AMERICAN ACADEMY OF OTOLARYNGOLOGY–HEAD AND NECK SURGERY FOUNDATION

Otolaryngology–Head and Neck Surgery
1–8

DOI: 10.1177/0194599816654139
http://otojournal.org
SAGE

Gene Methylation Profiling in Sinonasal Adenocarcinoma and Squamous Cell Carcinoma

Maria Costales, MD, PhD[1], Alejandro López-Hernández, MSc[1], Cristina García-Inclán, PhD[1], Blanca Vivanco, PhD[2], Fernando López, MD, PhD[1], José Luis Llorente, MD, PhD[1], and Mario A. Hermsen, PhD[1]

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objective. To identify epigenetic events in intestinal-type sinonasal adenocarcinoma (ITAC) and sinonasal squamous cell carcinoma (SNSCC) and to evaluate their relation to clinicopathologic features and follow-up data.

Study Design. Retrospective study.

Setting. Academic research hospital.

Subjects and Methods. The methylation status of 23 genes in 50 ITACs and 32 SNSCCs was analyzed by methylation-specific multiplex ligation-dependent probe amplification and its relation to clinicopathologic features and follow-up data.

Results. Gene methylation was observed in 50% of all tumors. Recurrent methylated genes in SNSCC were *RASSF1* and *CDH13* (for both, 6 of 32 cases), *CHFR* (4 of 32 cases), and *TIMP3* (2 of 32 cases). None of these genes showed significant correlation to clinicopathologic features or overall survival. In ITAC, recurrent methylated genes were *CDH13* (18 of 50 cases), *ESR1* (13 of 50 cases), *APC* (7 of 50 cases), *TIMP3* (5 of 50 cases), *CASP8* (3 of 50 cases), and *HIC1* and *RASSF1* (for both, 2 of 50 cases). Papillary and colonic ITAC subtypes carried a mean of 1.26 gene methylations per tumor versus 0.63 in solid and mucinous subtypes. Methylation of *TIMP3* was associated with a significantly worse survival in ITAC patients.

Conclusion. ITAC carries a higher number and a different profile of gene methylations as compared with SNSCC. Gene methylation plays a greater role in papillary and colonic ITAC subtypes, which may indicate a different tumorigenic pathway for these ITAC subtypes. These findings could be used as prognosticators and may have implications for future individualized therapies based on epigenetic changes.

Keywords

sinonasal cavities, squamous cell carcinoma, adenocarcinoma, gene methylation, prognosis

Received February 25, 2016; revised May 4, 2016; accepted May 20, 2016.

A wide variety of histologic types of tumors originate in the sinonasal area. The most common are sinonasal squamous cell carcinomas (SNSCCs) and intestinal-type sinonasal adenocarcinomas (ITACs), which account for, respectively, 50% to 80% and 10% to 20% of all nasal epithelial tumors.[1-3] The sinonasal area is close to sensitive structures, such the eyes and the brain, thereby complicating surgical and postoperative radiotherapeutic procedures. Sometimes, mutilation and aesthetic deformities are difficult to avoid. Current treatment approaches include surgery, which may be accompanied by radiotherapy and, in some patients, chemotherapy.[2-6] Despite improvements in the field of surgery and radiotherapy, the prognosis is still poor, with a 5-year overall survival of 30% to 50%.[5,7-10] Therefore, new therapeutic approaches are needed to improve these figures.

SNSCC and ITAC are etiologically related to wood and leather dust and other occupational exposures[2,3,11]; however, the tumorigenesis is still poorly understood. Apart from aiming to find the genes involved in tumor development, identifying ''druggable'' genetic alterations is becoming increasingly important in aiding clinical management by novel anticancer treatments.[3] Gene mutations, chromosomal translocations, gene copy number gains and losses, and epigenetic changes can activate or inactivate cellular signalling pathways that can be specifically targeted by small molecule inhibitors or monoclonal antibodies for cancer therapy.

[1]Department of Otolaryngology, Instituto Universitario de Oncología del Principado de Asturias, Hospital Universitario Central de Asturias, Oviedo, Spain
[2]Department of Pathology, Hospital Universitario Central de Asturias, Oviedo, Spain

Corresponding Author:
Mario A. Hermsen, PhD, Department of Otolaryngology, Instituto Universitario de Oncología del Principado de Asturias, Hospital Universitario Central de Asturias, Avenida de Roma s/n, 33011 Oviedo, Asturias, Spain.
Email: mhermsen@hca.es

Epigenetic abnormalities in cancer consist of genome-wide hypomethylation and local hypermethylation at specific genes. Hypermethylation of CpG islands within the regulatory region of tumor suppressor genes is one of the earliest and most frequent alterations in cancer development, and it is associated with transcriptional repression. In recent years a number of epigenetic alterations have been described in head and neck squamous cell carcinomas (HNSCCs), affecting genes such as *p16*, *p15*, *DAPK1*, and *RASSF1A*, *RARβ2*, *MGMT* and *E-cadherin*.[12-16]

To our knowledge, no gene methylation studies have been performed on sinonasal carcinomas. The aim of this study was to analyze epigenetic events in 32 SNSCCs and 50 ITACs and to evaluate their relation to clinicopathologic features and follow-up data.

Material and Methods

Samples

Between 1990 and 2009, 32 surgical tissue specimens from patients who were diagnosed with SNSCC were collected at the otolaryngology departments at Central University Hospital of Asturias (Oviedo, Spain) and Gregorio Marañon General University Hospital (Madrid, Spain), and 50 surgical tissue specimens from patients who were diagnosed of ITAC were collected at the Department of Otolaryngology at Central University Hospital of Asturias. The patients in this study met the following criteria of inclusion: (1) a complete clinical history, (2) pathologic diagnosis of SNSCC or ITAC, (3) a sample of tissue in optimal conditions for genetic analysis, (4) no prior history of head and neck cancer, and (5) no prior chemotherapy or radiotherapy. All 32 SNSCC samples proceeded from paraffin-embedded tissue, and all 50 ITAC samples were obtained from surgical resection specimens of nonnecrotic tumor areas that were immediately stored in liquid nitrogen after surgery. Written informed consent for the collection, storage, and analysis of specimens was obtained from all patients. The study received approval from our institutional ethical committees (Comité de Ética de la Investigación del Principado de Asturias and Comité Ética de Investigación Clínica del Instituto de Investigación Sanitaria del Hospital Gregorio Marañón).

Clinical Variables

Of 32 SNSCC patients, 23 were men (72%), and 9 were women (28%). The mean patient age was 68 years (range, 49-91 years). Twenty-five tumors were localized in the maxillary sinus (78%) and 7 in the ethmoid sinus (22%). The series comprised 12 well- (38%), 8 moderately (24%), and 12 poorly differentiated tumors (38%). According to the TNM system for tumor classification,[17] 3 tumors were stage II (6%); 9, stage III (28%); 14, stage IVa (44%); and 6, stage IVb (19%). Twenty-four patients (75%) received radiotherapy after radical surgery. The median follow-up was 15 months (range, 1-168 months).

All 50 ITAC patients were men, and the tumor was localized in the ethmoid sinus; 48 had profession-related exposure to wood dust. The mean patient age was 66 years (range, 49-92 years). According to the World Health Organization histologic classification,[18] our series comprised 3 (6%) papillary, 28 (56%) colonic, 5 (10%) solid, and 14 (28%) mucinous tumors. Sixteen tumors (32%) were stage I; 5 (10%), stage II; 15 (30%), stage III; 4 (8%), stage IVA; and 10 (20%), stage IVB. No patient had lymph node or distant metastases at the time of diagnosis. Thirty-seven patients (54%) received radiotherapy after radical surgery. Follow-up information was available with a median of 39 months (range, 1-259 months). A summary of all clinical data is given in **Table 1**.

Table 1. Patient and Tumor Characteristics.

	Patients, n (%)	
	SNSCC (n = 32)	ITAC (n = 50)
Sex		
Female	9 (28)	0 (0)
Male	23 (72)	50 (100)
Tumor site		
Maxillary sinus	25 (78)	0 (0)
Ethmoid sinus	7 (22)	50 (100)
Disease stage		
I	0 (0)	16 (32)
II	3 (9)	5 (10)
III	9 (28)	15 (30)
IVa	14 (44)	4 (8)
IVb	6 (19)	10 (20)
Histologic differentiation		
Well differentiated	12 (38)	
Moderately differentiated	8 (24)	
Poorly differentiated	12 (38)	
Histologic subtype		
Papillary		3 (6)
Colonic		28 (56)
Solid		5 (10)
Mucinous		14 (28)
Recurrence		
No	7 (22)	24 (48)
Yes	25 (78)	26 (52)
Metastasis		
No	28 (88)	45 (90)
Yes	4 (12)	5 (10)
Patient status		
Alive	5 (16)	20 (40)
Died of disease	22 (68)	22 (44)
Died of other causes	5 (16)	8 (16)

Abbreviation: ITAC, intestinal-type sinonasal adenocarcinoma; SNSCC, sinonasal squamous cell carcinoma.

DNA Extraction

Tumor purity was evaluated via a hematoxylin and eosin–stained section of the sample and was accepted when tumor cells were present at $\geq$70%, to minimize contamination by normal cells. Tumor and normal control DNA was extracted

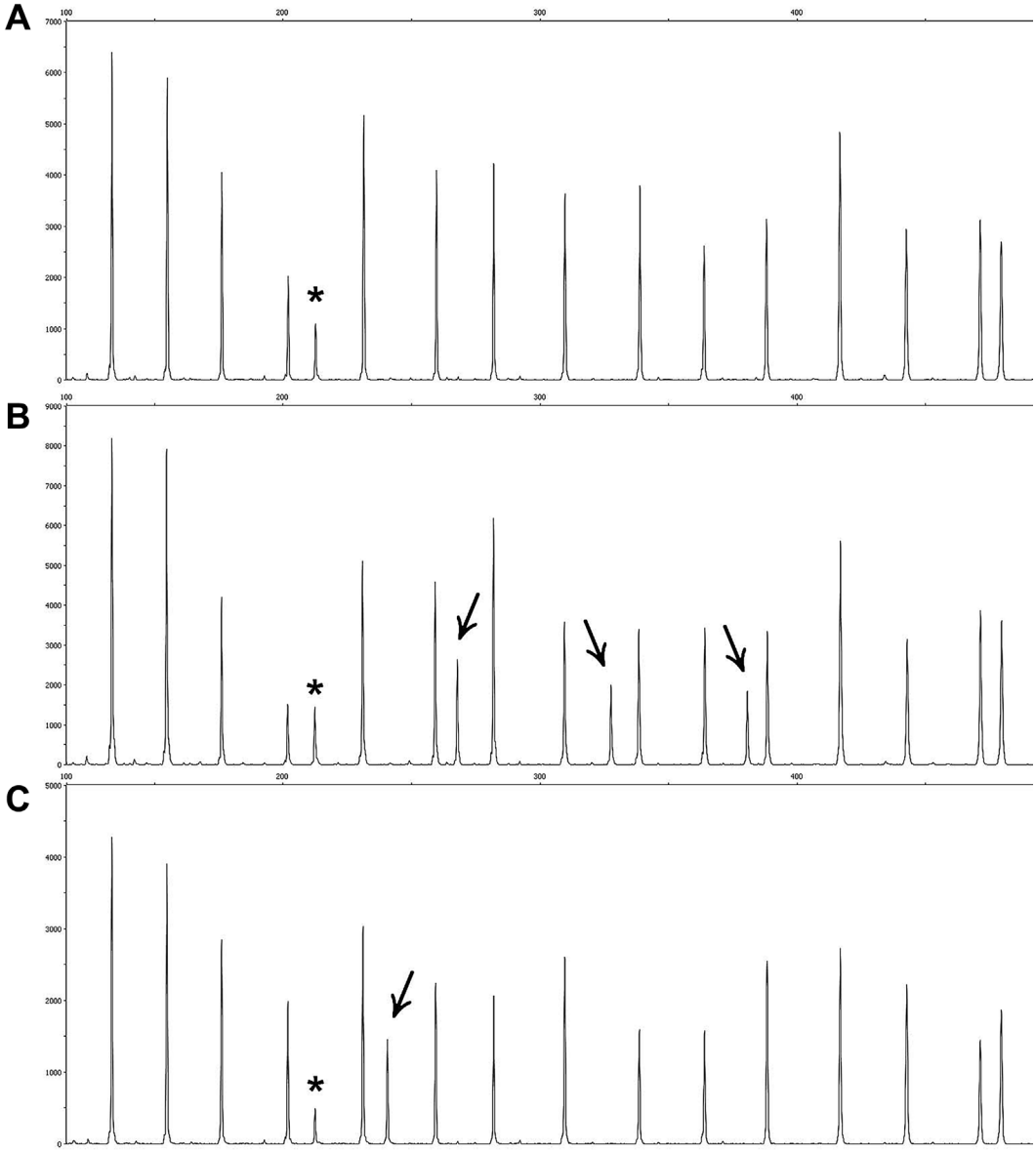

Figure 1. Examples of methylation-specific multiplex ligation-dependent probe amplification analysis in normal DNA (A), intestinal-type sinonasal adenocarcinoma (B), and sinonasal squamous cell carcinoma (C). Arrows indicate methylation of *CASP8* and *RASSF1* (B) and *CHFR* (C). *CDKN2B* (asterisks) was excluded from analysis.

from 3 consecutive 50-μm sections of paraffin-embedded tissue samples through Qiagen extraction kits (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. DNA from archival material can be partly degraded and cross-linked; the extent of which depends on the pH of the formaldehyde and the time of the fixation before paraffin embedding. To obtain good quality DNA, we applied an elaborate extraction protocol especially for paraffin tissues, which includes incubation in sodium thiocyanate to reduce crosslinks and prolonged digestion in proteinase K lysis buffer.[19] With this protocol, most samples yielded DNA with A260/A280 values between 1.7 and 2.0 and lengths between 2000 and 20,000 base pairs. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) on tumor DNA samples was analyzed with normal DNA also obtained from paraffin blocks of normal tissue (negative lymph nodes) as reference.

Methylation-Specific Multiplex Ligation-Dependent Probe Amplification

MS-MLPA was performed as described previously in detail,[14] with the SALSA MS-MLPA ME001-C1 Tumor Suppressor-1 Kit (MRC-Holland, Amsterdam, The Netherlands). This kit contains 26 MS-MLPA probes to analyze the methylation status of the promoter region of 24 tumor suppressor genes. In addition, 15 reference probes are included, which are not methylation sensitive.[20] The probe for *CDKN2B* was found to be sensitive to improper Hha1 digestion in DNA obtained from paraffin embedded tissues and was therefore excluded from analysis (**Figure 1**), leaving a total of 23 analyzed genes.

Statistical Analysis

Possible correlations between genetic and clinical parameters were statistically analyzed by SPSS 12.0 software for Windows (SPSS, Inc, Chicago, Illinois), based on the Pearson chi-square test and Fisher's exact test. Kaplan-Meier analysis was performed for estimation of survival, comparing distributions of survival through the Mantel-Cox log-rank test. Values of $P < .05$ were considered significant.

Results

Follow-up

During the course of follow-up of the 32 SNSCC patients, 25 developed local recurrences (78%), 4 of whom also developed distant metastasis (12%). At the time of the current report, 5 patients remained disease-free (16%). The overall 5-year survival rate was 13%, and the 1- and 5-year disease-free survival rates were 23% and 12%, respectively. The main causes of death in our series were local recurrences and distant metastasis. Five patients died during the postoperative period or because of intercurrent causes. The overall survival of tumor stages I, II, and III versus IVa and IVb is shown in **Figure 2**.

Of 50 ITAC patients, 26 patients (52%) developed local recurrence and 5 patients, distant metastasis (4%). At the time of writing, 20 (40%) patients remained disease-free. The overall 5-year survival was 51%, and the 1- and 5-year disease-free survival rates were 58% and 4%, respectively. The main cause of death in our series was local recurrence and intracranial invasion; however, 8 patients died during the postoperative period or due to intercurrent causes. Overall survival was significantly related to tumor stage (log rank = 13.184, $P = .001$; **Figure 2**), while mucinous and solid types showed a tendency toward worse survival as compared with colonic and papillary types.

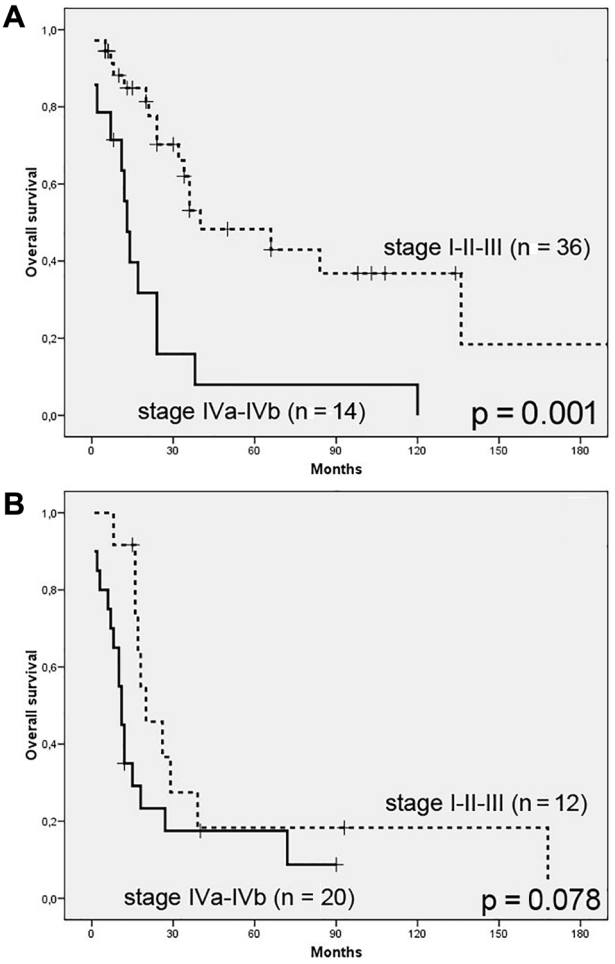

Figure 2. Kaplan-Meier survival analysis: overall survival according to disease stage of (A) 50 patients with intestinal-type sinonasal adenocarcinoma and (B) 32 patients with sinonasal squamous cell carcinoma.

MS-MLPA Data and Correlation with Clinicopathologic and Follow-up Data

Methylation in ≥1 genes was observed in 15 of 32 SNSCCs (47%; mean, 0.63; range, 1-2 genes) and in 26 of 50 ITACs (52%; mean, 1.02; range, 1-6 genes). Recurrent methylated genes in SNSCC were *RASSF1* and *CDH13* (both 6 cases), *CHFR* (4 cases), and *TIMP3* (2 cases). In ITAC, these were *CDH13* (18 cases), *ESR1* (13 cases), *APC* (7 cases), *TIMP3* (5 cases), *CASP8* (3 cases), and *HIC1* and *RASSF1* (both 2 cases). All results are presented in **Table 2**.

SNSCC cases with ≥1 methylated genes were not different with regard to sex, localization, stage, differentiation, the development of recurrence or metastasis, or overall survival, as compared with cases with none of the 23 genes methylated. Recurrent specific methylated genes did not correlate to sex, age, localization, tumor stage, differentiation, or the development of recurrence or metastasis or overall survival.

ITAC cases with ≥1 methylated genes were not different with regard to stage, development of recurrence or metastasis, or overall survival, when compared with cases with no methylation. The papillary and colonic subtypes showed 18 of 31 (58%) cases with ≥1 gene methylation versus 8 of 19 (42%) for the solid and mucinous subtypes. In addition, papillary and colonic subtypes carried a mean number of 1.26 gene methylations per tumor, while solid and mucinous subtypes showed a mean number of 0.63. However, this difference did not reach statistical significance ($P = .095$). Apart from the higher frequency of gene methylation, papillary and colonic ITACs did not show a different profile of methylated genes (**Table 2**). Five cases that harbored methylation of *TIMP3* had a significantly worse overall and disease-free survival (**Figure 3**). In addition, 3 of 5 cases that developed metastasis, as opposed to 2 of 45 cases that did not, carried *TIMP3* methylation (χ^2: $P = .005$), and 4 of 5 cases with *TIMP3* methylation developed recurrence. Other recurrent specific methylated genes did not correlate to sex, age, localization, tumor stage, differentiation, or the development of recurrence or metastasis or overall survival.

Discussion

There are several methods to study methylation at specific gene promotors—from methylation-specific polymerase chain

Table 2. Frequencies of Gene Methylation in 32 SNSCCs and 50 ITACs.[a]

		ITAC		
	SNSCC (n = 32)	Total (n = 50)	PAP-COL (n = 31)	SOL-MUC (n = 19)
≥1 gene methylation	15 (47)	26 (52)	18 (58)	8 (42)
CDH13	6 (19)	18 (36)	13 (42)	5 (26)
ESR1		13 (26)	10 (32)	3 (16)
APC	1 (3)	7 (14)	6 (19)	1 (5)
TIMP3	2 (6)	5 (10)	3 (10)	2 (11)
HIC1		2 (4)	1 (3)	1 (5)
CASP8		2 (4)	1 (3)	1 (5)
RASSF1	6 (19)	2 (4)	1 (3)	1 (5)
RARB		1 (2)	1 (3)	
TP73		1 (2)	1 (3)	
IGSF4		1 (2)	1 (3)	
FHIT		1 (2)		1 (5)
CHFR	4 (13)			
DAPK1	1 (3)			

Abbreviations: ITAC, intestinal-type sinonasal adenocarcinoma; PAP-COL, papillary or colonic subtype; SNSCC, sinonasal squamous cell carcinoma; SOL-MUC, solid and mucinous subtype.
[a]Values presented as n (%). The following genes were not represented among the samples: *CDKN2A, MLH1, ATM, BRCA1, CDKN1B, PTEN, BRCA2, CD44, VHL, GSTP1*.

reaction after bisulfite treatment, analyzing 1 gene per assay, to dedicated microarray chips, analyzing thousands of genes simultaneously. We used MS-MLPA, a multiplex polymerase chain reaction assay that uses the methylation sensibility of the HhaI restriction enzyme to assess multiple genes in a single experiment.[20] This technique is especially suitable for DNA obtained from paraffin-embedded tissues, as only short fragments of DNA are required. In recent years, many studies have compared MS-MLPA with other methods of DNA methylation analysis, demonstrating a very good concordance with these assays[14]; therefore, in this study we did not repeat the methylation analysis by other techniques.

The relatively low number of cases in this study presents statistical limitations that cannot be overcome, as sinonasal cancer is a rare disease with an annual incidence of approximately 1 per 100,000 inhabitants. Nevertheless, knowledge on the genetic changes involved in ITAC and SNSCC is increasing,[3,19,21-28] which is relevant with regard to clinical decision making and possibilities for the application of molecularly targeted cancer therapies.[29-31] To our knowledge, this report is the first to describe epigenetic changes in sinonasal carcinomas. In addition, we obtained all clinical, pathologic, and follow-up data, which appeared similar to those described in clinical series published in the literature.[1-3,7-10] Therefore, we believe that our results offer valid and interesting observations.

While there was no difference in the proportion of cases with ≥1 gene methylations—52% of ITAC and 47% of SNSCC—we found that the 2 tumor types have different epigenetic profiles: ITAC carried an average of 2.0 methylations affecting 11 genes, whereas SNSCC harbored 1.3 methylations affecting 6 genes. Our figures are lower than those reported for HNSCC.[12-16] Using the same method, López et al[14] observed promoter methylation in 68% of 53 laryngeal squamous cell carcinomas. Given the relatively low number of the 2 tumor types analyzed, it is difficult to match clinical features, such as patient age, tumor stage, and etiology, with studies on HNSCC. Therefore, many factors may have influenced the observed differences. However, it may be speculated that the difference could be related to etiology. A majority (>90%) of HNSCC patients are tobacco smokers,[32] while in sinonasal cancer approximately 40% of cases are attributed to occupational exposure to several industrial compounds.[2,3,11] Among the ITAC, papillary and colonic cases carried more gene methylations than did the solid and mucinous tumors (58% vs 42%), which may indicate that these subtypes develop through a different process of tumorigenesis.

Recurrent methylated genes shared by ITAC and SNSCC were *RASSF1, CDH13*, and *TIMP3*. Additionally, ITAC showed recurrent methylation in *ESR1, APC, HIC1*, and *CASP8* and SNSCC, in *CHFR*. These genes may have clinical relevance as diagnosis and treatment biomarkers in sinonasal carcinomas.

The *RASSF1* gene encodes a protein similar to the RAS effector proteins and plays a major role in the regulation of mitosis. Loss or altered expression of this gene has been associated with the pathogenesis of a variety of cancers, which suggests the tumor suppressor function of this gene.[33]*RASSF1* methylation has been frequently reported in lung cancers and, to a lesser extent, in breast, ovarian, and HNSCC cancer.[34,35] The frequency of 13% to 18% *RASSF1* methylation in HNSCC is similar to that of SNSCC in this study.[35] In renal clear cell carcinoma, methylation of

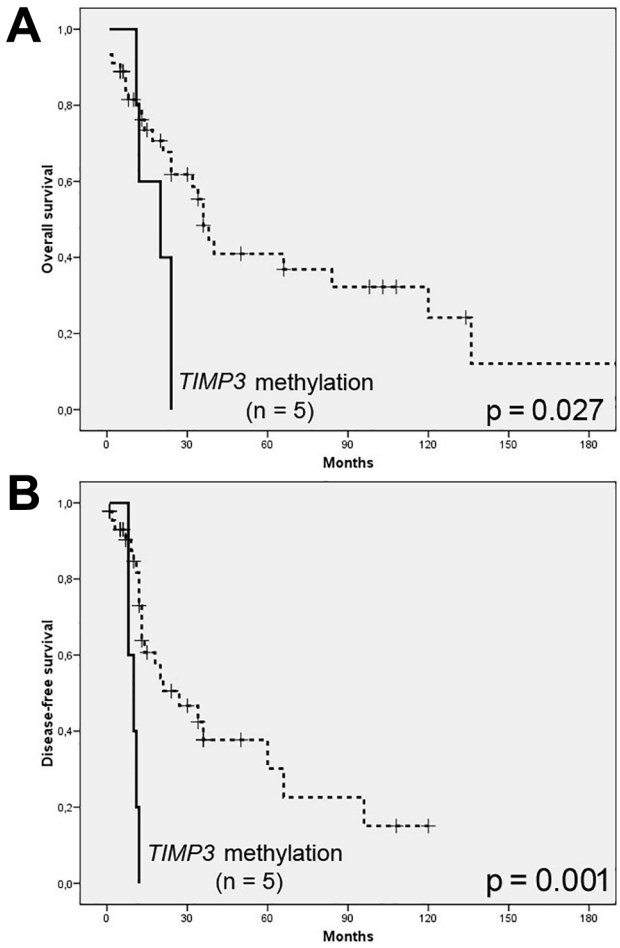

Figure 3. Kaplan-Meier survival analysis: (A) overall survival and (B) disease-free survival of 50 patients with intestinal-type sinonasal adenocarcinoma according to the presence of *TIMP*3 methylation.

RASSF1 correlated with worse prognosis.[36] In our study, we did not find any correlation between methylation of this gene and clinicopathologic parameters.

The gene *CDH13* (H-cadherin) encodes a member of the cadherin superfamily. The protein acts as a negative regulator of axon growth during neural differentiation and protects vascular endothelial cells from apoptosis in oxidative stress–associated atherosclerosis. Gene methylation has been found in nasopharyngeal carcinoma and esophageal adenocarcinoma.[37,38] In non–small cell lung cancer, *CDH13* methylation was more frequent in adenocarcinomas than in squamous carcinomas,[39] analogous to the difference between ITAC and SNSCC in this study.

CHFR is a gene involved as a mitotic checkpoint that delays chromosome condensation in response to mitotic stress. Its function is impaired by promotor hypermethylation in a significant proportion of human cancer cell lines, and treatment with the methyltransferase inhibitor 5-aza-20-deoxycytidine induced reexpression of *CHFR*.[40] We found aberrant methylation of *CHFR* in 13% of SNSCC cases, similar to laryngeal squamous cell carcinoma.[14] Cancer cells that lack *CHFR* expression have shown to be more susceptible to paclitaxel. Therefore, methylation of *CHFR* could be clinically useful as predictor of sensitivity to particular chemotherapeutic agents.[16]

TIMP3 is a membrane-bound inhibitor of TNF-α-converting enzymes and induces programmed cell death through the stable cell surface TNF-α receptor.[41] *TIMP3* shows loss of function aberrations in cancer, including mutations, deletions, methylation, and chromosomal translocation.[42] Prognostic value for *TIMP3* methylation has been reported in gastric, colon, prostate, and hepatic cancer, with all being adenocarcinomas.[42] *TIMP3* methylation was also related to worse survival and more frequent recurrences and metastases in patients with adenocarcinoma (ITAC) in the present study (**Figure 1**). Interestingly, gene copy number loss of *TIMP2* has been related to poor prognosis in ITAC as well.[43] These data suggest an important role of TIMP proteins in the progression of ITAC and so warrant a more detailed study.

Promoter hypermethylation of *ESR1* and *APC* has been described in adenocarcinoma of different localizations, and in this study, they occurred almost exclusively in this type of tumor (ITAC). *ESR1* (estrogen receptor 1) is an important tumor suppressor gene in breast adenocarcinoma.[44] Methylation of *ESR1* has been described in colorectal and esophageal adenocarcinoma, including as early event in premalignant Barrett's lesions.[45,46] Also *APC* inactivation, by either mutation or methylation, occurs as an early genetic event in tumorigenesis of colorectal cancer.[47] Our data did not show differences in *ESR1* or *APC* methylation by ITAC tumor stage; however, we observed more frequent methylation of both genes in the less aggressive papillary and colonic ITAC subtypes. In the case of *APC*, this finding is in agreement with a previous observation that Wnt-Bcatenin pathway activation is especially frequent in these 2 ITAC subtypes as compared with solid and mucinous subtypes.[22] Another interesting aspect of *ESR1* and *APC* methylation in colorectal cancer is their association with chronic inflammation.[46,48] Chronic inflammation has also been claimed to play a role in the development of ITAC.[3,49] It may be speculated that gene methylation is an important genetic event in the tumorigenesis of chronic inflammation-associated cancers, such as colorectal cancer (ulcerative colitis), esophageal cancer (Barrett's esophagus), and perhaps also ITAC.

A number of studies on HNSCC have reported several methylated genes in relation to poor survival, including *DCC, EDRNB*, and *HOXA9*[50]; *DAPK1, RASSF1, CDKN2A*, and *MINT31*[51]; *CCNA1, CASP8*, and *SYBL1*[52]; and *TIMP3* and *CDKN2B*.[14] However, there appear no common methylated genes among these studies. With regard to overall methylation frequencies, some reports claimed worse survival associated with higher methylation rates,[14,50-52] while others observed better survival[53] or no relation.[54] It may be speculated that gene methylation often occurs early in tumorigenesis; however, certain methylation events may confer a more aggressive phenotype and thus be associated with unfavorable clinical outcome.

In conclusion, promotor hypermethylation plays a role in approximately 50% of sinonasal tumors. Moreover, ITAC

and SNSCC show differences in methylation frequency and in the specific genes involved. *CDH13, TIMP3, ESR1*, and *APC* are more important in ITAC, whereas *RASSF1* and *CHFR* are more frequent in SNSCC, confirming methylation patterns in adenocarcinomas and squamous cell carcinomas of other organs. *TIMP3* methylation was associated with worse survival in ITAC. It is difficult to understand why other frequently methylated genes did not associate with survival. It may be speculated that *TIMP3* plays a role in processes such as invasion and metastasis. This is supported by our finding that cases with recurrence and metastasis carry more frequent *TIMP3* methylations. The other methylated genes may be more important in tumor initiation—for example, for *APC* in colorectal cancer, this is well known. These findings could be used as prognosticators and may have implications for future individualized therapies based on epigenetic changes.

Acknowledgments

We thank Bartolomé Scola, Gregorio Marañon General University Hospital (Madrid, Spain), for his collaboration and Sira Potes Ares for her technical support.

Author Contributions

Maria Costales, tissue and clinical data collection, DNA extraction, data analysis, writing; **Alejandro López-Hernández**, DNA extraction, methylation-specific multiplex ligation-dependent probe amplification, data analysis, editing; **Cristina García-Inclán**, DNA extraction, methylation-specific multiplex ligation-dependent probe amplification, editing; **Blanca Vivanco**, pathologic classification, editing; **Fernando López**, tissue and clinical data collection, editing; **José Luis Llorente**, study design, tissue collection, editing; **Mario A. Hermsen**, study design, data analysis, writing.

Disclosures

Competing interests: None.

Sponsorships: None.

Funding source: Instituto Carlos III: PI08-1599, PI11-929, and PI13-646. Red Temática de Investigación Cooperativa en Cáncer: RD12/0020/0034.

References

1. Turner JH, Reh DD. Incidence and survival in patients with sinonasal cancer: a historical analysis of population-based data. *Head Neck*. 2012;34:877-885.
2. Lund VJ, Stammberger H, Nicolai P, et al; on behalf of the European Rhinologic Society Advisory Board on Endoscopic Techniques in the Management of Nose, Paranasal Sinus and Skull Base Tumors. European position paper on endoscopic management of tumors of the nose, paranasal sinuses and skull base. *Rhinol Suppl*. 2010;22:1-143.
3. Llorente JL, López F, Suárez C, Hermsen MA. Sinonasal carcinoma: clinical, pathological, genetic and therapeutic advances. *Nat Rev Clin Oncol*. 2014;11:460-472.
4. Dulguerov P, Allal AS. Nasal and paranasal sinus carcinoma: how can we continue to make progress?*Curr Opin Otolaryngol Head Neck Surg*. 2006;14:67-72.
5. Nicolai P, Schreiber A, Villaret AB, et al. Intestinal type adenocarcinoma of the ethmoid: outcomes of a treatment regimen based on endoscopic surgery with or without radiotherapy [published online June 3, 2015]. *Head Neck*.
6. Nibu K, Sugasawa M, Asai M, et al. Results of multimodality therapy for squamous cell carcinoma of maxillary sinus. *Cancer*. 2002;94:1476-1482.
7. Youlden DR, Cramb SM, Peters S, et al. International comparisons of the incidence and mortality of sinonasal cancer. *Cancer Epidemiol*. 2013;37:770-779.
8. Ansa B, Goodman M, Ward K, et al. Paranasal sinus squamous cell carcinoma incidence and survival based on Surveillance, Epidemiology, and End Results data, 1973 to 2009. *Cancer*. 2013;119:2602-2610.
9. Cantu G, Solero CL, Miceli R, et al. Anterior craniofacial resection for malignant paranasal tumors: a monoinstitutional experience of 366 cases. *Head Neck*. 2012;34:78-87.
10. Vergez S, du Mayne MD, Coste A, et al. Multicenter study to assess endoscopic resection of 159 sinonasal adenocarcinomas. *Ann Surg Oncol*. 2014;21:1384-1390.
11. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Arsenic, metals, fibres, and dusts. *IARC Monogr Eval Carcinog Risks Hum*. 2012;100:11-465.
12. Hennessey PT, Ochs MF, Mydlarz WW, et al. Promoter methylation in head and neck squamous cell carcinoma cell lines is significantly different than methylation in primary tumors and xenografts. *PLoS One*. 2011;6:e20584.
13. Stephen JK, Chen KM, Shah V, et al. DNA hypermethylation markers of poor outcome in laryngeal cancer. *Clin Epigenetics*. 2010;1:61-69.
14. López F, Sampedro T, Llorente JL, et al. Utility of MS-MLPA in DNA methylation profiling in primary laryngeal squamous cell carcinoma. *Oral Oncol*. 2014;50:291-297.
15. Pierini S, Jordanov SH, Mitkova AV, et al. Promoter hypermethylation of *CDKN2A, MGMT, MLH1* and *DAPK* genes in laryngeal squamous cell carcinoma and their associations with clinical profiles of the patients. *Head Neck*. 2014;36:1103-1108.
16. Chen K, Sawhney R, Khan M, et al. Methylation of multiple genes as diagnostic and therapeutic markers in primary head and neck squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg*. 2007;133:1131-1138.
17. Sobin LH, Gospodarowicz MK, Wittekind C, eds. *TNM Classification of Malignant Tumors*. 7th ed.New York, NY: Wiley-Blackwell; 2009.
18. Franchi A, Santucci M, Wenig B. Adenocarcinoma. In: Barnes L, Eveson JW, Reichart P, Sidransky D, eds. *Pathology and Genetics of Head and Neck Tumors: World Health Organization Classification of Tumors*. 5th ed.Lyon, France: IARC; 2005:20-23.
19. López F, Llorente JL, García-Inclán C, et al. Genomic profiling of sinonasal squamous cell carcinoma. *Head Neck*. 2011; 33:145-153.
20. Nygren AO, Ameziane N, Duarte HM, et al. Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucl Acids Res*. 2005;33:e128.

21. Hermsen MA, Llorente JL, Pérez-Escuredo J, et al. Genome-wide analysis of genetic changes in intestinal-type sinonasal adenocarcinoma. *Head Neck*. 2009;31:290-297.
22. Díaz-Molina JP, Llorente JL, Vivanco B, et al. Wnt-pathway activation in intestinal-type sinonasal adenocarcinoma. *Rhinology*. 2011;49:593-599.
23. Pérez-Escuredo J, Martínez JG, Vivanco B, et al. Wood dust-related mutational profile of TP53 in intestinal-type sinonasal adenocarcinoma. *Hum Pathol*. 2012;43:1894-1901.
24. García-Inclán C, López F, Pérez-Escuredo J, et al. *EGFR* status and *KRAS*/*BRAF* mutations in intestinal-type sinonasal adenocarcinomas. *Cell Oncol (Dordr)*. 2012;35:443-450.
25. López F, García Inclán C, Pérez-Escuredo J, et al. *KRAS* and *BRAF* mutations in sinonasal cancer. *Oral Oncol*. 2012;48: 692-697.
26. Franchi A, Palomba A, Fondi C, et al. Immunohistochemical investigation of tumorigenic pathways in sinonasal intestinal-type adenocarcinoma: a tissue microarray analysis of 62 cases. *Histopathology*. 2011;59:98-105.
27. Holmila R, Bornholdt J, Heikkila P, et al. Mutations in *TP53* tumor suppressor gene in wood dust-related sinonasal cancer. *Int J Cancer*. 2010;127:578-588.
28. Schröck A, Göke F, Wagner P, et al. Fibroblast growth factor receptor-1 as a potential therapeutic target in sinonasal cancer. *Head Neck*. 2014;36:1253-1257.
29. Bossi P, Perrone F, Miceli R, et al. *TP53* status as guide for the management of ethmoid sinus intestinal-type adenocarcinoma. *Oral Oncol*. 2013;49:413-419.
30. Sundvall M, Karrila A, Nordberg J, Grénman R, Elenius K. *EGFR* targeting drugs in the treatment of head and neck squamous cell carcinoma. *Expert Opin Emerg Drugs*. 2010;15:185-201.
31. Udager AM, Rolland DC, McHugh JB, et al. High-frequency targetable *EGFR* mutations in sinonasal squamous cell carcinomas arising from inverted sinonasal papilloma. *Cancer Res*. 2015;75:2600-2606.
32. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer*. 2011;11:9-22.
33. Donninger H, Vos MD, Clark GJ. The *RASSF1A* tumor suppressor. *J Cell Sci*. 2007;120:3163-172.
34. Hesson LB, Cooper WN, Latif F. The role of *RASSF1A* methylation in cancer. *Dis Markers*. 2007;23:73-87.
35. Hogg RP, Honorio S, Martinez A, et al. Frequent 3p allele loss and epigenetic inactivation of the *RASSF1A* tumor suppressor gene from region 3p21.3 in head and neck squamous cell carcinoma. *Eur J Cancer*. 2002;38:1585-1592.
36. Klacz J, Wierzbicki PM, Wronska A, et al. Decreased expression of *RASSF1A* tumor suppressor gene is associated with worse prognosis in clear cell renal cell carcinoma. *Int J Oncol*. 2016;48:55-66.
37. Sun D, Zhang Z, Van do N, et al. Aberrant methylation of *CDH13* gene in nasopharyngeal carcinoma could serve as a potential diagnostic biomarker. *Oral Oncol*. 2007;43:82-87.
38. Jin Z, Cheng Y, Olaru A, et al. Promoter hypermethylation of *CDH13* is a common, early event in human esophageal adenocarcinogenesis and correlates with clinical risk factors. *Int J Cancer*. 2008;123:2331-2336.
39. Xue R, Yang C, Zhao F, Li D. Prognostic significance of *CDH13* hypermethylation and mRNA in NSCLC. *Onco Targets Ther*. 2014;7:1987-1996.
40. Rodríguez-Rodero S, Fernández-Morera JL, Fernandez AF, Menéndez-Torre E, Fraga MF. Epigenetic regulation of aging. *Discov Med*. 2010;10:225-233.
41. Fata JE, Leco KJ, Voura EB, et al. Accelerated apoptosis in the Timp-3-deficient mammary gland. *J Clin Invest*. 2001;108: 831-841.
42. Guan Z, Zhang J, Song S, Dai D. Promoter methylation and expression of *TIMP3* gene in gastric cancer. *Diagn Pathol*. 2013;8:110.
43. Pérez-Escuredo J, López-Hernández A, Costales M, et al. Recurrent DNA copy number alterations in intestinal-type sinonasal adenocarcinoma [published online April 23, 2016]. *Rhinology*.
44. Robinson DR, Wu YM, Vats P, et al. Activating *ESR1* mutations in hormone-resistant metastatic breast cancer. *Nat Genet*. 2013;45:1446-1451.
45. Eads CA, Lord RV, Kurumboor SK, et al. Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. *Cancer Res*. 2000;60:5021-5026.
46. Arasaradnam RP, Khoo K, Bradburn M, Mathers JC, Kelly SB. DNA methylation of ESR-1 and N-33 in colorectal mucosa of patients with ulcerative colitis (UC). *Epigenetics*. 2010;5:422-426.
47. Ding Z, Jiang T, Piao Y, Han Y, Xie X. Meta-analysis of the association between *APC* promotor methylation and colorectal cancer. *Onco Targets Ther*. 2015;8:211-222.
48. Scarpa M, Scarpa M, Castagliuolo I, et al. Aberrant gene methylation in non-neoplastic mucosa as a predictive marker of ulcerative colitis-associated CRC [published online February 4, 2016]. *Oncotarget*.
49. Llorente JL, Pérez-Escuredo J, Alvarez-Marcos C, Suárez C, Hermsen M. Genetic and clinical aspects of wood dust related intestinal-type sinonasal adenocarcinoma: a review. *Eur Arch Otorhinolaryngol*. 2009;266:1-7.
50. Hayashi M, Wu G, Roh JL, et al. Correlation of gene methylation in surgical margin imprints with locoregional recurrence in head and neck squamous cell carcinoma. *Cancer*. 2015;121: 1957-1965.
51. Choudhury JH, Ghosh SK. Promotor hypermethylation profiling identifies subtypes of head and neck cancerwith distinct viral, environmental, genetic and survival characteristics. *Plos One*. 2015;10:e0129808.
52. Colacino JA, Dolinoy DC, Duffy SA, et al. Comprehensive analysis of DNA methylation in head and neck squamous cell carcinoma indicates differences by survival and clinicopathologic characteristics. *Plos One*. 2013;8:e54742.
53. Kostareli E, Hielscher T, Zucknick M, et al. Gene promotor methylation signature predicts survival of head and neck squamous cell carcinoma patients. *Epigenetics*. 2016;11:61-73.
54. Kempen PMW, Van Bockel L, Braunius WW, et al. HPV-positive oropharyngeal squamous cell carcinoma is associated with *TIMP3* and *CADM1* promoter hypermethylation. *Cancer Med*. 2014;3: 1185-1196.