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RESEARCH

Low Prevalence of K-RAS, EGF-R and BRAF Mutations in Sinonasal Adenocarcinomas. Implications for Anti-EGFR Treatments

Alessandro Franchi · Duccio Rossi Degli Innocenti · Annarita Palomba · Lucia Miligi · Fabiola Paiar · Ciro Franzese · Marco Santucci

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Abstract We have previously shown that a subset of sinonasal intestinal-type adenocarcinomas (ITAC) shows activation of the epidermal growth factor-receptor (EGFR) pathway. In this study we examine the status of the EGFR, KRAS and BRAF genes in a series of sinonasal intestinal (ITAC) and non-intestinal type adenocarcinomas (non-ITAC). Eighteen ITACs and 12 non-ITACs were studied immunohistochemically for EGFR expression. Point mutations were analyzed for EGFR exons 19 and 21, KRAS exon 2 and BRAF exon 15 by direct sequencing. Non-ITACs showed significantly higher expression of EGFR ($p=0.015$). Mutation analysis revealed one ITAC with EGFR and one ITAC with KRAS mutation, while two non-ITACs presented mutation of BRAF. We conclude that a subset of sinonasal adenocarcinomas shows overexpression of EGFR, while activating mutations of the signaling cascade downstream of EGFR are rare, suggesting that these tumors could be good candidates for anti-EGFR therapies.

Keywords Sinonasal adenocarcinoma · Intestinal type adenocarcinoma · EGFR · KRAS · BRAF

A. Franchi (✉) · M. Santucci
Section of Pathological Anatomy, Department of Surgery and Translational Medicine, University of Florence, Largo Brambilla 3, 50134 Florence, Italy
e-mail: franchi@unifi.it

D. R. D. Innocenti
Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy

A. Palomba
Unit of Histopathology, Azienda Ospedaliera Universitaria Careggi, Florence, Italy

L. Miligi
Unit of Environmental and Occupational Epidemiology, ISPO – Cancer Prevention and Research Institute, Florence, Italy

F. Paiar · C. Franzese
Unit of Radiation Oncology, Azienda Ospedaliera Universitaria Careggi, Florence, Italy

Introduction

Sinonasal adenocarcinomas are a rare group of tumors accounting for approximately 20 to 30 % of all malignancies at this anatomic sites [1, 2]. Significant geographic differences have been noted in their incidence, possibly in relation with different exposure to risk factors [3]. Indeed, a high relative risk for specific chemical exposures and occupational settings has been reported, particularly for epithelial tumors. Sinonasal adenocarcinomas are known to be associated with exposure to hardwood dust, but an excess risk for these tumors has also been observed in leather workers [3].

Overall, the clinical outcome of patients with sinonasal carcinomas remains poor, and has not changed significantly over the last 3 decades [2]. Treatment options resulting in a better survival include surgery or a combination of surgery and radiotherapy [2]. However, the morbidity associated with these treatment modalities, as well as the high rate of local recurrence, remain a problem still to be solved. For these reasons, the attention has been focused on the possible introduction of molecular targeted therapies aimed to interfere with specific molecular mechanisms involved in tumor growth. In recent years, several drugs have been developed which can interfere successfully with neoplastic growth, including molecules that target tyrosine kinase receptor proteins, such as

HER-2, KIT, PDGFR, ABL and EGFR, from which promising results have been obtained in the treatment of different types of cancers.

The human epidermal growth factor receptor (HER)-erbB family of receptor tyrosine kinases includes the EGFR (ERB-1 or HER-1), HER2/C-neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). These receptors are involved in the regulation of key processes controlling tumor progression, including proliferation, angiogenesis, invasion and metastases. The binding with the respective ligands activates a mitogenic signalling cascade through two main pathways, the KRAS–RAF-mitogen-activated protein kinase (MAPK) pathway, and the lipid kinase phosphatidylinositol 3-kinase (PI3K), which promotes Akt-mammalian target of rapapycin (mTOR) activation, responsible for anti-apoptosis and prosurvival signals. Therefore, EGF-R appears to be a rational target for molecular therapeutic strategies and the use of monoclonal antibodies that inactivate the extracellular domain of EGF-R and inhibit the down-stream signaling pathways, has shown promising results in the treatment of malignant tumors, including colorectal cancer [4]. Considering the phenotypic and molecular similarities existing between ITAC and colorectal cancer, it seems reasonable to investigate the EGFR signaling cascade in sinonasal adenocarcinomas, as a possible key pathway for new therapeutic options. However, clinical response to these treatments has been variable and predictive factors have not yet been identified with certainty.

We previously examined a series of sinonasal ITAC to assess the frequency of EGFR protein expression by immunohistochemistry and gene status by fluorescence in situ hybridization (FISH) [5]. We observed that a subset of ITAC, mostly occurring in woodworkers, demonstrate expression of high levels of EGFR and this is often associated with either gene amplification or chromosome 7 polysomy. Here, we further investigate the EGFR pathway in sinonasal adenocarcinomas, extending our observations to the status of the EGF-R, K-RAS and BRAF genes, in a series of sinonasal adenocarcinomas including both intestinal and non-intestinal subtypes.

Materials and Methods

Tumor Specimens and Tissue Microarray Construction

In this study we investigated 30 sinonasal adenocarcinomas, including 18 ITACs and 12 non-ITACs. All the available histological slides were retrieved and examined by two pathologists (AF and AP). Immunohistochemical staining for cytokeratin 20 and CDX2 was used to confirm the diagnosis of ITAC. These tumors were further classified in three grades (G1, G2, G3) or defined as mucinous, when more than 50 % of the lesion showed intra or extra-cellular mucous formation.

For the construction of the tissue microarray, areas of interest rich in nonnecrotic tumor glands, were identified on corresponding hematoxylin and eosin-stained sections and marked on the source paraffin block. The source block was cored and a 1 mm core transferred to the recipient master block using the Beecher Tissue Microarrayer (Beecher Instruments, Silver Spring, MD). Three cores from different areas of the same tissue block were arrayed for each case. Five micrometers thick sections were obtained from the block, which were stained with hematoxylin and eosin, or employed for the immunohistochemical analysis.

Immunohistochemistry

Immunohistochemical analysis was performed as previously described [5] using the EGFR Pharm Dx kit K 1494 (Dako Cytomation, Glostrup, Denmark). The immunostaining was scored according to the following scale: negative 0; +1 weak reactivity that was membranous, cytoplasmic, or both; +2 circumferential membrane staining with intermediate intensity and frequent cytoplasmic reactivity that was of weaker intensity than the membrane reactivity; and +3 complete strong circumferential staining, usually associated with cytoplasmic staining of weaker intensity.

DNA Extraction

DNA was isolated from three 10 μm formalin-fixed paraffin-embedded tissue sections after microdissection of tumor areas from each specimen. After dewaxing tissue sections and overnight proteinase K (200 μg/ml) digestion at 50 °C, the DNA was heated to 96 °C for 15 min to destroy proteinase K activity. The DNA was purified with MasterPure™ DNA Purification (Epicentre Biotechnologies, WI—USA) according to the manufacturer's protocol and the DNA concentration was assessed spectrophotometrically.

KRAS, EGFR and BRAF Sequencing

KRAS, EGFR and BRAF genes were analyzed by direct sequencing. PCR reaction was performed using the forward and reverse primers indicated in Table 1.

Table 1 Forward and reverse primers used in this study

KRAS for	ACTGAATATAAACTTGTGGTAGTTGGACCT
KRAS rev	TAATATGTCGACTAAAACAAGATTTACCTC
EGFR19 for	GCACCATCTCACAATTGCCAGTTA
EGFR19 rev	GAGGTTCAGAGCCATGGACCC
EGFR21 for	CCATGATGATCTGTCCCTCACA
EGFR21 rev	AGGAAAATGCTGGCTGACCTAAAG
BRAF for	TGCTTGTGATAGGAAAATG
BRAF rev	CCACAAAATGGATCCAGACA

The reaction for KRAS gene was carried out with 2 mM MgCl2 and 25 pmol of each primer with 200–350 ng of DNA template. The cycle conditions were: 40 s at 96 °C, 40 s at 55 °C and 30 s at 72 °C for 40 cycles. The reactions for EGFR gene were performed with 1.5 mM MgCl2 and 150 pmol of each primer and with 300 ng of DNA template. The cycle conditions were: 60 s at 96 °C, 60 s at 58 °C and 60 s at 72 °C for 35 cycles. The reaction for BRAF was carried out with 1.25 mM MgCl2 and 20 pmol of each primer with 200 ng of DNA template. The cycle conditions were: 30 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C for 35 cycles.

All the amplifications were performed in a 2720 Thermal Cycler (Applied Biosystems, USA).

Table 2 Clinico-pathologic characteristic and mutation status of 30 patients affected by sinonasal adenocarcinoma

Case	Sex	Age	EGFR IHC score	EGFR Mutational analysis	KRAS	BRAF	Histopathologic subtype	Occupational exposure	Follow up status
1	M	61	0	WT	WT	WT	ITAC mucinous	Unknown	Lost to follow-up
2	F	62	0	WT	WT	WT	ITAC G3	Wood dust	Recurrence at 9 m; NED at 19 m
3	F	63	0	WT	g.35G>A (p.G12D)	WT	ITAC G2	Leather dust	Recurrence at 7 m; NED at 19 m
4	M	63	2	WT	WT	WT	ITAC G3	Wood dust	Recurrence at 4 m; DOD at 10 m
5	M	77	1	WT	WT	WT	ITAC G2	Wood dust	NED at 25 m
6	M	50	0	WT	WT	WT	ITAC mucinous	None	Recurrence at 26 m; DOD at 32 m
7	M	72	0	WT	WT	WT	ITAC G3	None	DOD at 6 m
8	M	73	0	WT	WT	WT	ITAC G1	Leather dust	Recurrence at 10 m; DOD at 78 m
9	M	68	0	WT	WT	WT	ITAC G1	Wood dust	DOD at 24 m
10	F	58	0	Exon 21, codon 858, T>G, Leu>Arg	WT	WT	ITAC G1	None	NED at 44 m
11	M	75	2	WT	WT	WT	ITAC mucinous	Leather dust	NED at 54 m
12	M	56	1	WT	NV	NV	ITAC G1	Unknown	NED at 24 m
13	M	84	0	WT	WT	NV	ITAC G1	Leather dust	NED at 9 m
14	M	86	0	WT	WT	WT	ITAC G2	Unknown	NED at 6 m
15	M	76	1	NV	NV	NV	ITAC G1	Wood dust	NED at 50 m
16	M	50	0	WT	WT	WT	ITAC G3	Leather dust	DOD at 10 m
17	M	67	0	WT	WT	WT	ITAC G2	Leather dust	NED at 8 m
18	M	59	3	WT	WT	WT	ITAC G2	Unknown	Lost to follow-up
19	M	59	3	WT	WT	WT	non ITAC G3	None	Lost to follow up
20	F	39	0	WT	WT	WT	non ITAC G1	None	NED at 26 m
21	M	48	2	WT	WT	WT	non ITAC G1	None	NED at 30 m
22	M	53	1	WT	WT	T>A, Val>Glu	Low grade tubulopapillary AC	None	Recurrence at 5 m; NED at 34 m
23	M	64	1	WT	WT	T>A, Val>Glu	Low grade tubulopapillary AC	None	NED at 30 m
24	F	42	2	WT	WT	WT	Myoepithelial carcinoma	Unknown	NED at 6 m
25	M	57	1	NV	NV	NV	Adenoid cystic carcinoma	None	NED at 6 m
26	M	75	1	WT	WT	WT	Adenoid cystic carcinoma	None	NED at 63 m
27	F	60	1	WT	WT	NV	Adenoid cystic carcinoma	None	Recurrence at 120 m; NED at 140 m
28	F	58	1	WT	WT	WT	Adenoid cystic carcinoma	None	Recurrence at 23 m; NED at 60
29	F	66	3	WT	WT	WT	Adenoid cystic carcinoma	None	NED at 21 m
30	M	63	3	WT	WT	WT	Adenoid cystic carcinoma	None	NED at 8 m

Abbreviations: *F* female, *M* male, *WT* wild type, *ITAC* intestinal type adenocarcinoma, *NED* no evidence of disease, *DOD* dead for disease, *m* months

The amplification products were purified with MSB® Vario Cleanup Kit (Invitek, Berlin, DE) according to the manufacturer's protocol. Subsequently we performed cycle sequencing reaction of purified PCR products using the KRAS/EGFR/BRAF forward primers and BigDye® Teminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's protocol. The sequencing products were purified using DyeEx® 2.0 Spin Kit (Qiagen, Hilden, DE). Then 10 μl of purified sequencing reactions were added to 20 μl formamide and was heated to 95 °C for 5 min to allow the DNA denaturation. The samples were analyzed using the AbiPrism 310 Genetic Analyzer (Applied Biosystems, USA). The sequence results for each sample were analyzed using Seqscape® Software v2.5 (Applied Biosystems, USA) to verify the sequencing results and identify the possible mutations.

Results

Patients

The clinico-pathological features of this series are summarized in Table 2. There were 23 males and 7 females, ranging in age between 39 and 86 years (mean 62.8 years). Occupational history was known for 26 patients: 6 patients affected by ITAC had been employed in leather industry, 5 in wood industry, and 3 had no significant work exposure, while none of the patients affected by non-ITAC had significant occupational history. Follow-up information was available for 28 patients. Eight patients experienced local recurrence (5 in the group of ITAC and 3 in the group of non-ITAC), and 5 patients, all affected by ITAC, died of disease.

Immunohistochemistry for EGFR

Overall, 17 (56.6 %) adenocarcinomas showed EGFR immunoreactivity (Table 2, Fig. 1). There were 6 positive cases in the group of ITACs (33.3 %), 3 of which were scored as 1+, 2 as 2+ and 1 as 3+. Among non-ITACs, 11 out of 12 tumors (91.6 %) were positive for EGFR, with a score distribution of 6 grade 1+, 2 grade 2+ and 3 grade 3+. Statistical analysis (Pearson chi-square) showed that EGFR expression was significantly higher in non-ITACs ($p=0.015$), which showed a score 2 or 3 in 5 of 12 cases (41.6 %), while similar expression levels were noted only in 3 ITACs (16.6 %).

Mutational Analysis

The results are summarized in Table 2. Direct sequencing of EGFR (exons 19 and 21), and KRAS (exon 2, codon 12 and 13) genes was successful for 28 of 30 FFPE samples. We found absence of EGFR mutation in 29 cases, whereas only one case showed a L858R mutation (T<G; Leu<Arg) of EGFR (Fig. 2). This was a well differentiated ITAC occurring in a patient with no history of occupational exposure to wood or leather dusts.

KRAS alteration occurred in one sample and consisted of a g.35G>A (p.G12D) mutation. This was a moderately differentiated ITAC occurring in a patient with history of occupational exposure to leather dusts (Fig. 3).

Sequencing analysis of BRAF exon 15 (V600) was successful for 26 of 30 FFPE samples and showed wild type results in all cases of ITAC. Two low grade adenocarcinomas out of 12 non-ITAC carried the BRAF exon 15 (V600E) mutation (T>A, Val>Glu) (Fig. 4). Notably, these two lesions were previously reported together as they share some unusual histopathological features, including bland morphology with

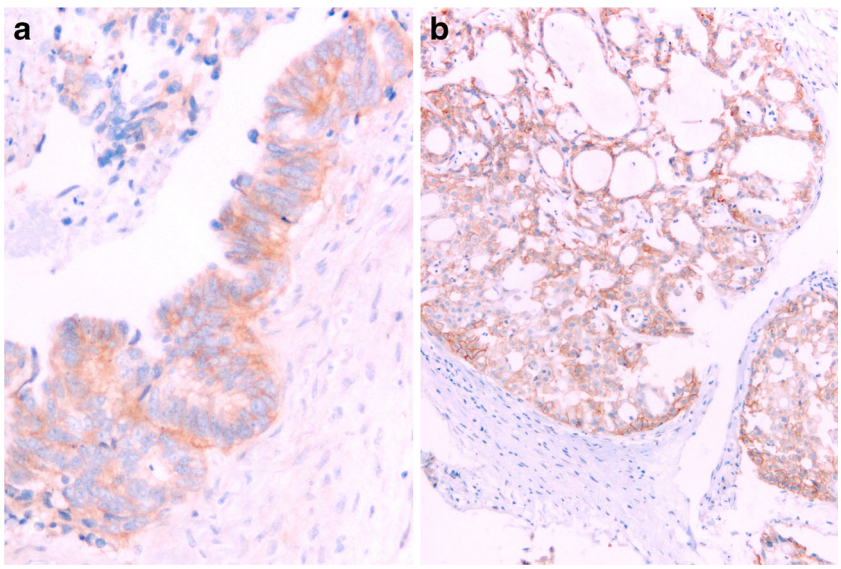

Fig. 1 Representative images of EGFR immunostaining (score 3) in ITAC (**a**) and non-ITAC (**b**)

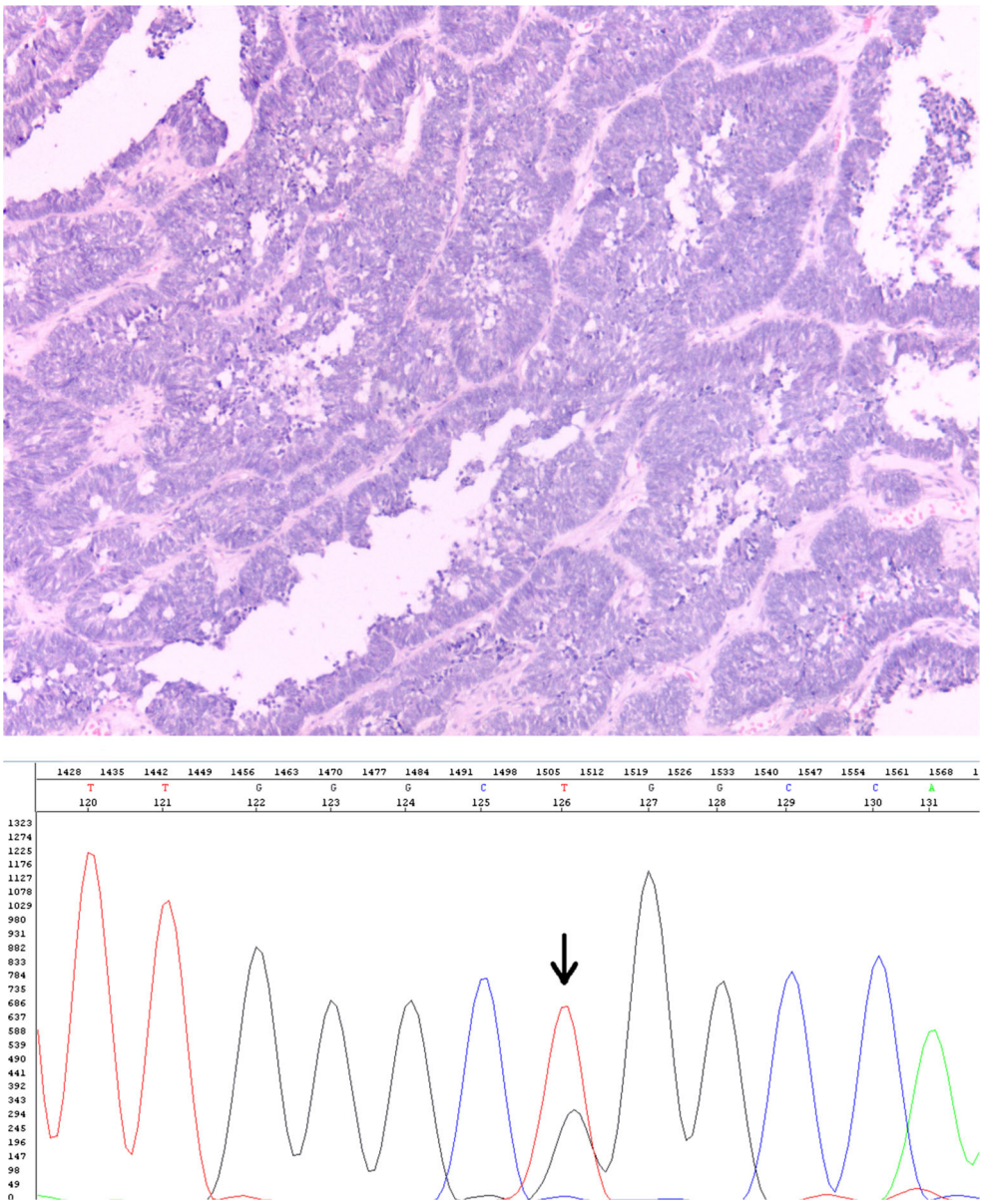

Fig. 2 Sequence analysis of part of EGFR exon 21. Well differentiated ITAC (*top*) showing a T<G missense mutation at codon 858 (*bottom*)

tubulo-papillary architecture, absence of cytokeratin 20 and CDX-2, and positivity for cytokeratin 7 and myoepithelial markers [6].

Discussion

The group of sinonasal adenocarcinomas encompasses several entities with different epidemiology, clinical behavior and pathological findings. Their development therefore most likely occurs according to different molecular pathways, which to date have been only partially investigated, in studies mainly focused on the intestinal subtype. In this study we have examined the status of the EGFR-RAS-RAF-signaling cascade in a series of sinonasal ITAC and non-ITAC. This is a key pathway for tumor growth because it is implicated in proliferation, differentiation and survival of cancer cells. Moreover, therapies aimed to block EGFR signaling cascade are now available and have been applied in retrospective and prospective clinical trials. The response to these treatments depends on several and only partially understood factors, including the expression of EGFR and the status of the EGFR gene, but also of downstream players such as KRAS and BRAF genes [4]. Indeed, activating KRAS mutations determine a constitutively active protein, which renders the downstream pathway permanently "switched on", and the blockage of EGFR will not elicit any suppressive effects. Similarly, activating BRAF gene mutations, which in

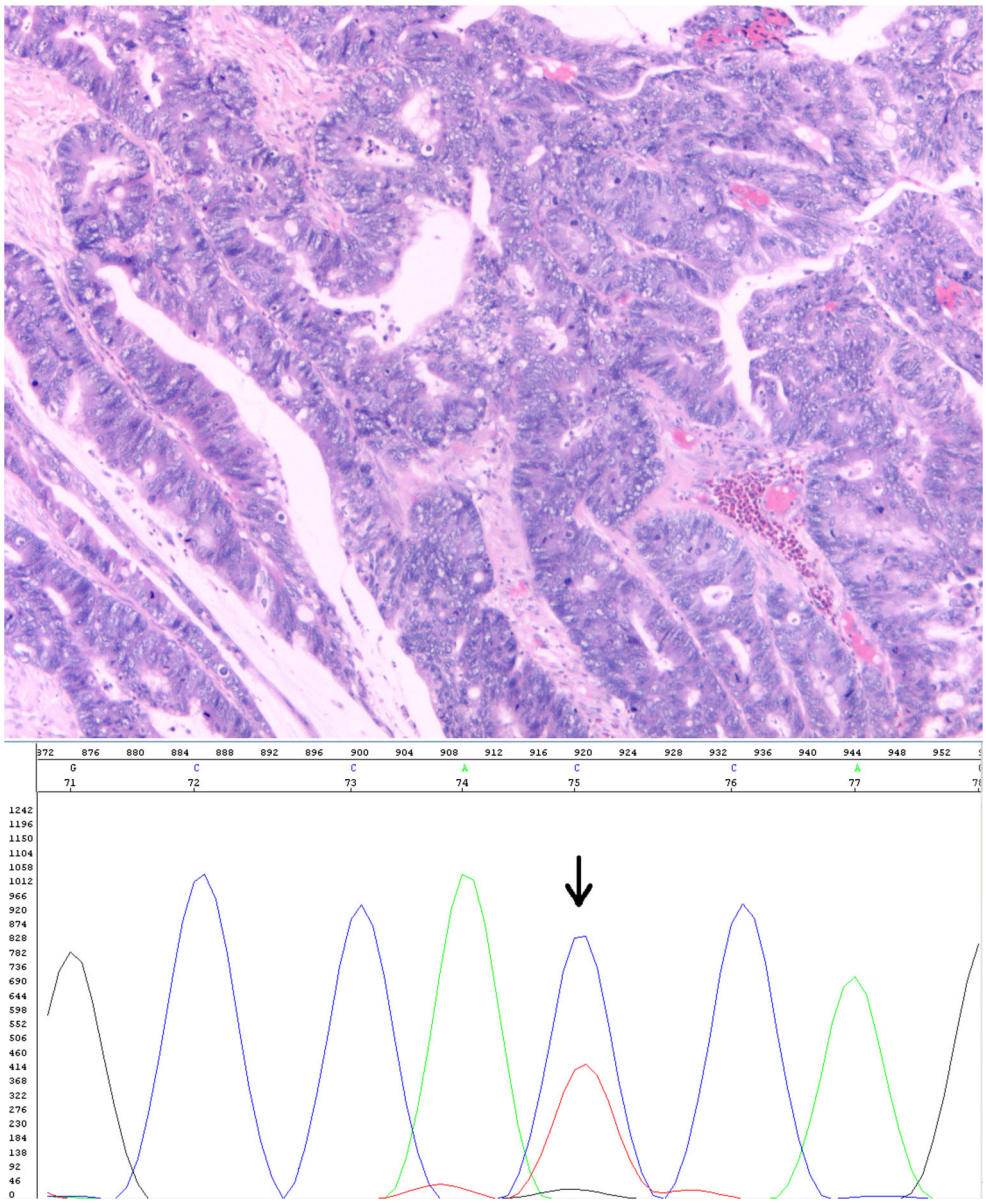

Fig. 3 Sequence analysis of part of KRAS exon 2. Moderately differentiated ITAC (*top*) of a patient with leather dust exposure, showing a G>A missense mutation at codon 12 (*bottom*)

colorectal carcinoma are present only in tumors that do not carry KRAS mutations, confer resistance to anti-EGFR treatments [4].

In our previous study employing immunohistochemistry and FISH analysis, EGFR overexpression was observed in 14.5 % of 55 ITACs analyzed, and most of these cases showed either chromosome 7 polysomy or gene amplification [5]. Recently, Garcia-Inclan and coworkers reported the presence of EGFR copy number gains in 45 % of 98 ITACs studied by FISH, while protein over-expression was detected in 21 % of their cases [7]. In the study by Szablewski and coworkers, 30 % of ITACs exhibited a high expression level of EGFR by immunohistochemistry [8]. In the present analysis, immunohistochemistry for EGFR showed similar results for the group of ITACs, while we show for the first time that non-ITACs have a significantly higher expression of the receptor, with almost half of the cases showing either a score 2 or 3 for the expression of the receptor. However, it should be noted that EGFR expression as detected by immunohistochemistry is not considered an significant predictor of response to gefitinib treatment in non-small cell lung cancer.

We then extended these observations by testing the presence of activating mutations of the EGFR gene in sinonasal adenocarcinomas. In non-small-cell lung cancer the presence of EGFR activating mutations correlates with clinical responsiveness to the tyrosine kinase inhibitor gefitinib and improved survival [9, 10]. Conversely, in colorectal adenocarcinoma EGFR somatic mutations occur at low frequency and are not associated with response to anti-EGFR treatments. In agreement with these observations, in our cohort of sinonasal

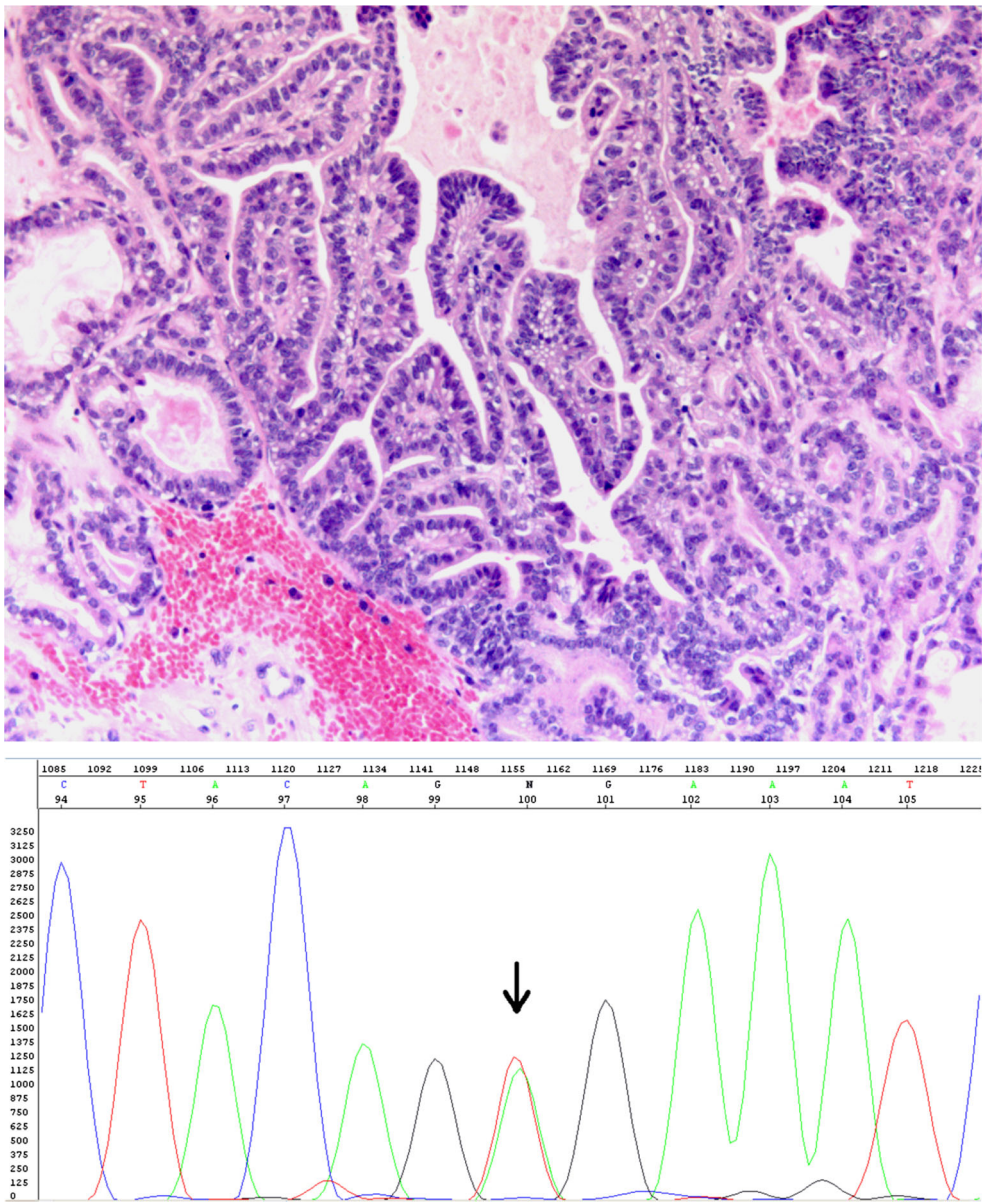

Fig. 4 Non-intestinal type sinonasal adenocarcinoma (*top*), showing BRAF exon 15 (V600E) T>A mutation (*bottom*)

adenocarcinomas only one ITAC presented a mutation previously identified in non-small-cell lung cancer and colorectal adenocarcinoma, while no mutations were identified in non-ITACs. Interestingly, this L858R mutation is present in the non-small cell lung cancer H3255 cell line, which is extraordinary sensitive to gefitinib in comparison with other adenocarcinoma cell lines with wild type EGFR [11]. This mutation has also been associated with good response to gefitinib in patients affected by lung adenocarcinoma [11]. No mutations in the EGFR gene were found in the cohort of patients affected by ITAC analyzed by Garcia-Inclan et al. [7].

KRAS mutations have been detected with variable, generally low frequency in sinonasal carcinomas, being more frequent in adenocarcinomas (0–50 % of cases) than in squamous cell carcinomas (1 % of cases) [8–10, 12–15]. In a recent study analyzing KRAS status in wood-dust related sinonasal carcinomas, mutations were identified in 13 % of adenocarcinomas, whereas only 1 % of squamous cell carcinomas were mutated [16]. Similar frequencies were observed in a study of 115 sinonasal carcinomas, where 12 % of ITACs and none of the squamous cell carcinomas harbored KRAS mutations [17]. KRAS mutations tended to occur more frequently in woodworkers, and in histologically less aggressive ITAC subtypes [16, 17]. A significantly better overall survival has also been observed in patients affected by ITACs with KRAS mutations [8].

Interestingly, in these studies KRAS mutations consisted prevalently of G → A transitions, a type of mutation typically produced by alkylating agents in experimental systems, and this could be related to a combination of exposure to tobacco,

wood dust, and possibly other occupational agents [13]. In our series, only one ITAC and none of the non-ITACs showed KRAS mutation. This discrepancy may be due to the small number of tumors analyzed, as well as to the limited number of adenocarcinomas arising in wood workers present in our series. However, with the exception of one study [15], the frequency of KRAS mutation in sinonasal ITAC appears to be significantly lower than in colorectal adenocarcinoma, where this genetic change has been detected in 35 % to 45 % of metastatic CRC patients [4].

KRAS mutations are currently considered a major predictor of resistance to anti-EGFR treatments in metastatic colorectal adenocarcinoma. Their relatively low frequency in sinonasal adenocarcinoma should not therefore preclude the possibility of investigating the effects of anti-EGFR treatments in these tumors, also in the light of our previous observation regarding the expression of EGFR by tumor cells as demonstrated by immunohistochemical analysis and the presence of chromosome 7 polysomy or EGFR gene amplification [5].

The BRAF gene encodes for a serine-threonine protein kinase that is a downstream effector of activated KRAS. Activating BRAF mutations are observed in approximately 4–15 % of sporadic microsatellite instability low colorectal adenocarcinoma and in up to 70 % of sporadic microsatellite instability-high colorectal adenocarcinoma. The most frequent mutation occurs at codon 600 and consists of a thymidine to adenine transversion (V600E). The presence of BRAF activating mutations affects the response to therapies based on EGFR-targeting, but sensitivity may be restored by the multikinase inhibitor sorafenib [18]. We observed no BRAF V600E mutations in our series of ITAC, and this is in agreement with previous studies, which showed that this mutation is either absent or extremely rare in these tumors [7, 8, 15, 17]. Conversely, two low grade non-ITACs carried the same V600E BRAF mutation. Interestingly, these two lesions share several histological and immunophenotypic features, and were previously reported as low grade tubulo-papillary adenocarcinomas showing a dual epithelial secretory and myoepithelial neoplastic component [6]. Moreover, no mutation had been previously identified in TP53 exons 4–9 in these two lesions [6]. Considering these peculiar features, and the presence of the same V600E BRAF mutation, we suggest that these tumors may represent a separate subset of sinonasal non-intestinal type low grade adenocarcinomas.

In conclusion, our results indicate that sinonasal adenocarcinomas frequently show overexpression of EGFR, while mutations determining constitutive activation of the signaling cascade downstream of EGFR are rare, suggesting that these tumors could be good candidates for anti-EGFR therapies. In addition, low grade non-intestinal type adenocarcinomas bearing BRAF V600E mutations could be potential candidates for treatment with BRAF inhibitors.

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