

EGFR mutation and HPV infection in sinonasal inverted papilloma and squamous cell carcinoma*

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Rhinology 58: 4, 368 - 376, 2020

<https://doi.org/10.4193/Rhin19.371>

***Received for publication:**

October 24, 2019

Accepted: January 23, 2020

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Abstract

Background: To evaluate the involvement of EGFR signalling and HPV infection in a cohort of inverted sinonasal papilloma (ISP) and sinonasal squamous cell carcinoma (SNSCC) and their value for prognosis and clinical treatment.

Methods: We analysed 55 ISP, 14 SNSCC associated with ISP (SNSCC-isp) and 60 SNSCC not associated with ISP (SNSCC-novo) for EGFR gene mutation and copy number gain, protein expression of EGFR and phosphorylated EGFR (pEGFR), and HPV-infection and KRAS mutation. Findings were correlated to clinico-pathological and follow-up data.

Results: We found EGFR exon 20 mutations in 38% (7/18) ISP, in 50% (6/12) SNSCC-isp and in 5% (1/19) SNSCC-novo. EGFR was expressed in 92% of ISP, while pEGFR was observed in 54% (21/39). SNSCC-isp and SNSCC-novo demonstrated comparable expression of EGFR (57% and 33%) and of pEGFR (44% and 38%). We observed an inverse relation between EGFR exon 20 mutation and pEGFR expression. Four of 39 (10%) ISP carried HPV-16. Oncogenic HPV was detected in 3/12 (25%) SNSCC-isp and in 1/8 (13%) SNSCC-novo. KRAS mutations were not detected in any of the samples. HPV infection was inversely correlated with pEGFR expression but not with EGFR mutation. ISP with EGFR activation by mutation or by phosphorylation had longer ISP-free survival, however, neither EGFR exon 20 mutation, pEGFR expression nor HPV infection demonstrated prognostic value in SNSCC.

Conclusions: EGFR exon 20 mutation is frequent in ISP and SNSCC-isp, while activation of EGFR through phosphorylation also plays an important role. Our data indicate that a large proportion of SNSCC patients could benefit from therapy with modern EGFR inhibitors.

Key words: sinonasal squamous cell carcinoma, inverted sinonasal papilloma, EGFR mutation, HPV infection, treatment

Introduction

Sinonasal squamous cell carcinoma (SNSCC) represents approximately 50% of all sinonasal malignancies. The incidence is less than 1 case per 100,000 inhabitants per year, occurring predominantly among men with a mean age of presentation of 50 to 60 years^(1,2). SNSCC occurs predominantly in the respiratory epithelium of the maxillary sinus and the nasal cavity and approximately 30% of cases are etiologically related to professional exposure to textile, leather, wood or aluminium^(3,4), whereas tobacco does not appear to play a key role. Histological changes

that precede the development of SNSCC include squamous metaplasia and subsequent dysplasia⁽⁵⁾. In addition, inverted sinonasal papilloma (ISP) has been associated with an estimated 5-15% of SNSCC^(6,7). Although distant and lymph node metastasis are exceptional, SNSCC carry a poor prognosis, with an overall 5-year survival at 30-50%^(1,2,8,9) and new therapeutic options are needed.

Genetic data on these rare tumours are limited. Frequent TP53 mutation and p53 overexpression has been reported in up to 70% of SNSCC^(10,11). HPV infection has been described in 38%

of benign and 31% malignant tumours^(6,7,12). In addition, 21% of SNSCC overexpressed p16INK4A (cyclin-dependent kinase inhibitor 2A), often used as surrogate marker of HPV-positive cases. However, the finding of many non-oncogenic or low-risk HPV subtypes made a clear role in malignant transformation less likely^(6,7,12,13). Other indications for ISP as precursor to invasive SNSCC came from X chromosome inactivation analysis, although in one case where both ISP and synchronous SNSCC could be analysed, the two samples showed X inactivation of different alleles, indicating that they arose from different clones⁽¹⁴⁾. Finally in 2015, Udager et al were the first to positively demonstrate a clonal relationship between ISP and synchronous and metachronous SNSCC by detecting identical EGFR mutations⁽¹⁵⁾. Moreover, they reported a high frequency of mutations, especially affecting EGFR exon 20, in 88% of ISP and in 77% of SNSCC-isp. Two later studies confirmed the clonal EGFR mutations in pairs of ISP and SNSCC-isp, albeit in varying frequencies, from 30% up to 91% of tumours^(16,17).

It is possible that EGFR mutation and HPV infection represent two different pathways of progression from ISP to SNSCC. As the number of cases studied are still low, it remains uncertain whether EGFR mutation or HPV infection can predict the progression of ISP into SNSCC, or whether SNSCC with and without EGFR mutation or HPV infection carry a different prognosis.

Our aim was to study EGFR pathway activation and HPV infection in a series of ISP and SNSCC, and to correlate the findings to clinico-pathological characteristics and follow-up data. EGFR pathway activation was evaluated either through EGFR gene mutation, EGFR gene copy number gain, phosphorylation of EGFR protein or, alternatively, by mutation of KRAS in the pathway downstream of EGFR itself.

Material and methods

Samples and clinical data

Our cohort is composed of 129 patients treated between 1989 and 2017 at the department of Otolaryngology, Hospital Universitario central de Asturias (Oviedo, Spain). Tumour samples were recollected retrospectively from the biobank archives of our hospital. All patients had signed an informed consent for the collection, analysis and storage of their biological material and the study was approved by the ethical committee (approval number 66/15 for the project PI15/01629 and 07/16 for project CICPF16008HERM). All specimens were primary tumours, being 55 inverted papillomas (ISP), 14 SNSCC associated with ISP (SNSCC-isp) and 60 SNSCC without ISP association (SNSCC-novo). All SNSCC were of the keratinizing type. Treatment and follow-up data for all cases are described in Table 1.

Of 55 ISP patients, 19 were female (35%) and 36 male (55%). Mean age was 60 years (range 27-81). According with the Krouse T Stage there were 18 T1 (33%), 29 T2 (53%) and 8 T3 (14%).

None presented T4 stage. Eightteen originated in the maxillary sinus (33%), 3 frontal sinus (5%), 10 ethmoid (18%) and 24 nasal cavity (44%). Mean follow-up time was 141 months (range: 5-402). Recurrent disease in the form of a new ISP was observed during follow-up in 16 (29%) cases. None of the 55 developed a SNSCC.

The SNSCC-isp group consisted of 6 metachronous and 8 synchronous cases. Nine were women (64%) and 5 men (36%). Mean age was 58 years (22-82 range). Eight tumours were located in the maxillary sinus (57%), 4 ethmoid (29%) and 2 nasal cavity (14%). Two carcinomas were T2 (13%), 4 T3 (29%), 4 T4a (29%) and 4 T4b (29%). At the time of diagnosis, 3/14 (21%) patients harboured lymph node metastasis. Mean follow-up time was 33 months (range: 3-159). During follow-up, 12/14 (86%) developed recurrent SNSCC.

Among the 60 SNSCC-novo patients, 16 were female (26%) and 44 male (74%), with a mean age of 66 years (42-92 range). Forty-seven tumours were localized in maxillary sinus (78%), 1 sphenoid sinus (2%), 10 ethmoid (17%) and 2 nasal cavity (3%). One case was tumour stage T1 (2%), 5 T2 (8%), 16 T3 (27%), 30 T4a (50%) and 8 T4b (13%), and 48 N0 (80%), 10 N1 (17%) and 2 N2 (3%). At the time of diagnosis, 10 (21%) patients harboured lymph node metastasis. Mean follow-up time was 28 months (range: 1-216). During follow-up, 48/60 (80%) developed recurrent SNSCC.

DNA extraction

DNA was extracted from paraffin-embedded tissues with DNA QIAmp DNA Mini KIT (Qiagen GmbH, Hilden, Germany), according to the manufacturer's recommendations using an elaborate deparaffinization and lysis protocol published previously⁽¹⁸⁾. Normal blood DNA used as reference in the MLPA analysis was isolated with Roche High Pure Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany), following the supplier's guidelines.

EGFR and KRAS mutation analysis

Mutation in EGFR exon 20 and KRAS exon 2 (codons 12 and 13) were analysed by direct sequencing. While it is true that activating mutations may also occur in EGFR exons 18, 19 and 21, and in KRAS exon 3, the great majority occur in EGFR exon 20 and KRAS exon 2. Therefore, we decided to analyse only these regions. Amplification was carried out in a Simplicam Thermal Cycler VXA24811 in standard conditions. PCR Primers and cycling conditions are detailed in Supplementary Table 1. PCR products were purified with Exo-BAP Mix (EURx Ltd, Gdansk, Poland) in accord with the manufacturer's specifications, and analysed by Sanger sequencing using the ABI PRISM 3100 and 3730 Genetic Analyser, (Applied Biosystems, Foster City, CA, USA). PCR conditions were as follows: EGFR exon 20: (5min/95°C + (15" 95°C / 30" 57°C / 1min 72°C) x 35 cycles + 7min/72°C +

Table 1. Clinical and follow-up data of all tumours.

		55 ISP	14 SNSCC-isp	60 SNSCC-novo
Gender	Female	19/55(35%)	9/14 (64%)	16/60 (26%)
	Male	36/55 (65%)	5/14 (36%)	44/60 (74%)
Age at Diagnosis	Range	27-81 years	22-82 years	42-92 years
	Mean	60 years	58 years	66 years
Differentiation	Poor	n.a	6/14 (42%)	25/60 (42%)
	Moderate	n.a	4/14 (29%)	12/60 (20%)
	Well	n.a	4/14 (29%)	23/60 (38%)
T Stage	T1	n.a	0 (0%)	1/60 (2%)
	T2	n.a	2/14 (13%)	5/60 (8%)
	T3	n.a	4/14 (29%)	16/60 (27%)
	T4a	n.a	4/14 (29%)	30/60 (50%)
	T4b	n.a	4/14 (29%)	8/60 (13%)
N Stage	N0	n.a	11/14 (79%)	48/60 (80%)
	N1	n.a	3/14 (21%)	10/60 (17%)
	N2	n.a	0 (0%)	2 (3%)
Krouse ISP T Stage	T1	18/55 (33%)	n.a	n.a
	T2	29/55 (53%)	n.a	n.a
	T3	8/55 (14%)	n.a	n.a
	T4	0 (0%)	n.a	n.a
Localization	Maxillary sinus	18/55 (33%)	8/14 (57%)	47/60 (78%)
	Frontal sinus	3/55 (5%)	0 (0%)	0 (0%)
	Sphenoid sinus	0 (0%)	0 (0%)	1/60 (2%)
	Ethmoid	10/55 (18%)	4/14 (29%)	10/60 (17%)
	Nasal cavity	24/55 (44%)	2/14 (14%)	2/60 (3%)
Treatment	None	1/55 (2%)	0(0%)	0 (0%)
	Surgery	54/55 (98%)	2/14 (14%)	15/60 (25%)
	Surgery+RT	0 (0%)	11/14 (79%)	42/60 (70%)
	Surgery+CRT	0 (0%)	1/14 (7%)	1/60 (2%)
	CRT	0 (0%)	0(0%)	2/60 (3%)
Surgery	Open	6/54 (11%)	10/14 (71%)	57/58 (98%)
	Endoscopic	48/54 (89%)	4/14 (29%)	1/58 (2%)
Recurrence	ISP	16/55 (29%)	n.a	n.a
	SNSCC	n.a	12/14 (86%)	48/60 (80%)
Follow-up	Median	115 months	20 months	15 months
Status	NED	42/55 (76%)	2/14 (14%)	5/60 (8%)
	DOD	0/55 (0%)	12/14 (86%)	47/60 (79%)
	DOC	13/55 (24%)	0 (%)	8/60 (13%)

n.a.: not applicable, NED: no evidence of disease, DOD: died of disease, DOC: died of other causes.

4°C) and KRAS: (5min/95°C + (30" 95°C / 45" 60°C / 1min 72°C) x 30 cycles + 7min/72°C + 4°C).

Immunohistochemistry

Immunohistochemistry (IHC) was performed on an automatic staining workstation (Dako Autostainer Plus; DakoCytomation).

The antibodies used for IHC were: anti-EGFR clone 2-18C9 (DAKO, Glostrup, Denmark) and anti-pEGFR clone D7A5 (Cell Signaling Technology, Cambridge, UK). The immunostaining was evaluated by two experienced investigators (MM and BV). EGFR and pEGFR immunostaining was considered positive when moderate to strong membranous, cytoplasmic, or both staining

Table 2. Genotype of the EGFR exon 20 mutations and HPV status.

Patient	Tumour Type	Mutation	Described before	HPV infection
8	SNSCC-isp Synchronous	p.S778_D770 dup SVD / c.2303_2311 dup GTGTGGACA	ref 15,21	Negative
69	SNSCC-isp Synchronous	p.D770_N771 ins G / c.2310_2311 ins GGT	ref 15,21	Negative
77	SNSCC-isp Synchronous	p.D770_N771 ins GL / c.2310_2311 ins GGGTTA	ref 15,21	HPV 16
126	ISP	p.D770_N771 ins SVD / c.2311_2312 ins GCGTGGACA	ref 21	n.d.
33	ISP	p.D770_N771 ins SVD / c.2311_2312 ins GCGTGGACA	ref 21	n.d.
18	ISP	p.D770_N771 ins SVD / c.2311_2312 ins GCGTGGACA	ref 21	Negative
16	ISP	p.N771 delins GS / c.2310_2311 AA > GGGTC	NO	n.d.
5	ISP and SNSCC-isp Metachronous	p.N771 delins GY / c.2310_2311 A>GGGT	ref 15	Negative
4	SNSCC-isp Synchronous	p.N771 delins GY / c.2310_2311 A>GGGT	ref 15	HPV 42
22	ISP	p.N771_P772 ins V / c.2313_2314 ins GTC	ref 15	Negative
76	SNSCC-novo	p.P772_H773 dup PDN / c.2315_2323 dup GGACAACCC	NO	HPV 16
6	SNSCC-isp Metachronous	p.H773_V774 ins NPH / c.2319_2320 ins AACCCCCAC	ref 21	HPV 16
30	ISP	p.H773_V774 ins NPH / c.2319_2320 ins AACCCCCAC	ref 21	HPV 16
31	ISP	p.H773_V774 ins NPH / c.2319_2320 ins AACCCCCAC	ref 21	n.d.

ref: reference; n.d.: no data.

was observed in at least 10% of tumour cells; tumours with no or weak staining were regarded as negative.

Multiplex Ligation-dependent probe amplification (MLPA)

MLPA was performed as described in detail previously⁽¹⁹⁾ using SALSA P105D-2 Glioma-2 (MRC-Holland, Amsterdam, The Netherlands) that contains 56 probes (including 10 for reference and 4 as internal control). This mix allows the detection of aberrant copy number of EGFR using 11 specific probes. Relative copy number values, including standard deviation, higher than 1.25 were considered as gains and 2.0 or higher as amplifications.

HPV DNA detection

The quality of the extracted DNA was checked by PCR amplification of β -globin (forward primer 5'-ACACAACCTG-TGTGTTCACTAGC-3' and reverse primer 5'-CAAATTCATC-CACGTTACAC-3'). PCR with MY11/GP6+ primers (site-directed L1 fragment of HPV) was performed in order to detect a broad spectrum of HPV genotypes^(11,20). Briefly, the PCR was performed in 25 μ l of reaction mixture containing 1x PCR buffer, 2 mmol/L $MgCl_2$, 50 μ mol/L of each deoxynucleoside, 0.5 μ mol/L of sense and antisense primers, 10 μ l of DNA sample and 1 U Taq DNA polymerase (Promega Biotech Iberica S.L. Madrid, Spain), by thermal profile of 35 cycles: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, with an initial denaturation at 94°C for 5 min and a final extension at 72°C for 10 min. The amplified DNA fragments of approximately 200 bp were identified by electrophoresis in 1.5% agarose gel with ethidium bromide. All positive specimens for L1 fragment

were tested by hybridization assays using type-specific probes for HPV (Supplementary Table 1).

Statistical analysis

SPSS 12.0 software (SPSS Inc., Chicago, IL, USA) was used to analyse correlations existent between clinical and genetic factors using Pearson chi-square and Fisher tests. Continuous variables were analysed with Student's t test. Kaplan-Meier curves were undertaken for the evaluation of survival by application of logarithmic range test (Log-range test) considering significance for values under $p=0.05$.

Results

Genetic alterations in EGFR, KRAS, and HPV detection

DNA was extracted from all 129 samples included in this study. Unfortunately, interpretable EGFR exon 20 sequences were only obtained in 49 cases due to the bad quality and low quantity of DNA obtained from paraffin-embedded tumour material. In total 14 samples, 7/18 (39%) ISP, 6/12 (50%) SNSCC-isp and 1/19 (5%) SNSCC-novo, carried mutations in EGFR (Supplementary Table 2). All mutations exclusively concerned in-frame insertions affecting the region S768-V774 in exon 20 (Table 2). To our knowledge, two mutations had not been described before in the literature on sinonasal tumours (Supplementary Figure 1). EGFR gene copy number gain as evaluated by MLPA was very infrequent, observed in only 1/17 (6%) ISP, 1/5 (20%) SNSCC-isp and 6/32 (19%) of SNSCC-novo (Supplementary Table 2). EGFR protein expression was observed in 34/37 (92%) ISP, 6/10 (60%) SNSCC-isp and in 24/54 (44%) SNSCC-novo, while EGFR activation by phosphorylation (pEGFR) was observed in 21/39

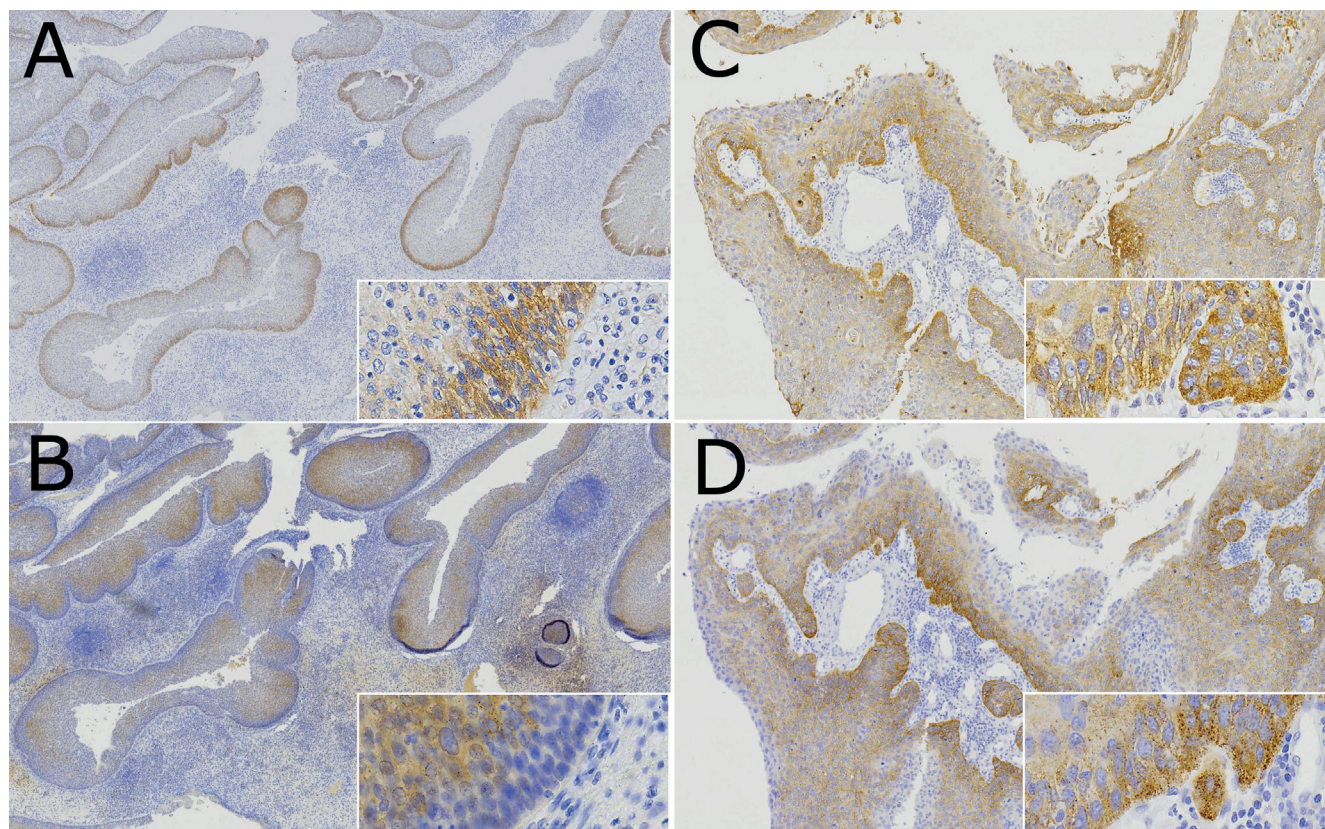


Figure 1. Photomicrographs of immunohistochemical expression of EGFR (A and C) and pEGFR (B and D) detected in the cytoplasm and the cell membrane of an ISP (A and B; original magnification 4x and insert 40x) and a SNSCC-novo (C and D; original magnification 10x and insert 40x).

(54%) ISP, 4/12 (33%) SNSCC-isp and 20/52 (38%) SNSCC-novo (Figure 1). Four of 40 (10%) ISP samples harboured HPV-16 and no low-risk HPV types were detected. Among the 13 analysed SNSCC-isp, two cases showed presence of HPV type 16, one type 18 and two low-risk types 42 and 61. In contrast, only 1/50 (2%) SNSCC-novo was found positive with HPV type 16 (Supplementary Table 2 and Figure 2). No KRAS mutations were detected in all of the analysed ISP, SNSCC-isp and SNSCC-novo samples (Supplementary Table 2).

Correlations between genetic alterations and clinical data

Univariate analysis of clinical and genetic characteristics of 14 SNSCC-isp and 60 SNSCC-novo showed that the former occurred more frequently in women (64% versus 26%, Fisher exact χ^2 , $p=0.012$), at a younger age of onset (58 years versus 66 years, Student's t -test, $p=0.031$) and more often (though not significantly) in the ethmoid sinus than SNSCC de novo (29% versus 15%). There were no significant differences in T stage, N-stage, degree of differentiation, nor in clinical treatment or clinical course (Table 1). EGFR exon 20 mutations were found significantly more frequently in SNSCC-isp than SNSCC-novo, respectively 6/12 (50%) versus 1/19 (5%) (Fisher exact χ^2 , $p=0.007$), whereas EGFR and pEGFR expression and also EGFR copy number gains were similar in the two groups of tumours.

HPV infection (all subtypes) occurred significantly more frequent in SNSCC-isp than in SNSCC-novo, respectively 5/13 (38%) versus 1/50 (2%) (Fisher exact χ^2 , $p=0.001$). This difference was also significant considering only the oncogenic subtypes, respectively 3/13 (38%) versus 1/50 (2%) (Fisher exact χ^2 , $p=0.025$). Multivariate logistic regression analysis (Table 3) of the four variables that were significantly different in univariate analysis showed only EGFR mutation as an independent significant factor differentiating SNSCC-isp and SNSCC-novo (adjusted odds ratio 25.953, $p=0.033$).

Analysing all 74 SNSCC, we found no correlation between EGFR mutation and T stage, N stage, grade of differentiation, tumour localization, age, or overall, disease-specific or disease-free survival. Also EGFR or pEGFR expression and HPV infection were not related to any of the clinical parameters or survival. Similarly among the 55 ISP, there was no relation between EGFR mutation, EGFR or pEGFR expression or HPV infection and Krouse T stage, tumour localization, age, or gender. However, Kaplan Meier analysis showed longer ISP-free survival in cases with EGFR pathway activation, either by mutation or by phosphorylation (Log rank 3.636, $p=0.057$) (Figure 3A). Longer ISP-free survival was also observed in cases with HPV infection (Log rank 1.043, $p=0.307$) (Figure 3B).

Finally, including all 55 ISP and all 74 SNSCC cases, we analysed

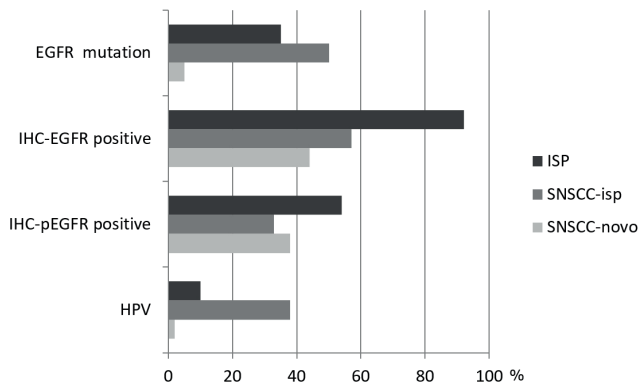


Figure 2. Graphic representation of the frequencies of EGFR mutation, EGFR and pEGFR protein expression, and HPV infection according to type of tumour sample.

possible relations between the genetic data. We observed an inverse (although not completely exclusive) relation between EGFR mutation and pEGFR expression, with 3/12 (25%) EGFR mutated cases versus 18/27 (67%) EGFR wildtype cases also expressing pEGFR (Fisher exact χ^2 , $p=0.035$). Also the presence of oncogenic HPV occurred exclusively in cases with no pEGFR expression (0/4 (0%) HPV-positive cases versus 34/82 (67%) HPV-negative cases expressing pEGFR, Fisher exact χ^2 , $p=0.149$). However, we found oncogenic HPV-positivity co-occurring with EGFR mutation in two ISP and also two SNSCC-isp (Table 2 and Supplementary Figure 2).

Discussion

In the present study we found EGFR exon 20 insertion mutations in an elevated proportion of ISP and SNSCC-isp, but not in SNSCC-novo, confirming previous studies^(15-17,21). However, in contrast to these studies, HPV infection was infrequent and did not occur mutually exclusive with EGFR mutation. In addition, we found few cases with EGFR copy number gains, however, activated pEGFR expression occurred frequent in ISP and SNSCC-isp and does appear mutually exclusive with EGFR mutation. The number of cases SNSCC-isp in this study is relatively small, however, it must be taken into account that SNSCC is a rare tumour and, within this group of tumours, those associated with inverted papillomas are even less frequent. Comparable series in the literature included 17-23 cases⁽¹⁵⁻¹⁷⁾. The clinical features of our cohort of ISP and SNSCC were similar, to those published in previous studies^(15-17,21). Our data confirm the earlier age of onset of SNSCC-ISP compared to SNSCC-novo ($p=0.031$)^(16,22). We also found a higher proportion of women in the SNSCC-isp group (64%) as opposed to the other groups, however, this was not observed in other studies. The frequency of EGFR mutations in ISP (39%) was notably less than in previous studies that reported 72-91% of mutated tumours^(15-17,21). Among the SNSCC-isp this was different; we identified 50% of tumours with mutation,

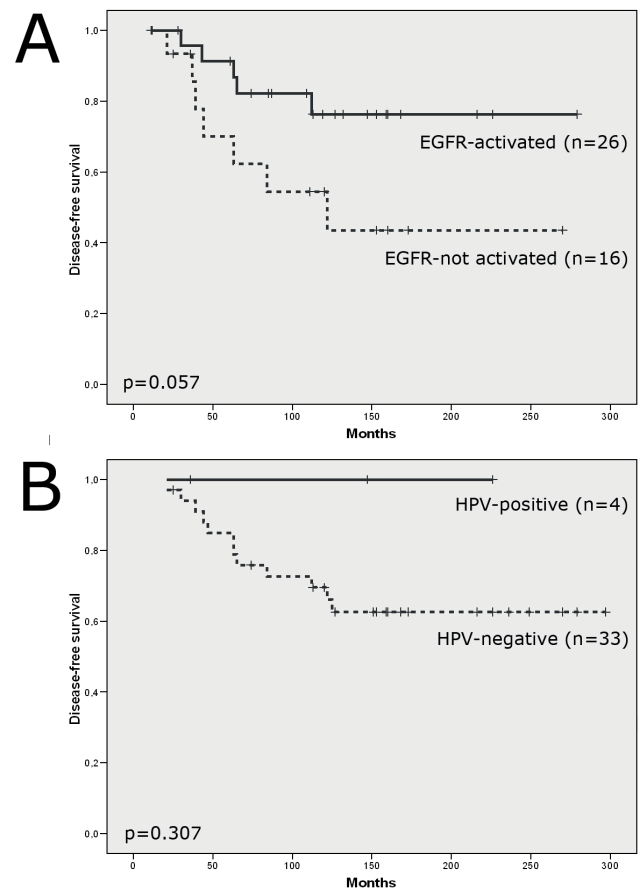


Figure 3. A) Disease-free survival of inverted sinonasal papillomas, 26 with and 16 without EGFR activation. B) Disease-free survival of inverted sinonasal papillomas, 4 with and 33 without HPV infection.

while a Japanese study found 30%⁽¹⁷⁾ and American and Italian studies respectively 77% and 72%^(16,22). We have no explanation for these differences. Sasaki et al.⁽¹⁷⁾ investigated possible geographic or ethnic factors, also taking into consideration frequencies of EGFR mutation in lung cancer, but concluded that there is no evidence for that.

Protein expression of EGFR and the activated form pEGFR was not studied before in ISP or SNSCC-isp. Our data showed that 92% ISP, 60% SNSCC-isp and 44% SNSCC-novo expressed EGFR. A proportion of these tumours also showed pEGFR positivity. Interestingly, the activation of EGFR by mutation and by phosphorylation appeared inversely correlated, suggesting that the EGFR pathway plays an important role both in SNSCC-isp and SNSCC-novo, although activation of the pathway may occur through different mechanisms.

Wild-type EGFR needs binding of its ligand for homo- or heterodimerization and subsequent autophosphorylation to become activated and regulate cell migration, proliferation and survival. EGFR mutations in exon 19 and L858R can cause constitutive dimerization and autophosphorylation without the need of ligand binding⁽²³⁾, however, exon 20 insertion mutations lead to a pro-

Table 3. Multivariate logistic regression analysis of variables in SNSCC-isp and SNSCC-novo.

	SNSCC-isp	SNSCC-novo	Univariate		Multivariate	
			Statistical test	Significance	Adjusted odds ratio	Significance
EGFR-mutation	6/12 (50%)	1/19 (5%)	Fisher Exact Chi ²	p=0.007	25.953	p=0.033
Gender (female/male)	9/5	16/44	Fisher Exact Chi ²	p=0.012	12.847	p=0.057
Age (years)	58	66	Student t-test	p=0.031	1.016	p=0.641
HPV-infection	5/13 (38%)	1/50 (2%)	Fisher Exact Chi ²	p=0.001	0.533	p=0.650

tein conformation change that activates signalling independent of dimerization and autophosphorylation⁽²⁴⁾. This may explain our finding that EGFR exon 20 mutation and expression of phosphorylated EGFR occur in a mutually exclusive manner. We also found that EGFR gene copy number gain never co-occurred with EGFR mutation, while it was more frequent in cases with EGFR and pEGFR immunopositivity, suggesting that gains partly may be responsible for the elevated expression.

HPV infection has long time been considered as an important oncogenic factor in SNSCC^(7,12-13). High risk HPV subtypes have been related with SNSCC-novo, while lower risk subtypes would be more frequent in ISP or SNSCC-isp^(16,22). Moreover, the existence of HPV infection and EGFR mutation was considered almost exclusive⁽²²⁾. The results from our cohort are not in agreement with these studies. We found that 100% (4 of 4) ISP and 60% (3 of 5) SNSCC-isp to be HPV-positive with subtypes 16 or 18, while 1 SNSCC-novo case harboured HPV type 16. In fact, only two samples were found with low-risk HPV (subtypes 42 and 61) and these were SNSCC-isp. In addition, we did not find that HPV infection and EGFR mutation were mutually exclusive. In four samples (two SNSCC-isp and two ISP), we detected simultaneous HPV-16 and EGFR exon 20 mutation. We believe that a role for oncogenic HPV in the tumourigenesis of SNSCC, either through premalignant ISP or in SNSCC-novo, still remains to be demonstrated.

The presence of EGFR mutation in ISP has been correlated with a lower risk of developing SNSCC^(16,22). Although our study did not include ISP samples of patients that later developed SNSCC, we did find that cases with EGFR pathway activation (by mutation or phosphorylation) carried lower risk of developing recurrent ISPs (Figure 3A). Somehow in contrast with these data, Sasaki et al.⁽¹⁷⁾ observed a worse 5-year overall survival of SNSCC carrying EGFR mutation compared to EGFR wildtype (although not significant, 48% versus 65%, respectively). In addition, Takahashi et al.⁽²⁵⁾ found correlation between EGFR protein expression and worse overall and disease-free survival in 67 SNSCC. However, in a similar study on 54 SNSCC, Lopez et al found no such correlation⁽¹⁹⁾. In our present study we found neither worse overall nor worse disease-free survival for SNSCC with EGFR mutation or EGFR activation through phosphorylation. This may in part

be explained by the 5-year disease-specific survival of 23% in our series of 74 SNSCC, which is low compared to the literature reporting an average of 50%. Indeed our cohort contains a high proportion (62%) of stage T4a/T4b tumours and a recurrence rate of 81% (Table 1). This possibly reflects the fact that our hospital is a center of referral for sinonasal and skull base tumours, therefore receiving a relatively high number of complicated cases. The majority of patients were treated with open surgical approaches. Although endoscopic surgery has become common for sinonasal tumours, in most SNSCC an open approach is more appropriate, particularly when the tumour has infiltrated the hard palate, external maxillary wall, orbital fat or extraocular muscles, orbital apex, brain, or facial soft tissue. HPV-positive ISP have been reported to have a significantly higher risk of progression to SNSCC⁽²²⁾. Our data only allowed to evaluate the risk of ISP to develop recurrent ISP and showed oncogenic HPV infection only in 4 cases with no recurring ISP (Figure 3B). This again demonstrates that the role of HPV in ISP, SNSCC-isp and SNSCC-novo still need further study.

Apart from a possible prognostic value of EGFR exon 20 mutations, the fact that an elevated percentage of ISP and SNSCC-isp carry these mutations opens up the possibility for therapy with specific inhibitors. All mutations found in our series, including the two novel mutations not described before in the literature on sinonasal tumours (Table 2, Supplementary figure 1), were situated exclusively in the S768-V774 region of the protein kinase region of EGFR (enclosing codons 18 to 24) promoting a more active state that triggers the pathway in a ligand- and autophosphorylation-independent manner. EGFR exon 20 insertion mutation inhibitors as neratinib, dacomitinib, afatinib specifically targeting this region have yielded only minimal responses⁽²³⁾. Osimertinib, a third generation EGFR exon 20 inhibitor has shown more promising results and has recently been approved as first-line therapy in NSCLC⁽²⁶⁾. As new exon 20 insertion-selective inhibitors are being developed and preclinically tested, at the present moment perhaps the most promising compound being tested in an ongoing phase II clinical trial is poziotinib⁽²⁷⁾, which may also be effective against exon 20 insertion mutations in ERBB2. To date, there are no data on the usefulness of these inhibitors in SNSCC. Using an EGFR exon 20 mutated SNSCC

tumour cell lines as model, Udager et al did show that second generation inhibitors, especially neratinib, caused strong growth inhibition, as opposed to first generation inhibitors⁽¹⁵⁾. This finding demonstrates the potential usefulness of treatment for patients with SNSCC. While KRAS and BRAF mutations could be negative response predictors for anti-EGFR therapies, fortunately our study and also previous studies have shown that neither ISP nor SNSCC-isp or SNSCC-novo carry these mutations^(28,29).

Conclusion

In conclusion, we found EGFR exon 20 insertion mutations in 39% ISP and 50% SNSCC-isp. Protein expression of the activated form of EGFR was also observed frequently in these tumours as well as in SNSCC-novo, while HPV infection does not appear to play a critical role. As none of these tumours carry KRAS mutation, these results show that a large proportion of patients with SNSCC could be treated with EGFR antagonists, either irreversible small molecule inhibitors targeting exon 20 mutation in SNSCC-isp, monoclonal antibodies anti-EGFR in SNSCC-novo, or alternatively, with inhibitors downstream in the EGFR signalling pathway.

Acknowledgements

The authors thank Santiago Melón for the aid with the HPV analysis, and the technicians Eva Allonca and Aitana Vallina for retrieving and processing the paraffin embedded tissue samples and part of the immunohistochemical stainings. This study was supported by grants PI15-1629 from the Fondos de Investigación Sanitaria (FIS), grant CICPF16008HERM of Fundación AECC, and grant CB16/12/00390 from the Centro de Investigación Biomédica en Red de Cancer (CIBERONC), Spain. Plan Nacional de I+D+I 2013-2016 of the Plan Estatal cofinanced by the FEDER Funding Program from the European Union.

Authorship contribution

VNC, MM, JLL, CAM and MAH contributed to the study conception and design. MM, FL, CAM and JLL were responsible for tissue sample and clinical data collection. Material preparation, data collection and analysis were performed by VNC, MM, BV, SPA, LSF, CR, RGM and VBL. The first draft of the manuscript was written by VNC and MAH and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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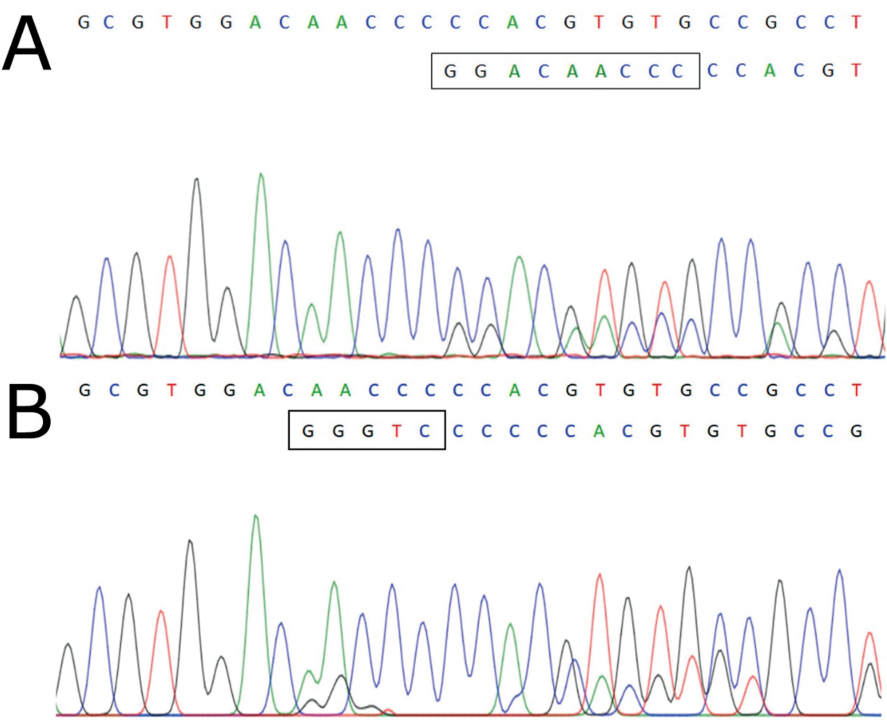
SUPPLEMENTARY MATERIAL

Supplementary Table 1. Primers used for PCR Sanger sequencing of EGFR and KRAS and HPV genotyping.

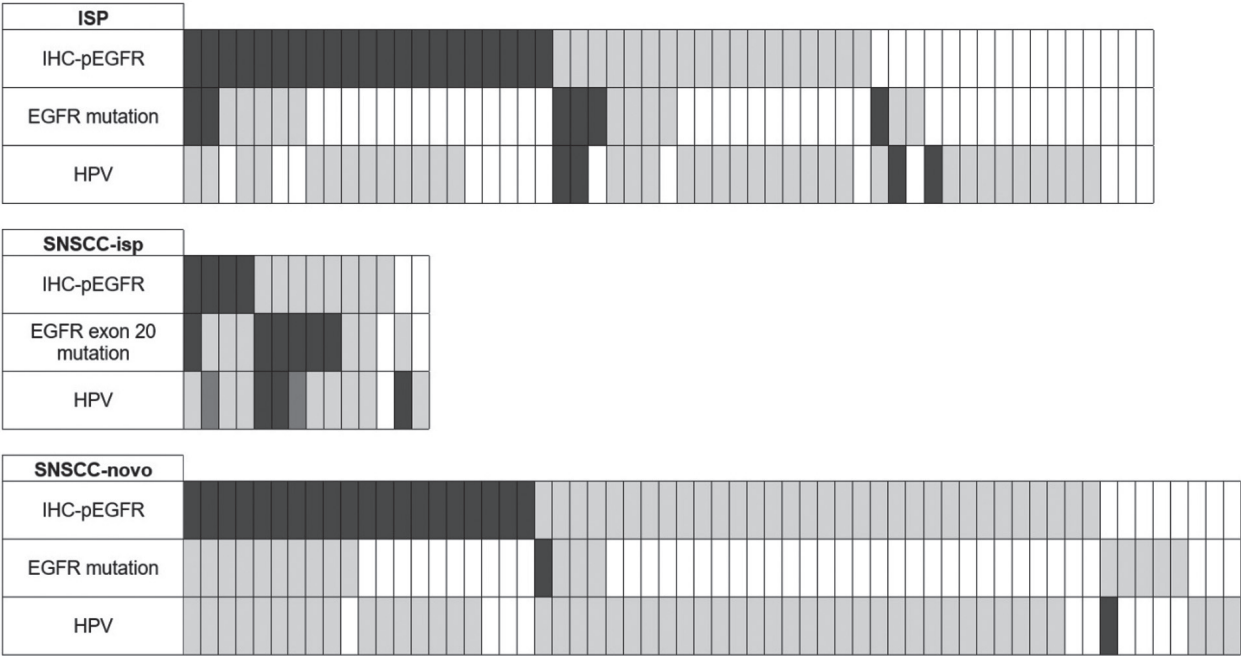
Primers	Sequence 5' to 3'
KRAS Exon 2 Fw	TACTGGTGGAGTATTGATAGTG
KRAS Exon 2 Rv	CTGTATCAAAGAATGGTCCTG
EGFR Exon 20 Fw	CCCTGTGCTAGGTCTTTTG
EGFR Exon 20 Rv	GTCTTTGTGTTCCCGGACAT
HPV type-specific primers	HPV-6 (MY12: 5'CATCCGTAACATCTTCCA-3')
	HPV-11 (MY13: 5'-TCTGTGTCTAAATCTGCTACA3')
	HPV-16 (MY14: 5'-CATACACCTCCAGCACCTAA-3')
	HPV-18 (WD74: 5'GGATGCTGCACCGGCTGA-3')
	HPV-31 (WDB128: 5'TTGCAAACAGTGATACTACATT-3')
	HPV-33 (MY16: 5'CACACAAGTAAGTAGTGACAG-3')
	HPV-35 (5'-CTGCTGTGTCTTCTAGTGA-3')
	HPV-39 (5'ATAGAGTCTTCCATACCTTC-3')
	HPV-45 (MYB69: 5'ATACTACACCTCCAGAAAAGC-3')
	HPV-51 (5'-TGCTGCGGTTTCCCAA-3')
	HPV-52 (5'-GAATACCTTCGTCATGGC-3')
	HPV-56 (5'-TGTCTACATATAATTCAAAGC-3')
	HPV-58 (SANTI58: 5'TGAAGTAACTAAGGAAGGTACA-3')
	HPV-66 (5'-AGCTAAAAGCACATTAATAA-3')
	HPV-68 (5'CTGAATCAGCTGTACCAAT-3')

Supplementary Table 2. Frequencies of EGFR mutation, protein expression and gene copy number, and HPV infection and KRAS mutation, according to type of tumour sample.

Analysis	55 ISP	14 SNSCC-isp	60 SNSCC-novo
EGFR mutation	7/18 (39%)	6/12 (50%)	1/19 (5%)
IHC-EGFR positive	34/37 (92%)	6/10 (60%)	24/54 (44%)
IHC-pEGFR positive	21/39 (54%)	4/12 (33%)	20/52 (38%)
MLPA-EGFR gain	1/17 (6%)	1/5 (20%)	6/32 (19%)
HPV 16 positive	4/40 (10%)	2/13 (15%)	1/50 (2%)
HPV 18 positive	0/40 (0%)	1/13 (8%)	0/50 (0%)
HPV 42 positive	0/40 (0%)	1/13 (8%)	0/50 (0%)
HPV 61 positive	0/40 (0%)	1/13 (8%)	0/50 (0%)
KRAS mutation	0/28 (0%)	0/9 (0%)	0/46 (0%)



Supplementary Figure 1. Two new EGFR exon 20 mutations not described before in the literature. A) In-frame 9 bp insertion: p.P772_H773dupPDN c.2315_2323dupGGACAACCC detected in a SNSCC-novo; B) In-frame deletion-insertion: p.N771delinsGS c.2310_2311 AA>GGGTC detected in an ISP.



Supplementary Figure 2. Case-by-case representation of pEGFR expression, EGFR mutation and HPV infection in 55 ISP, 14 SNSCC-isp and 60 SNSCC-novo. Dark bars indicate presence and grey bars absence of pEGFR-positivity, EGFR mutation or HPV-infection; white bars represent cases that could not be analysed. Low-risk HPV infection (two cases SNSCC-isp) are indicated by dark-grey bars. Activated pEGFR expression and EGFR mutation occur mostly mutually exclusive, however, in contrast to previous publications, EGFR mutation and oncogenic HPV do co-occur in this cohort.