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Promoter methylation in head and neck tumor genesis

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Summary

In addition to genetic alterations of gains and losses, epigenetic events of promoter methylation appear to further undermine a destabilized genomic repertoire in squamous head and neck carcinoma (HNSCC). This review provides an overview of frequently methylated tumor suppressor genes in benign head and neck papillomas, primary HNSCC tumors, and HNSCC cell lines and their relevance as epigenetic markers in head and neck tumorigenesis.

Keywords

promoter methylation; benign; malignant; primary; cell lines

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 alone, it accounts for nearly 3.2% of all newly diagnosed cancers (1). Not only one of the most ubiquitous, HNSCC is also one of most lethal cancers responsible for 2.1% of all cancer deaths in the United States and is noted as the sixth most common malignant disease worldwide (1). HNSCC carries a high mortality rate despite advances in chemotherapy and radiation therapies. This is due mainly to the highly heterogeneous nature of the disease, both morphologically and genetically. 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.

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. These processes are natural and essential to many organism functions, but if they occur improperly, there can be major adverse health and behavioral effects.

Epigenetic regulation is central to the biological function of all cells. 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, (2, 3) imprinting (4) and X chromosome inactivation (5).

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However, recent studies have identified hypermethylation as a probable cause in the development of various cancers (6–8). 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 (9). Gene transcriptional inactivation via hypermethylation at the CpG islands within the promoter regions is an important mechanism (10). This anomalous hypermethylation has been noted in a variety of tumor-suppressor genes (TSGs), whose inactivation can lead many cells down the tumorigenesis continuum (10–12). 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 (13–15). 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.

Studies of sequential molecular alterations and genetic progression of pre-invasive HNSCC have not been clearly defined. A tissue field of somatic genetic alterations precedes the histopathological phenotypic changes of carcinoma (16). 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. A few studies have shown recurring alterations of chromosome 9p21 in the early stages of HNSCC (17–19). 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. Therefore, 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.

DNA methylation in HNSCC

Numerous tumor suppressor genes have been implicated as targets for methylation in other cancers (13–15). Promotor hypermethylation of genes in HNSCC have been reported for *p16, p14, DAP-K*, *RASSF1A* (20–26), *RARβ2* (27–29), *MGMT*, a DNA repair gene that functions to remove mutagenic (O^6-guanine) adducts from DNA (30), and *E-cadherin*, a Ca^{2+}- dependent cell adhesion molecule that functions in cell-cell adhesion, cell polarity, and morphogenesis (31).

Historically, the molecular pathogenesis of cancer has been teased out one gene at a time. The development of several new high throughput analytical methods for the analysis of DNA, mRNA, and proteins within a cell (32–35) have permitted a more detailed molecular characterization of the cancer genome. In HNSCC, recent comprehensive high-throughput methods from our group and others have underscored the contribution of both genetic (36–38) and epigenetic events (26, 39–43), often working together (44), in the development and progression of HNSCC. In HNSCC, methylation of *p16*, *RARβ*, and *MGMT* suggested early events, with incidences of methylation in HNSCC cell lines and primary tumors being similar (27, 43–46).

Aberrant DNA methylation patterns in HNSCC have served as powerful diagnostic, prognostic, and risk assessment biomarkers. Promoter hypermethylation pattern of the *p16*, *MGMT*, *GSTP1,* and *DAPK* genes have been used as molecular markers for cancer cell detection in the paired serum DNA and almost half of the HNSCC patients with methylated tumors were found to display these epigenetic changes in the paired serum (26).

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A: HNSCC Cell lines

The majority of published epigenetic data in HNSCC comes from methylation specific PCR following bisulfite treatment (MSP) (47). 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 (48) and multi-candidate gene applications (44). Recently, using a multi-candidate gene approach, the Methylation Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) assay (MS-MLPA, Figure 1) (44, 49), 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 (Figures 2–4)(44).

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 (Figure 3). Gene silencing through promoter hypermethylation was observed in 5/6 cell lines and contributed to primary and progressive events in HNSCC (44). In addition to genetic alterations of gains and losses, epigenetic events appear to further undermine a destabilized genomic repertoire in HNSCC.

B: Primary HNSCC tissue

Subsequently (27), we evaluated aberrant methylation status in 28 primary HNSCC using MS-MLPA (Figures 5, 6) and confirmed aberrant promoter methylation using conventional Methylation Specific PCR (MSP) (47) (gel electrophoresis separation of products, Figures 7,8) and real time PCR following bisulfite treatment, Figure 9). MS-MLPA promoter methylation profiling of 22 tumor suppressor genes (Table 1), many of which are involved in head and neck cancer, identified *RARβ*, *APC*, and *CHFR* as frequent epigenetic events. These preliminary findings of promoter hypermethylation of *RARβ* and *APC* in both early and late stage tumors and of *CHFR* by MS-MLPA and MSP assays 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. The alterations of *RARβ*, *APC*, and *CHFR* via DNA hypermethylation have several implications in HNSCC. 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 (50). 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 (51). The examination of the prevalence and pattern of *CHFR* inactivation in human tumors found CpG methylation-dependent silencing of CHFR expression in 40%of primary colorectal cancers, 53% of colorectal adenomas, and 30% of primary HNSCC (52). We reported *CHFR* as a solely late stage 4 event, occurring in 7/28 HNSCC (27), 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 (52). Additionally because cancer cells that lack CHFR expression have shown to be more susceptible to the microtubule inhibitor taxol (52), silencing of *CHFR* by methylation can serve as a marker for predicting sensitivity to particular chemotherapeutic agents. *APC* (adenomatosis polyposis coli), a tumor suppressor gene, was originally implicated in colon cancer. Promoter hypermethylation of *APC* has been reported in 25% of oral cancers (53) and in Barrett's metaplasia and dysplasia (54). In

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our primary HNSCC cohort (27), *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.* Validation of these findings in larger HNSCC cohorts would further support these genes as relevant epigenetic biomarkers of cancer therapy given the reversible nature of epigenetic gene silencing.

C. Delineating an epigenetic continuum in HNSCC

A tissue field of somatic genetic alterations precedes the histopathological phenotypic changes of carcinoma (16). 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 pre-invasive HNSCC have not been clearly defined. A few studies have shown recurring alterations of chromosome 9p21 in the early stages of HNSCC (17–19). 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.

Benign Papillomas

Papillomas are benign neoplasms of epithelium on a connective tissue core(55). 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 (56). Inverted SP are associated with invasive squamous cell carcinoma (SCC)(57) and a small percentage of RRP cases also progress to malignancy (58).

Human papilloma virus (HPV) is frequently associated with sinonasal (59, 60) and laryngeal (61–63) papillomas. Most HPV-positive cases of SP are of the inverted type (64). 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(65). Studies on HPV typing in benign laryngeal papillomas have demonstrated an association of HPV-11 with a more aggressive course of the disease(66, 67). HPV infection in inverted papillomas (68) and in particular HPV-11 infection in RRP(69) may be an early event in a multistep process of malignant transformation.

Sinonasal Papillomas

Sinonasal papillomas have been categorized histologically as inverted, fungiform (exophytic), and cylindrical cell papillomas (70). Inverted papillomas are the most commonly occurring sinonasal papillomas followed by exophytic(57). 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(71). Malignant transformation occurs in 7 to 10% of cases (57, 72). 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(73).

Benign inverted papillomas were reported as monoclonal but lacking common genetic alterations associated with squamous head and neck cancer(73). Therefore 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. We

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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.* The *CDKN2B* gene, detected by MS-MLPA (Figure 10), was a consistent target of aberrant methylation and was confirmed by MSP (Figure 11).

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. Inactivation of the *CDKN2B* and *CDKN2A* genes at the genomic and epigenetic level is a frequent event in human oral SCCs (74) and in HNSCC (37, 44, 75). *TP73* and *ESR1* were aberrantly methylated in 2 of the 7 cases. *TP73* is involved in cell cycle regulation and can activate TP53-responsive proteins, inhibit cell growth and induce apoptosis (76). We have reported *TP73* hypermethylation in HNSCC to be a primary as well as a disease progression event (44). *ESR1* has metastasis-suppressor properties in breast cancer cells (77), suggesting a tumor-suppressor role (78). *ESR1* is methylated in Barrett's metaplastic and dysplastic samples as well as in some adenocarcinoma samples suggesting that DNA hypermethylation is an early epigenetic event in the progression of esophageal adenocarcinomas (EAC) (54). These findings support a role for epigenetic events of promoter hypermethylation in the pathogenesis of benign inverted and exophytic papillomas. As a consistent target of aberrant promoter hypermethylation, *CDKN2B* may serve as a useful biomarker and a potential therapeutic target for gene reactivation studies and in disease monitoring for progression.

Recurrent respiratory (laryngeal) papillomas (RRP)

Recurrent respiratory (laryngeal) papillomas (RRP) present primarily as tiny warts on the vocal cords and can be potentially life-threatening due to airway obstruction(56). Human papillomavirus types 6 and 11 account for 80–90% of RRP(79). Laryngeal papillomas usually run a benign but recurrent course. In the spontaneous transformation of RP or RRP to 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. Therefore we investigated alterations in DNA methylation in recurrent biopsies from patients with RRP to asses the contribution of promoter methylation-mediated epigenetic events in RRP tumorigenesis. Samples from 15 subjects who had 1 to 6 subsequent biopsies were interrogated by MS-MLPA. Aberrant methylation of *CDKN2B* and *APC* genes were most frequent, occurring in 8 of 14 cases, with dissimilar epigenetic events in the remaining cases (Table 3). There were 5 cases that had at least one abnormally methylated gene in a recurrent biopsy, of which the *CDKN2B* gene showed consistent hypermethylation in all 5 cases (Table 4). One case demonstrated aberrant methylation of *APC* and *VHL* promoter regions in all three biopsies.

In precancerous oral tissues(75) aberrant methylation of *CDKN2B* has been implicated as an early event in the pathogenesis of oral lesions. *APC* 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 oral squamous cell carcinomas(53). *VHL* is a tumor suppressor gene that is responsible for the Von Hippel-Lindau syndrome which is an inherited familial cancer syndrome that makes patients susceptible to a variety of cancers, malignant and benign. It has been found that treatment of methylated *VHL* tumors with a demethylating agent results in re-expression of the *VHL* transcripts(80). Persistence of the same aberrantly methylated gene in 36% of multiple recurrent biopsies (5/14) in our study supports a monoclonal origin for RRP and permits the tracing of an epigenetic continuum implicating key tumor suppressor genes in RRP. The high frequency of epigenetic events

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points to the utilization of gene silencing mechanisms as one of the driving forces behind the growth of recurrent laryngeal papillomas.

Conclusion

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.

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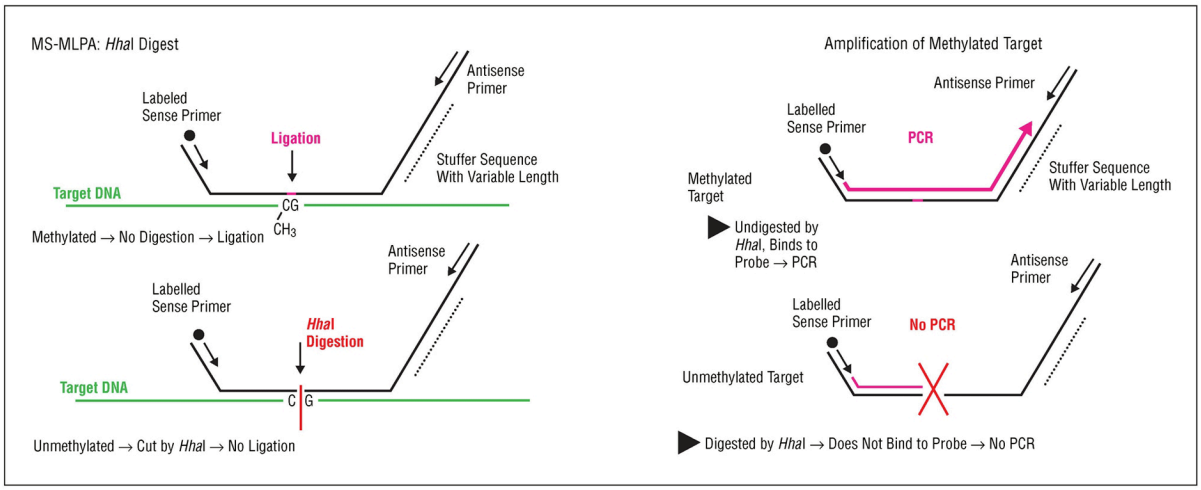

Figure 1.
Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) with and without HhaI. CH3 indicates methyl group; PCR, polymerase chain reaction.

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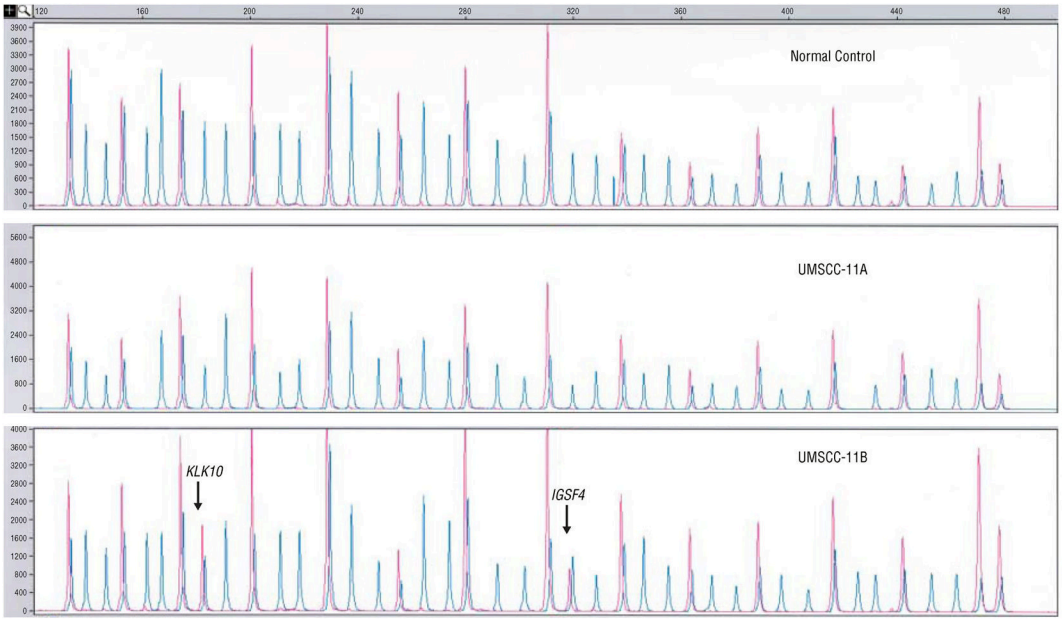

Figure 2.
Multiplex ligation-dependent probe amplification peaks with (red) and without (blue) *Hha*I for the normal DNA, UMSCC-11A, and UMSCC-11B.

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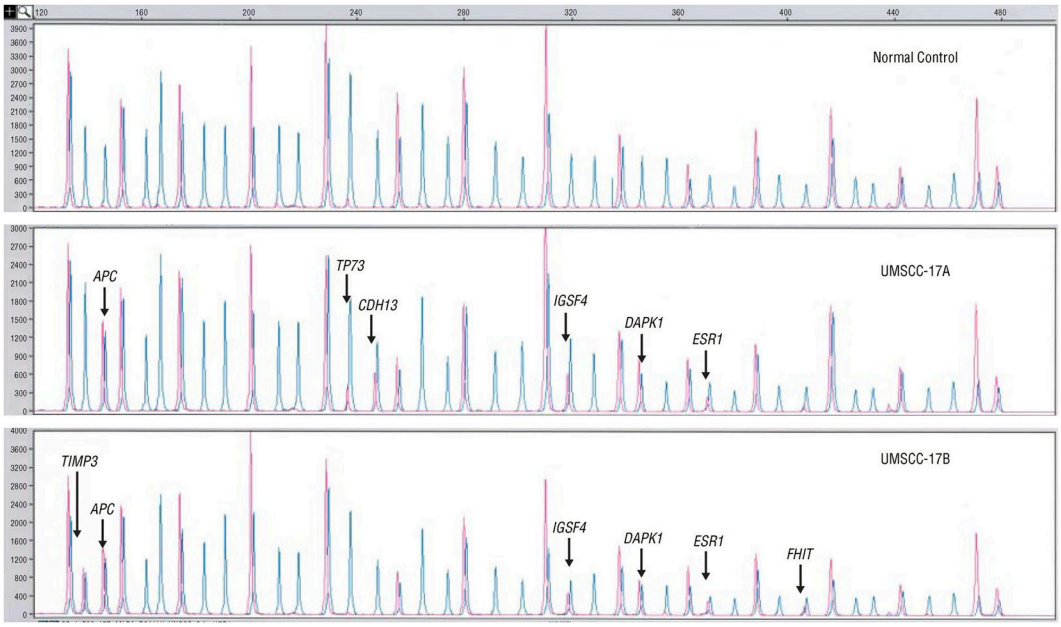

Figure 3.
Multiplex ligation-dependent probe amplification peaks with (red) and without (blue) *Hha*I for the normal DNA, UMSCC-17A, and UMSCC-17B.

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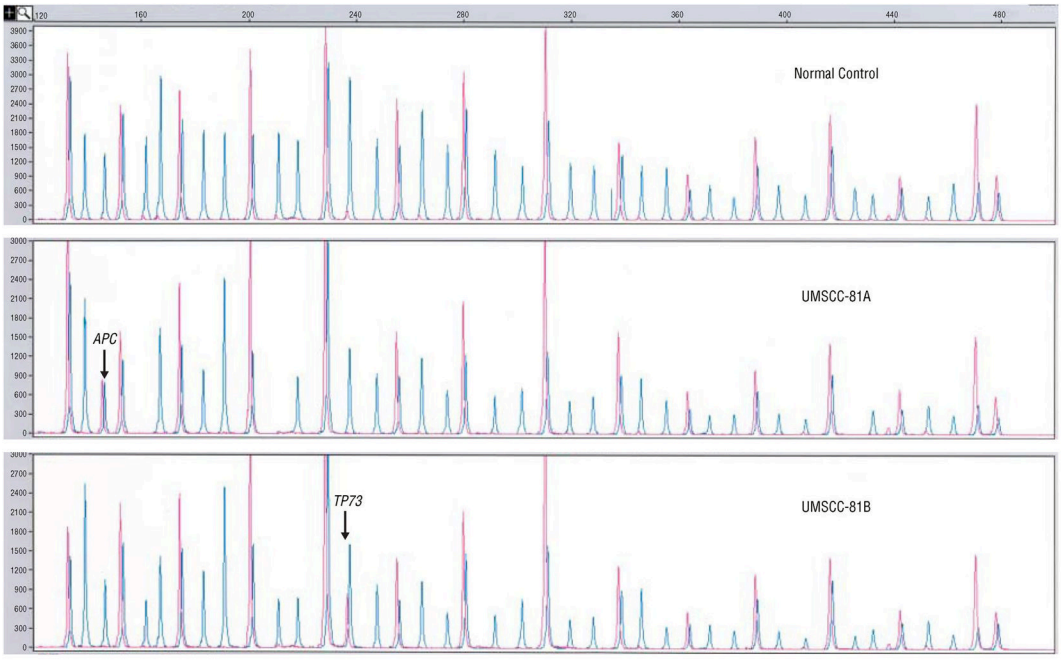

Figure 4.
Multiplex ligation-dependent probe amplification peaks with (red) and without (blue) *Hha*I for the normal DNA, UMSCC-81A, and UMSCC-81B.

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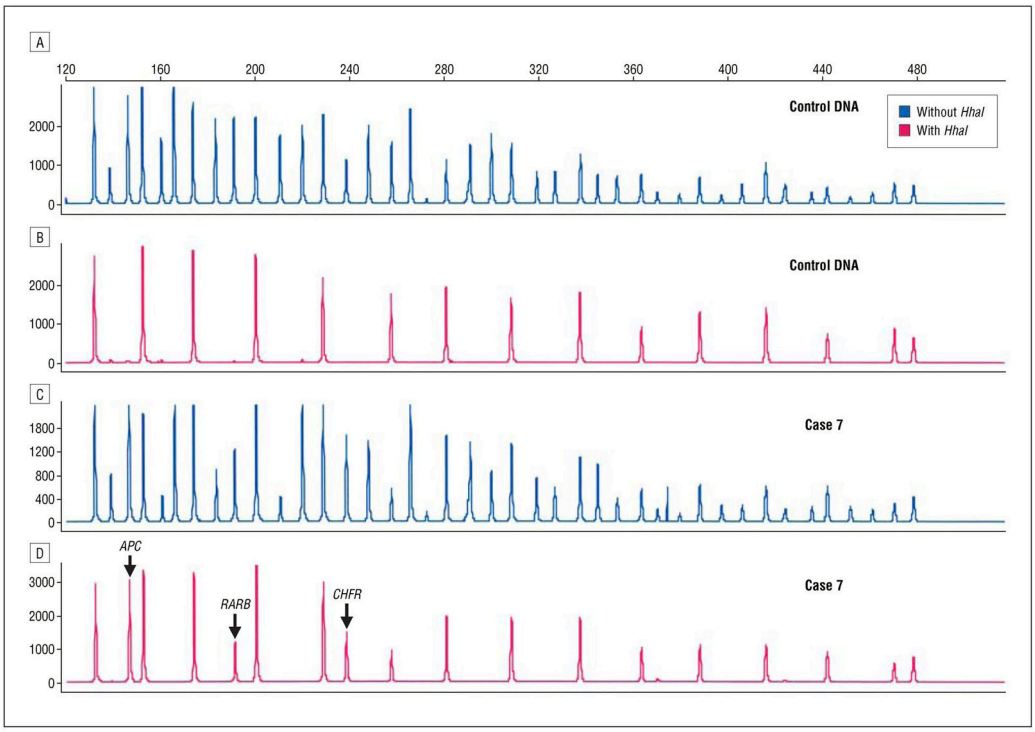

Figure 5.

Methylation-specific multiplex ligation-dependent probe amplification probe mix without (A and C) and with (B and D) *Hha*I enzyme. Fifteen peaks are seen in the control DNA sample (B). The methylation peaks in case 7 (D) that are not present in the control DNA (B) represent promoter hypermethylation *APC*, *RARB*, and *CHFR*.

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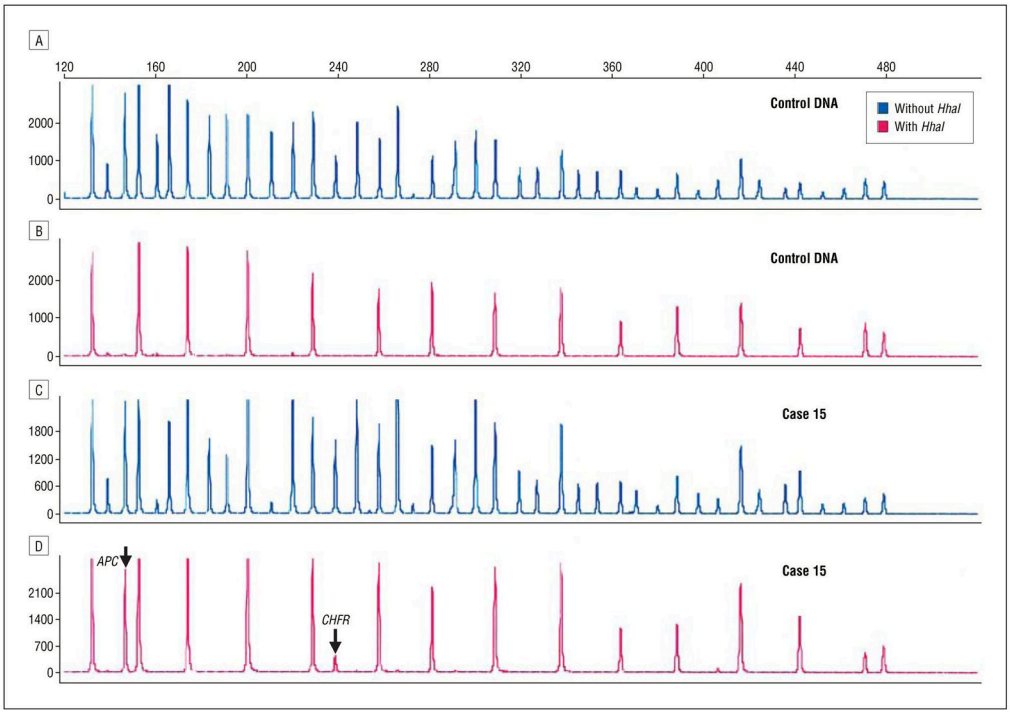

Figure 6.

Methylation-specific multiplex ligation-dependent probe amplification probe mix without (A and C) and with *Hha*I enzyme (B and D) in DNA control DNA and DNA from case 15. Methylation of *APC* and *CHFR* is seen in case 15 with *Hha*I digestion (D).

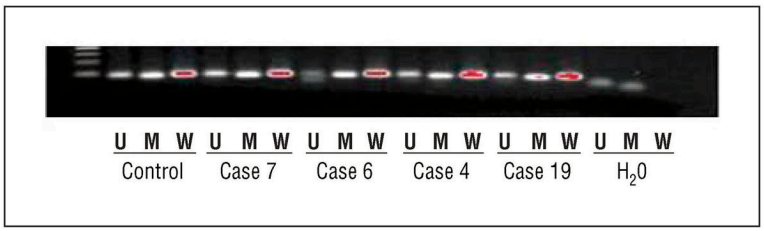

Figure 7.
Gel electrophoresis methylation-specific polymerase chain reaction results for *RARB*. Note the presence of the 84–base pair (bp) methylation (M) band and the unmethylated (U) 94-bp product in cases 4, 6, 7, and 19; the latter indicates an admixture of normal and tumor cells. H2O indicates water; W, wild types.

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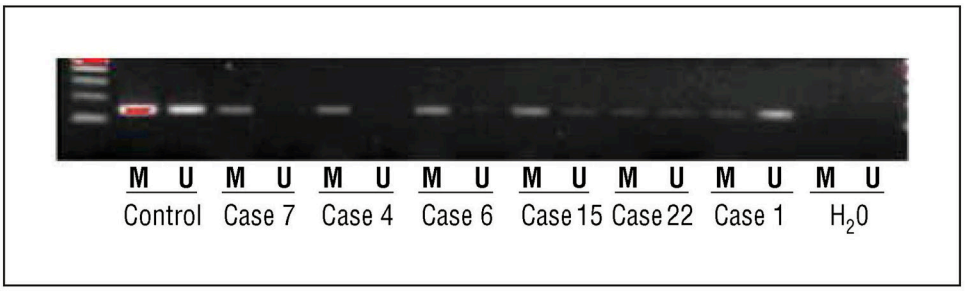

Figure 8.

Gel electrophoresis methylation-specific polymerase chain reaction (MSP) results for *CHFR*. Note the presence of the 155–base pair (bp) methylation (M) band in cases 1, 4, 6, 7, 15, and 22 and the unmethylated (U) 155-bp product in cases 1, 15, and 22; the latter indicates an admixture of normal and tumor cells. H2O indicates water.

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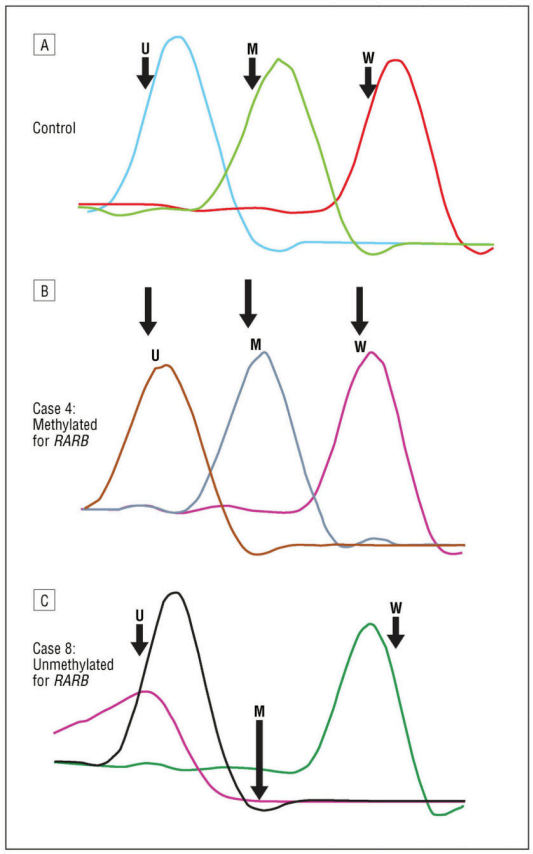

Figure 9.

Real-time polymerase chain reaction (PCR) with methylation-specific PCR (MSP) methylated (M) and unmethylated (U) primers for *RARB* for the control specimen (A), case 4 (B), and case 8 (C). Specific melting temperature peaks are seen for control U, M, and wild-type (W) DNA. The presence of the M melting temperature peak in case 4 indicates promoter hypermethylation of *RARB*, supported by MSP gel electrophoresis (Figure 4). The absence of an M melting temperature peak in case 8 is supported by MSP gel electrophoresis and MS multiplex ligation-dependent probe amplification.

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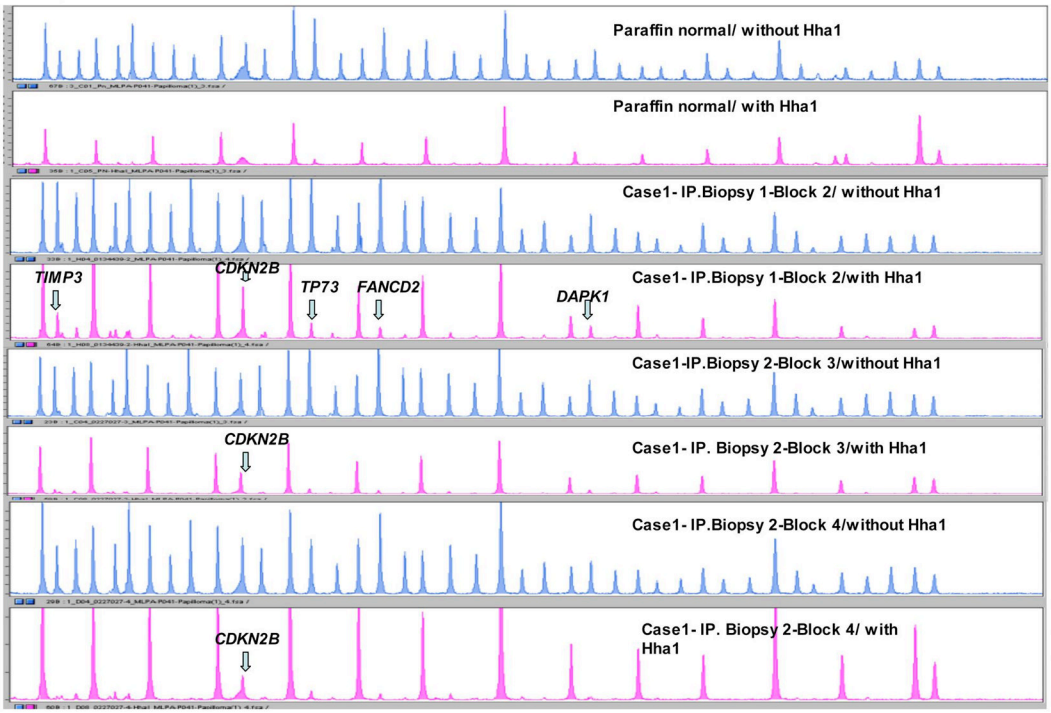

Figure 10.

MS-MLPA probe mix with and without *Hha*I enzyme (DNA sequencer - ABI 3130). Results for Case 1 - biopsy 1 block 2 and biopsy 2 blocks 3 and 4. Note 15 peaks in the control DNA sample with *Hha*I. Presence of a peak in biopsies 1 and 2 (blocks 3 and 4) not present in the control DNA is that of aberrantly methylated *CDKN2B* gene. Presence of peak for aberrantly methylated *DAPK1* in biopsy 1 block 2 and biopsy 2 block 3 not present in the control DNA.

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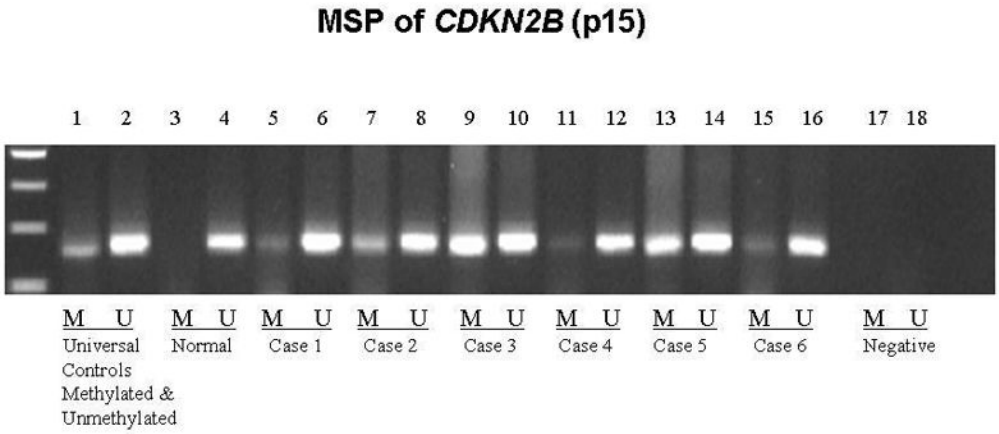

Figure 11.

Methylation Specific PCR (MSP) confirmation of aberrant methylation detected by MS-MLPA for *CDKN2B*. Lane 1: universal methylated control; Lane 2: universal unmethylated control; Lanes 3 & 4: normal control, note presence of only unmethylated PCR product; Lanes 5–16 span Cases 1–6. Note presence of methylated and unmethylated product, the latter indicating admixture of normal and papilloma cells; Lanes 17 & 18: negative control.

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Table 1

Methylation-Specific MLPA Probe Panel (ME001)

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

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#	Gene probe	Chrom Loc
24	***HIC1***	17p13.3
25	***BRCA1***	17q21
	BCL2	18q21.3
	KLK3	19q13
26	*TIMP3*	22q12.3

Bolded=probes with *Hha*I site (n=26 probes);

* genes with multiple probes in the promoter region

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Table 2

Clinical characteristics of cohort with MS-MLPA results

	Biopsies	Location	TIMP3	APC	CDKN2A	MLH1	CDKN2B	TP73	FANCD2	DAPK1	ESR	GSTP1
Case 1—IP	1—reference	Nasal	M			M	M	M	M	M		
	2–10 months	Ethmoid and nasal maxillary-block 3				M				M	M	
	2–10 months	Ethmoid and nasal maxillary-block 4				M						
Case 2—IP	1—reference	Nasal cavity			M		M	M				M
	2–6 months	Ethmoid sinus					M					
Case 3—IP	1—reference	Ethmoid sinus					M					
Case 4—IP/EP	1—reference	Nasal mucosa					M				M	
Case 5—IP/EP	1—reference	Nasal vestibule					M					
Case 6—EP	1—reference	Frontal sinus			M		M					
Case 7—EP	1—reference	Nasal cavity		M								
		Total	1/7	1/7	2/7	1/7	6/7	2/7	1/7	1/7	2/7	1/7

EP, exophytic papilloma; IP/EP, inverted and exophytic papilloma; IP, inverted papilloma; M, methylated.

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Table 3

Case Summary and Methylation Status

	Patient No.														
	1[a]	2[a]	3[a]	4[a]	5[a]	6[c]	7[b]	8[a]	9[a]	10[a]	11[b]	12[b]	13[b]	14[a]	15[a]
No. of biopsies	7	6	2	6	4	2	4	2	2	3	3	3	5	2	2
TIMP3[d]							M			M	M	M	M		
APC[d]			M	M	M		M	M					M	M	M
CDKN2A[d]					M		M				M	M		M	
MLH1					M		M								
KLK10										M		M			M
MEN1				M											
CDKN2B[d]		M		M	M		M			M	M	M	M		
VHL[d]			M	M							M	M	M		
TP73				M							M		M		
FANCD26				M											
BRCA2	M			M											
IGSF4				M			M								
RASSF1														M	
DAPK1[d]				M					M			M	M		
HIC1[d]				M	M			M			M				
ESR1		M										M			
CDKN1B										M					
BRCA1									M	M					
GSTP1[d]				M	M		M		M						

Abbreviation: M, methylated.

[a]Cases with dissimilar epigenetic events in multiple biopsy specimens.

[b]Cases with similar epigenetic events in multiple biopsy specimens.

[c]Case with absence of M genes.

[d]Commonly M genes (present in >3 cases).

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Table 4

Epigenetically Linked Recurrent Laryngeal Papilloma Cases

Patient No.	Biopsy	*APC*	*CDKN2B*	*VHL*	*TP73*	*GSTP1*
4	1 [Reference]		M			
	2 (10 mo)		M		M	
	3 (30 mo)		M		M	
7	1 [Reference]	M	M			M
	2 (3 mo)		M			
	3 (6 mo)	M	M			M
11	1 [Reference]		M			
	2 (15 mo)		M			
12	1 [Reference]		M			
	2 (14 mo)		M			
13	1 [Reference]	M		M		
	2 (1 mo)	M	M	M		
	3 (3 mo)	M	M	M		

Abbreviation: M, methylated.