POLITECHNICAL UNIVERSITY OF THE MARCHE

FACULTY OF MEDICINE AND SURGERY

Ph.D in Biomedical Sciences

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XXXII cycle

miRNoma ANALYSIS IN SINONASAL INTESTINAL-TYPE ADENOCARCINOMA: IDENTIFICATION OF microRNAs PROFILE AND ITS CLINICAL AND PROGNOSTIC SIGNIFICANCE

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Academic year 2018/2019

Sommario

1. ABSTRACT

Background. There are few informations about the molecular events involved in the pathogenesis of sinonasal intestinal-type adenocarcinomas (ITACs). Moreover, although some of the classic prognostic factors (advanced pT classification, high-grade, and positive surgical margins) continue to be very useful in predicting the clinical behavior of ITACs, it is still unclear why some patients have a better prognosis than others with the same type of lesion in terms of histological characteristics and clinical stage. The purpose of this study was to identify in a well-characterized and homogeneous cohort of patients with ITAC, through a miRNoma analysis, a specific microRNAs (miRNAs) profile, ~~a~~nd to assess their clinical and prognostic significance.

Methods. Patients with ITAC who underwent endoscopic surgery ± RT at two centers were retrospectively reviewed. miRNoma analysis has been performed using Next Generation Sequencing (NGS) technology in tumor and adjacent healthy tissue; among these, based on the expression and the distribution in characteristic clusters of the pathology, 4-miRNAs (miR-34c, miR-192, miR-205, miR-449a) were selected, which were subsequently validated on the population of ITAC patients. In order to assess the clinical value, the expression of the 4-miRNAs has been associated with the outcome of the patients. In addition, overall survival (OS) and disease-free survival (DFS) were analyzed using the Kaplan-Meier curve and miRNA groups (below and above the median) were compared using the log-rank test. Cox multivariate regression analysis was used to evaluate independent survival prediction factors. A value of $p < 0.05$ was considered statistically significant. All statistical analyzes were performed using SPSS for Windows version 19.0 (SPSS Inc, Chicago, IL).

Results. Fortythree patients were included. The results of our study showed that both miR-205 and miR-449a are independent biomarkers for recurrence in patients with sinonasal ITAC. In particular, the over-

expression of the two miRNAs was associated with a higher risk of recurrence and therefore with a worse prognosis. The multivariate analysis confirms their independent predictive value.

Conclusion. Our results highlights that an over-expression of miR-205 and miR-449a in sinonasal ITAC is associated with an increased risk of recurrence (reduced DFS) and reduced OS, suggesting that mutation of these miRNAs in combination with additional genetic events, could play a significant role in the pathogenesis of ITAC.

2. INTRODUCTION

2.1. SINONASAL INTESTINAL-TYPE ADENOCARCINOMA

The sinonasal tract is the location for a wide variety of benign and malignant tumors; per cubic centimeter, it gives rise to a greater diversity of neoplasms than any other site in the human body. The diversity is partly due to the anatomic complexity and highly varied tissues in this compact area. Sinonasal cancers (SNCs) are rare and aggressive neoplasms, accounting for 5% of head and neck malignancies and less than 1% of all tumors [1] with an incidence of 0.1–1.4 new cases/year/100,000 inhabitants.[2,3] Adenocarcinomas account for 10–20% of all primary malignant neoplasms of the sinonasal tract.[4] The World Health Organization histological classification divides sinonasal adenocarcinoma in 2 histologic forms according to Barnes classification: intestinal-type adenocarcinoma (ITAC) and nonintestinal-type adenocarcinoma (non- ITAC).[5] ITAC is one of the most frequent epithelial, nonsquamous cell tumor of the sinonasal tract, around 8–25% of all SNCs.[6] The preferential site of origin is commonly identified in the nasoethmoidal complex, but according to the observations of Jankowski et al[7] the tumor specifically originates from the olfactory cleft. Another typical feature of the sinonasal ITAC is the quite constant association with occupational exposure to hardwood and leather dusts. [8] Exposure to wood and leather dusts increases the risk of adenocarcinoma by 500- fold. [9,10] ITACs are aggressive tumors characterized by frequent local recurrences, low incidence of distant metastases, and an overall mortality of approximately 53%. [11]

In the last decades, a number of refinements in the treatment of ITAC have emerged, including transnasal endoscopic surgery (TES), heavy ion radiation therapy, and chemotherapy as a valuable tool especially in the neoadjuvant setting. [12] Surgery represents the mainstay of treatment for ITAC, usually followed by adjuvant intensity-modulated radiotherapy (IMRT). [12]

Although some of the classic prognostic factors (advanced pT classification, high-grade, and positive surgical margins) continue to be of great utility in predicting the clinical behavior of ITACs it is still not clear why some patients with ITACs have a better prognosis than others with the same type of lesion in terms of histological characteristics and clinical stage.

Conventional clinic pathological parameters do not accurately reflect the clinical outcome of patients with this tumor. Therefore, there is an urgent need of new prognostic factors able to predict the outcome in patients affected by ITACs. The molecular events involved in the pathogenesis of ITAC [4,6,13-19] are still unknown despite the increasing informations about the molecular mechanisms involved in the pathogenesis of head and neck squamous cell carcinomas (HNSCC). [20-30] Recently, regulatory epigenetic mechanisms have intensively been investigated in many fields and a number of studies showed that epigenetic alterations play a critical role in HNSCC carcinogenesis. [31-34]

2.2. EPIDEMIOLOGY

ITAC is a rare tumor with heterogeneous worldwide incidence and sex distribution. In Europe, the age-standardized incidence per 100 000 person-years accounts for 0.26 cases in men and 0.04 in women, [35] whereas in the United States, these values are 0.058 in men and 0.034 in women. [36] Accordingly, ITAC is one of the most frequent SNCs in Europe, whereas it is much rarer in the United States. Similarly, the prevalence in men is remarkably variable, with a men-to-women ratio ranging from 1.07 to 1.39 in the United States up to 6.68 in Italy (Europe). [35-37] Median age at diagnosis is 64–68 years, with a peak of incidence at 60–69 years in the United States and 75–84 years in Italy (Europe). [35,36] Worldwide, the prevalence of ITAC overtime is stable; however, a rising incidence has been observed in some countries (e.g., Denmark). [35,38] The disease is associated with a moderately increased risk of occurrence (odds-ratio: 1.47) and worse prognosis in blacks compared with whites. [36,37] The large majority of ITAC (88%) can be attributed to occupational exposure. [35]

The most important risk factor is exposure to wood dust [relative risk (RR): 29.4], followed by products in the textile industry (RR: 3.5). Cumulative time of exposure and latency are widely variable, as reported by Nicolai's group: 2–66 and 11–72 years, respectively. [39] Conversely, exposure to formaldehyde, nickel/chromium compounds, or asbestos, which has been identified as risk factor for sinonasal cancer, was not confirmed to play a role in the development of adenocarcinoma. [40,41] Alcohol, tobacco, and human papillomavirus do not seem to be risk factors for the development of ITAC, in contrast to squamous cell carcinoma of the head and neck. [42]

2.3. HISTOPATHOLOGY

Two main pathological classifications of ITAC are currently used. [43] According to Barnes, [11] five categories can be identified: papillary (18%), colonic (40%), solid (20%), mucinous (14%), and mixed (8%) (Figure 1).

Papillary-type ITACs are well-differentiated, presenting a simple papillary pattern with a thin villous stromal support covered by a layer of tall columnar monomorphic cells with low number of mitotic figures.

Colonic-type ITACs consist of moderately differentiated cells with a mixed tubulo-papillary pattern and an increased mitotic rate.

Solid-type ITACs are poorly differentiated and can be characterized by a solid growth pattern with hardly any tubules and nuclei that are highly hyperchromatic.

Mucinous-type ITACs generally show a diffuse pattern formed by alveolar goblet or signet ring cells and mucin lakes.

Finally, some tumors present more than one type and are termed mixed or transitional type.

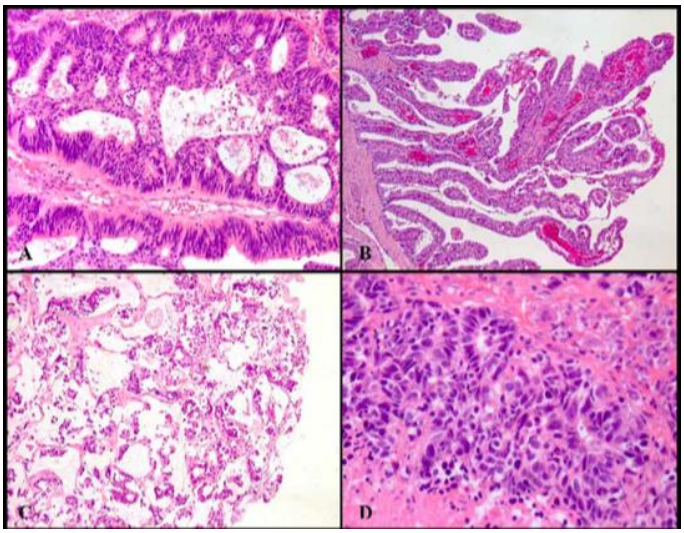


FIGURE 1. Pathological subtypes of intestinal-type adenocarcinoma (ITAC): (A) colonic; (B) papillary; (C) mucinous; and (D) solid. The mixed subtype contains features of the 4 ITAC subtypes that are shown in this figure. [42]

Kleinsasser and Schroeder [45] subdivided ITAC into four variants: papillary-tubular cylinder cell, (graded from I to III), alveolar goblet, signet-ring cell, and transitional.

Because the Schneiderian mucosa is of ectodermal origin and normally shows no evidence of intestinal differentiation, ITAC probably develops through intestinal metaplasia induced by hardwood and leather dust. It is hypothesized that normal respiratory mucosa first undergoes cuboidal metaplasia, followed by intestinal metaplasia, and then possibly through dysplasia develops into ITAC. [45,46]

Histologic diagnosis is relatively simple, and the differential diagnosis includes a secondary localization of colorectal adenocarcinoma and low-grade nonintestinal sinonasal adenocarcinoma. [47] ITAC and adenocarcinomas of the intestines show a similar immunophenotypical spectrum: at immunohistochemistry, both stain positive for CK20, CDX-2, villin, and MUC2. [47] However, chromogranin A and synaptophysin expression, which can be found in neuroendocrine cells within the tumor, is typical of primary ITAC. [47,48] Positive staining for CK7 supports the diagnosis of sinonasal ITAC over that of intestinal cancer metastasis,

although with low sensitivity. [49] However, only the integration of pathological and clinical information can differentiate ITAC from metastatic colorectal adenocarcinoma, which accounts for 6.1% of secondary sinonasal tumors. [47,50]

Differential diagnosis of ITAC from sinonasal nonintentional adenocarcinomas is guided by immunohistochemistry, as CK20, CDX-2, and villin only stain positive in ITACs. [47]

2.4. CARCINOGENESIS

Although the carcinogenesis of ITAC is still far from being fully elucidated, some advances have been made in the last years.

Genetics

Foci of intestinal metaplasia have been described in close vicinity to ITAC (27.5%), with a high rate of dysplasia (75%). [51] TP53 over-expression and mutation are related to wood exposure in both normal mucosa and ITAC. [42,52] This evidence suggests that carcinogen(s) contained in wood dust serves as initiation factor(s) for cells of the sinonasal mucosal glands, with carcinogenesis occurring in a multistep fashion similar to other cancer models. The transition from the most differentiated subtype (papillary) to the least differentiated ITAC (mucinous) seems to be related to the total amount of mutations. [53] In recent years, the mutational landscape of ITAC has been better characterized. The spectrum of copy number alterations is variable (gains at 1q, 3q, 5p, 7, 8q, 11q, 12p, 13q, 20q, and 22 and loss at 3p, 4, 5q, 6q, 8p, 9p, 10q, 16q, 17p, 18q, 19p, and 21), [42] including chromosomal regions usually involved in most of the epithelial malignancies (gains at 7p, 8q, and 20q and losses at 8p and 17p) and others that are typical of ITAC (gains at 3q, 5p, 12p, and 18p and losses at 4q, 19p, and 22q). [54] EGFR copy number gain is frequent (45%; with protein overexpression in 13–

21% of cases), especially in the colonic subtype. [55] EGFR protein overexpression is more common in woodworkers (42.8%) than in leatherworkers (9.5%) and patients without professional exposure (0%). [56] Mutations in EGFR (0– 5.5%), K-RAS (5.5–12%), and BRAF (0–5.9%) are rarely found, are mutually exclusive, and are not associated with EGFR overexpression. [57-59]

This feature differentiates ITAC from colorectal and lung adenocarcinomas, which are commonly associated with EGFR (only lung), K-RAS, and BRAF mutations. [54] The mutation in codon 12 or 13 of K-RAS is thought to be specifically related to wood dust cause. [42] H-RAS is mutated in 16% of cases and associated with worse prognosis. [17,42]

TP53 mutations and p53 protein over-expression is found in 18–57% of cases and is related to the subtype of ITAC. [20,42] Nonfunctional p53 protein is frequent and associated with reduced chemosensitivity and worse prognosis compared with patients with a functional protein. [60,61] MET is overproduced in 64% of ITACs. [62]

Epigenetic Mechanisms (MicroRNAs)

Regulatory epigenetic mechanisms have been intensively investigated in many fields, and a number of studies have shown that epigenetic alterations play a critical role in HNSCC carcinogenesis. [32,33] microRNAs (miRNAs), a class of small non-protein-coding RNA molecules consisting of 19–25 nucleotides that control expression of target genes, are now recognized as crucial components of the epigenome, orchestrating events ranging from organogenesis to immunity, and they are known to be critical in the development of many diseases, including cancer. [34,63] They regulate gene expression at the post-trascriptional level by binding to partially complementary sites in the 3'-UTR (untranslated regions) of their messenger RNA targets. miRNAs function as gene expression repressors at post-transcriptional level, affecting the translation or causing the degradation of the mRNA targets. [64] miRNAs regulate the expression of oncogenes and tumor-suppressor genes to affect cell proliferation, apoptosis, migration, invasion, and differentiation. Many miRNAs have been shown to be associated with specific types or stages of tumors, or with patient survival,

and may be developed either as diagnostic or as prognostic evaluation markers. [65] Each miRNA can regulate up to 200 mRNAs and each mRNA can have multiple recognition sites that are targeted by different miRNAs. An accumulation of evidence suggests that miRNAs inhibit transcription by degrading mRNAs or blocking their translation. miRNAs are also involved in several diseases, including cancer, coronary, neurodegenerative, and autoimmune diseases. [66] Several studies have focused on searching for potential therapeutic agents by identifying differences in miRNA expression between cancer tissues and paired neighboring noncancerous tissues. Certain miRNAs have been reported to show an aberrant expression in tumors, which suggests that they may have significant impacts on the diagnosis, clinical treatment, and prognostic evaluation of cancers. [67]

In the ENT field, previous studies have demonstrated the expression of miR-21, miR-34c and let-7a in laryngeal neoplastic tissue. [25] miR-34c-5p belongs to the miR-34 family, consisting of three conserved members, miR-34a, miR-34b, and miR-34c, with important tumor suppressor functions linked to the p53 pathway, and previously identified as suppressors in a number of human cancers. [31] Let-7a may act as a tumor suppressor, which is poorly expressed in lung and colon cancer [68,69] and down-regulated of the let-7 miRNA expression has been reported to have a prognostic impact on the survival of surgically treated lung cancer patients. [68] miR-21 is one of the best-studied miRNA with a clear role in carcinogenesis and has several confirmed targets. [70,71] miR-21 has been suggested to function as an oncogene because it is over-expressed in many types of tumors compared with normal tissues, [72,73] including head and neck cancers. [74]

2.5. DIAGNOSIS AND STAGING

When a malignant lesion of the sinonasal tract is suspected based on history and endoscopic findings, diagnostic work-up should include morphological imaging followed by biopsy. Contrast-enhanced magnetic resonance imaging (MRI) is the gold standard. It affords precise soft tissue characterization with high-contrast resolution, which discriminates neoplastic tissue from nasal secretions and inflammatory changes, and defines any possible critical extension (dural/transdural involvement, perineural spread, intraorbital extension, etc.). Computed tomography (CT) provides additional information on tissue density (i.e., calcifications or bony structures), but is frequently unnecessary in the hands of an expert radiologist. On MRI, the signal intensity of adenocarcinomas varies according to their mucin content, cellularity, and presence of hemorrhagic-necrotic areas. Mucin-producing adenocarcinomas usually show spontaneous hyperintensity on T2-weighted images and gradual enhancement on contrast-enhanced T1-weighted sequences, whereas adenocarcinomas without mucin production show isointensity to hypointensity on T2-weighted images. However, the imaging features of adenocarcinomas are often indistinguishable from those of other sinonasal cancers. [75] Sinonasal ITAC has a low tendency to lymphatic dissemination: nodal metastases are rare, accounting for 2.4 – 8.0% at presentation and 1.8 – 2.4% during follow-up, being distinctive of an aggressive behavior of the tumor. [39,76,77]

Cervical metastases mostly occur at level II and must be confirmed with ultrasound with or without fine-needle aspiration cytology. Retrolateropharyngeal nodal metastases are likely underestimated, but can be easily detected with MRI or CT. Distant metastases are identified in 1.7% of cases at presentation and 12.8% of cases during follow-up. [78] At positron emission tomography (PET), ITAC shows a high-standard uptake value, which is second only to sinonasal undifferentiated carcinoma. [79] Consequently, primary regional and distant staging as well as follow-up may benefit from PET, especially in advanced-stage and high-grade ITACs. [39] Total body CT associated with bone scintigraphy can be a reasonable alternative.

2.6. TREATMENT

Surgery represents the mainstay of treatment for ITAC, usually followed by adjuvant intensity-modulated radiotherapy (IMRT).

Surgery

Surgical planning is guided by both preoperative imaging and intraoperative findings. Whatever the approach, the aim is to perform radical resection using frozen sections to map the surgical margins. In the last 15 years, TES has emerged as the surgical approach of choice for most sinonasal cancer located in the nasoethmoidal complex. [39,80,81] External or combined approaches (craniofacial and cranioendoscopic resection, respectively) still have a role in selected cases (i.e., massive brain infiltration, supraorbital dural extension, nasal bones/facial invasion, massive involvement of the lacrimal system, and/or orbital content). [82] Endoscopic resection with transnasal craniectomy is indicated when the tumor is in contact or crosses the anterior skull base, to provide an adequate margin of resection. After anterior skull base resection is performed, a watertight reconstruction of the dura is required to avoid cerebrospinal fluid leak. Although the use of pedicled flaps has dramatically decreased this occurrence, [83] they are rarely available due to either direct tumor infiltration or the need to sacrifice the vascular pedicle. On the other hand, three-layer reconstruction with iliotibial tract grafts has been validated as a reliable and safe technique for anterior skull base defects. [57,58] Morbidity of endoscopic surgery for sinonasal malignancies is limited, with excellent outcomes in terms of quality of life, hospitalization time, morbidity, and mortality compared with traditional external approaches. [84,85] The concept of 'field of cancerization' as the result of wood dust (or other factors) inhalation led to consider bilateral resection of the ethmoidal box a paradigm in surgery of ITAC, regardless of the extent of primary tumor. [86] Against this philosophy, some authors reported good oncological outcomes even with unilateral resection applied to strictly lateralized lesions. [87] However, as data in the literature are not robust enough to draw definitive conclusions, more stringent post-treatment surveillance

is mandatory when a limited resection is performed. In view of the low rate of cervical lymph node metastases at presentation and during follow-up, elective neck treatment is not indicated. [78,88]

Radiotherapy

Photon radiation therapy is usually administrated as adjuvant treatment after surgery in high-grade and/or advanced-stage tumors. [39] Recent reports seem to confirm that early-stage (T1–2), low-grade (papillary/colonic) ITACs can be adequately treated with radical surgery alone. [89,90] Given the proximity to highly radiosensitive anatomical structures (i.e., orbital content, optic nerves, brain), the use of IMRT is strongly recommended over conventional radiation techniques, with the intent to minimize treatment toxicity. [91] Recently, the role of heavy ion therapy, such as proton beam radiation therapy, is also emerging: adjuvant proton beam radiation for sinonasal cancers was reported to provide a local control superior to conventional radiotherapy or IMRT. [92,93]

This is likely due to the possibility to better sharp the dose gradient in view of the physical features of heavy particles, thus optimizing the balance between the need for high dose on the tumor bed and low dose on neighboring noble structures. Like-wise, carbonion radiation therapy is a promising strategy for sinonasal adenocarcinoma. [94] In case of non resectable tumors, radiotherapy with photons and/or neutrons, possibly associated with chemotherapy, provides a valuable 5-year overall survival (OS); however, specific data on ITAC are lacking. [95]

Chemotherapy

The only data available on chemotherapy for sinonasal ITAC are based on the Italian experience at the 'Istituto Nazionale dei Tumori' in Milan. [60,61] Neoadjuvant chemotherapy with cisplatin, 5-fluorouracil, and leucovorin (PFL) resulted in a pathological complete response (CR) in up to 40% of cases. The probability to obtain a CR to chemotherapy was significantly higher in ITACs with functional p53 (58% of cases), including

both the wild-type and the mutated protein with function preservation or gain. Based on these data, neoadjuvant chemotherapy with PFL scheme is indicated only for ITACs bearing functional p53 protein. [96] Apart from this isolated experience, the role of chemotherapy in the neoadjuvant or adjuvant setting in the management of ITAC has not been systematically investigated.

Biotherapy

The progresses in biological and genetic profiling of ITAC are paving the way toward new target therapies. Although no clinical data are available on biotherapy for ITAC, the biological profile resulting from several preclinical studies and the availability of an immortalized tumor cell line encourages the employment of target therapies in the management of this tumor. [42,97] By virtue of a low incidence of EGFR, K-RAS, and BRAF mutations and high rate of EGFR copy number gain, the mutational profile of ITAC seems particularly fitting to anti-EGFR drugs. [58,59]

Likewise, the high rate of MET mutation (64%) makes MET inhibitors another interesting option. [42,62] The subgroup of patients with H-RAS mutation (16%) may benefit from the administration of MAPK/ERK pathway inhibitors, alone or combined with inhibitors of the cyclin-dependent kinase-4/6. [42,98] Specific studies are warranted to explore the real benefit of these drugs in a clinical setting.

2.7. PROGNOSTIC FACTORS AND ONCOLOGIC OUTCOMES

Oncologic outcomes are influenced by several factors, such as patient characteristics or biological features of the tumor. Age higher than 75 years, male sex, and black race have been identified as adverse prognostic factors. [99] From a histopathologic perspective, mucinous and solid subtypes are associated with poor prognosis. [78,80,99] High proliferation index and several other biological markers are significantly associated with worse prognosis. [100] Surgery with free margins and overall adequacy of primary management are of

utmost importance, as positive margins, local failure, and inadequate previous treatments are independently associated with poor prognosis. [39,62,80,101,102] In the largest published series, 5-year overall, disease-specific, and disease-free survivals are 53% – 83%, 82%–83%, and 62%–74%, respectively. [39,79,80,103,104] The recurrence rate after definitive treatment ranges from 17.6% to 49.6%, with local, regional, and distant relapse accounting for 16.0% – 38.2%, 1.8% – 2.4%, and 6.5% – 12.8%, respectively. [39,77,78,80,104] It is worth mentioning that recurrent ITAC has a remarkably aggressive behavior, which is similar to that of high-grade histologies (e.g., mucosal melanomas, sinonasal undifferentiated carcinomas, sinonasal neuroendocrine carcinomas, sarcomas). [105] K-Ras o H-Ras mutations, such as c-erbB-2 expression, are associated with more aggressive tumor behavior. [19,106]

Although some of the classic prognostic factors (advanced pT classification, high-grade, and positive surgical margins) continue to be of great use in predicting the clinical behavior of sinonasal ITAC, it is still unclear why some patients with ITAC have a better prognosis than others with the same type of lesion in terms of histological characteristics and clinical stage. The pathological parameters of the conventional clinic do not accurately reflect the clinical outcome of patients with this tumor.

Therefore, there is an urgent need for new prognostic factors that can predict the outcome in ITAC patients.

2.8. FOLLOW-UP

Postoperative surveillance should mainly focus on the primary site, by including endoscopic examination and MRI (or contrast-enhanced CT) at 3-month and 6-month intervals for the first 24 months, respectively; beyond this period, both outpatient and imaging evaluations can be performed every 6 months until year 5 and subsequently once a year, [107–109] in view of the possibility for ITAC to develop late local recurrences. Similarly to primary staging, PET/CT imaging can be considered for high-grade/high-stage ITACs also during follow-up.

2.9. AIM OF THE STUDY

To gain further insight into the phenotype and possible mechanisms of ITACs, miRNoma analysis has been performed, and specific miRNAs involved in the pathology have been identified. The selected miRNAs were then evaluated in a well-characterized and homogeneous cohort of patients with ethmoidal ITACs, treated with transnasal endoscopic surgery ± radiotherapy (RT). Subsequently, the miRNA expression has been associated with clinic outcome of patients and prognostic value evaluated.

3. MATERIALS AND METHODS

3.1. STUDY POPULATION

Patient and specimen selection

In this retrospective multicenter cohort study, the medical charts of all patients with primary ITACs of the ethmoid, undergoing curative treatment with endoscopic resection ± RT at the Otorhinolaryngology Department of the University Hospital - Ospedali Riuniti of Ancona and the ENT Department of Metropolitan Area, Bellaria Hospital, Bologna in the period 2011-2017, were retrieved and reviewed from a dedicated database; the following clinical data were collected: age at diagnosis, sex, occupational history, previous treatment, preoperative workup, histologic findings, surgery, adjuvant treatment, relapse of the disease, re-treatment, and follow-up [disease-free survival (DFS) and overall survival (OS)].

Inclusion criteria were: complete clinical data, a minimum of 3 follow-up years and the availability of normal formalin-fixed paraffin-embedded (FFPE) tissue samples.

The exclusion criteria included: patients with previous or synchronous second malignancies and/or with previous treatments radio/chemotherapy; patients in precarious clinical conditions for important concomitant pathologies that had conditioned the implementation of the established therapeutic protocol.

Representative tissue blocks of both centers, have been analyzed at the Section of Pathological Anatomy and Histopathology, University Hospital - Ospedali Riuniti of Ancona, Ancona, Italy.

The paired samples from tumor tissues and adjacent normal tissues were obtained from patients with ITACs underwent surgical resection. The diagnosis and assessment of the histological findings, according to WHO (2005) classification of head and neck tumors, [110] were made on 4-6µm-thick paraffin tissue sections stained with conventional hematoxylin and eosin.

In all cases the tumors diagnosis of ITAC was confirmed as described by Barnes. [11]

Tumor extension (T), nodal involvement (N) and clinic-pathologic stage were determined according to AJCC/UICC TNM classification (7th edition 2010). [111]

Ethics approval and consent to participate

All subjects filled a questionnaire including their informed consent. The study was carried out according to the Helsinki Declaration, and the samples were processed under approval of the written consent statement by the Ethical Committee of the University Hospital of Marche (Ospedali Riuniti di Ancona), Italy, Protocol Number 211226, 23/06/2011. The cell lines used in the study do not require ethic approval.

Patients cohort and workup

Overall 43 patients met the inclusion criteria. All patients had undergone complete clinical examination, and were staged by multiplanar CT and contrast enhanced MRI (or contrast-enhanced CT whenever an MRI could not be obtained), chest X-ray, and PET/CT in advanced-stage lesions. After imaging evaluation, a biopsy with the patient under local anesthesia was obtained. Treatment planning was discussed by the local multidisciplinary team at the 2 centers, based on a common management strategy.

Surgery

All patients were treated by surgery [endoscopic resection without or with transnasal craniectomy (ERTC) or cranioendoscopic resection] based on the local extent of disease.

Surgical techniques are described in detail elsewhere. [112-114]

Lesions without any critical relationship with the orbit and anterior skull base were considered amenable to endoscopic resection. Contact with or limited infiltration of the anterior skull base, focal infiltration of the dura, with or without limited intradural extension, were considered indications for ERTC, which included the resection of the anterior skull base between the 2 laminae papyracea and from the posterior wall of the

frontal sinus back to the planum sphenoidale, the overlying dura, and olfactory bulbs and tracts. Lesions with anterior or lateral involvement of the frontal sinus, infiltration of the dura far over the orbital roof, or extensive infiltration of the brain were managed by combining the endoscopic approach with subfrontal craniotomy (cranioendoscopic resection).

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, were considerate absolute contraindications for endoscopic approach. [39]

Treatment of the neck was performed only in patients who presented clinical and radiological positive nodes at diagnosis.

During surgery, a biopsy sample of tumor tissue and an adjacent sample of healthy tissue were taken from each patient. The biopsy sample was immersed in physiological solution, immediately frozen at -20°C, and subsequently stored at -80 ° C.

Histological Evaluation

Tissue blocks were retrieved and histologic slides were examined by a senior pathologist (C.R.) to confirm the diagnosis of ITAC and assess the grade of histopathological differentiation of tumor, according to World Health Organization criteria, [110] as follows: G1= well–differentiated, G2= moderately differentiated and G3= poorly differentiated.

Surgical and histologic reports were analyzed, and all lesions were retrospectively staged according to the TNM classification. [111]

Adjuvant therapy

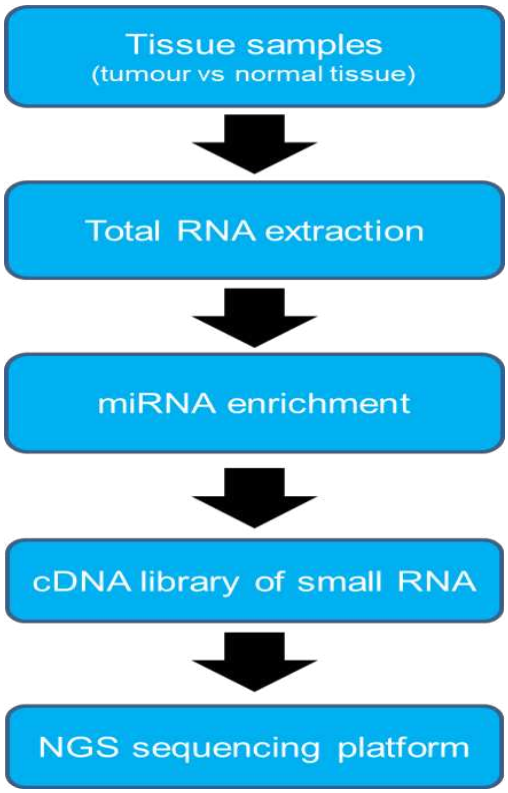
Although advanced stage, poor differentiation, and presence of positive surgical margins were the main factor considered, the indication for adjuvant RT was discussed for each patient by the multidisciplinary team, also taking into account age, comorbidities, previous treatment and, especially for low-stage ITAC, the availability of the patient for adequate follow-up.

Follow-up

All patients were undergoing to 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. [39]

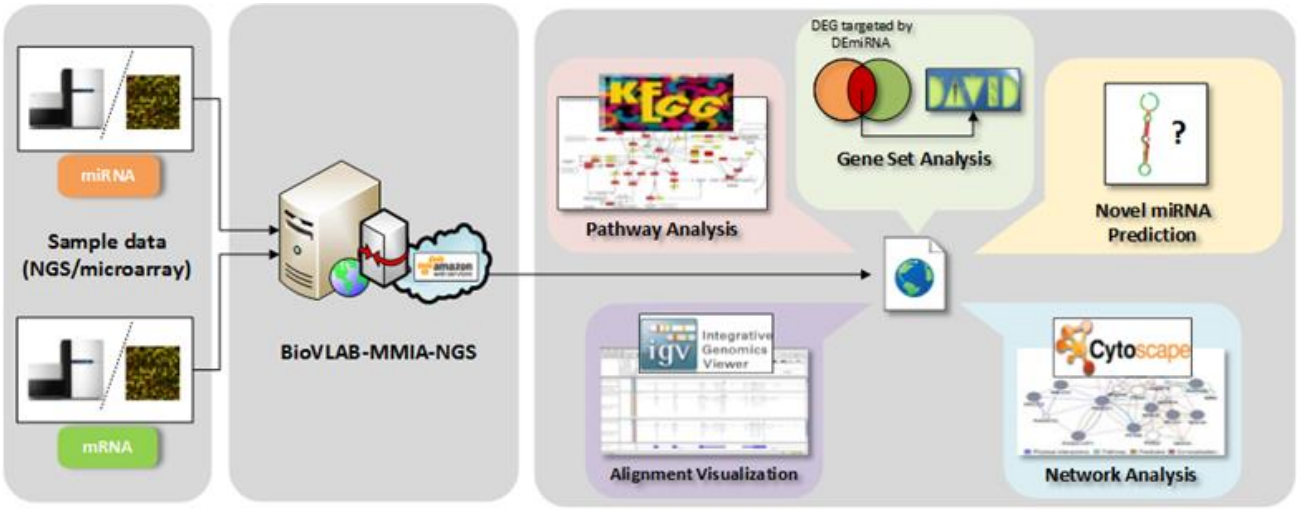
3.2. miRNoma ANALYSIS

The first phase of the study consisted in the identification of deregulated miRNAs in malignant neoplastic tissue in relation to the adjacent non-malignant counterpart of the nasal cavity. For this purpose, all miRNAs (miRNoma) have been analyzed by NGS technology 'Next Generation Sequencing '. The miRNoma analysis was performed in a subgroup of ITAC patients (n = 5). The patients had an average age of 69.8 ± 7.8 years, 4 males and 1 female, of whom 4 were smokers. Total RNA was extracted from the biopsy samples (tumour and adjacent normal tissue) of the selected patients using a commercial RNeasy Mini Kit (Qjagen). After analysing the purity and integrity of total RNA using the Qubit Flex Fluorometer (ThermoFisher), the samples were sequenced with NGS technology according to the steps illustrated in Scheme 1.



Scheme 1. Graphical representation of the various phases for the analysis of miRNA by NGS technology.

miRNA-seq libraries were assessed by capillary electrophoretic analysis with the Agilent 4200 Tape station and sequenced on an Illumina NextSeq500 generating 1X75bp-reads generating about 8 million fragments per sample. Starting from raw FASTQ files, quality of reads obtained from each sample was assessed using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters were trimmed and reads with length <18bp or >27bp were filtered out with cutadapt (1.16). Filtered reads were aligned to the miRBase 21 using SHRiMP2 (2.2.3) with the "mirna" option activated. Differential miRNA expression analysis was performed with DESeq2 (1.16.1) as previously described Krauskopf J, et al. [115]



The miRNA levels recovered from the miRDeep2 output, were analyzed using the R programming language (version 3.2.2). The DESeq2 package (version 1.8.1) was used to detect the differential miRNA levels between the different conditions. The results were filtered for significantly altered miRNAs by applying a falsehood rate of less than 5%. For the quantification of isomiR, Isomirage was used to map all the readings on an isomiR database derived from a miRBase. The readings were converted proportionally and the predominant isomiR for miRNA was identified for each condition. The remaining readings from the miRDeep2 sequencing were used to predict new miRNAs.

3.3. DETECTION OF miRNAS BY QUANTITATIVE RT-PCR

Total RNA was extracted from biopsy tissue samples (10-100 µg) using the RNeasy (Qiagen) kit according to the manufacturer's instructions. The concentration and purity of RNA were determined with the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). The cDNA was synthesized using the reverse transcription kit TaqMan®Advanced miRNA cDNA Synthesis Kit (item no. A25576; Life Technologies, Grand

Island, NY). Quantitative RT-PCR (qPCR) was performed using the TaqMan Fast Advanced Master Mix (Applied Biosystems) using miR-99b as the 'housekeeping' normalizing gene. PCR assays were performed using the Mastercycler EP Realplex (Eppendorf) at the following conditions: 50°C for 2 minutes, 95°C for 20 s, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds, followed by a suspension at 4°C. The raw data were analyzed using the automatic cycle threshold setting (CT) to assign the baseline and the threshold for the determination of the Ct. The samples were analyzed in duplicate and miRNAs with a CT value >35 were excluded. The results were expressed as a relative expression ($2^{-\Delta CT}$) or as a Fold-Change ($2^{-\Delta\Delta CT}$).

3.4. STATISTICAL ANALYSIS

Results are expressed as mean ± standard deviation or as median, quartile and confidence interval (CI). The categorical variables were reported as fractions or percentages and compared with the chi-square method. Comparisons between groups were performed using Student's t-test two-tailed (two groups) or by analysis of variance (ANOVA) followed by post-hoc Tukey analysis (greater than two groups). OS and DFS were estimated using Kaplan-Meier curves and the log-rank test was used to evaluate the difference between the subgroups. The OS was defined as the time interval between the date of surgery and the date of death or the date of last follow-up for surviving patients. The DFS was considered as the time from surgery until the recurrence of the disease. The Cox regression multivariate analysis was used to evaluate independent predictors of survival. Insignificant prognostic factors were

excluded from the model using Wald-Backward elimination. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the SPSS statistical package (SPPS Inc. Chicago, IL).

4. RESULTS

4.1. PATIENTS DATA

Overall 43 patients met the inclusion criteria. Patients population consisted most of males (93%), with a mean age of 69.3±13 years (range 33– 90 years) (Table 1).

The majority of patients (86%) had a history of professional exposure to wood or leather. As shown in Figure 2, the most representative work tasks were related to the working of wood (carpenter) and leather (shoemakers).

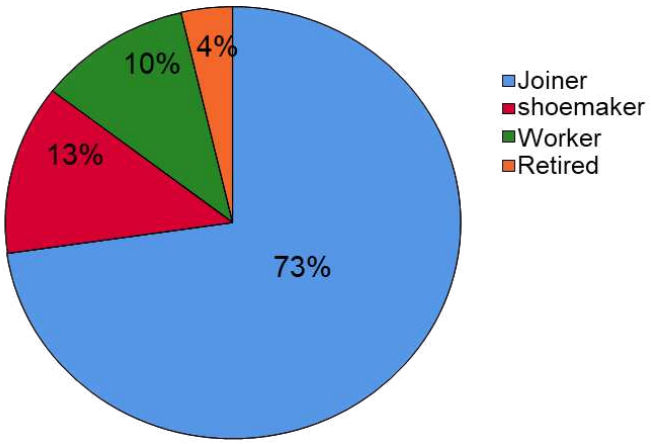


Figure 2. Distribution of patients according to the occupational tasks

The intestinal-type adenocarcinoma was localized in all cases in the ethmoid region as confirmed by endoscopic and imaging (TC and/or MRI) evaluation. No patients presented clinical and radiological positive nodes at diagnosis.

Surgery

Endoscopic resection without or with transnasal craniectomy (ERTC) was the most commonly adopted procedure for treatment of the primary lesion.

No elective treatment of the neck was performed.

Histologic findings and post-operative staging

Pathologic TNM classification was distributed as follows:

2 (5%) pT1N0M0, Stage I; 13(30%) pT2N0M0, Stage II; 22 (51%) pT3N0M0, Stage III; 5 (12%) pT4aN0M0, Stage IVa; 1 (2 %) pT4bN0M0, Stage IVb (Table 1).

Distribution by histologic grade was as follow: 6 patients (14%) had a well-differentiated lesion (Grade1), 28 patients (65%) had a moderately differentiated lesion (Grade 2) and 9 patients (21%) had a poorly differentiated lesion (Grade 3) (Table 1).

Immunohistochemistry: the cases classified as ITAC showed a variable cellular appearance and were composed of a mixture of tall columnar cells, atypical stratified cylindrical cells similar to the cells seen in conventional colorectal adenocarcinoma, globet cells, and large round to polygonal non-descriptive epithelial cells.

Adjuvant therapy

Adjuvant radiotherapy on the primary site with different techniques was delivered in 20 of 43 patients (47%) (Table 1).

Follow-up

No patient was lost to follow-up. The median follow-up was 41.9 [22.8- 61.0] months. After a median of 29.9 [22.6-37.2] months, 18 patients (42%) developed local relapse. All patients with local recurrence were treated with curative intent by surgery ± RT or RT and 18 patients (42%) died of disease with a survival median of 41.9 [6.7-296.20] months; the surviving patients (58%) were without evidence of active disease at the end of the follow-up time. The OS and DFS are shown in Figure 3.

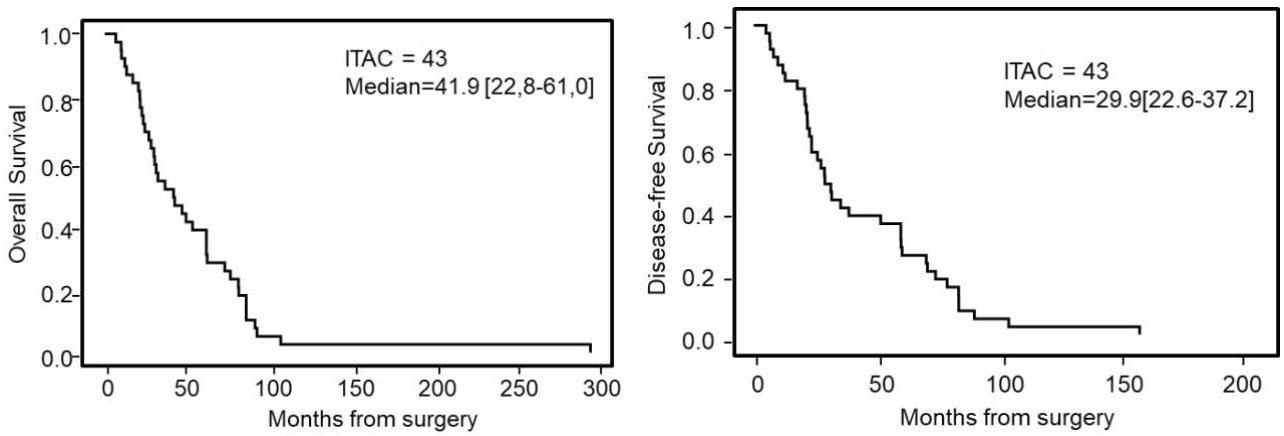


Figure 3. Overall survival and disease-free survival of patients affected by ITAC.

Table 1. Demographic and clinicopathological characteristics, adjuvant therapy, recurrence, and patients status at the end of the follow-up in 43 patients with sinonasal intestinal-type adenocarcinoma

Characteristic	No. of patients	%
Total	43	
Age (mean years)	69 ± 13	
Sex		
Male	40	93
Female	3	7
Exposure type		
None	8	19
Wood dust	28	65
Leather dust	7	16
Smocking		
No	14	48
Yes	10	35
Former	5	17
pT classification		
T0	0	0
T1	2	5
T2	13	30
T3	22	51
T4a	5	12
T4b	1	2
pN classification		
N0	43	100
N1	0	0
N2a	0	0
N2b	0	0
N2c	0	0
N3	0	0
Grade		
1	6	14
2	28	65
3	9	21
Adjuvant therapy		
None	23	53
RT	20	47
Recurrence		
No	24	60
Yes	16	40
Status		
NED	25	58
AWD	0	0
DOC	0	0
DOD	18	42
OS (median)	41.9 [6.7-296.2]	
DFS (median)	30.0 [5.0-162.0]	

Abbreviations: NED, no evidence of disease; AWD, alive with disease; DOC, dead of other causes; DOD, died of disease; OS, overall survival; DFS, disease-free survival

4.2. miRNA ANALYSIS

Study design

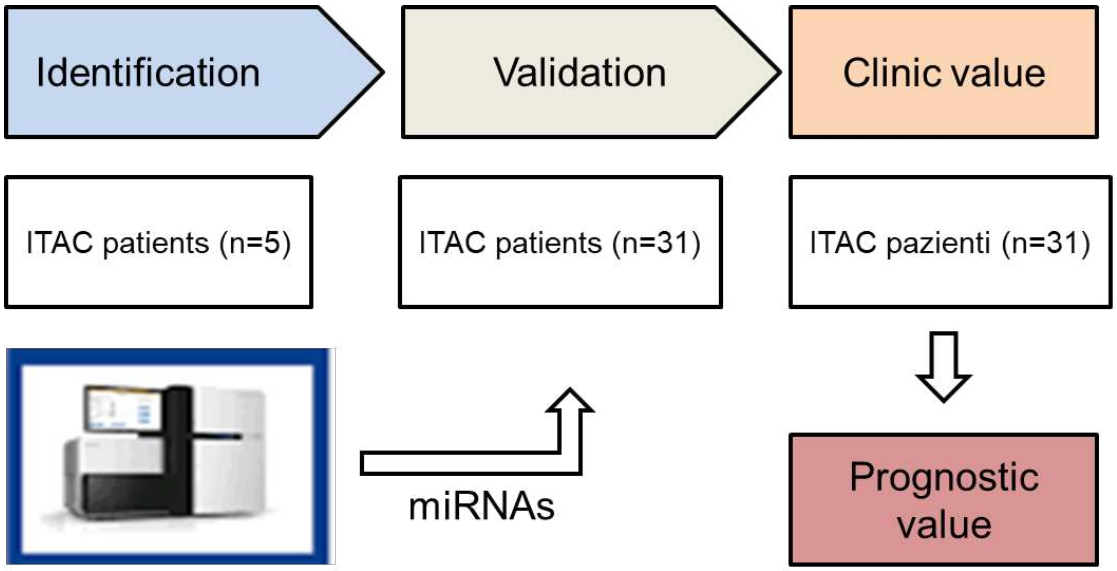
The study included three phases:

Phase I: identification of the miRNAs that are regulated in the tumour tissue with respect to the adjacent non-malignant counterpart.

Phase II: validation of the selected miRNAs.

Phase III: clinical value.

The experimental procedure is summarized in Scheme 2.



Scheme 2. Representation of the phases of the study

Phase I : miRNAs identification

From the sequencing analysis using the NGS method, 40 deregulated miRNAs in the tumour tissue with respect to the corresponding normal counterpart were identified. As shown in Figure 4, the majority of miRNAs were over-expressed in tumor tissue (62%) compared to non-malignant tissue.

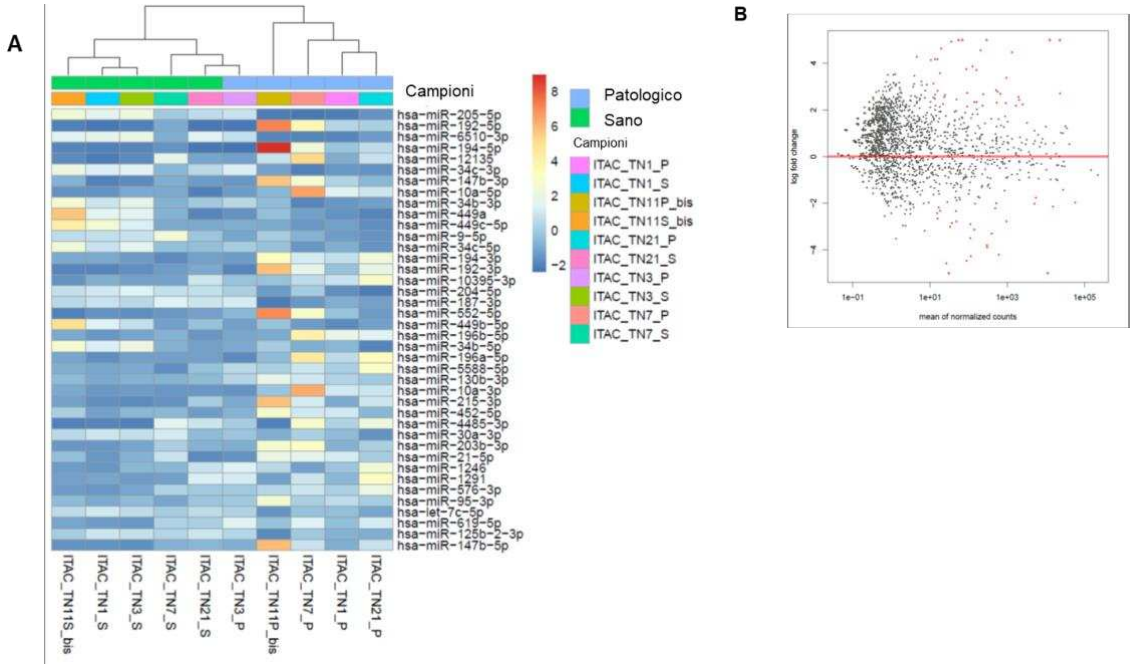


Figure 4. Hierarchical cluster analysis based on miRNA expression (miRNoma). A) Heatmap of significantly deregulated miRNA between the two groups (malignant and adjacent non-malignant counterpart). Intensity of color indicates expression level (red-orange, high expression; sky-blue, low expression). B) Volcano plot between cancer and normal tissue. There were relatively upregulated/downregulated miRNAs with statistical significance (adjusted $p < 0.05$).

Based on the statistical significance, the number of times under or over-expressed in the malignant tissue with respect to the non-malignant counterpart (Fold-Cange), and based on the formation of characteristic groups of the pathology (clusters), four miRNAs were selected (Figure 5).

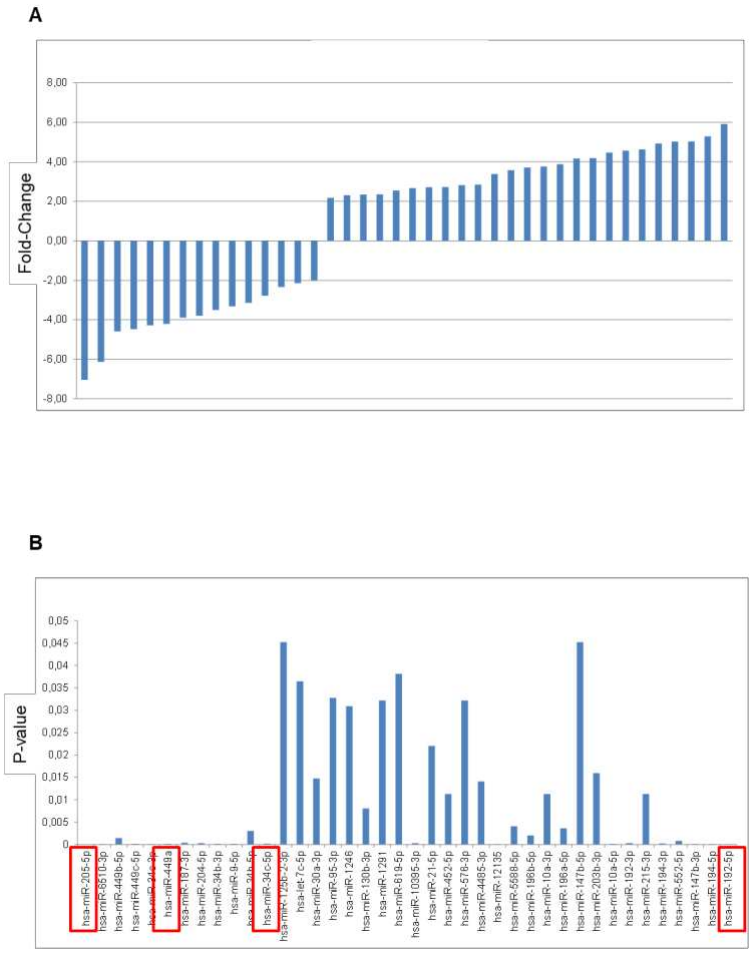


Figure 5. Distribution of deregulated miRNAs in ITAC. Down- and up-regulated miRNA in malignant tissues respect to non-malignant tissues according to fold-change (A) and p-value (B). Differences with $p<0.05$ were considered statistically significant.

The miR-205 and the miR-192 were selected as they were significantly under and over-expressed in tumors ($p = 1.8 \times 10^{-11}$ and $p= 8.2 \times 10^{-7}$, respectively). The clusters of the miR-449 (miR-449a, miR-449b, miR-449c) and of the miR-34 (miR-34b, miR-34c) were also evaluated, as they have been described as two typical families of miRNAs characteristic of the ciliated epithelium of the nasal mucosa. [116]

Phase 2: Validation of the selected miRNAs

The identified miRNAs by NGS technique, and subsequently selected, were then validated in a larger population of patients with ITAC.

From the expression analysis of the identified miRNAs, it was observed that miR-34c, miR-205, and miR-449a, were significantly down-regulated in malignant tissues with respect to their non-malignant counterparts, while miR-192 was up-regulated (Figure 6).

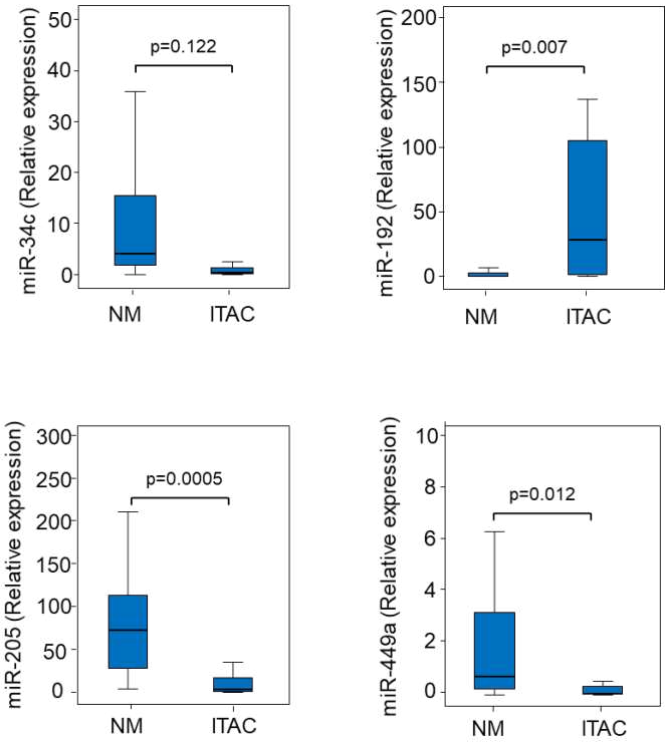


Figure 6. Distribution of selected miRNAs in ITAC. Expression of miR-34c, miR-192, miR-205 and miR-449a in malignant (ITAC) and non-malignant (NM) tissues. Comparisons between groups were determined by t-test analysis. Differences with $p<0.05$ were considered statistically significant.

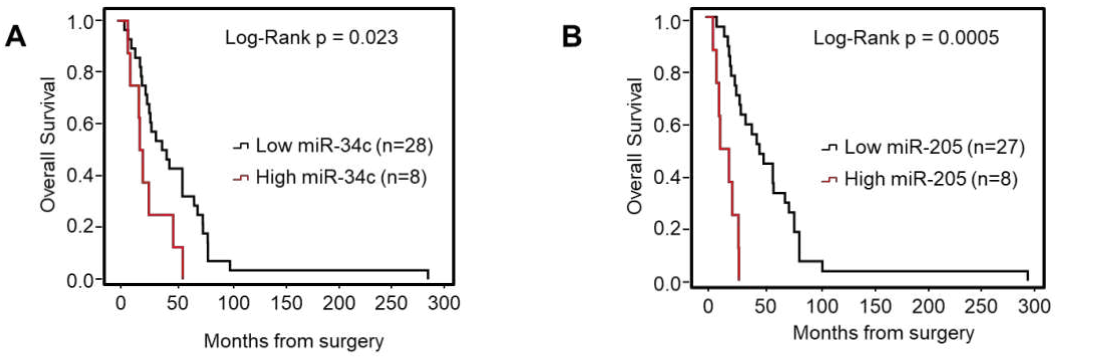
Phase 3: Clinical value of miRNAs

ITACs are very aggressive tumors, and despite the multidisciplinary management with multimodal treatment (surgery and radiotherapy) are characterized by a generally very poor prognosis. [115]

In this context, identification of new diagnostic and prognostic biomarkers is critical to improving the outcome of these patients. In order to assess their clinical value, the expression of the 4-miRNAs of ITAC patients was correlated with the outcome of the patients with sinonasal ITAC.

Furthermore, OS and DFS were analyzed using the Kaplan-Meier curve and miRNA groups (below and above the median) were compared using the log-rank test. Statistically significant variables were entered into a multivariate Cox regression model.

From the univariate analysis, both the miR-34c and miR-205 showed a statistically significant prognostic value (Figure 7 A,B). In particular, patients with low expression of miRNAs showed a better prognosis (OS, median 42.6 [20.4-64.7] months versus 21.3 [16.0-26.7] months, p = 0.023 for miR-34c and OS, median 49.5 [31.1-67.9] months versus 13.2 [0.59-25.8] months, for miR-205).



miR-34c	Overall Survival		
Groups	Median	SE	95% CI min-max
Low miR-34c	42,6	11,3	20,4-64,7
High miR-34c	21,3	2,7	16,0-26,7
Total	32,4	9,1	14,5-50,2

miR-205	Overall Survival		
Groups	Median	SE	95% CI min-max
Low miR-205	49,5	9,4	31,1-67,9
High miR-205	13,2	6,4	0,59-25,8
Total	32,4	7,5	17,7-47,1

Figure 6. Kaplan-Meier survival curves for ITAC subdivided by miR-34c and miR-205 expression. Low and high expression of miR-34c (A) (cut-off median value = 1.4) and miR-205 (B) (cut-off median value = 23) was associated with overall survival. Comparisons between groups were made using log-rank test and two-sided $p < 0.05$ were considered statistically significant. The median values and the 95% confidential interval (CI) [minimum-maximum] are summarized in the down panel.

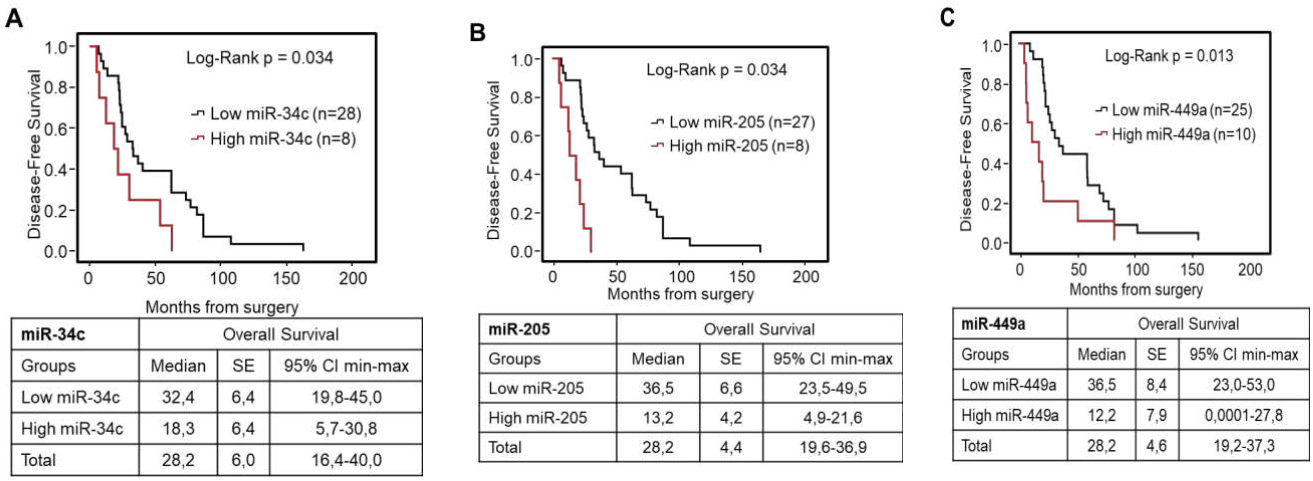
Multivariate analysis confirms the clinical value of miR-205 as an independent prognosis biomarker together with tumor grading (Table 2).

Table 2. Multivariate Cox regression analysis associated with OS

miR-34c			
Variable	HR	95% CI (HR)	p-value
Age	0.98	0.94-1.03	0.526
Gender	1.31	0.11-15.89	0.830
Smoking	0.57	0.10-3.41	0.540
Grading	0.23	0.02-2.74	0.244
Staging	0.99	0.18-5.47	0.992
miR-34c	0.616	0.15-2.47	0.547
miR-205			
Variable	HR	95% CI (HR)	p-value
Age	0.98	0.94-1.03	0.445
Gender	8.82	0.51-151.16	0.133
Smoking	0.27	0.01-1.33	0.108
Grading	**0.07**	**0.007-0.788**	**0.031**
Staging	0.15	0.02-1.49	0.106
miR-205	**0.015**	**0.002-0.136**	**0.0005**

Regression model with stepwise Wald-backward adjusted for age, gender, smoking, grading, staging and miRNA. Abbreviations: OS, Overall Survival; HR, hazard ratio; CI, confidence interval.

Furthermore, the miR-34c, miR-205 and miR-449a were significantly associated with DFS (Figure 7). In particular, high levels of the miRNAs were associated with a higher risk of recurrence and therefore with a worse prognosis.



miR-34c	Overall Survival		
Groups	Median	SE	95% CI min-max
Low miR-34c	32,4	6,4	19,8-45,0
High miR-34c	18,3	6,4	5,7-30,8
Total	28,2	6,0	16,4-40,0

miR-205	Overall Survival		
Groups	Median	SE	95% CI min-max
Low miR-205	36,5	6,6	23,5-49,5
High miR-205	13,2	4,2	4,9-21,6
Total	28,2	4,4	19,6-36,9

miR-449a	Overall Survival		
Groups	Median	SE	95% CI min-max
Low miR-449a	36,5	8,4	23,0-53,0
High miR-449a	12,2	7,9	0,0001-27,8
Total	28,2	4,6	19,2-37,3

Figure 7. Kaplan-Meier survival curves for ITAC subdivided by miRNA expression. Low and high miRNA expression of miR-34c (A), miR-205 (B) and miR-449a (C) were associated with disease-free survival. Comparisons between groups were made using log-rank test and two-sided $p < 0.05$ were considered statistically significant. The median values and the 95% confidential interval (CI) [minimum-maximum] are summarized in the down panel.

The multivariate analysis confirmed only the miR-205 and miR-449a as independent biomarkers for recurrence (Table 3).

Table 3. Multivariate Cox regression analysis associated with DFS

miR-34c			
Variable	HR	95% CI (HR)	p-value
Age	1.00	0.96-1.05	0.882
Gender	0.22	0.02-2.95	0.252
Smoking	0.38	0.07-2.24	0.285
Grading	**0.08**	**0.01-0.98**	**0.048**
Staging	1.35	0.25-7.20	0.725
miR-34c	0.87	0.24-3.16	0.835
miR-205			
Variable	HR	95% CI (HR)	p-value
Age	1.00	0.96-1.04	0.794
Gender	1.49	0.07-32.83	0.802
Smoking	0.35	0.08-1.56	0.168
Grading	**0.041**	**0.004-0.469**	**0.010**
Staging	0.204	0.015-2.716	0.229
miR-205	**21.67**	**2.05-229.13**	**0.011**
miR-449a			
Variable	HR	95% CI (HR)	p-value
Age	0.99	0.95-1.04	0.835
Gender	-	-	-
Smoking	0.42	0.09-1.90	0.257
Grading	0.38	0.02-7.00	0.516
Staging	0.34	0.04-3.36	0.358
miR-449a	**7.53**	**1.13-50.43**	**0.037**

Regression model with stepwise Wald-backward adjusted for age, gender, smoking, grading, staging and miRNA. Abbreviations: DFS, Disease-Free Survival; HR, hazard ratio; CI, confidence interval.

5. DISCUSSION

Despite their histological similarity to colorectal adenocarcinomas, there is very little information about the molecular events involved in the pathogenesis of sinonasal intestinal-type adenocarcinomas (ITACs). Several crucial pathways of cancerogenesis have been identified in colorectal adenocarcinomas. These pathways involve the mutation and inactivation of multiple oncogenes, tumor suppressor genes, and DNA mismatch repair genes including K-ras, APC, p53, MLH1, and MSH2. [14,15,117]

Working on the hypothesis that morphological similarities to colorectal adenocarcinomas might reflect equivalent genetic alterations, several authors have investigated the presence of activating mutations of Ras oncogenes and TP53 mutations in ITAC. TP53 mutations and p53 protein over-expression have been found in 18-44% of mostly occupational ITACs, whereas, K-Ras mutations were found in 10-15% of ITACs. The results of these studies suggest that mutations of K-Ras and other Ras genes are relatively uncommon in ITAC and similary, TP53 mutations in ITACs have not been widely demonstrated. [14,15,16,118,119]

Other studies have shown that K-Ras mutation and C-erb-2 expression could be associated to more aggressive ITACs. [17]

Licitra et al[60] investigated the prediction of TP53 status in ITAC for primary cisplatin fluorouracil chemotherapy in the neoadjuvant setting. The results of this study indicate that there are two genetic ITACs subgroups, defined by differences in TP53 mutational status or protein functionality, that strongly influence pathologic response to primary chemotherapy and prognosis. They found a significantly higher complete response rate (83%) and disease-free survival rate in patients carrying tumors with wild type TP53. By contrast, 89% of patients bearing mutated TP53 tumors did not achieve a complete response.

Perez-Ordonez and al[120] evaluated the possible role of DNA mismatch repair (MMR) gene defects or disruptions of E-cadherin/β-catenin complex in ITAC by investigating the immunohistochemical expression of the MMR gene products, E-cadherin and β-catenin, in a group of sporadic ITACs. The preserved nuclear

expression of MLH1, MSH2, MSH3, and MSH6 suggested that mutations or promoter methylation of MMR genes do not play a role in the pathogenesis of ITAC.

Kennedy and al[121] found that sinonasal ITACs have a distinctive phenotype, with all cases expressing CK20, CDX-2, and villin and most ITACs also expressing CK7, so that the expression pattern of CK7, CK20, CDX-2, and villin positive may be useful in separating these tumors from other non ITAC adenocarcinomas of the sinonasal tract.

Currently data in the literature demonstrate that miRNAs may be directly involved in the carcinogenesis process and that more than 50% of miRNAs are located in cancer-associated genomic regions or in fragile sites. [122,123] Some miRNAs appear to promote, while others inhibit cell proliferation and survival and these two classes of miRNAs may play an important role in cancer development as novel oncogenes and tumor suppressors, respectively. [124,125]

The potential utility of measuring miRNAs expression as a biomarker, useful in cancer diagnosis, prognosis and therapeutics, is becoming increasingly evident. Many studies have reported significant associations between miRNA profiles and important clinical features concerning head and neck tumors and patient survival. [19,21-24,27-30]

At present, due to the rarity of this type of cancer, there are very few studies in the literature [6] evaluating the expression of miRNAs in intestinal-type adenocarcinomas of the paranasal sinuses.

Given these findings, to explore other pathways involved in the molecular pathogenesis of sinonasal ITACs and to investigate their potential roles in tumor development, in a well-characterized series of patients with ethmoidal ITACs treated by primary surgery $\pm$ RT we performed a miRnoma analysis using Next Generation Sequencing (NGS) technology and selected 4 deregulated miRNAs (miR-34c miR-192, miR-205, miR-449a). To analyse the prognostic role of these miRNAs, their expression levels were then retrospectively correlated with clinical pathological characteristics of the tumor itself and with the patient outcome to evaluate their independent prognostic relevance.

The results of our study demonstrated that both miR-205 and miR-449a are independent biomarkers for recurrence in patients with sinonasal ITAC. In particular, patients with high expression of both miR-205 and miR-449a have a shorter disease-free period (short-term recurrence risk) ($p = 0.003$) and therefore a worse prognosis. The multivariate analysis confirms their independent predictive value.

miR-205 and miR-449a are involved in the organization of epithelial tissues while miR-205 regulates the structure of the epithelium, miR-449a is essential in the development of ciliated epithelia. [126] In particular, the miR-449 cluster has some similarities in sequence with the family of miR-34, suggesting similar mRNA targets,[127-131] and are classified as a single miRNA family involved in the proper differentiation of airway epithelium, and in the first line of defense of the respiratory tract against toxic agents. [132] All members of this miRNA family are capable of mediating cell cycle arrest and apoptosis and might thereby contribute to tumor suppression; underlying mechanisms include the downregulation of histone acetyl transferases and consecutive activation of p53, but also the targeting of cyclin dependent kinases and their association partners. [126]Particularly, miR-449a, which has been reported to inhibit tumor growth, invasion, and metastasis, and to promote apoptosis and differentiation through the transforming growth factor-β activated kinase 1, NOTCH, nuclear factor-κB/P65/vascular endothelial growth factor, retinoblastoma-E2F, mitogen-activated protein kinase signaling pathways, WNT–β-catenin signaling, tumor protein P53, and androgen receptor signaling pathways. [133]

According to what we reported, miR-449a has been found over-expressed in malignant breast tissue where its expression was significantly associated with a higher incidence of recurrence of the patient, reduction in the OS and reduction of DFS. [134] In contrast, miR-449a was found to be under-expressed in numerous other cancers including lung, colorectal, gastric, cervix, prostate cancer. [135-139]

Regarding miR-205, various evidences support its dual role in cancerogenesis acting either as a tumor suppressor through inhibiting proliferation and invasion, or as an oncogene through facilitating tumor initiation, proliferation, and resistance to anti-tumor therapy depending on the specific tumor context and

target genes. [140-142] In addition, miR-205 is speculated to potentially target over 2000 genes if all available algorithms are taken into account, and a micro network may be established between miR-205 and its multiple targets. [143,144] It is likely that in a micro network established between miR-205 and its targets, a homeostasis is maintained by miR-205 and its antagonists and synergists. [140]

Several studies found high expression of miR-205 in head and neck squamous cancer cell lines and esophageal squamous cell carcinoma, [145,146] as well as in lung squamous cell carcinoma and bladder cancer [147-150] but none of them made a correlation with the outcome of the patients. In our study, the high expression of miR-205 was associated with worse prognosis (reduction of DFS) and the multivariate analysis confirmed miR-205 as independent biomarker of OS in patients with sinonasal ITAC. This data imply that the over-expression of miR-205 may play an essential role in sinonasal ITAC carcinogenesis. In contrast, in breast cancer, [151,152] prostate, [153,154] renal [155] and in malignant melanoma, [157] has been found a low expression of miR-205, eliciting its tumor suppressive function.

These observations suggest that the onco-suppressor or onco-promoter effect of miRNAs depends on several factors including the type of tumor and the biological characteristic.

The current study presents some limitations: first of all, although we tried to evaluate a homogeneous patient cohort in terms of stage and treatment, ours is a retrospective study and the patient cohort remains heterogeneous in some critical aspects. Moreover, the study did not conduct a sensitivity analysis due to the small sample size (n=43).

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

6. CONCLUSIONS

The present study provides the first evidence that an over-expression of miR-205 and miR-449a in sinonasal ITAC is associated with an increased risk of recurrence (reduced DFS) and reduced overall survival, suggesting that mutation of these miRNAs, in combination with additional genetic events, could play a significant role in the pathogenesis of ITAC.

The analysis performed on a small sample of patients will necessarily be extended to a larger series and our results, should request validation through larger perspective and multicenter randomized series.

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