

# A pilot study to evaluate the expression of microRNA-let-7a in patients with intestinal-type sinonasal adenocarcinoma

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**Abstract.** Despite its histological resemblance to colorectal adenocarcinoma, there is little information about the molecular events involved in the pathogenesis of intestinal-type sinonasal adenocarcinoma (ITAC). The present study investigated the possible role and clinical value of microRNA (miR)-let-7a, a head and neck squamous cell carcinoma-related miR, in a well-characterized and homogeneous cohort of patients with ethmoidal ITAC associated with occupational exposure, treated by primary surgery. miR-let-7a expression levels were analyzed in 23 pairs of ethmoidal ITAC and adjacent normal formalin-fixed paraffin-embedded tissues by reverse transcription-quantitative PCR. The expression was evaluated in tumor and healthy tissues according to: Tumor grade (G) of differentiation and extension, and pTNM stage, and presence/absence of recurrence. Comparisons within and between groups were performed using two-tailed Student's paired t-test and one-way ANOVA with Tukey's post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference. miR-let-7a expression in ethmoidal ITAC tissues was significantly lower than that in adjacent normal tissues ( $P < 0.05$ ; mean expression level  $\pm$  SD,  $1.452707 \pm 1.4367189$  vs.  $4.094017 \pm 2.7465375$ ). miR expression varied with pT stage. miR-let-7a was downregulated

( $P < 0.05$ ) in advanced stages (pT3-pT4) compared with earlier stages (pT1-pT2). Furthermore, downregulation of miR-let-7a in ITAC was associated with poorly-differentiated (G3) cancer ( $P < 0.05$ ). No other associations were observed between miR-let-7a expression and the other clinicopathological parameters, including disease-free survival. In conclusion, downregulation of miR-let-7a in ITAC was associated with advanced-stage (pT3 and pT4) and poorly-differentiated (G3) disease, suggesting that the mutation of this gene, combined with additional genetic events, could serve a role in ITAC pathogenesis.

## Introduction

Malignant neoplasms of the nasal cavity and paranasal sinuses represent 0.2% of all human primary malignant tumors, and their incidence is about 0.1-1.4 new cases/year/100,000 inhabitants (1,2). Among primary malignant neoplasms of the sinonasal tract nasal, sinonasal adenocarcinomas account for 10-20% (3); the majority of them show a salivary gland origin, while others show histological features resembling those of colon adenocarcinoma. One particular subtype of sinonasal adenocarcinoma was named intestinal-type adenocarcinoma (ITAC), which is responsible for less than 4% of total malignancies in this region (4), and can occur sporadically or associated with specific workers' categories that are exposed to hardwood and leather dust (5): in fact, these high-risk individuals show an approximately 500-fold higher incidence (6,7), and ITACs arising in subjects with occupational dust exposure are more often diagnosed in men, and show a significant propensity to develop in the ethmoid sinuses (8-10). In addition, the ITACs connected with occupational exposure (hardwood dust, leather dust) are preceded by the intestinal metaplasia of the respiratory mucosal tract.

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ITAC represent a clinically aggressive entity that is typically associated with a tendency to recur locally, a low incidence of distant metastases, and an overall mortality of approximately 53% (10). Several studies reported how the most important prognosticators in patients with ITAC are the histopathological grading, and the pT classification (9-12). Surgery combined with postoperative radiotherapy (RT) and, in some cases, with adjuvant chemotherapy (CH) is the best treatment option (13).

Although the classic prognostic factors maintain a great utility in predicting the clinical behavior of ITAC (13,14), nevertheless it is not clear why some ITACs present a more aggressive behavior in comparison to others with the same type of features in terms of histological subtype and clinical stage (15-25). In the last ten years, information about the molecular mechanisms involved in the pathogenesis of head and neck squamous-cell carcinomas (HNSCC) are rapidly increasing (26-36); recently, some authors showed that epigenetic alterations have a critical role in HNSCC carcinogenesis (37-39). MicroRNAs (miRNAs), a type of small non-protein-coding RNA molecules that modulate the expression of target genes, operating as gene expression repressors at post-transcriptional level, and affecting the translation or causing the degradation of mRNA targets, are now considered as crucial components of the epigenome, orchestrating events ranging from organogenesis to immunity, and they are known to be important in influencing the origin of many diseases, including malignant tumors (40-54).

To achieve further knowledge about the phenotype and possible mechanisms of ethmoidal ITAC development, for the first time we investigated the role and the prognostic value of miR-let-7a, a head and neck squamous-cell carcinoma (HNSCC) related microRNA (53-55) in a well-characterized and homogeneous cohort of patients affected by primary ethmoidal ITAC, associated with occupational exposure and treated by primary surgery (56,57).

## Materials and methods

**Patients and specimen selection.** This retrospective cohort study analyzed consecutively the medical charts of all patients with primary ITAC, treated by surgery with curative intent at the Department of Otorhinolaryngology, Umberto I University General Hospital (Marche Polytechnic University, Ancona, Italy) between January 2000 and January 2010. From the medical records, the following data were collected: age at the diagnosis, gender, occupational history, site of the tumor, post-operative staging (pTNM classification), histological findings, disease-free survival (DFS), and overall survival (OS).

**Inclusion criteria:** complete clinical data, 5 follow-up years at least, uniformity of histological differentiation throughout the tumor sample, and the availability of normal formalin-fixed paraffin-embedded (FFPE) tissue samples.

**Exclusion criteria:** patients with previous or synchronous second malignancies, or patients who underwent previous radiation therapy or chemotherapy, or patients who died of postoperative complications. As additional, specific exclusion criteria were applied regarding the surgical approach for removing the tumors as previously described and suggested in the literature (13,58,59). Absolute contraindications for an endoscopic approach were erosion of nasal bones or floor of the

nasal cavity, extensive involvement of the nasal pathway (except the nasolacrimal duct), infiltration of the walls of the maxillary sinus (except the medial one), and invasion of the orbital content.

Length of survival was calculated from the date of surgery to the date of the latest clinical follow-up, or to the date of death by disease or other causes. Representative tissue blocks were collected from the archives of the section of Pathology Department of Marche Polytechnic University (Ancona, Italy). The paired samples from tumor tissues and adjacent normal tissues were obtained from patients with ITAC who had undergone surgical resection. The diagnosis and assessment of the histological grading (G, from G1 to G3) of tumor differentiation were made on 4-6  $\mu$ m-thick paraffin tissue sections stained with conventional hematoxylin and eosin according to WHO Classification of Head and Neck Tumors (56).

In all the cases, ITAC tumor diagnosis was confirmed, as described by Barnes (10).

The identification of the anatomical site of the tumor (T, from T1 to T4), nodal involvement (N0, N1, N2), and clinical-pathologic stage were determined according to AJCC/UICC TNM classification (7th edition) (57).

Written informed consent was obtained at the time of surgery from each patient included in the study, and samples were processed after approval of the Ethical Committee of the Marche Regional Hospital, Ancona, Italy, Rec. no. 501 of November 29, 2011.

**Patient cohort and workup.** The clinical data were collected prospectively from patients, then updated retrospectively after the follow-up review. All patients underwent complete clinical examination and were staged by multiplanar CT and by contrast-enhanced MRI (or contrast-enhanced CT whenever an MRI could not be obtained). After imaging evaluation, a biopsy with the patient under local anesthesia was performed. Treatment planning was discussed by the local multidisciplinary team.

**Surgery.** All patients were treated by surgery that was, depending on the position and the extension of the tumor or a craniofacial and cranioendoscopic resection or a transnasal endoscopic approach with or without transnasal craniectomy. In all cases we used surgical techniques already described in the literature (13,58,59).

Patients who had absolute contraindication to endoscopic approach as describe in the exclusion criteria performed traditional open surgical approach (13).

**Histological evaluation.** Tissue blocks were collected, and the histological slides were examined by a senior pathologist (C.R.) to confirm the diagnosis of ITAC and to assess the grade of histopathological differentiation of each tumor, according to WHO criteria [56], as follows: G1=well-differentiated, G2=moderately differentiated, and G3=poorly-differentiated.

Surgical and histological reports were analyzed, and all the lesions were retrospectively staged according to the TNM classification (57).

**Adjuvant therapy.** Although advanced stage, poor differentiation, and presence of positive surgical margins were the main considered factors, the indication for adjuvant RT and /or CHT was discussed for each patient by the multidisciplinary team,

Table I. Overview of the clinical and pathological characteristics of patients with primary ethmoidal intestinal-type sinonasal adenocarcinoma.

Patient	Age, years	Sex	TNM stage	Grade	CH	CHT	RT
1	70	M	T2N0M0	3	Yes	No	No
2	55	M	T3N0M0	2	Yes	No	Yes
3	67	M	T3N0M0	2	Yes	No	Yes
4	60	F	T1N0M0	1	Yes	No	No
5	62	M	T3 N0M0	3	Yes	No	Yes
6	54	M	T3N0M0	2	Yes	No	Yes
7	54	M	T3N0M0	3	Yes	No	Yes
8	74	M	T3N0M0	1	Yes	No	Yes
9	74	M	T2N0M0	2	Yes	No	Yes
10	58	M	T1N0M0	1	Yes	No	No
11	74	M	T2N0M0	3	Yes	No	Yes
12	70	M	T2N0M0	2	Yes	No	Yes
13	72	M	T3N0M0	1	Yes	No	Yes
14	68	M	T3N0M0	3	Yes	No	Yes
15	77	M	T3N0M0	2	Yes	No	Yes
16	67	M	T3N0M0	3	Yes	No	Yes
17	77	M	T2N0M0	2	Yes	No	No
18	65	M	T3N0M0	3	Yes	No	Yes
19	63	M	T2N0M0	1	Yes	No	Yes
20	61	F	T1N0M0	1	Yes	No	No
21	65	M	T2N0M0	2	Yes	No	No
22	61	M	T4aN0M0	3	Yes	No	Yes
23	76	M	T4bN0M0	3	Yes	No	Yes

CH, chemotherapy; CHT, adjuvant chemotherapy; F, female; M, male; RT, radiotherapy.

also considering age, comorbidities, previous treatment and, especially for low-stage ITAC, the availability of the patient for adequate follow-up.

**Follow-up.** All patients were followed according to our institutional protocols by endoscopic evaluation and MRI every 2 and 4 months, respectively, during the first year, both endoscopic evaluation and MRI every 6 months until the fifth year, and clinical evaluation and MRI yearly thereafter; this follow-up protocol was the same applied in literature on large sample of patients (59).

**miRNA detection by reverse transcription-quantitative PCR (RT-qPCR).** Expression levels of miR-let-7a were measured in 23 FFPE samples of ethmoidal ITAC and in the corresponding adjacent healthy normal tissues considered as control (CTR).

The expression of miR-let-7a was also evaluated according to: tumor grade of differentiation G (G1 or G2 vs. G3), tumor extension (T1 or T2 vs. T 3 or T4), tumor stage (I or II vs. III or IV), and presence or absence of recurrence.

Total RNA was extracted from FFPE samples using FFPE RNA/DNA purification Kit (Norgen, Canada). MiRNAs were quantified by RT-qPCR using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) according to the Manufacturer's protocol. Data were analyzed with the iCycler (Bio-Rad Laboratories, Segrate, Milan, Italy) with an

automatic setting for assigning the baseline. RT-qPCR data were standardized to RNU48.

The  $2^{-\Delta\Delta C_t}$  method was used for quantification (60). Relative miR expression obtained from RT-qPCR was calculated using Ct (cycle threshold), i.e., the fractional cycle number where a fluorescent signal reaches the detection threshold. Levels of miRs expressed with reference to RNU48 were turned into linear form using the formula  $2^{-DCT}$ ,  $DCT=Ct\ miR-X-Ct\ RNU$ , and reported as arbitrary units (a.u.). Ct values from RT-qPCR assays >35 were considered as not expressed. The intra- and inter-assay variability of miR measurements were <5% and <10%, respectively.

**Statistical analysis.** Results are expressed as mean  $\pm$  standard deviation (SD) or as median, quartile and confidence interval (CI). Comparisons between and among groups were performed using two-tailed Student's paired t-test (two groups) and analysis of variances (one-way ANOVA), followed by post-hoc Tukey analysis, respectively.  $P<0.05$  was considered statistically significant. All statistical analyses were performed using the SPSS statistical package (SPSS Inc. Chicago, IL).

## Results

**Patient data.** Overall, twenty-three patients met the inclusion criteria. Patient population consisted of twenty-one (91.3%)

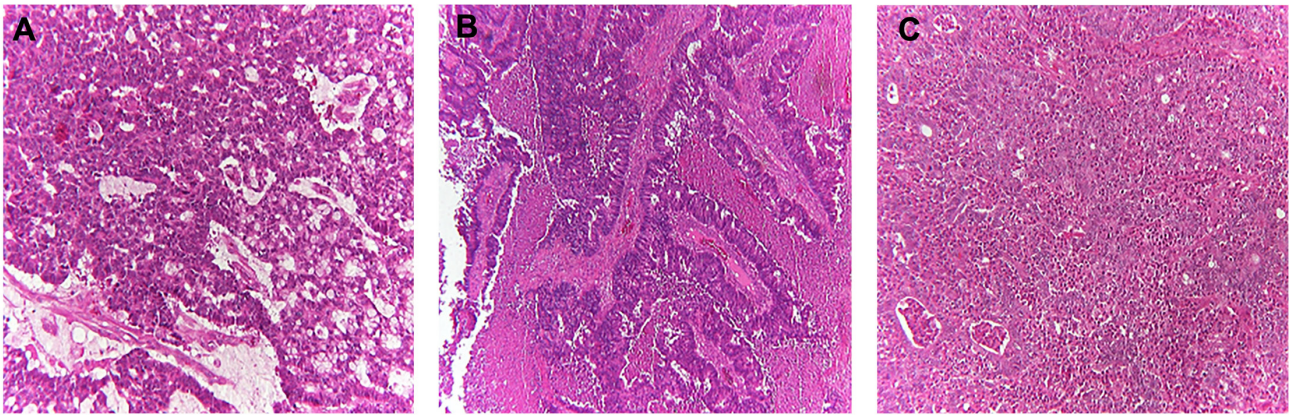


Figure 1. Histology of the tumor. (A) Well-differentiated (G1), (B) moderately differentiated (G2) and (C) poorly-differentiated (G3) intestinal-type sinonasal adenocarcinoma.

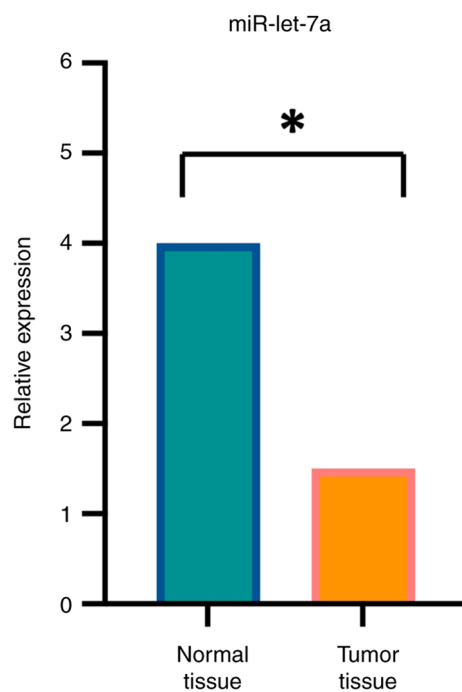


Figure 2. miR-let-7a expression in ethmoidal intestinal-type sinonasal adenocarcinoma tissues was significantly lower than that in adjacent normal tissues (mean expression level  $\pm$  SD, 1.452707 $\pm$ 1.4367189 vs. 4.094017 $\pm$ 2.7465375). \*P<0.05. miR, microRNA.

males and two (8.7%) females, with a mean age of 66.3 years (range 54-77 yr). All the patients had a known history of occupational exposure to hardwood dust and the ITAC was in the ethmoid region in all cases, as confirmed by endoscopic and imaging (enhanced TC and/or MRI) evaluation. No patients presented clinical and radiological (cN) positive lymph nodes at diagnosis. The main clinicopathological features of the patients included in the study are summarized in Table I. No patients underwent selective neck dissection (ND).

*Histological findings, post-operative staging (pTNM), and adjuvant therapy.* The patients following pTNM classification were distributed as follows: three (13%) in stage I, seven (30.4%) in stage II, eleven (47.8%) in stage III, and two (8.7%) in stage IV.

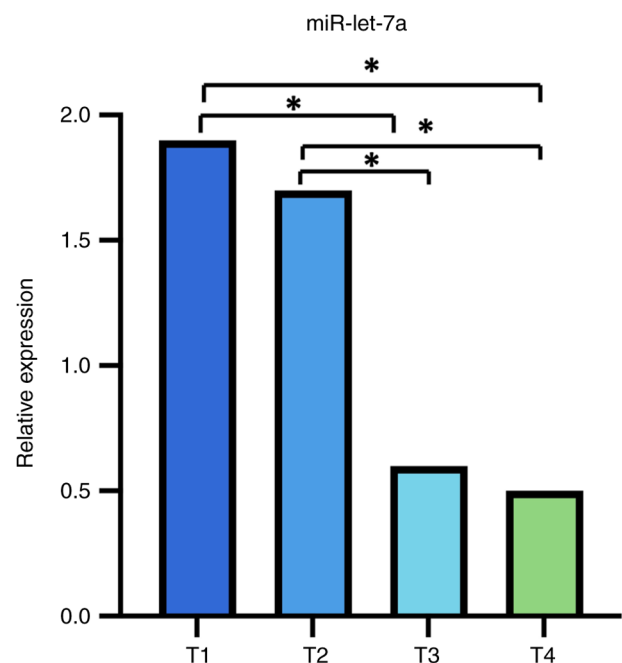


Figure 3. miR-let-7a varies with the T stage of the tumor, being downregulated in the more advanced stages (T3 and T4) compared with earlier stages (T1 and T2) [mean expression level  $\pm$  SD, 0.584179392 $\pm$ 0.581234561 (T3 and T4) vs. 1.926450065 $\pm$ 1.627465375 (T1 and T2)]. \*P<0.05. miR, microRNA.

Looking at the severity of the tumor (histological grade), the patients were distributed as follows: six patients affected by Grade 1 (well-differentiated) (Fig. 1A), eight by Grade 2 (moderately differentiated) (Fig. 1B), and nine suffering from Grade 3 tumor (poorly differentiated) (Fig. 1C).

The immunohistochemistry analysis on the tissue showed that cases classified as ITAC had variable cellular appearance, and consisted of a mixture of tall columnar cells, atypical stratified cylindrical cells like the cells seen in conventional colorectal adenocarcinoma, goblet cells, and large round to polygonal non-descriptive epithelial cells.

None of the patients had a tumor 'within' (R1) or 'close to' (<1 mm, Rclose) the surgical margins. Nineteen (83%) of the patients underwent post-operative conventional RT on the primary site; nobody was treated by adjuvant CH.

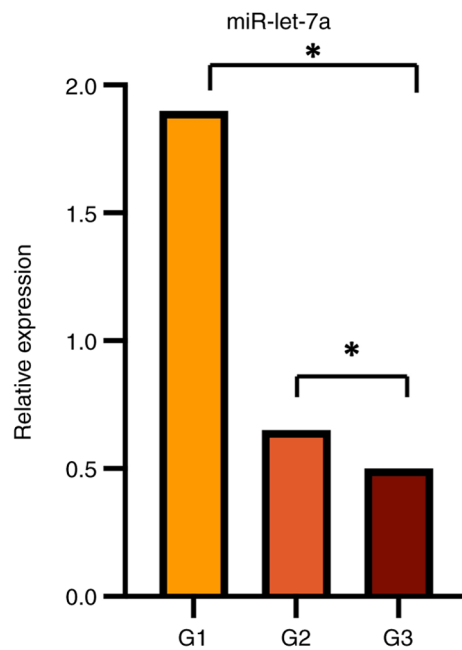


Figure 4. miR-let-7a expression in poorly differentiated ethmoidal ITAC tissues (G3) was significantly lower than that in well- and moderately differentiated ethmoidal ITAC tissues (G1 and G2) [mean expression level  $\pm$  SD, 0.583269371 $\pm$ 0.580274566 (G3) vs. 1.938231067 $\pm$ 1.857467372 (G1 and G2)]. \*P<0.05. ITAC, intestinal-type sinonasal adenocarcinoma; miR, microRNA.

**miR-let-7a expression.** miR-let-7a expression levels in ethmoidal ITAC tissues were significantly lower than in adjacent normal tissues (P<0.05) (Fig. 2). Moreover, miR-let-7a varies with the pT stage of the tumor, being lower-expressed in the more advanced stages (pT3-pT4) compared to earlier stages (pT1-pT2) (mean expression level  $\pm$  SD; 1.452707 $\pm$ 1.4367189 vs. 4.094017 $\pm$ 2.7465375; P<0.05) (Fig. 3). Moreover, there was a statistically significant relationship between miR-let-7a down-expression and G3 histological grading (P<0.05) (Fig. 4). No other significant findings were found between miR-let-7a expression and the other clinicopathological parameters, including DFS.

## Discussion

The results of our study showed, for the first time, that the overall expression levels of miR-let-7a were significantly down-regulated in tumor tissues as compared with the adjacent non-pathologic tissues (approximately three times lower in tumor samples than in normal tissue) (P<0.05). The down-regulation of miR-let-7a was associated with the pT stage, reaching the minimum levels of expression in pT3 and pT4 samples (P<0.05).

Furthermore, we found a down-regulation of miR-let-7a expression (P<0.05) in poorly-differentiated tumors (G3) compared with moderately- and well-differentiated tumors (G1-G2), indicating a potential role of miR-let-7a in the cell differentiation of ITACs. No other statistically significant variances were found looking at the up/down regulation of miR-let-7a related to other clinicopathological parameters, including DFS.

Despite their histological similarity to colorectal carcinomas, there is a scarce amount of data about the molecular

events that are involved in ITAC pathogenesis. A wide number of tumorigenesis pathways have been identified in colorectal adenocarcinomas, and these pathways are related to mutation and inactivation of various oncogenes, tumor suppressor genes, and DNA mismatch repair genes, including K-ras, APC, p53, MLH1, and MSH2 (16,17,61).

Assuming that the morphological similarities to colorectal adenocarcinomas might reflect equivalent genetic alterations, the presence of activating mutations of Ras oncogenes and TP53 mutations in ITAC were investigated in many studies. TP53 mutations were found in 18-44% of mostly occupational ITACs, whereas K-Ras mutations were observed in 10-15% of ITACs. The results of these studies suggested that mutations of K-Ras and other Ras genes are relatively uncommon in ITAC and, similarly, TP53 mutations in ITACs have not been widely demonstrated (18,19,20,62,63). Other authors found that K-Ras mutation and C-erb-2 expression might be associated with a more aggressive behavior and poorer outcome (21).

Licitra *et al* (64) described two genetic ITACs subgroups, characterized by differences in TP53 mutational status or protein functionality, that significantly influence the pathological response to primary CH and, ultimately, the prognosis.

Perez-Ordóñez and colleagues investigated the role of DNA mismatch repair (MMR) gene defects or disruptions of E-cadherin/ $\beta$ -catenin complex in ITAC by testing the immunohistochemical expression of the MMR gene products, E-cadherin and  $\beta$ -catenin, in a cohort of patients with sporadic ITACs, and they found that the nuclear expression of MLH1, MSH2, MSH3 and MSH6 were preserved in these tumors, suggesting that mutations or promoter methylation of MMR genes do not play a role in ITAC pathogenesis (65).

An interesting finding was performed by Kennedy and al., who found that sinonasal ITACs have a distinctive phenotype, with all the cases expressing CK20, CDX-2, villin, and most ITACs also expressing CK7. So the expression pattern of CK7, CK20, CDX-2, and villin positive may be used to distinguish these tumors from other non-ITACs of the sinonasal tract (66).

Furthermore, published data showed how miRNAs may have an important role in the carcinogenesis process because more than 50% of miRNAs were found in cancer-associated genomic regions or fragile sites (67,68). Some miRNAs seem to promote the cancer onset, while others inhibit the cell proliferation and survival. Basically, both miRNA classes are showing important connection with cancer development, being able to act like novel oncogenes or tumor suppressors, respectively (69,70). Measuring miRNAs expression as a biomarker may bring an important advantage in cancer diagnosis, prognosis and therapy. Also, concerning head and neck tumors there are many studies that have reported significant associations among miRNA profiles and patient survival (23,27-30,33-36).

At present time, due to the rarity of this type of cancer, there is still a lack of literature evaluating the expression of miRNAs in ITAC of the paranasal sinuses (25). Our research team previously investigated the status of MiR-126 and we found that it was reduced in ITACs compared with benign tumors, suggesting the potential role of this miRNA acting as a circulating biomarker for the detection of malignant transformation (25).

On the basis of these findings, to explore other pathways involved in the molecular pathogenesis of ITACs, in the current

study we tried to investigate the expression of miR-let-7a, which is an HNSCC related microRNA (53,55). To analyze the prognostic role of this miRNA, its expression levels were then retrospectively correlated with clinicopathological characteristics of the tumor itself and the patient's outcome to evaluate its independent prognostic relevance.

The let-7 gene family consists of 11 very closely related genes and miR-let-7a is currently the best characterized member. Recently the miR-let-7a expression was found to be reduced in different tumor model tissues, compared to adjacent healthy tissue and, therefore, miR-let-7a could probably act as a tumor suppressor miRNA (55,71-75).

miR-let-7a was found poorly expressed in many types of malignancies such as lung, colon, thyroid, and renal cancer (70-74). Furthermore, data from recent published works suggested that the down-regulation of let-7 miRNA family gene, targeting RAS oncogene, may be related with poor survival and relapse in surgically treated non-small cell lung cancer (53,71-72). The expression of miR-let-7a is also reduced in gastric cancer and relates to tumor cells differentiation degree. A xenograft model of mice showed that miR-let-7a acts as suppressor for the growth of gastric cancer *in vivo* and *in vitro* (76).

Long *et al.* (55) evaluated the expression of miR-let-7a in a sample of 48 patients surgically treated for laryngeal primary carcinoma, and compared miR-let-7a levels among tumor tissue and adjacent healthy tissue. Results showed that in 37 out of 48 patients a statistically significant reduction in miR-let-7a expression level was present in all tumors with different clinical stages, compared with normal larynx tissues.

miR-let-7a could be a tumor suppressor in laryngeal cancer by inhibiting cell growth and inducing cell apoptosis. This action would be possible by down-regulating the protein expression of oncogenes such as RAS and c-MYC (target genes). Ras proteins are membrane associated GTPase, signaling proteins that regulate cellular growth and differentiation, while MYC is an evolutionarily conserved nuclear protein also involved in the control of cell proliferation and differentiation (55). An inverse correlation was observed among RAS/c-MYC protein and miR-let-7a expression in laryngeal tumor, suggesting that increased RAS and c-MYC protein expressions may be caused by the loss of miR-let-7a expression (55).

A down-regulation of tumor miR-let-7a suppressor family exists also in tumors of the Ewing's sarcoma family (ESFT). The mechanism by which miR-let-7a expression modulates the growth of ESFT has been shown to be mediated by its target gene HMGA2. An overexpression of miR-let-7a and the consequent repression of HMGA2 inhibit the tumorigenicity of ESFT cells (77).

The present study shows some limitations. In this work we did not consider the prognostic value of the expression of miR-let-7a, which will be the object of a second retrospective analysis. Second, although we tried to evaluate a homogeneous cohort of patients in terms of pTNM stage and treatment, our data were achieved from a retrospective cohort study and the patients cohort remains heterogeneous in some crucial clinical aspects (different pTNM classification, non-uniform surgical approach, mode, and effectiveness of complementary protocol treatment, follow-up). Moreover, the study did not conduct a sensitivity analysis due to the small sample size (n=43).

Finally, understanding the connections between miRNAs deregulated in cancer and cellular signaling pathways involved in cancer was hindered by our limited knowledge of miRNA target recognition.

Despite these limitations, we presented a pilot study through highly standardized retrospective analysis of a single head and neck cancer institution.

In conclusion, this study provides the first evidence that a down-regulation of miR-let-7a in ethmoidal ITAC is associated with advanced stage (pT3 and pT4) disease, and with poorly differentiated tumors (G3). Our data suggest that the specific mutation of this gene, in combination with additional genetic events, could play a role in ITAC pathogenesis.

The analysis performed on a small sample of patients will necessarily be extended to a larger cohorts, and our single-institution results would require validation through a broader prospective and multicenter analysis.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

FMG and MR conceived the study. FMG wrote the first draft. ADS and PDL analyzed the data, defined the conclusions, revised the manuscript and helped write the manuscript. ADS and MS were involved in image curation by selecting the images and drawing the graphs. AC, AP, GI, AS and MS collected clinical data, and were involved in the analyses of data and definition of the results. CR, MT, MS and FO were involved in collection and analysis of histologic findings. FMG, FO and MR analyzed the data and participated in the definition of the conclusions. FMG and MR confirm the authenticity of all the raw data. All authors were involved in critical review of the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The study was approved by the Ethical Committee of the Marche Regional Hospital, Ancona, Italy, Rec. no. 501 of November 29, 2011. All patients provided written informed consent to participate in the study.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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