ORIGINAL ARTICLE

Consistent DNA Hypermethylation Patterns in Laryngeal Papillomas

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Abstract

Introduction: This study examined the contribution of promoter hypermethylation to the pathogenesis of respiratory papillomatosis (RP), including recurrences (RRP) and progression to squamous cell carcinoma (SSC).

Materials and methods: A retrospective cohort of 25 laryngeal papilloma cases included 21 RRP, two of which progressed to SCC. Aberrant methylation status was determined using the multigene (22 tumor suppressor genes) methylation-specific multiplex ligation-dependent probe amplification assay and confirmed using methylation specific PCR.

Results: Twenty genes had altered DNA methylation in 22 of 25 cases. Aberrant methylation of *CDKN2B* and *TIMP3* was most frequent. Promoter hypermethylation of *BRCA2*, *APC, CDKN2A* and *CDKN2B* was detected in 2 RRP cases with subsequent progression to SCC. Of the 25 cases, 22 were positive for HPV-6, 2 for HPV-11 and 1 for HPV-16 and 33.

Conclusion: Consistent aberrant methylation of multiple tumor suppressor genes contributes to the pathogenesis of laryngeal papillomas. Persistent aberrant DNA methylation events in 2 RRP cases that progressed to cancer indicate an epigenetic monoclonal progression continuum to SCC.

Keywords: Laryngeal papillomas, recurrent papillomas, DNA methylation, squamous cell carcinoma.

INTRODUCTION

Papilloma is a benign exophytic neoplasm of epithelium on a connective tissue core.[1] 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.[1] 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.[2,3] Transformation of laryngeal papillomas to malignant neoplasms range from 1.25 to 42.9%.[4,5]

The human papilloma virus (HPV), which is associated with genital papillomas, has also been associated with laryngeal papillomas as an etiologic agent,[6-8] particularly HPV types 6 and 11.[9] Studies on HPV typing in benign laryngeal papillomas have demonstrated an association of HPV type 11 with a more aggressive course of the disease.[10,11] According to Lele et al,[12] HPV-11 infection may be an early event in progression of RRP to carcinoma.

Epigenetics is the regulation of changes in gene expression by mechanisms that do not involve changes in DNA sequence. Establishment and maintenance of epigenetic control (gene silencing) has several aspects, which include promoter region hypermethylation, methyl-binding proteins, DNA methyltransferases, histone deacetylases and chromatin state. Aberrant methylation of CpG islands is a hallmark of human cancers and is found early during carcinogenesis.[13] Epigenetic events of DNA

hypermethylation contribute to RRP pathogenesis, some of which are initiating clonal alterations in the recurrence continuum.[14] Aberrant methylation of *CDKN2B* and *APC* genes was most frequent, followed by *CDKN2A*, *TIMP3*, *VHL*, *DAPK1*, *HIC1*, and *GSTP1*.[14]

Recurrent genomic aberrations are good indicators of genes that are causally associated with transformation, cancer development or progression. To asses the contribution of promoter methylation in RP tumorigenesis, we investigated an expanded retrospective cohort of 25 papilloma cases with an initial laryngeal papilloma biopsy between the years 1994 and 2004, with follow-up for subsequent transformation to carcinoma *in situ*, or SCC through August 2009. Aberrant promoter methylation of 22 unique methylation-prone tumor suppressor genes was evaluated using the high-throughput methylation-specific multiplex ligation dependent probe amplification (MS-MLPA) assay (41 gene probes, 35 unique genes, including control probes) and methylation specific PCR (MSP).

MATERIALS AND METHODS

Patient Cohort

The laryngeal papilloma cohort of 25 subjects (21 Caucasian American {CA} and 4 African American {AA}), comprised 5 females and 20 males, all adult onset, ranging in age from 19 to 73 years and 1 female juvenile onset (1-year-old). Of the 25 cohort subjects, 4 were nonrecurrent papillomas (RP) and 21 were RRP. Of the 21 RRP cases, DNA from multiple biopsies were available from 15 RRP for methylation assays. The number of recurrent biopsies ranged from 2 to 22 biopsies from the initial primary biopsy (follow-up through August 2009). The interval between biopsies for these subjects ranged from 23 days (shortest) to 102 months (longest).

When the primary RP biopsy was not available for DNA analysis, the first available RP became the reference biopsy. Of the 25 cases, there were 19 primary and 6 reference biopsies. Primary and reference biopsies included lesions with benign squamous papilloma (16 cases), mild dysplasia (5 cases), moderate dysplasia (1 case), and moderate/severe dysplasia (3 cases). Lesions with mixed moderate and severe dysplasia were classed separately from progression lesions of purely severe dysplasia, carcinoma *in situ* (CIS), and SCC.

DNA Extraction

Whole 5 micron formalin-fixed tissue sections or microdissected papilloma tissue, and subsequent transformation to severe dysplasia, CIS, or SCC lesions (2 cases), were processed for DNA extraction as previously described.[15,16]

HPV Detection

HPV status was identified using the Linear Array HPV Genotyping kit (Roche, Indianapolis, IN) in all cases. PCR using HPV type primers for HPV 6, 11, 16, 31 and 33 especially designed to amplify less than 120 base pair DNA fragment lengths (Table 1) was also used to detect HPV status in some cases.

Table 1: HPV PCR primers

GenBank accession #	*Start to end (bp)*	*Forward and reverse (5' to 3')*	*PCR length*	*Annealing temp*	*Regions*
AF335604.1: HPV6-137	1080 to 1217	F: ACATGCGTCATGTGGAAGAG R: AGGCGATAACCCAAAGTTCC	137bp	52°C	L1
M14119.1: HPV 11-182	578 to 760	F: CCTGCAGCCTCCTGACCCTGT R: CTCCGTCTGTGCACTCCACAA	182bp	60°C	E7
M14119.1: HPV 11-230	291 to 521	F: TGCAGCGTGTGCCTGTTGCTT R: AGCAACGACCCTTCCACTGGT	230bp	60°C	E6
NC001526.1: HPV 16-101	497 to 597	F: TGGACCGGTCGATGTATGT R: CATATATTCATGCAATGTAGGTGTA	101bp	54°C	E6 & E7
NC001526.1: HPV 16-173	425 to 597	F: AAGCCACTGTGTCCTGAAGAA R: CATATATTCATGCAATGTAGGTGTA	173bp	54°C	E6 & E7
NC001526.1: HPV 16-258	382 to 640	F: AATACAACAAACCGTTGTGTGATT R: CAGTAGAGATCAGTTGTCTCTGGTTGC	258bp	58°C	E6 & E7
J04353.1: HPV 31-124	3861 to 3962	F: TTTGCTTTGCTTTTGTGTGCTAC R: TGGAGAGGTTGCAATAACCCATA	124bp	52°C	E5
M12732.1: HPV 33-149	424 to 572	F: TGTCAAAGACCTTTGTGTCCTC R: GGCGTTTTTACACGTCACAG	149bp	54°C	E6

The Methylation-Specific Multiplex Ligation Dependent Probe Amplification (MS-MLPA) Assay

The Multiplex Ligation-Dependent Probe Amplification assay allows for the relative quantification of approximately 41 different DNA sequences in a single reaction requiring only 20 ng of human DNA. The standard use of the technique to observe quantitative changes in copy number (MLPA)[16,17] and adaptation of MLPA to detect aberrant methylation (MS-MLPA) has been detailed elsewhere.[14,16,18]

Bisulfite Modification and Methylation-Specific Polymerase (MSP) Chain Reaction Assay

Genomic DNA (100 ng) from formalin-fixed paraffin embedded papilloma tissue and control universal methylated DNA (Chamicon International, Inc) and control unmethylated DNA (normal genomic DNA) were modified using the EZ DNA methylation gold kit (Zymo Research, Orange, CA) during which methylated DNA is protected and unmethylated cytosine is converted to uracil.[18] The modified DNA served as a template using primers specific for the methylated or modified unmethylated sequences (Table 2). MSP amplification was performed using 3ul of bisulfite modified DNA in a final volume of 25 ul PCR mix containing 1X PCR buffer, 2.5 mM dNTP, 1 mM $MgCl_2$ and 1U Amp gold Taq DNA polymerase, 0.5 uM primer followed by 38 cycles at 95ºC 45 seconds, 62ºC 45 seconds, 72ºC 1 min.[18] The resultant PCR products were separated on 2% agarose gel stained with ethidium bromide and visualized under UV illumination.

RESULTS

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).[14] MSP confirmed aberrant methylation of *CDKN2B* in RRP cases 4, 7, 11 and 12 in multiple recurrent biopsies (MSP for Case 13 was not performed).

Progression to SCC occurred in RRP cases 1 and 5 (Table 3). 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). For case 5, the laryngeal subsite for the reference biopsy and the subsequent recurrent lesions was the right true vocal cord.

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

Table 2: Methylation and unmethylation MSP primer sequences for laryngeal papillomas

Gene		Methylation specific primers	Unmethylation specific primers	Product size
BRCA2	Forward	5'-GACGGTTGGGATGTTTGATAAGG	5'-AGGGTGGTTTGGGATTTTTAAGG	M - 337bp
	Reverse	5'-AATCTATCCCCTCACGCTTCTCC	5'-TCACACTTCTCCCAACAACAACC	U - 250bp
APC	Forward	5'-TATTGCGGAGTGCGGGTC	5'-GTGTTTTATTGTGGAGTGTGGGTT	M - 97bp
	Reverse	5'-TCGAAGAACTCCCGACGA	5'-CCAATCAACAAACTCCCAACAA	U - 108bp
GSTP1	Forward	5'-TTCGGGGTGTAGCGGTCGTC	5'-GATGTTTGGGGTGTAGTGGTTGTT	M - 91bp
	Reverse	5'-GCCCCAATACTAAATCACGACG	5'-CCACCCCAATACTAAATCACAACA	U - 97bp
CDKN2A^{ARF}	Forward	5'-TATCGAGTTTTTTGTGTTTAGTCC	5'-TATTGAGTTTTTTGTGTTTAGTTT	M - 112bp
	Reverse	5'-AACGACCAACAAAAAAAAAAAACG	5'-CAACAAAAAAAAAACAACCAAC	U - 124bp
CDKN2B†	Forward	5'-GAAGGTGCGATAGTTTTTGGAAGTCGGCGC	5'-TGGAGAAGGTGTGATAGTTTTTGGAAGTTGGTGT	M - 160bp
	Reverse	5'-GACGATCTAAATTCCAACCCCGATCCGCCG	5'-CATCAACAATCTAAATTCCAACCCCAATCCACCA	U - 169bp

M = Methylated product
U = Unmethylated product
[†]Nygren, AO, et al, Methylation-specific MLPA (MS-MLPA): Simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. Nucleic Acids Res, 2005. 33(14):e128.

CDKN2A (biopsies 5 and 6). MSP and MS-MLPA were concordant for lack of methylation *APC*, *GSTP1*, and *CDKN2A*, and *CDKN2B* (Table 3).

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 3, Fig. 1). MSP confirmed MS-MLPA methylation of *APC* (biopsies 1 and 4) and *CDKN2A* (biopsies 1 to 3). MSP also confirmed absence of methylation for *CDKN2B* (biopsies 1 and 4) and *GSTP1* (biopsies 2 to 4) detected by MS-MLPA (Fig. 2).

HPV was identified in all 25 cases by either the Roche Linear Array and/or by PCR. Among the 21 HPV positive RRP cases, 18 were HPV-6, 1 was HPV-16 and 33 (Case 1), and 2 were HPV-11 positive (Cases 5 and 7). The remaining 4 RP were positive for HPV-6. Case 1 was positive for HPV-16 and 33 in the primary SCC but was not detected in the recurrent SCC lesion. HPV-6 status in case 1, biopsy 5, detected by the Roche Linear Array was not confirmed by PCR. Case 5 biopsies were negative for HPV by the Roche Linear Array. However, HPV-11 by PCR, using two different primer sets, identified HPV-11 only in the first (reference) biopsy and confirmed lack of HPV for biopsies 2 through 4 (Table 3).

DISCUSSION

Recurrent genomic aberrations are good indicators of genes that are causally associated with cancer development, transformation or progression. Our previous studies[14,16] have demonstrated that epigenetic events of DNA hypermethylation underlie the pathogenesis of benign sinonasal and recurrent laryngeal papillomas, establishing a monoclonal origin for RRP. Our current findings reiterate consistent DNA hypermethylation events in a larger cohort of laryngeal papillomas and trace a progression continuum to SCC. The results further support a monoclonal progression for malignant transformation in 2 RRP cases.

Spontaneous transformation of RP to laryngeal squamous cell carcinoma (LSCC) is not easily characterized by a histologic progression through dysplasia over time, making these lesions difficult to diagnose histologically and clinically early on in the course of the transformation of the disease. Several studies have attempted to identify markers that can predict which patients with RP are at a higher risk

Table 3: Epigenetically linked progressive laryngeal cases

RRP	Lesion type	Biopsy	Time interval	BRCA2	APC	CDKN2A	CDKN2B	HPV (Roche)	HPV (PCR)
Case 1	Squamous papilloma	1	Primary	M*	M†	U*	U*	16	16
	Squamous papilloma with severe dysplasia	2	4 months	M†	M†	U*	U		16
	Primary SCC, Block 1 tumor	3T	6 months	M†	U*	U*	U		16 and 33
	Primary SCC, Block 1 dysplastic papilloma	3P	6 months	M†	M†	U*	U		16
	Recurrent severe dysplasia	4	50 months	M†	M*	M†	U*	Neg	Neg
	Carcinoma in situ	5	51 months	M†	M†	M*	U*	6	Neg
	Recurrent SCC	6	53 months	M†	M†	M*	U, NR by MSP	Not informative	Neg
Case 5	Squamous papilloma with moderate to severe dysplasia	1	Reference	M†	M*	M*	U*	Neg	11
	Squamous papilloma with mild to moderate dysplasia	2	2 months	M†	M†	M*	M	Neg	Neg
	Primary SCC	3	9 months	M†	M†	M*	M	Neg	Neg
	Carcinoma in situ	4	11 months	M†	M*	M†	U*	Neg	Neg

RRP = Recurrent respiratory papilloma
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

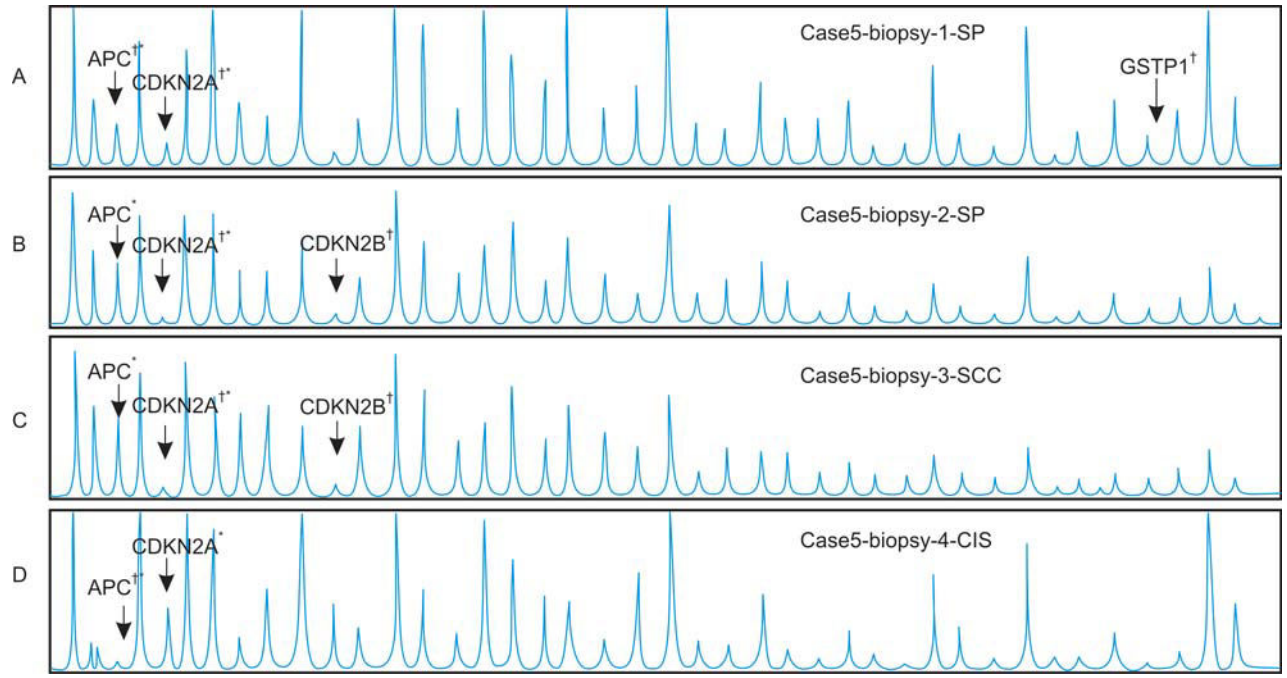


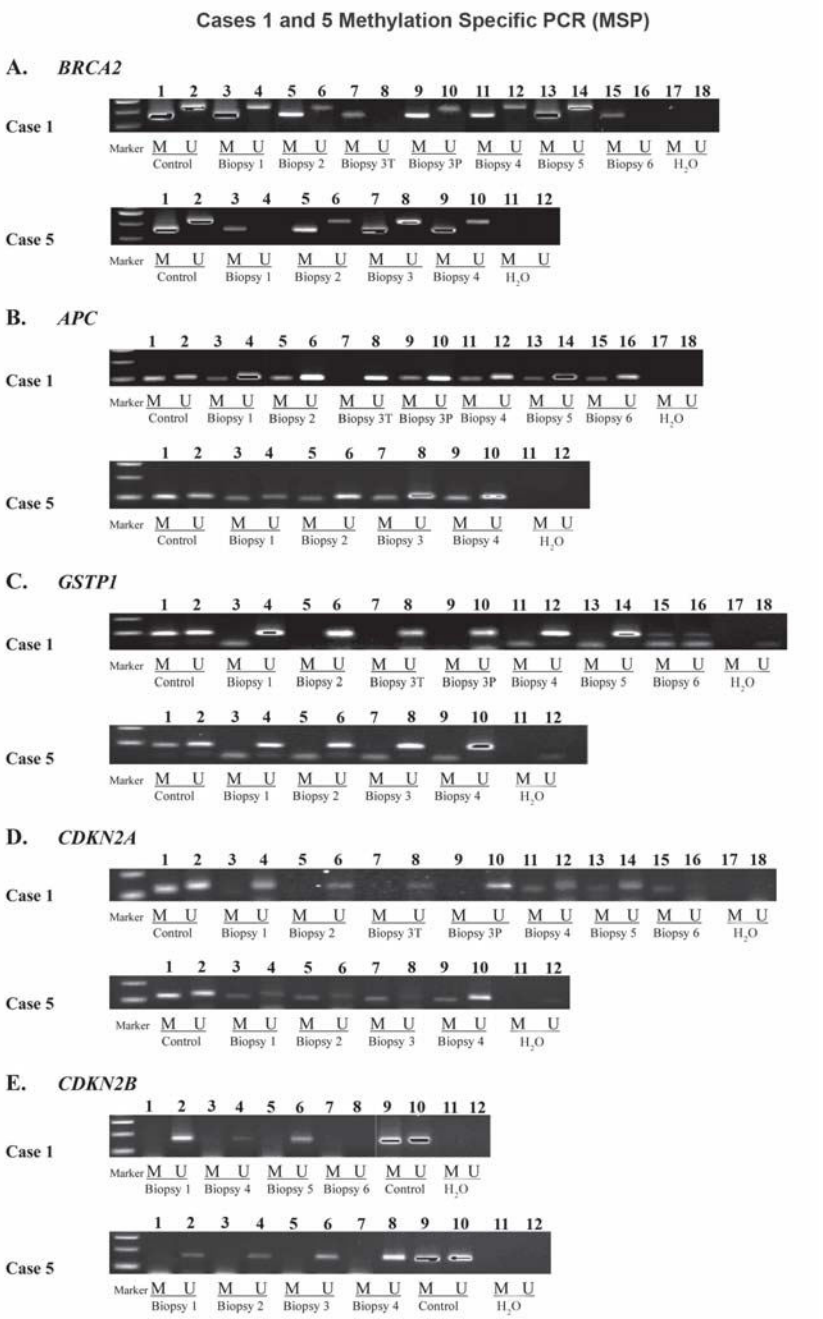
Fig. 1: Case 5 results of MS-MLPA. Note methylation of *APC* by MS-MLPA with confirmation by MSP (*APC*†*) in biopsies 1 and 4. MSP alone detected *APC* methylation in biopsies 2 and 3 (*APC**). Note methylation of *CDKN2A* by MS-MLPA with confirmation by MSP (*CDKN2A*†*) in biopsies 1, 2 and 3. MSP alone detected *CDKN2A* methylation in biopsy 4 (*CDKN2A**). Note methylation of *CDKN2B* in biopsies 2 and 3 and *GSTP1* in biopsy 1 by MS-MLPA only (*CDKN2B*†, *GSTP1*†) (SP–squamous papilloma, CIS–carcinoma in situ, SCC–squamous cell cancer)

for more frequently recurring aggressive disease or malignant transformation. However, results in both benign laryngeal lesions (papillomatosis) and malignant lesions have not been definitive.[12,19-22] Currently, there are no biomarkers of aggressive RP that predict benign recurrence and transformation to malignancy from RP.

In this study, *TIMP3* was the most frequently methylated gene (13/22 cases), followed by *CDKN2B*, *CDKN2A*, *APC*, *VHL*, *TP73, GSTP1, HIC1, MLH1*and *DAPK 1*. *TIMP3* induces apoptosis,[23] inhibits angiogenesis,[24] impedes cell migration,[25] and is a physiological regulator of inflammation.[26] Promoter methylation of *TIMP3* has been observed in many tumor types[27,28] and is involved in the genesis of esophageal adenocarcinoma notably during progression from dysplasia to carcinoma.[29,30]

CDKN2B and *CDKN2A* were hypermethylated in 11 of 22 and 9 of 22 cases, respectively. Genetic alterations in *CDKN2A* and *CDKN2B* genes, which map to 9p21, have been linked to malignant progression in HNSCC.[31-33] 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.[17,34,35] One study reported aberrant methylation of *CDKN2B (p15)* and *CDKN2A (p16)* in more than 50% of the oral squamous cell carcinomas (OSCC).[36] The presence of aberrant methylation of *p15* and *p16* in precancerous oral tissues [35] implicates methylation of *p15* and *p16* as early events in the pathogenesis of oral lesions. In undifferentiated nasopharyngeal carcinoma (NPC), preferential methylation of *CDKN2B* has been shown to be a useful tumor marker for NPC.[37] In case 5, aberrant methylation of *CDKN2A* in the reference biopsy and *CDKN2B* in biopsy 2 and subsequent transformation biopsies occurs as an early event and provides evidence of a monoclonal progression continuum to SCC.

Hypermethylation of the *APC* gene was detected in multiple biopsies in 8/15 RRP cases and 1 RP. *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.[38] previously, reported mutations and/or deletions of *APC* in primary OSCC and suggested that loss of *APC* function



Figs 2A to E: Methylation specific PCR (MSP) for *BRCA2, APC, GSTP1, CDKN2A* and *CDKN2B*: *A(BRCA2)* Lanes 1 and 2: universal methylated and unmethylated controls; *Case 1*: Lanes 3-16 span biopsies 1-6. Note presence of methylated product in all biopsies. Lanes 17 and 18: negative control. *Case 5*: Lanes 3-10 span biopsies 1-4. Note presence of methylated product in all biopsies. Lanes 11 and 12: negative control, *B(APC)* Lanes 1 and 2: universal methylated and unmethylated controls; *Case 1*: Lanes 3-16 span biopsies 1-6. Note presence of methylated product in biopsies 1, 2, 3P, 4, 5 and 6 *(Table 3)*. Note absence of methylated product in biopsy 3T. Lanes 17 and 18: negative control. *Case 5:* Lanes 3-10 span biopsies 1-4. Note presence of methylated product in all biopsies. Lanes 11 and 12: negative control**,** *C(GSTP1)* Lanes 1 and 2: universal methylated and unmethylated controls; *Case 1:* Lanes 3-16 span biopsies 1-6. Note presence of methylated product in biopsy 6. Note absence of methylated product in biopsies 1-5. Lanes 17 and 18: negative control. *Case 5:* Lanes 3-10 span biopsies 1-4. Note absence of methylated product in all biopsies. Lanes 11 and 12: negative control, *D(CDKN2A)* Lanes 1 and 2: universal methylated and unmethylated controls; *Case 1*: Lanes 3-16 span biopsies 1-6. Note presence of methylated product in biopsies 4, 5 and 6. Note absence of methylated product in biopsies 1-3P. Lanes 17 and 18: negative control. *Case 5:* Lanes 3-10 span biopsies 1-4. Note presence of methylated product in all biopsies. Lanes 11 and 12: negative control, *E(CDKN2B): Case 1:* Lanes 1-8 span biopsies 1, 4-6. Note absence of methylated product in biopsies 1, 4 and 5. No reaction in biopsy 6 due to insufficient DNA. MSP was not performed on biopsies 2, 3T and 3P. Lanes 9 and 10: universal methylated and unmethylated controls. Lanes 11 and 12: negative control. *Case 5:* Lanes 1-8 span biopsies 1-4. Note absence of methylated product in all biopsies. Lanes 9 and 10: universal methylated and unmethylated controls. Lanes 11 and 12: negative control

contributes to carcinogenesis in the oral region. *APC* inactivation as a result of promoter hypermethylation occurred in 25% of OSCC cell.[38] Hypermethylation of *APC*, observed in the initial and subsequent biopsies in RRP cases 1 and 5 is an early event and supports a monoclonal progression continuum to SCC.

BRCA2 (Breast cancer 2, early onset) is a tumor suppressor gene whose mutations are strongly associated with an elevated risk of breast and ovarian cancers.[39] Mutations in *BRCA2* gene are associated with an increased risk of prostate, pancreas, stomach, melanoma, lung, and bladder cancers.[40] Chromosome instability may be caused by failure in the repair of DNA double-strand breaks (DSB)[41] and *BRCA2* is involved in maintaining genome stability. Aberrant promoter hypermethylation of *BRCA2* was detected in 42% of nonsmall cell lung cancer (NSCLC) compared to absent or low methylation in their matched normal lung tissue.[42] In this study, aberrant methylation of *BRCA2* in the initial and subsequent transformation biopsies in RRP cases 1 and 5, similar to *APC*, occurred early with retention in the progression continuum.

The study cohort, drawn from a multiethnic primary care patient population with nearly 40% AAs, revealed a nearly 5:1 predominance of CA with RP. CA race predilection for RP is supported by the Moore et al.[21] study, which reported a 4:1 CA predominance for cohort subjects drawn from a mostly tertiary care patient population setting. We found a preponderance of male RP patients as compared to female RP (20 males: 5 females). This is a deviation from previous reports that indicate approximately equal gender distribution for RP.[21,43]

In RP, human papillomavirus types 6 and 11 account for 80 to 90% of RP.[44] In our cohort, types 6 and 11 account for 96% of the cases with 22 cases positive for HPV-6 and 2 cases positive for HPV-11. HPV-11 appears to confer a more aggressive neoplastic phenotype than HPV-6 and is associated more often with atypia and frequent recurrence.[45] 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.[21] 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.[21] Oncogenic HPV, particularly HPV-16, has been established as a causative agent for 25% of head and neck squamous cell carcinoma (HNSCC)[20] and the development of laryngeal carcinoma is associated with HPV infection.[19,20]

MSP for the most part confirmed promoter hypermethylation detected by MS-MLPA. MSP did not confirm MS-MLPA methylation of *CDKN2B* observed in case 1 and case 5 biopsies. While a distinct advantage of MS-MLPA is the ability to examine aberrant promoter methylation in multiple cancer genes in a single assay run, multiplex PCR of a large number of gene probes (22 unique genes) inherently encounters competitive amplification and detection algorithms may miss hypermethylation events that do not reach the threshold for detection.[34] In contrast, MSP examines only one gene at a time[18] and therefore, is more sensitive than MS-MLPA[18] and is underscored by aberrant methylation of *BRCA2* in case 1 and case 5 biopsies by MSP alone. In cases where MSP did not confirm MS-MLPA methylation, background noise presenting as spurious peaks may be a contributing factor. Spurious peaks (background noise) may be attributed to challenges posed to DNA from formalin-fixed tissue, the quality of which is dependent on tissue fixation variables. Regardless, MS-MLPA profiling of multiple genes for aberrantly methylated promoter regions is a valuable screening tool to determine frequency and pattern of promoter methylation in neoplasia. These epigenetic signatures, upon subsequent validation as diagnostic or prognostic biomarkers, can become reduced to a more definitive candidate gene panel of only a few key genes. The latter would be amenable to higher detection sensitivities using a targeted 3 or 4 MS-MLPA gene probe panel or by MSP alone.

Malignant transformation rates of benign laryngeal papillomas can range from 1.25 to 42.9%[4,5] and larger benign RP cohorts will be key to providing more accurate progression rates. Though this study is limited in its sample size (25 patients) and the number of cases that progressed to SCC (2 cases), it closely mirrors other larger study cohorts with similar transformation rates.[21] In the two cases with progression to SCC, promoter methylation occurred as an early event and persisted in initial and subsequent biopsies for cases 1 and 5 with progression to cancer supporting an epigenetic monoclonal progression continuum to SCC.

The high frequency of DNA hypermethylation events in this study supports the utilization of gene silencing

mechanisms as one of the driving forces behind the growth of laryngeal papillomas, reiterating DNA hypermethylation events as hallmarks of RP pathogenesis, some of which are initiating clonal alterations in the recurrence continuum in some RRP cases.[14] Aberrant methylation of *BRCA2, APC*, *CDKN2A* and *CDKN2B*, confirmed by MSP and detected in the initial and all subsequent transformation biopsies in RRP cases 1 and 5, 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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