This finding supports the idea that smoking induces an epigenetic field of cancerization, a term that was previously described for breast, colon, liver, and stomach cancers, and is used to denote epigenetic modifications that occur during the early stages of carcinogenesis. The use of molecular chaperones as therapeutic targets for malignant tumors emerges as an exciting idea, and several Hsp90 inhibitors are currently being investigated as potential anticancer agents.

Increasingly, new revelations about epigenetic modifications promise to transform all facets of cancer biology and to provide prophylactic, diagnostic, and therapeutic benefits. Epigenetic modifications could, in addition, become one of the missing links between infectious diseases and cancer. The ability of certain viruses, bacteria, parasites, and protozoa to cause malignant transformation represents one of the most fascinating topics in life sciences. This connection was regularly re-discovered throughout the past century, it repeatedly fell into oblivion and, historically, demonstrating causality often proved challenging. It is currently estimated that approximately 20% of all cancers worldwide are linked to pathogens, and the involvement of epigenetic changes in shaping this connection could soon lead to new chapters in cancer biology, establishing links that we never would have thought could exist.

2. DNA methylation and gene dysregulation in oral carcinogenesis

Oral squamous cell carcinoma (OSCC) encompasses all the malignancies that originate in the oral tissues, which include cancer of the lip, tongue, gingiva, floor of the mouth, buccal mucosa, palate, and the retromolar trigone. It is the largest group of cancers that fall into the head and neck squamous cell cancer (HNSCC) category, making it the 6th most common cancer in the world (Silverman, 1998).

Oral cancer begins as a focal clonal overgrowth of altered progenitor cells near the basement membrane, expanding upward and laterally, replacing the normal epithelium.

Carcinogenesis in human OSCC is a multistep phenomenon (Scully et al, 2000), which is usually associated with or preceded by potentially malignant oral disorders. A series of genetic hits are required for a cell to progress through dysplasia, carcinoma in situ, invasion, and metastasis. Crucial genetic events that trigger carcinogenesis include the activation of oncogenes and inactivation of tumor suppressor genes. The current understanding in the molecular pathogenesis of oral cancer suggests that both the genetic and epigenetic alterations are implicated in this multistep process as they are known to complement each other during successive stages of oral carcinogenesis. A change in gene expression profiles is evident in cancer cells at the epigenetic level via transcriptional inactivation owing to DNA methylation (Jones,2003). Among the various epigenetic alterations that lead to altered gene expression, the most important are believed to be DNA methylation and chromatin remodeling by histone modification (Baylin & Herman, 2000). DNA methylation markers stand out for their potential to provide a unique combination of specificity, sensitivity, high information content, and applicability to a wide variety of clinical specimens. Methylation markers are particularly suited for situations where sensitive detection is necessary, such as when tumor DNA is either scarce or contaminated by excess normal DNA.

While the effects of genotoxic agents such as tobacco smoke and alcohol are the most important risk factors for the development of oral cancers, the interaction of epigenetic factors and genotoxic agents may synergistically increase the risk of oral carcinogenesis. It may be argued that an epigenetic disruption in progenitor cells might be a common event in human cancer and the epigenome is a logical target for early events in carcinogenesis. The presence of methylated CpG islands in the promoter region of human genes can suppress their expression because of the presence of 5-methylcytosine, which interferes with the binding of transcription factors or other DNA-binding proteins repressing transcription activity. Inactivation of some tumor suppressor genes, such as p16, is an initial event in head and neck cancers (Von Zeidler et al, 2004), which is frequent during early oral carcinogenesis and more so in the later stages (Lopez et al, 2003). Although many important genes and gene products have been identified through DNA methylation changes, no single unifying pathway has been identified that accounts for all OSCC. Aberrant promoter hypermethylation of tumor-associated gene leads to their inactivation, as it may inactivate one or both the alleles of the proven tumor suppressor genes in sporadic cancers and can potentially act as a second hit during the development of hereditary cancer.

Cell cycle regulation, which is coordinated by cyclin-dependent kinases, its binding partners, and the inhibitory molecules such as p16, pRb, and p15 have been widely studied in oral cancer (Chang et al, 2004; Nakahara et al, 2001; Lee et al, 2004; Soni et al, 2005). Cellular signal transduction involves the conversion of one signal or stimulus (mechanical or chemical) to another. The transduction process is usually performed by enzymes in association with second messengers (Oshiro et al, 2005; Mhaweck, 2005; Nakajima et al, 2003; Gasco et al, 2002). Wnt signaling pathway is a network of proteins best known for their role in embryogenesis and cancer. E-cadherin, Adenomatosis polyposis coli APC, β -catenin, CDH3, SFRP family, WIF1, and DKK3 are some of the genes of Wnt pathway, which are epigenetically silenced in cancer. Wnt pathway is also involved in calcium-dependent cell adhesion through the interaction of E-cadherin and β -catenin (Nakayama et al, 2001; Uesugi et al, 2005; Gao et al, 2005). One of the hallmarks of cancer is the down-regulation of genes involved in DNA repair pathways. O-6-methylguanine-DNA methyltransferase (MGMT), MutL homolog 1 (MLH1), and fragile histidine triad (FHIT) are most widely studied in oral

cancer. Frequent inactivation of some of these repair genes in cancer has been reported to be due to promoter hypermethylation (Mikami et al, 2007; Murakami et al, 2004; Rohatgi et al, 2005; Kato et al, 2006; Rodriguez et al, 2007; Kim et al, 2004; Paradiso et al, 2004; Baer-Desurmont et al, 2007).

Failure of cells to undergo apoptosis is a common and frequent event in carcinogenesis. Resistance to apoptosis of cancer cells has great clinical significance as these cells are resistant to chemo- and radiotherapies. Thus, identifying the checkpoints of apoptosis in cancer may offer newer therapeutic modalities for the treatment of cancer. The genes, which are involved in the apoptosis, such as Death-associated protein kinase 1, p73, and RASSF2, have shown to be down-regulated in oral cancer because of promoter hypermethylation (Hasegawa et al, 2002). In head and neck cancers, a statistically significant correlation was detected between the presence of DAP kinase gene promoter hypermethylation, lymph node involvement, and advanced disease stage. Tobacco smoking was slated to play an important role in the occurrence of promoter methylation and in delineating the precise pathway that eventually resulted in a tumorigenic phenotype (Araki et al, 2002). Some of the molecular changes characteristic of early oral cancer development have been identified in immortal oral dysplasia cultures and are associated with the loss of the expression of RAR-beta and the cell cycle inhibitor p16INK4A. RAR-beta and / or p16 reexpression could be reinduced by treatment with 5-aza-2'-deoxycytidine in some immortal dysplasias, and the possibility of reversing the immortal phenotype of some dysplasias by 5-aza-2'-deoxycytidine was considered to be of clinical usefulness (McGregor et al, 2002).

The ability to determine methylation states in primary tumors, saliva, and serum may have a potential clinical application in creating methylation gene panels for cancer screening. Gene methylation in saliva is a promising biomarker for the follow-up and early detection of still curable relapses of patients with HNSCC (Righini et al, 2007). Sanchez-Cespedes et al. (2000) studied the promoter hypermethylation patterns of the p16, MGMT, and DAP-K genes in tumor DNA of head and neck primary tumors and paired saliva samples to test whether the cells with tumor-specific aberrant DNA methylation might be found in the saliva of affected patients. Promoter methylation in saliva DNA was found in all tumor stages and more frequently in tumors located in the oral cavity. Moreover, none of the saliva from patients with methylation-negative tumors displayed methylation of any marker. This assay allowed sensitive and accurate detection of tumor DNA in saliva and may be potentially useful for detecting and monitoring recurrence in patients with head and neck cancers.

3. Human papillomavirus-16 DNA methylation patterns

This year marks a century since Francis Peyton Rous' seminal discovery that laid the foundations of a new field at the crossroads between microorganisms and human cancer. In 1911, Rous showed that cell-free filtrates from birds with sarcoma were able to cause tumors in healthy birds. The idea that viruses are causally linked to cancer was viewed at the time with skepticism, and fell into oblivion for decades, but re-emerged later. Fifty-five years after his groundbreaking discovery, Rous received the 1966 Nobel Prize in Physiology or Medicine.

A century after this major discovery, over 20% of the cancers have been causally linked to human pathogens, including viruses, bacteria, and parasites (Zur Hausen, 2009). Why an

acute infectious disease is sometimes controlled, while on other occasions it progresses to malignant tumors, is still a mystery, and the mechanisms of how pathogens may accomplish this are still elusive but, for increasing numbers of pathogens, epigenetic modifications have been implicated (Paschos & Alladay, 2010).

Three of the most extensively studied oncogenic viruses, the hepatitis B virus (HBV), human papilloma viruses (HPV), and the Epstein-Barr virus (EBV) are, at the same time, major public health concerns. HBV infects over 2 billion people worldwide, and causes chronic infection in approximately 350 million of them (Fernandez & Esteller, 2010). Human papillomavirus (HPV) is thought to be the most frequent infectious agent that causes cancer. Specific human papillomavirus serotypes were causally linked to more than 70% of cervical cancers, to most anal cancers, and to respiratory papillomatosis. In addition, this virus emerges as an increasingly important causal agent of head and neck cancers, particularly in younger patients, and has also been associated with breast cancer (Westra, 2009).

To dissect the link between inflammation and cancer, Iliopoulos et al (Iliopoulos 2009, 2010) fused the estrogen receptor ligand-binding domain to the Src kinase oncoprotein to create a tamoxifen-inducible fusion protein. By using this system, the authors found that a transient inflammatory signal generated by an induction as short as 5 minutes triggered, in an NF- κ B (transcription factor)-dependent manner, an epigenetic switch similar to the one that controls tissue differentiation during development. This switch established a positive feedback loop, and as a result, nontransformed human mammary epithelial cells were becoming transformed, as revealed 24 to 36 hours later. The transformed state was propagated for many generations in the absence of the initiating inflammatory signal. Subsequently, the authors found several microRNAs that are differentially regulated as part of this positive feedback loop. The 2 most up-regulated ones, miR-21 and miR-181b-1, are key regulators of tumor-suppressor genes (Stein, 2011).

Infection with HPV, notably with high-risk HPV types as HPV-16 and 18, is a necessary step in the etiology of anogenital cancers, specifically carcinoma of the cervix uteri. DNA diagnosis confirmed the presence and transcription of HPV genomes in all cervical carcinomas, and the study of HPV oncoproteins catalyzed a variety of molecular mechanisms that convert normal into malignant cells (Munoz et al, 2003). In case of head and neck squamous cell carcinomas (HNSCC), approximately 80% of all HNSCC do not contain HPV genomes, and must therefore originate from HPV independent etiological processes, likely including mutational events triggered by tobacco and alcohol consumption. Among head and neck sites, squamous cell carcinomas of the tonsils have the strongest statistical support for an HPV dependent etiology (Syrjjanen, 2005). There is less strength and consistency for a linkage between HPV infection and carcinogenesis at sites of the oral mucosa such as tongue, palate, floor of mouth and gingiva. Analysis of some HPV containing oral carcinomas revealed recombination between HPV genomes and cellular DNA as well as HPV oncogene expression (Ragin et al, 2004), properties that are generally viewed as support of carcinogenic processes under the influence of HPV. On the basis of these observations one must conclude that infection with high-risk HPV plays an etiological role in at least a proportion of malignancies of the oral cavity (Balderas-Loaeza et al, 2007).

With the recent reported increase in young HNSCC cases many of them nonsmokers, the question arises as to whether these cases have the same genetic alterations as seen in the classic progression model. Toner and O'Regan (2009) studied HNSCC in young adults using

array comparative genomic hybridization on a cohort of predominantly nonsmoking young adults and compared them with a cohort of mostly smoking older adults. Results from this study showed that when stratified by age the young cohort do not have the genetic alterations that are seen so consistently in older HNSCC. In fact, the mean number of aberrations in the young nonsmokers was less than 50% of that in the older smokers. Molecular alterations at the p53 gene have been documented as being the most frequent genetic alteration observed in carcinomas and has been found to be altered in over 70% of HNSCC (Gillison et al, 2000). It has been shown that p53 sequence alteration decreased in the setting of HPV infection, since there is an alternative means of p53 silencing with the production of E6 (Mork et al, 2001). Overexpression of p16^{INK4} has also been reported in head and neck cancers, and it is believed that HPV infection, via inactivation of retinoblastoma gene, accounts for these high levels of p16^{INK4} expression [Mork et al, 2001]. Toner and O'Regan (2009) found that p16 methylation is a more common event in those younger than 40 years in contrast to p16 deletions, which are more common in those older than 40 years. Consequently, it appears that specific modes of inactivation of p16 in HNSCC are related to specific patient risk profiles. After detailed study of p16 mRNA expression and p16 immunohistochemistry in this cohort, it was clear that all HPV mRNA positive cases showed p16 overexpression. In relation to copy gain and loss, previous studies have found only occasional chromosomal loss in HPV16 positive cases, suggesting that HPV16 infection is an early event in HNSCC development.

Inverted papillomas are benign, rare sinonasal lesions well known for their local recurrence, invasiveness and predisposition for malignant transformation. Recurrence rates for inverted papillomas range from 6 to 33% and malignant transformation occurs in 7-10% of cases (Batsakis, 2001). Endophytic sinonasal papillomas, comprising inverted papillomas and cylindrical cell papillomas according to the World Health Organization classification, show malignant progression in up to 25% of cases (Gujrathi et al, 2003).

The exact nature and biological evolution of sinonasal papillomas is not well known. The suggested hypothesis includes viral infections, chronic inflammation, and proliferation of nasal polyps, allergy, and environmental carcinogens. In view of its epitheliotropic nature and increasing evidence, Human Papilloma Virus (types 6 and 11) is postulated to be the most probable aetiological factor. Causative factor for malignant transformation in a papilloma is still unknown. It has been seen that HPV 16 and 18 are more carcinogenic than HPV 6 and 11. Over-expression of p53 & reduced expression of CD44 has been seen in carcinomas associated with inverted and exophytic papillomas. Overexpression of p53 may serve as a marker for malignant transformation of inverted papilloma (el-Deiry, 1998). Induction of p21waf1/cip1 is associated with terminal differentiation, senescence, and apoptosis in several tissues (Yook & Kim, 1998). Expression of p21waf1/cip1 has been detected in head and neck cancers, in particular oral SCCs and its precursors (Ng et al, 1999).

Epigenetic alterations of promoter hypermethylation have not been previously reported in sinonasal papillomas. Stephen et al. (2007) investigated whether epigenetic events of aberrant promoter hypermethylation in genes known to be involved in squamous head and neck cancer underlie the pathogenesis of sinonasal papillomas. Ten formalin-fixed paraffin DNA samples from three inverted papilloma cases, two exophytic (everted) papilloma cases, and two cases with inverted and exophytic components were studied. DNA was

obtained from microdissected areas of normal and papilloma areas and examined using a panel of 41 gene probes, designed to interrogate 35 unique genes for aberrant methylation status (22 genes) using the methylation-specific multiplex-ligation-specific polymerase assay. Methylation-specific PCR was employed to confirm aberrant methylation detected by the methylation-specific multiplex-ligation-specific polymerase assay. All seven cases indicated at least one epigenetic event of aberrant promoter hypermethylation. The *CDKN2B* gene was a consistent target of aberrant methylation in six of seven cases. Methylation-specific PCR confirmed hypermethylation of *CDKN2B*. Recurrent biopsies from two inverted papilloma cases had common epigenetic events. Promoter hypermethylation of *CDKN2B* was a consistent epigenetic event. Common epigenetic alterations in recurrent biopsies underscore a monoclonal origin for these lesions. Epigenetic events contribute to the underlying pathogenesis of benign inverted and exophytic papillomas. As a consistent target of aberrant promoter hypermethylation, *CDKN2B* may serve as an important epigenetic biomarker for gene reactivation studies (Stephen et al, 2007).

4. Epigenetic changes in nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is rare in most part of the world but prevalent in southern China, including Guangdong and Hong Kong, and Southeast Asia, with an incidence rate of 20 to 30 per 100 000 people/year (Li et al, 2011). The unique ethnic and geographic distribution of NPC indicates its unusual etiology. Three major etiologic factors, genetic, environmental, and viral factors – Epstein-Barr virus (EBV), have been identified to lead to multiple genetic and epigenetic alterations during NPC pathogenesis by either acting alone or in synergy.

EBV is a prototype of gamma herpes virus which infects >90% of the world adult population (Liebowitz, 1994). Humans are the only natural host for EBV. Primary infection with EBV normally occurs in early childhood and is usually asymptomatic in most underdeveloped countries. But when the exposure to EBV is delayed until adolescence it occasionally presents as mononucleosis. Long-term EBV coexists with most human hosts without overt serious consequences. However, in some individuals, the virus is implicated in the development of malignancy. EBV has a strong tropism for human lymphocytes and for epithelium of the upper respiratory tract (Young & Rickinson, 2004). EBV was the first human virus identified to be associated with human lymphomas as well as epithelial tumors, such as post-transplant lymphoma, AIDS-associated lymphomas, Burkitt lymphoma, Hodgkin's disease, T-cell lymphoma, nasopharyngeal carcinoma, parotid gland carcinoma, and gastric carcinoma. (Murray & Young, 2002)

Tumorigenesis of nasopharyngeal carcinoma is a multistep process. EBV may play an important role in the progression of NPC, involving activation of oncogenes and/or the inactivation of tumor suppressor genes (TSGs). Early genetic changes may predispose the epithelial cells to EBV infection or persistent maintenance of latent cycle.

Nasopharyngeal carcinoma also distinguishes itself from other tumors by the number of genes targeted for silencing by promoter methylation. The key tumor suppressor genes like p53 or Rb which are found to be mutated in 50% of all the tumors were rarely found to be mutated in nasopharyngeal carcinoma (Wensing & Farrel, 2000). On the contrary, hypermethylation of known or candidate tumor suppressor genes involved in various

fundamental pathways has been reported in NPC, such as apoptosis, DNA damage repair, tumor invasion and metastasis. Many TSGs were aberrantly methylated in their 5' CpG islands: 84% of the RASSF1A, 80% of the RAR β 2, 76% of the DAP-kinase, 46% of the p16, 89,7% of the CDH13, 65% of the CHFR, 50,9% of the RASSF2A (Li et al, 2011). DNA methylation may play an important role in the maintenance of specific EBV latency programmes in the nasopharyngeal carcinoma cells. Methylation of both viral and cellular genes may be involved in the transformation of nasopharyngeal epithelial cells. Induction of epigenetic alterations in certain cellular genes was proposed as one of the mechanisms for enhancing the transformation of nasopharyngeal epithelial cells by EBV infection.

Loss of heterozygosity at 3p21 is common in various cancers including nasopharyngeal carcinoma (NPC). BLU is one of the candidate tumor suppressor genes (TSGs) in this region. Ectopic expression of BLU results in the inhibition of colony formation of cancer cells, suggesting that BLU is a tumor suppressor. Qiu and colab. (2004) identified a functional BLU promoter and found that it can be activated by environmental stresses such as heat shock, and is regulated by E2F. The promoter and first exon are located within a CpG island. BLU is highly expressed in testis and normal upper respiratory tract tissues including nasopharynx. However, in all seven NPC cell lines examined, BLU expression was downregulated and inversely correlated with promoter hypermethylation. Biallelic epigenetic inactivation of BLU was also observed in three cell lines. Hypermethylation was further detected in 19/29 (66%) of primary NPC tumors, but not in normal nasopharyngeal tissues. Treatment of NPC cell lines with 5-aza-20-deoxycytidine activated BLU expression along with promoter demethylation. Although hypermethylation of RASSF1A, another TSG located immediately downstream of BLU, was detected in 20/27 (74%) of NPC tumors, no correlation between the hypermethylation of these two TSGs was observed (Qiu et al, 2004).

Aberrant "epigenetic code" of cell signaling facilitates the subsequent selection of genetic mutations of certain signaling pathways in the initiation and progression of NPC. As more epigenetic alterations of cell signaling genes are found, we will obtain systematic understanding of the molecular features of NPC. Study of epigenetically silenced cell signaling regulators in NPC will lead to the further development of clinical strategies of NPC prevention and therapy. Moreover, promoter methylation of cell signaling regulators could serve as diagnostic biomarkers for NPC risk assessment, early detection, and prognosis (Li et al, 2011).

5. Epigenetic changes in laryngeal cancer

Laryngeal cancers are the most frequent cancers of the head and neck region and their occurrence is strongly associated with the exposure to cigarette smoke and the consumption of strong alcohols. Almost all laryngeal cancers are squamous cell carcinomas (Paluszczak et al, 2011).

The chondroitin/dermatan sulfate fine chemical structure is altered in laryngeal carcinomas (Kalathas et al, 2009) as well as in most cancers. In healthy larynx, chondroitin/dermatan sulfate (C-6 and C-4 sulfated) in the cartilaginous parts is present in greater amounts compared to cancer. Moreover, the decrease in cancer is more abrupt in C-6 sulfation; C-4 sulfation is diminished gradually to the advanced stages of cancer. These alterations may be due to differential biosynthesis of core protein precursors, to differences in the substrates

pool, and to differential expression of the enzymes involved in chondroitin/dermatan sulfate biosynthesis.

The purpose of Kalathas and colab. (2010) was to examine the expression of the various chondroitin/dermatan sulfate synthesizing and modifying enzymes in laryngeal cartilage in healthy, macroscopically normal and cancerous specimens by RT-PCR analysis and western blotting. Furthermore, methylation specific PCR (MSP) was used to find out if DNA methylation is a regulative mechanism of their expression in laryngeal cancer. C4ST1 gene expression was very low in healthy specimens (20 times lower compared to the GAPDH gene), and increased in patients' specimens as indicated by both RT-PCR and western blotting. Its expression seemed to be controlled via methylation of a CpG island, since hypomethylation of the gene was observed in the pathologic samples compared to the macroscopically normal samples. D4ST1 gene was about equally expressed with the GAPDH gene and possessed its highest expression in the healthy tissues. In cancer, its expression was decreased 4 to 5 times and it was about equal between normal and pathologic samples. The CpG island near the promoter region was fully unmethylated therefore it did not affect the enzyme expression. DSE expression was not detected in the macroscopically normal samples, and the highest levels of it were observed in the pathologic samples, as indicated by RT-PCR, being about 10-times more compared to healthy. DSE expression seemed to be controlled by methylation of the promoter region in certain samples; the pathologic samples were hypomethylated compared to the macroscopically normal. The differential modification of the various glycosaminoglycans during cancer reflected differential expression of the enzymes involved in their biosynthesis. In their study, the clearest observations in laryngeal cancer were the significant decrease of CHSY3, CHST3 and D4ST1, and the significant increase of DSE. DSE is responsible for the epimerization of glucuronic acid in dermatan sulfate chains, which in addition require D4ST1 for their sulfation. The differential expression of only these two enzymes, which are highly responsible for the biosynthesis of dermatan sulfate, a glycosaminoglycan with tumor-inhibitory activity, indicates that a simple imbalance in enzymes' expression may affect tumor progression.

Different environmental factors are able to modulate the epigenetic information. It is widely accepted that the aberrant DNA methylation changes can be induced by both cigarette smoke and alcohol. Alcohol consumption and smoking induced the hypermethylation of p15 in the upper respiratory tract cells (Chang et al, 2004), while the hypermethylation of MGMT, p16 and DAPK was associated with tobacco-chewing induced oral cancers (Vuillemonet et al, 2004). A correlation was also found between cigarette smoking and the hypermethylation of CDH1, RARbeta and FHIT (Van Engeland et al, 2003). Alcohol consumption was associated with the methylation of APC, p14, p16, MGMT, RASSF1A and hMLH1 (Van Engeland et al, 2003). Since alcohol and tobacco are the major risk factors for the development of laryngeal squamous cell carcinomas (LSCC), one can predict the frequent occurrence of the methylome aberrations in LSCC patients.

Since Slaughter's proposal of a genetic field defect concept for the explanation of the local relapse occurrence, much evidence has accumulated for its confirmation. The last ten years brought evidence that the genetic changes in the field are frequently accompanied by epigenetic aberrations. The epigenetic field defect was observed for oesophageal (Oka et al, 2009), lung (Guo et al, 2004) or stomach (Ushijima, 2007) cancers. Similarly, epigenetic

changes in normal mucosa cells derived from surgical margins were detected in head and neck carcinomas (Martone et al, 2007). However, so far such changes have not been observed specifically in laryngeal cancers. In Paluszczak's paper (2011) evidence of a widespread occurrence of the aberrations in the profile of DNA methylation in laryngeal cancer patients is presented. Less than ten percent of cancer cases did not show any epigenetic changes in the normal mucosa samples. Gene methylation frequency was only slightly lower in the normal epithelium of epiglottis or trachea than in tumor cells. However, it should be taken into account that all the patients were exposed to similar laryngeal cancer risk factors. As discussed earlier, tobacco and alcohol are associated with the aberrations in the DNA methylation profile. The long-term exposure of patients to these factors could be responsible for the common appearance of epigenetic defects in a large field of upper respiratory tract mucosa. The existence of the epigenetically changed field in laryngeal cancers seems to be confirmed especially by such cases where lack of gene methylation in tumor cells was accompanied by the presence of hypermethylation in the normal epithelial cells although the percentage of patients with such gene methylation pattern was rather low.

6. Epigenetics and environment exposure

For many decades, it was assumed that chemicals are able to cause cancer only if they mutate the DNA. However, a growing body of scientific evidence reveals that this "carcinogenesis equals mutagenesis" paradigm is no longer accurate. Twenty years ago, Ashby and Tennant (1991) examined 301 chemicals tested by the US National Toxicology Program, and found that from 162 (53%) that were carcinogens, 64 (40%) were not genotoxic, illustrating the importance to focus on carcinogenic mechanisms other than genotoxicity. For many chemical agents, it has become increasingly clear that biological perturbations leading to neoplastic transformation may occur even in the absence of mutagenesis, via non-genotoxic, epigenetic changes. In addition, epigenetic changes may be relevant for tumor development and progression and ways that years ago seemed unimaginable. For example, Tanemura et al. (2009) revealed, for the first time, that CpG methylation in cutaneous melanoma is associated with tumor progression, and Nobeyama et al. (2007) demonstrated that tissue factor pathway inhibitor-2 (*TFPI-2*), a encoding a protein that suppresses the invasiveness of malignant melanoma, was methylated in 29% of metastatic lesions but in none of the primary tumors examined, pointing towards differences in gene expression and phenotypic characteristics between metastatic tumors and the primary tumor they originated from, a finding with significant therapeutic applications. The risk factors for sinonasal carcinoma include wood dust exposure, occupational exposure to chromium and nickel and its organic compounds (Luce & Leclerc, 2002)

Very high relative risks have been invariably found and 10-45 fold risks have been indicated for the sinonasal adenocarcinoma cell type in association to occupational exposure to hardwood dust, the risk related to softwood dust exposure is less clear. (Demers et al, 1997) Exposure to wood dust in the occupational setting is a common occurrence. It has been estimated that in the year 2000-2003 about 3.6 million workers were occupationally exposed to inhalable wood dust in the European Union and over half a million of these workers were estimated to be exposed to high levels (exceeding 5mg/m³) of wood dust (Kauppinen et al., 2006). The terms "hardwood" and "softwood" refer to the taxonomic categorization of trees

and not necessarily to the hardness of the wood. Wood dust is a complex mixture of compounds including a wide variety of biologically active substances, also genotoxic and carcinogenic compounds (Hanahan & Weinberg, 2000). The particulate nature of the wood-dust exposure plays a role in generating reactive species of oxygen (ROS) within cells and inducing DNA damage and evoking an inflammatory response (Bornholdt & Saber, 2007). Multiple mechanisms of carcinogenesis have been proposed to be involved in the development of sinonasal cancer related to wood-dust exposure, but there is very little experimental or human data in the literature. The published findings have been based on a relatively limited number of cases, mostly involving adenocarcinomas. In these studies, high frequencies of DNA copy number changes as detected by comparative genomic hybridization have been detected (Ariza et al., 2004 and Korinth et al., 2005), while the mutation rates reported for the KRAS gene (Fratini et al., 2006 and Yom et al., 2005) and the p53 tumor suppressor gene have been lower (Perrone et al., 2003 and Licitra et al., 2004). Initially, KRAS and HRAS mutations were found to be quite frequent in sinonasal cancer, with implications for histogenetic and prognostic significance (Yom et al., 2005), but recent studies show that tumors with KRAS mutation might represent only a small proportion of all sinonasal carcinomas (Bornholdt et al., 2008). Most of the studies having as subject p53 mutation have concentrated on the intestinal type of adenocarcinoma, and the numbers of cases studied have been rather low. In these studies, a variable occurrence of p53 mutations has been reported (18-60%) (Licitra et al., 2004 and Perrone et al., 2003). Some of the studies have also examined the accumulation of p53 in the cell nucleus in adenocarcinoma type of sinonasal cancer. The accumulation of p53 may reflect a p53 mutation.

With the exception of chromium, which forms DNA adducts, most carcinogenic metals are weak mutagens and act by epigenetic mechanisms (Arita & Costa 2009). Nickel, a metal linked to occupational and environmental exposures, has carcinogenic effects, despite the fact that it is not known to be a mutagen (Kasprzak et al., 2003; Chen et al., 2006; Ellen et al., 2009). *In vitro* and *in vivo* experiments reveal that nickel compounds silence gene expression by causing DNA methylation, an effect explained by the ability of nickel ions to substitute magnesium in the DNA phosphate backbone and to increase heterochromatin condensation (Arita & Costa 2009). The ability of Ni²⁺ ions to displace Mg²⁺ and cause chromatin condensation, establishing dense regions of heterochromatin that prevent accessibility to the respective genomic region, was also revealed by Ellen and colleagues (2009) with atomic force microscopy and circular dichroism spectropolarimetry. Chromatin condensation could in addition trigger DNA methylation in the compacted region, which also affects gene expression. When the silenced chromosomal region contains genes that are relevant to cancer initiation or progression, such as tumor suppressor genes or senescence genes, their inactivation can lead to disease (Ellen et al., 2009).

Nickel compounds also induce global changes in histone acetylation, methylation, and ubiquitylation. Kang et al. (2004) linked nickel to hypoacetylation to apoptosis for the first time, when they found hypoacetylation and demethylation of histones as potential mechanisms leading to apoptosis. Golebiowski and Kasprzak (2005) reported decreased acetylation of histones H2A, H2B, H3 and H4, in a time- and concentration-dependent manner, in human and rat cell lines exposed to nickel. Chen et al. (2006) revealed that nickel decreased a specific histone demethylase and by this mechanism, it increased global H3K9 mono- and dimethylation in several cell lines, and strongly suggested that this increased methylation causes the silencing of a transgene. This effect was dependent, *in vitro*, on iron

and 2-oxoglutarate, and it is likely that it resulted from the nickel interfering with the iron moiety of the enzyme (Chen et al, 2006). In response to soluble nickel compounds at levels that had minimal cytotoxic effects, Ke et al. (2006) described three major histone modifications: H3K9 dimethylation, increased H2A and H2B ubiquitylation, and reduced histone acetylation, which was also associated with a transgene silencing.

An interesting example is provided by hexavalent chromium, to which half a million workers in the United States and several million individuals worldwide are occupationally exposed (Ali et al, 2011). Until recently, chromium was thought to cause cancer only through its ability to damage DNA (Arita & Costa 2009). Klein et al. (2002) reported for the first time that potassium chromate, a carcinogen, causes aberrant DNA methylation and silences a reported gene in a mammalian cell line. Ali et al. (2011) found higher rates of aberrant methylation in the promoters of three tumor-suppressor genes, APC (adenomatosis polyposis coli), MGMT (*O*₆-methylguanine-DNA methyltransferase), and hMLH1 in lung cancers of chromate workers as compared to non-chromate lung cancer controls (95% versus 52%), with concordant methylation of multiple loci observed in more chromate lung cancers than in nonchromate ones (48% versus 12%). Chromate was also linked to post-translational histone modifications. Sun et al. (2009) found that hexavalent chromium at 5-10 μ M concentrations causes global and local, gene-specific histone methylation changes in lung cancer, non-cancerous bronchial epithelial cells, and all respiratory tract which could impact tumorigenesis and tumor progression.

Benzene and aromatic hydrocarbons have increasingly emerged over the past few decades as environmental hazards. Exposure to benzene can occur in occupational settings, and also non-occupationally from coal or gasoline combustion products and cigarette smoke, which also contain polycyclic aromatic hydrocarbons. In the first study to show that low levels of a common environmental carcinogen are linked to epigenetic changes that occur in malignant human tumors, Bollati et al. (2007) reported that benzene, at low-level airborne exposures that are common in Western countries, causes epigenetic changes that reproduce modifications observed in human cancers. The authors examined 155 traffic police officers and gas station attendants, and 58 unexposed control subjects from Milan, Italy. The study revealed a dose-dependent global hypomethylation in the long interspersed nuclear element-1 (*LINE-1*) and *AluI* repetitive sequences, in addition to hypermethylation at specific promoters, such as p15, which is also hypermethylated in patients with acute myeloid leukemia, in addition to hypomethylation in *MAGE-1*, a gene that is hypomethylated in many malignant tumors.

As a group of chemicals, polycyclic aromatic hydrocarbons (PAHs) include thousands of compounds ubiquitously distributed in the environment. An interesting fact about PAHs is that for a long time, the focus has been on their ability to cause genotoxic damage while their potential to induce epigenetic modifications was largely ignored (Upham et al, 1998). Benzopyrene, a prototype PAH, is a classic carcinogen found in vehicle emissions and in cigarette smoke - the mainstream smoke of a filter cigarette contains approximately 10 ng benzopyrene (Sherer et al, 2000), and exposure can also occur occupationally. In the first study to show that benzopyrene causes epigenetic changes, Sadikovic et al. (2008) conducted a genome-wide analysis after exposure and found 775 genes that were hypoacetylated and 1456 that were hyperacetylated. These modifications occurred in genes important for fundamental cellular processes, such as DNA replication, repair, and carcinogenesis.

In the first study that examined the effects of smoking on miRNA expression, Schembri et al. (2009) found 28 differentially expressed miRNAs in smokers, 82% of which were down regulated. One of them, mir-218, is also down regulated in several cancers, and the authors revealed that modulation of miR-218 levels lead to changes in the expression of its target genes. Xi et al. (2010) found that cigarette smoke condensate causes a significant and early increase in miR-31 that was apparent within 24 hours after exposure and persisted for 20 days after removal of the exposure.

7. Challenges in epigenetic cancer therapy

Epigenetic mechanisms regulate the interpretation of genetic information. As such, our knowledge of these mechanisms is essential for understanding the phenotypic plasticity of cells, both in the context of normal cellular differentiation and in human disease (Freinberg, 2007). Research over the past two decades has identified two major levels of epigenetic modification: DNA methylation and covalent histone modifications (Strahl & Allis, 2000 and Klose & Bird, 2006). DNA methylation is mediated by a family of enzymes termed DNA methyltransferases (DNMTs) (Goll & Bestor, 2005), while histone modification patterns are established and maintained by a diverse set of enzymes that add or subtract acetyl-, methyl-, and other modifications to various amino acids of histone proteins (Kouzarides, 2007). Both regulatory mechanisms cooperate to determine the expression potential of individual genes.

For detection of cancer cells in body fluids, a high-sensitivity method is necessary. One way is mutation detection in cells because the exact location of a mutation within a gene is usually unknown, many primer sets are necessary for complete analysis. In contrast, aberrant methylation of DNA molecule of cancer cells, even in very few in number, can be sensitively detected by using Methylation-Specific PCR method (MSP), only with one set of PCR primer can be performed on chemically stable DNA, not on RNA (Herman et al, 1996 and Laird, 2003).

Considering that some aberrant DNA methylation is present in early stages of carcinogenesis, there is a possibility that such demethylating agents may protect against some cancers (Laird et al, 1995). Demethylating agents are including DNMT1 inhibitors group (Azacitadine, Decitabine, Zebularine and MG98), procainamide, procain and EGCG (epigallocatechin-3-gallate) (Fang et al, 2003 and Villar-Garea et al, 2003). Inhibitors of DNMTs have been widely used in cell culture systems to reverse abnormal DNA hypermethylation and restore silenced gene expression. However, only limited success has been achieved in clinical trials with these drugs (Thibault et al, 1998 and Goffin & Eisenhouer, 2002). Also, nucleosides analog inhibitors of DNMTs may promote genomic instability and increase the risk of cancer in other tissues, because have many potential side effects such as myelotoxicity, mutagenesis and tumorigenesis (Jones & Taylor, 1980 and Gaudet et al, 2003). There is an attractive alternative for possible clinical use of non-nucleoside analog DNMT inhibitors.

The use of these drugs raises questions regarding their potential to affect non-cancerous cells epigenetically. However, normal cells divide at a slower rate than malignant cells and incorporate less of these drugs into their DNA resulting in less of an effect on DNA methylation. Azacitadine and decitabine are labile and have acute hematological toxicities. Zebularine, a next generation DNA methylation inhibitor, might possibly overcome these

problems (Marquez et al, 2005 and Yoo et al, 2008). The non-nucleoside analogue inhibitors are not as potent as the nucleoside analogues and therefore this issue needs for improvement (Chuang et al, 2005).

Another prominent example for an epigenetic drug is the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA, vorinostat, Zolinza), which has been approved for the treatment of cutaneous T cell lymphoma (Marks & Breslow, 2007). Another HDAC inhibitor (Romidepsin, Istodax) has very recently been approved for the same indication. As of today, there are at least 20 structurally different HDAC inhibitors in clinical trials, either in monotherapy or in combination therapy trials for hematological and solid tumors. It should be noted that combination therapies of HDAC inhibitors with other anticancer drugs or with radiation therapy have shown a wide range of synergistic effects, both in preclinical models and in early clinical trials (Marks & Xu, 2009). The identification of tumor-specific epigenetic pathways represents a critically important step toward the establishment of targeted epigenetic cancer therapies. One possibility is the targeting of defined DNMTs with specific oncogenic functions (Linhart et al, 2007). Another possibility is the discovery of tumor-specific functions for enzymes with specific histone modification activities. A third option is the identification of tumor-specific interactions between epigenetic pathways, like interaction between DNMTs and HDACs through methyl-CpG binding proteins.

While the clinical application potential of the interaction between DNA methylation and histone hypoacetylation remains to be established, the results from preclinical experiments clearly suggest crosstalk between epigenetic silencing systems that warrants further investigation. A particular interesting finding in this context is the interaction between histone lysine methylation and DNA hypermethylation. Several independent studies have shown that genes that are marked by bivalent chromatin structures (i.e. the presence of both H3K4 and H3K27 methylation marks) in embryonic stem cells have a high probability of becoming de novo methylated in cancer (Schlesinger et al, 2007; Ohm et al, 2007; Widschwendter et al, 2007). The mechanistic details of these interactions are only beginning to be elucidated. The available data, however, raise the intriguing possibility that cancer-specific epigenetic mutations reflect the stem cell origin of tumors. As such, targeting of the interaction between bivalent chromatin structures and DNA hypermethylation might represent a highly specific approach toward erasing cancer-specific epigenetic mutations.

“Cancer has been the tip of the iceberg, but knowledge in cancer epigenetics is going to translate to other diseases. It is clear to me that there is no disease that is pure genetics, and there is no disease that is pure epigenetics. All diseases, from cancer to neurological disorders to cardiovascular conditions, are mixtures of genetics, epigenetics, and the environment” says Manuel Esteller, M.D., Ph.D., director of the Cancer Epigenetics and Biology Program in Barcelona.

Epigenomics-based diagnostic tools for early cancer detection represent an exciting development. Tumors shed their DNA into the blood, and epigenetic changes that occur early during tumorigenesis, sometimes even in premalignant lesions, can provide valuable biomarkers. More than ever, it is imperative to focus on understanding the mechanistic details of malignant transformation initiated by human pathogens, an area that promises exciting prophylactic, diagnostic, and therapeutic applications.

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9. References

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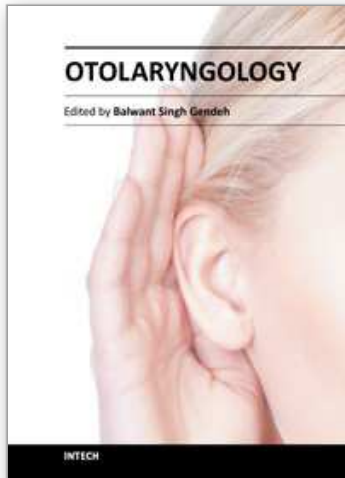
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This book emphasizes on different aspects of otolaryngology - the medical sciences of diagnosis and treatment of ENT disorders. "Otolaryngology" is divided into various clinical sub-specialities, namely otology, rhinology, laryngology, and head and neck. This book incorporates new developments, as well as future perspectives in otolaryngology. I would like to dedicate this book to those of you who will pick up the torch and by continued research, close clinical observation and the highest quality of clinical care, as well as by publication and selfless teaching, further advance knowledge in otolaryngology from this point forward. It is intended to be a guide to other books to follow. Otolaryngologists, researches, specialists, trainees, and general practitioners with interest in otolaryngology will find this book interesting and useful.

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