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Delineating an Epigenetic Continuum in Head and Neck Cancer

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Abstract

A tissue field of somatic genetic alterations precedes the histopathological phenotypic changes of carcinoma. Genomic changes could be of potential use in the diagnosis and prognosis of pre-invasive squamous head and neck carcinoma (HNSCC) lesions and as markers for cancer risk assessment. Studies of sequential molecular alterations and genetic progression of preinvasive HNSCC have not been clearly defined. Studies have shown recurring alterations at chromosome 9p21 (location of the *CDKN2A*) and *TP53* mutations in the early stages of HNSCC. However, gene silencing via hypermethylation is still a relatively new idea in the development of HNSCC and little is known about the contribution of epigenetics to the development of neoplasia, its transformation, progression, and recurrence in HNSCC. This review examines the role of promoter hypermethylation of tumor suppressor genes in the progression continuum from benign papillomas to malignancy in HNSCC.

Keywords

DNA hypermethylation; benign papillomas; progression; squamous cell carcinoma

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent cancers in the world with over 500,000 cases diagnosed annually. In the United States, approximately 52,140 new cases are expected in 2011 with an estimated 11,460 deaths for HNC of the oral cavity, pharynx, and larynx[1].

Despite considerable efforts, the 5-year survival rate for HNSCC has not changed significantly making accurate and reliable stratification for prediction of outcomes challenging. Much of this is attributed to the numerous anatomic sites and subsites from which tumors can arise and the diversity of histologic types of tumors in these locations[2].

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Conflict of Interest

None of the authors have any financial or other interests with regard to the submitted manuscript that might be construed as a conflict of interest.

Patients with advanced HNSCC are limited to a complete response of 50% and often require long-term rehabilitation. However, early HNSCC detection increases survival to 80%. To facilitate timely diagnosis and improve treatment, elucidation of early detection markers is crucial. A current shortcoming in the prognosis and treatment of HNSCC is a lack of methods and large study cohorts to adequately address the etiologic complexity and diversity of the disease.

1.1 Genomic Advances in HNSCC

Cancer is the result of transformation from a normal to a malignant cell that results from accumulated mutations. Acquisition of a fully malignant phenotype in colon cancer is thought to occur as a result of multiple steps whose targets are alterations of growth-promoting oncogenes and growth-inhibiting cancer suppressor genes [3]. The evolution in transformation from a normal squamous epithelial cell to a cancer cell is likewise assumed to require several steps, some defined by genetic alteration. However, the precursor lesion(s) and sequence of events are less clearly defined for head and neck squamous cell carcinoma (HNSCC).

1.2 Genetics of HNSCC

Early cytogenetic studies of HNSCC relied on analysis of later stage tumors and established cell lines. Recent short-term cell cultures have indicated similar genetic changes. Common sequences of SCC karyotype evolution appear to require initial loss of chromosome segments, followed by tetraploidization, and ultimately loss of previously uninvolved chromosomes from the tetraploid population [4; 5; 6]. A universal class of cytogenetic change is deletions, also observed as loss of heterozygosity (LOH). LOH /microsatellite instability at 3p, 9p, 17p, and 18q chromosomal locations[7] are among the most common [5; 6; 8; 9; 10; 11]. Patients with benign premalignant lesions that harbored HNSCC specific genetic loses and LOH had a significantly increased risk of developing cancer[12].

Mutations in the tumor suppressor *p53* gene occur in 45 to 70% of HNSCC and strategies targeting the *p53* gene and protein may halt or reverse the process of tumorigenesis [13]. Another important gene product in HNSCC pathogenesis is the $p16^{INK4a}$ (p16) protein made by the $p16^{INK4a}$ (*CDKN2A*) gene located at 9p21. p16 is a cyclin-dependent kinase inhibitor that inhibits phosphorylation of the retinoblastoma protein (pRb) and blocks cell cycle progression at the G1 to S check point[14]. Loss of p16 expression by deletion, mutation, or hypermethylation is common in HNSCC[15; 16] and is associated with worse prognosis in laryngeal squamous cell carcinomas[17].

1.3 Epigenomics and Cancer

The study of human disease has focused primarily on genetic mechanisms. Dispelling the belief that the only way to treat such conditions is by fixing or replacing damaged genes, scientists are instead focusing on the field of epigenetics--the study of changes in gene silencing that occur without changing the DNA sequence. Many types of epigenetic processes have been identified--they include methylation, acetylation, phosphorylation, ubiquitylation, and sumolyation. Epigenetic processes are natural and essential to many organism functions, but if they occur improperly, there can be major adverse health and behavioral effects.

Perhaps the best known epigenetic process, in part because it has been easiest to study with existing technology, is DNA methylation. This is the addition or removal of a methyl group (CH_3). Hypermethylation is a well described DNA modification that has been implicated in normal mammalian development, [18; 19] imprinting [20] and X chromosome inactivation [21]. However, recent studies have identified hypermethylation as a probable cause in the

development of various cancers [22; 23; 24]. Aberrant methylation by DNA-methyltransferases in the CpG islands of a gene's promoter region can lead to transcriptional repression akin to other abnormalities such as a point mutation or deletion [25]. Gene transcriptional inactivationvia hypermethylation at the CpG islands within the promoter regions is an important mechanism [26]. This anomalous hypermethylation has been noted in a variety of tumor-suppressor genes (TSGs), whose inactivation can lead many cells down the tumorigenesis continuum [26; 27; 28]. In many cancers, aberrant DNA methylation of so called “CpG islands”, CpG-rich sequences frequently associated with promoters or first exons, is associated with the inappropriate transcriptional silencing of critical genes [29; 30; 31]. These DNA methylation events represent an important tumor-specific marker occurring early in tumor progression and one that can be easily detected by PCR based methods in a manner that is minimally invasive to the patient.

1.4 Significance of DNA Methylation

When compared to the genomic , which is identical in every cell and tissue in the human body, the epigenome is highly variable over the life course, from tissue to tissue and from environment to environment [32]. Also, unlike genes that are inactivated by nucleotide sequence variation, genes silenced by epigenetic mechanisms are still intact and, thus, retain the potential to be reactivated by environmental or medical intervention[32]. There are several current human therapeutic intervention trials to reverse deleterious epigenetic changes. Some examples include epigenetic therapeutic trials to treat T-cell lymphoma based on reactivation of tumor suppressor genes[33] and similar trials to prevent colorectal cancer by inhibiting the enzyme responsible for DNA methylation[34]. Such therapies have shown promise in halting tumor growth by reactivation of the tumor suppressor gene or by blocking progression of precancerous epigenetic lesions. Additionally, demethylating drugs in combination with therapeutic HPV DNA vaccines have been found to control more effectively a variety of HPV-associated malignancies[35]. This is due to the fact that DNA methylation is capable of decreasing expression of the encoded antigen of the DNA vaccines[35]. In fact, preliminary studies already suggest that there is promise of improving preventative HPV DNA vaccine therapy by the addition of the demethylating drug 5-aza-2 deoxycytidine[35].

1.5 DNA Methylation in HNSCC

Promotor hypermethylation of genes in HNSCC have been reported for *p16*, *p14*, *DAP-K*, *RASSF1A* [36; 37; 38; 39; 40; 41; 42], *RARβ2* [43; 44; 45], *MGMT*, a DNA repair gene that functions to remove mutagenic (O^6-guanine) adducts from DNA [46], and *E-cadherin*, a Ca^{2+}-dependent cell adhesion molecule that functions in cell-cell adhesion, cell polarity, and morphogenesis [47].

Historically, the molecular pathogenesis of cancer has been teased out one gene at a time. The majority of published epigenetic data in HNSCC comes from methylation specific PCR (MSP) following bisulfite treatment, first described by Herman JG et. al. [48] (gel electrophoresis separation of products). The success of MSP has been attributed to its increased sensitivity, however, it generally relies on a pre-selected number of genes, assessed one gene at a time, as opposed to high-throughput microarray based methylation analysis [49] and multi- candidate gene applications [50]. In HNSCC, recent comprehensive high-throughput methods from our group and others have underscored the contribution of both genetic [15; 51; 52] and epigenetic events [42; 53; 54; 55; 56; 57], often working together [50], in the development and progression of HNSCC.

2. Delineating an Epigenetic Continuum in HNSCC

Gene silencing via hypermethylation is still a relatively new idea in the development of HNSCC. To assess the contribution of epigenetics to the development of neoplasia, its transformation, progression, and recurrence in HNSCC, we examined promoter hypermethylation of tumor suppressor genes along a progression continuum from benign papillomas to malignancy in HNSCC using a multi-candidate gene (Table 1) assay, the Methylation Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) assay (MS-MLPA, Figure 1) [50; 58]. The candidate gene panel comprises 22 tumor suppressor genes (**Table 1**), many of which are involved in head and neck cancer

2.1 Benign Papillomas

Papillomas are benign neoplasms of epithelium on a connective tissue core [59]. They can involve the nose and sinuses (sinonasal papillomas - SP) as well as the respiratory tract (respiratory papillomatosis - RP) to include the larynx, trachea, and bronchi. Both SP and RP have a tendency to recur. Recurrent respiratory (laryngeal) papillomatosis (RRP) is an extremely rare condition [60]. Inverted SP are associated with invasive squamous cell carcinoma (SCC) [61] and a small percentage of RRP cases also progress to malignancy [62].

Human papilloma virus (HPV) is frequently associated with sinonasal [63; 64] and laryngeal [65; 66; 67] papillomas. Most HPV-positive cases of SP are of the inverted type [68]. Benign papillomas are preferentially associated with the low-risk HPV types 6 and 11, whereas their malignant counterparts are exclusively positive for HPV-16 DNA [69]. Studies on HPV typing in benign laryngeal papillomas have demonstrated an association of HPV-11 with a more aggressive course of the disease [70; 71]. HPV infection in inverted papillomas [72] and in particular HPV-11 infection in RRP [73] may be an early event in a multistep process of malignant transformation.

2.1.1 Sinonasal Papillomas—Sinonasal papillomas have been categorized histologically as inverted, fungiform (exophytic), and cylindrical cell papillomas [74]. Inverted papillomas are the most commonly occurring sinonasal papillomas followed by exophytic [61]. Inverted papillomas are benign, rare sinonasal lesions well known for their local recurrence, invasiveness and predisposition for malignant transformation. Recurrence rates vary widely, ranging from 6% to 33%, despite management by different surgical treatment options [75]. Malignant transformation occurs in 7 to 10% of cases [61; 76]. Morphology is not useful in determining if a lesion will recur or acquire malignant changes. A general belief is that once excised, and in the absence of malignancy in the excised specimen, a recurrence is unlikely to convert to malignancy [77].

Benign inverted papillomas were reported as monoclonal but lacking common genetic alterations associated with squamous head and neck cancer [77]. To assess epigenetic alterations of promoter hypermethylation, not previously reported in sinonasal papillomas, we evaluated 7 patients with primary and recurrent sinonasal papillomas for aberrant promoter methylation status using MS-MLPA and confirmed aberrant methylation using conventional MSP[78]. We found all 7 cases had at least one epigenetic event of aberrant DNA hypermethylation with 10 of the 22 methylation-prone genes being methylated (**Table 2**). Commonly methylated genes included *CDKN2B, CDKN2A, TP73,* and *ESR1*. Recurrent biopsies from 2 inverted papilloma cases had common epigenetic events: aberrant methylation of *CDKN2B* and *DAPK1* in case 1, and *CDKN2B* in case 2, underscoring monoclonality for these lesions [78] (**Table 2**).

2.1.2 Laryngeal Papillomas—Respiratory papillomatosis (RP) is a benign disease characterized by unregulated growth of wartlike neoplasms of the larynx, trachea, and bronchi with propensity for recurrences (RRP). In the larynx, the stratified squamous variety is the commonest form of papilloma [59]. The histopathology is similar at all ages. Laryngeal papillomas usually run a benign but recurrent course. In the spontaneous transformation of RP or RRP to squamous cell carcinoma (SCC), a progression continuum to malignancy may not be histologically and clinically apparent, making these lesions difficult to diagnose early in the course of the transformation of the disease. Only a small percentage of RRP cases actually progress to malignancy [62; 79]. Transformation of laryngeal papillomas to malignant neoplasms range from 1.25% to 42.9% [80; 81].

Recurrent respiratory (laryngeal) papillomas (RRP) present primarily as tiny warts on the vocal cords and can be potentially life-threatening due to airway obstruction [60]. Human papillomavirus types 6 and 11 account for 80-90% of RRP [82].

The contribution of promoter hypermethylation to the pathogenesis of RP, including recurrences (RRP)[83] and progression to squamous cell carcinoma (SSC) was examined in a retrospective cohort of 25 laryngeal papilloma cases included 21 RRP, two of which progressed to SCC[84].

Promoter hypermethylation by MS-MLPA or by MSP was recorded in 22 of 25 cases. Twenty of 22 tumor suppressor genes in the multi-gene panel had altered DNA methylation in at least one RP biopsy. Aberrant methylation of *TIMP3* and *CDKN2B* genes was most frequent, occurring in 13 of 22 and 11 of 22 cases, respectively, followed by *CDKN2A, APC* and *VHL* genes in 9 of 22 cases, and *TP73, GSTP1, HIC1, MLH1* and *DAPK1* genes in 5 of 22 cases.

Of the 21 RRP cases, multiple biopsies were examined for aberrant methylation in 15 cases. Identical abnormally methylated genes were found in recurrent biopsies of 5 of 15 RRP cases and an aberrantly methylated *CDKN2B* gene linked all 5 cases (cases 4, 7, 11, 12, 13) [83] (**Table 3**).

Progression to SCC occurred in RRP cases 1 and 5 (**Table 4**). In RRP Case 1, the papillomas in biopsies 1 through 3 were located on both the left and right vocal folds. Subsequent dysplastic papillomas were located on both left and right true as well as false vocal cords (biopsies 4-6, **Table 3**). In RRP Case 1, aberrant methylation of *BRCA2* and *APC*, identified in the primary biopsy, was also present in the recurrent severe dysplasia, CIS, and recurrent SCC (**Table 3**). MSP confirmed MS-MLPA methylation of *BRCA2* (biopsy 1), *APC* (biopsy 4), *GSTP1* (biopsy 6), and *CDKN2A* (biopsies 5 and 6). MSP and MS-MLPA were concordant for lack of methylation *APC, GSTP1*, and *CDKN2A*, and *CDKN2B* (**Table 4**).

In RRP Case 5, aberrant methylation of *BRAC2, APC* and *CDKN2A* in the reference papilloma biopsy and *CDKN2B* in biopsy 2 were also identified in the subsequent progression lesions (**Table 4**). MSP confirmed MS-MLPA methylation of *APC* (biopsies 1 and 4) and *CDKN2A* (biopsies 1-3). MSP also confirmed absence of methylation for *CDKN2B* (biopsies 1 and 4) and *GSTP1* (biopsies 2-4) detected by MS-MLPA.

Of the 25 cases, 22 were positive for HPV-6, 2 for HPV-11 and 1 for HPV-16 and 33[84]. In RP, human papillomavirus types 6 and 11 account for 80-90% of RP [82]. In our cohort, types 6 and 11 account for 96% of the cases. Of the 25 cases, 22 were positive for HPV-6, 2 for HPV-11 and 1 for HPV-16 and 33[84]. HPV-11 appears to confer a more aggressive neoplastic phenotype than HPV-6 and is associated more often with atypia and frequent

recurrence [85]. Of the two RRP cases in this cohort positive for HPV-11, only Case 5 progressed to SCC. Though the majority of RP harbor low risk HPV 6 and 11, high-risk HPV types 16 and 18 have been reported and multiple HPV types were detected in 11.8% of RP [86]. RRP Case 1 with multiple HPV types (HPV-16 and 33 positive) progressed to SCC. High-risk HPV DNA alone may be sufficient to initiate tumorigenesis in the absence of traditional risk factors such as tobacco or alcohol use [86]. Oncogenic HPV, particularly HPV-16, has been established as a causative agent for 25% of head and neck squamous cell carcinoma (HNSCC) [87] and the development of laryngeal carcinoma is associated with HPV infection [87; 88].

2.2 HNSCC Tumors

2.2.1 HNSCC Cell lines—Recently, in paired HNSCC primary A) and recurrent or metastatic (B) UMSCC-11A/11B, UMSCC-17A/17B, and UMSCC-81A/81B cell lines, using MS-MLPA, we identified nine genes, *TIMP3, APC, KLK10, TP73, CDH13, IGSF4, FHIT, ESR1*, and *DAPK1* that were aberrantly methylated in paired HNSCC primary A) and recurrent or metastatic (B) UMSCC-11A/11B, UMSCC-17A/17B, and UMSCC-81A/81B cell lines[50].The most frequently hypermethylated genes were *APC* and *IGSF4* observed in 3/6 cell lines, and *TP73* and *DAPK1* observed in 2/6. For *KLK10* and *IGSF4, TIMP3* and *FHIT*, and *TP73*, in recurrent/metastatic cell lines, promoter hypermethylation was a disease progression event, indicating complete abrogation of tumor suppressor function for *KLK10, IGSF4*, and *TIMP3*, and gene silencing of one of two copies of *TP73*. Hypermethylation of *IGSF4, TP73, CDH13, ESR1, DAPK1*, and *APC* were primary events in UMSCC-17A. Gene silencing through promoter hypermethylation was observed in 5/6 cell lines and contributed to primary and progressive events in HNSCC [50]. In addition to genetic alterations of gains and losses, epigenetic events appear to further undermine a destabilized genomic repertoire in HNSCC.

2.2.2 Primary HNSCC Tissue—Subsequently [43] , we evaluated aberrant methylation status in 28 primary HNSCC using MS-MLPA and confirmed aberrant promoter methylation using conventional MSP and real time PCR. MS-MLPA promoter methylation profiling identified *RARβ, APC*, and *CHFR* as frequent epigenetic events. Promoter hypermethylation of *RARβ* and *APC* in both early and late stage tumors and of *CHFR* in only late stage tumors appear to suggest an epigenetic progression continuum, with *CHFR* as a late event and a putative diagnostic biomarker for late stage disease[43]. In another study of 79 primary laryngeal squamous cell carcinoma, aberrant methylation of *ESR1* and *HIC1* signified independent markers of poorer outcome[89].

3. A hypothetical epigenetic progression model

Based on the results of the described studies in benign papillomas, recurrent laryngeal papillomas with subsequent progression to SCC, and DNA methylation events in primary HNSCC and cell lines, a postulated hypothetical model is described in **Figure 1**. In benign and recurrent paillomas, frequently methylated genes included *CDKN2B, CDKN2A, APC, VHL, TP73, GSTP1, HIC1, MLH1*and *DAPK1*[78; 83]. Epigenetic events of progression from recurrent benign to squamous carcinoma were noted for promoter hypermethylation of *CDKN2B, CDKN2A, APC*, and *BRCA2* tumor suppressor genes[83].

In HNSCC paired cell lines, hypermethylation of *IGSF4, TP73, CDH13, ESR1, DAPK1*, and *APC* were primary events. For *KLK10* and *IGSF4, TIMP3* and *FHIT*, and *TP73*, in recurrent/metastatic cell lines, promoter hypermethylation was a disease progression event (**Figure 1**). DNA hypermethylation events in primary HNSCC tissue include *RARβ, APC, CHFR, CDKN2A, CDKN2B, BRCA2, HIC1*, and *ESR1*[43; 50; 89].

3.1 Significance of methylation events in the tumorigenesis progression continuum from benign and recurrent papillomas to squamous cell carcinoma

Genetic alterations in *CDKN2A* and *CDKN2B* genes, which map to 9p21, have been linked to malignant progression in HNSCC [15; 90; 91]. Inactivation of the *CDKN2B* (p15) and *CDKN2A* (*p14* and *p16*) genes at the genomic and epigenetic level is a frequent event in human oral SCCs and in HNSCC [50; 52; 92]. The presence of aberrant methylation of *CDKN2A* and *pCDKN2B* in precancerous oral tissues [92] implicates *methylation of these genes* as early events in the pathogenesis of oral lesions. *APC* (adenomatosis polyposis coli) is a tumor suppressor gene originally implicated in colon cancer. Genetic and epigenetic alterations in this gene have since been recognized in other malignancies including OSCC, gastric cancers and esophageal adenocarcinomas. Uesugi et al. [93] previously reported mutations and/or deletions of *APC* in primary OSCC and suggested that loss of *APC* function contributes to carcinogenesis in the oral region. *APC* inactivation as a result of promoter hypermethylation occurred in 25% of OSCC cell [93]. *APC* (adenomatosis polyposis coli) is a tumor suppressor gene originally implicated in colon cancer. Genetic and epigenetic alterations in this gene have since been recognized in other malignancies including OSCC, gastric cancers and esophageal adenocarcinomas. Aberrant methylation of *CDKN2A, CDKN2B, APC,* and *BRCA2* in initial benign, recurrent and subsequent transformation biopsies[83] indicate these as early events and provides evidence of a monoclonal progression continuum to SCC (**Figure 1**).

Alterations of *RARβ, APC,* and *CHFR* via DNA hypermethylation identified in primary HNSCC, have several implications. Decreased expression of *RARβ* has been associated with increased keratinizing squamous differentiation in HNSCC cells and pharmacological doses of retinoid ATRA (9-*cis*-RA) induced RARβ in HNSCC cells, resulting in restoration of a more normal differentiation [94]. More importantly, $RAR\beta_2$ silencing by promoter hypermethylation was shown to be an early event in head and neck carcinogenesis and 5-Aza-CdR restored $RAR\beta_2$ inducibility by ATRA in most cell lines [95]. We reported *CHFR* as a solely late stage 4 event, occurring in 7/28 HNSCC [43], suggesting a role for *CHFR* in tumor progression with potential utility as a biomarker of late stage disease. Treatment with the methyltransferase inhibitor 5-aza-2'-deoxycytidine induced re-expression of CHFR [96]. Additionally, because cancer cells that lack CHFR expression have shown to be more susceptible to the microtubule inhibitor taxol [96], silencing of *CHFR* by methylation can serve as a marker for predicting sensitivity to particular chemotherapeutic agents. *APC,* like *RARβ,* was hypermethylated in early and late stage tumors, suggesting DNA methylation of *APC* and *RARβ* as earlier epigenetic events, when compared to *CHFR*.

4. Concluding remarks and perspectives

Epigenetic events of promoter hypermethylation are emerging as one of the most promising molecular strategies for cancer detection and represent an important tumor-specific marker occurring early in tumor progression. In benign papillomas, the high frequency of DNA hypermethylation events supports the utilization of gene silencing mechanisms as one of the driving forces behind their growth, reiterating DNA hypermethylation events as hallmarks of sinonasal and laryngeal papilloma pathogenesis, some of which are initiating clonal alterations in the recurrence continuum in some sinonasal[78] and RRP cases [83]. Aberrant methylation of *BRCA2, APC, CDKN2A* (*p16*) and *CDKN2B,* detected in the initial and all subsequent transformation biopsies in some RRP, appears to be an early event in the pathogenesis of laryngeal papillomatosis tracing a monoclonal progression continuum to SCC.

Epigenetic alterations identified in precancerous lesions with biomarker potential would have high clinical significance in risk assessment and early detection, and may also serve as

molecular targets for chemopreventive interventions. Because promoter hypermethylation is potentially reversible, the molecules that regulate methylation status of DNA are considered promising targets for new cancer therapies.

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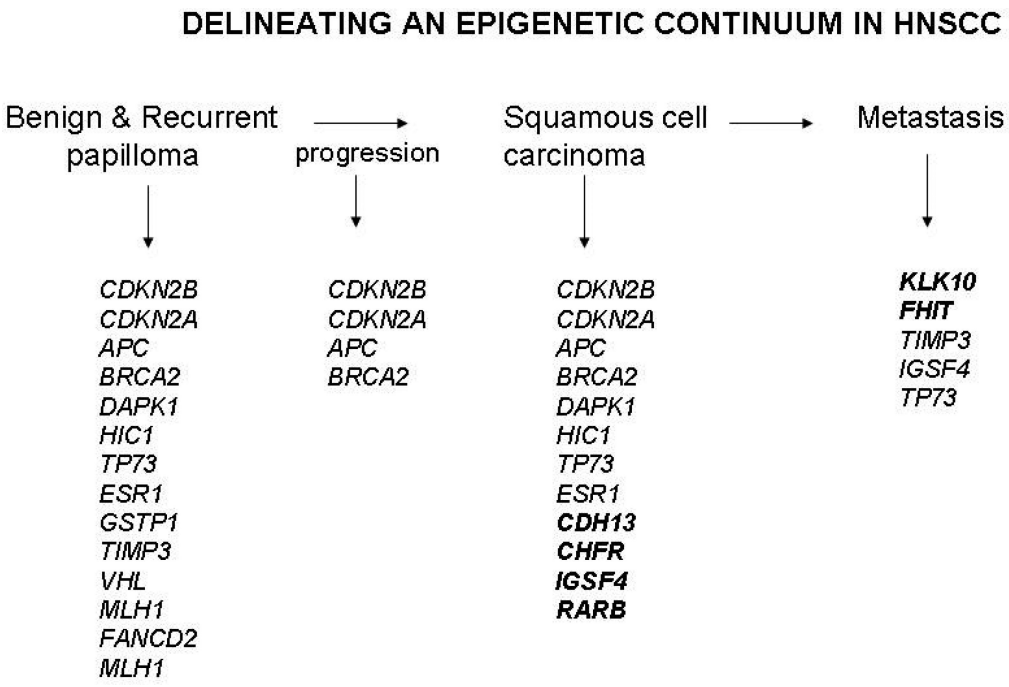

Figure 1. A postulated hypothetical epigenetic progression model

DNA hypermethylation of *CDKN2B, CDKN2A, APC, BRCA2, DAPK1, HIC1*, ***TP73***, and *ESR1* suggest early events in the tumorigenesis continuum from benign to primary SCC. *CDH13, CHFR, IGSF4*, and *RARB* (bolded) appear to be primary tumor-specific events and *KLK10* and *FHIT* progression to metastasis-specific events.

Table 1

Methylation-Specific MLPA Probe Panel (ME001)

#	Gene probe	Chrom Loc	#	Gene probe	Chrom Loc	#	Gene probe	Chrom Loc
1	***TP73***	01p36		*CDK6*	07q21.3		*PAH*	12q23
2	***CASP8***	02q22.3	12	***CDKN2A***	09p21	21	***CHFR***	12q24.33
3	***VHL***	03p25.3	13	***CDKN2B***	09p21	22	***BRCA2***	13q12.3
4	***RARB***	03p24	14	***DAPK1***	09q34.1		*BRCA2*	13q12.3
5	****MLH1***	03p21.1		*AI651963*	10p14		*MLH3*	14q24.3
6	***MLH1***	03p21.1		*CREM*	10p12.1		*TSC2*	16p13.3
	CTNNB1	03p22	15	***PTEN***	10q23.3		*CDH1*	16q22.1
7	****RASSF1***	03p21.3	16	***CD44***	11p12	23	***CDH13***	16q24.2
8	***RASSF1***	03p21.3	17	***GSTP1***	11q13	24	***HIC1***	17p13.3
9	***FHIT***	03p14.2	18	***ATM***	11q23	25	***BRCA1***	17q21
	CASR	03q21	19	***IGSF4***	11q23		*BCL2*	18q21.3
10	***APC***	05q21		*TNFRSF1A*	12p13		*KLK3*	19q13
11	***ESR1***	06q25.1		*TNFRSF7*	12p13	26	***TIMP3***	22q12.3
	PARK2	06q26	20	***CDKN1B***	12q13.1			

Chrom Loc: chromosomal location of probe

#number of probes (n=26 probes) with *HhaI* site (bolded)

*genes with multiple probes in the promoter region

Table 2

Methylation events in sinonasal papillomas

	Biopsies	*TIMP3*	*APC*	*CDKN2A*	*MLH1*	*CDKN2B*	*TP73*	*FANCD2*	*DAPK1*	*ESR1*	*GSTP1*
Case 1 - IP	1 - Reference	**M**			**M**	**M**	**M**	**M**	**M**		
(recurrent)	2 - 10 months					**M**			**M**	**M**	
	2 - 10 months					**M**					
Case 2 - IP	1 - Reference					**M**					
(recurrent)	2 - 6 months			**M**		**M**	**M**				**M**
Case 3 - IP	1- Reference					**M**					
Case 4 - IP/EP	1- Reference					**M**				**M**	
Case 5 - IP/EP	1- Reference					**M**					
Case 6 - EP	1- Reference			**M**		**M**					
Case 7 - EP	1- Reference		**M**								
Number of methylated genes/7 cases		1/7	1/7	2/7	1/7	6/7	2/7	1/7	1/7	2/7	1/7

M = methylated

IP = inverted papilloma

EP = exophytic papilloma

IP/EP = inverted and exophytic papilloma

Table 3

Epigenetically Linked Recurrent Laryngeal Papilloma Cases

Cases	Biospy	*APC*	*CDKN2B*	*VHL*	*TP73*	*GSTP1*
4	1 - Reference		**M**			
	2 - 10 months		**M**		**M**	
	3 - 30 months		**M**		**M**	
7	1 - Reference	**M**	**M**			**M**
	2 - 3 months		**M**			
	3 - 6 months	**M**	**M**			**M**
11	1 - Reference		**M**			
	2 - 15 months		**M**			
12	1 - Reference		**M**			
	2 - 14 months		**M**			
13	1 - Reference	**M**		**M**		
	2 - 1 month	**M**	**M**	**M**		
	3 - 3 months	**M**	**M**	**M**		

M = methylated

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Table 4

Epigenetic Progression Continuum to Squamous cell Carcinoma

RRP	Lesion Type	Biospy	Time Interval	*BRCA2*	*APC*	*CDKN2A*	*CDKN2B*
Case 1	Squamous Papilloma	1	Primary	**M***	**M†**	**U***	**U***
	Squamous Papilloma with severe dysplasia	2	4 months	**M†**	**M†**	**U***	**U**
	Primary SCC, Block 1 tumor	3T	6 months	**M†**	**U***	**U***	**U**
	Primary SCC, Block 1 dysplastic papilloma	3P	6months	**M†**	**M†**	**U***	**U**
	Recurrent Severe Dysplasia	4	50 months	**M†**	**M***	**M†**	**U***
	Carcinoma in Situ	5	51 months	**M†**	**M†**	**M***	**U***
	Recurrent SCC	6	53 months	**M†**	**M†**	**M***	**U, NR by MSP**
Case 5	Squamous Papilloma with moderate to severe dysplasia	1	Reference	**M†**	**M***	**M***	**U***
	Squamous Papilloma with mild to moderate dysplasia	2	2 months	**M†**	**M†**	**M***	**M**
	Primary SCC	3	9 months	**M†**	**M†**	**M***	**M**
	Carcinoma in Situ	4	11 months	**M†**	**M***	**M†**	**U***

SCC = Squamous Cell Carcinoma

M = methylation detected by MS-MLPA only

M† = methylation detected by MSP only

M* = MS-MLPA methylation confirmed by MSP

U = unmethylated by MS-MLPA only

U* = unmethylated by MS-MLPA and MSP

NR = no reaction by MSP because of insufficient DNA

neg = negative for HPV