Low levels of miR-34c in nasal washings as a candidate marker of aggressive disease in wood and leather exposed workers with sinonasal intestinal-type adenocarcinomas (ITACs)

Elisabetta Bigagli a, Giandomenico Maggiore b, Lorenzo Cinci c, Mario D’Ambrosio a, Luca Giovanni Locatello b, x, Cosimo Nardi c, Annarita Palomba d, Gianluca Leopardi e, Pietro Orlando b, Giuseppe Licci b, Oreste Gallo b, Cristina Luceri a, x

a Department of Neuroscience, Psychology, Drug Research and Child Health (NEUROFARBA) Section of Pharmacology and Toxicology, University of Florence, Florence, Italy
b Department of Otorhinolaryngology, Careggi University Hospital, University of Florence, Florence, Italy
c Department of Experimental and Clinical Biomedical Sciences, Radiodiagnostic Unit n. 2, Careggi University Hospital, Florence, Italy
d Section of Pathological Anatomy, Department of Health Sciences, Azienda Ospedaliero-Universitaria Careggi, University of Florence, Italy
e Ospedale S. Giuseppe Empoli

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ABSTRACT

Introduction: Sinonasal intestinal-type adenocarcinomas (ITACs) are rare and aggressive tumors, closely related to professional exposure to wood dusts or leather. Here we explored the role of non-coding RNAs controlling MUC2 in liquid biopsies and tumors from ITAC patients with the aim of identifying biomarkers and molecular mechanisms to improve early diagnosis, prognosis, and therapeutic approaches for this rare cancer.

Methods: MiR-34c-3p, lncRNA AF147447 and MUC2 were measured in tumors and normal mucosa, in nasal washings (NW) from the affected and non-affected nostril and in plasma from 17 ITAC patients. The Apparent Diffusion Coefficient (ADC) was also evaluated by Magnetic Resonance Imaging.

Results: MiR-34c was higher in ITACs compared to the corresponding normal mucosa (p = 0.021). Differentiated tumors exhibited higher miR-34c levels (p = 0.025) and lower ADC values (p < 0.001) compared to mucinous ones and these parameters were also inversely correlated (r = 0.87; p = 0.001). High MUC2 tumor expression was associated with orbital extension (p = 0.010). Low miR-34c levels in NW were associated with orbital (p = 0.009) and intracranial (p = 0.031) extension and with advanced TNM stage (p = 0.054). Functional analysis identified Wnt, Focal adhesion, MAPK and inflammatory signalings among the pathways most enriched in mir-34c targets.

Discussion: Our results suggest measuring miR-34c in NW as a biomarker for early diagnosis and monitoring of ITAC patients and for the surveillance of wood and leather exposed workers. Further research on the involvement of miR-34c regulated pathways in ITAC tumorigenesis may also allow the development of new therapeutic approaches for this rare cancer.
and non-coding RNAs (including long noncoding RNA (lncRNA) and microRNAs (miRNAs)), might be promising biomarkers in the early diagnosis and prognosis of cancer [7–12]. Thanks to their ability to concurrently target multiple genes involved in tumor development and progression, microRNA-based treatments, either as drugs or as drug targets, alone or in combination with chemotherapy and/or radiotherapy, showed promise to improve treatment strategies in some cancers [13, 14].

Only two studies evaluated miRNA expression in ITAC: reduced tissue and plasma miR-126 expression was reported in ITAC patients compared to controls, while miR-205 and miR-449a/miR-34c cluster showed promise to improve treatment strategies in some cancers [13, 14].

Progression, miRNA-based treatments, either as drugs or as drug targets, might be promising biomarkers in the early diagnosis and prognosis of cancer. However, only two studies evaluated miRNAs expression in ITAC: reduced tissue and plasma miR-126 expression was reported in ITAC patients compared to controls, while miR-205 and miR-449a/miR-34c cluster showed promise to improve treatment strategies in some cancers.

**Methods**

**Patients and samples collection**

This prospective study was approved by the local Institutional Review Board (Comitato Etico Area Vasta Centro, ref. 196,237/oss). In the period 2019–2021, a total of seventeen patients with histologically proven sinonasal ITACs were recruited at the Department of Otorhinolaryngology, Careggi University Hospital, Florence, after signing the written informed consent. In two cases of inoperable disease, no surgery was selected as reference miRNA due to its stability across samples. The calculation of relative expression was performed using 2–ΔΔCt for nasal washings and tissue samples and 2–ΔΔCt for plasma and expressed as log2.

For lncRNA AF147447 and MUC2 mRNA analyses, total RNA (500 ng) was reverse transcribed by using the RevertAid RT Kit (Thermo Scientific, Waltham, MA, USA); RT reaction was performed as follows: 4 µL 5X Reaction Buffer, 2 µL dNTPs Mix, 1 µL Random Hexamer Primer (N6), 1 µL RevertAid Reverse Transcriptase, 1 µL RiboLock RNA Inhibitor, 10 µL RNA, 1 µL RNase-free water. RT cycling program consisted in 60 min at 95 °C, 5 min at 70 °C and cooling at 4 °C. cDNA samples were stored at −20 °C. RT performed using the Gene Amp 9700 (Applied Biosystem, Waltham, MA, USA) cycler.

RT-quantitative PCR (qPCR) was performed using the SsoAdvanced Universal SYBR (Biorad, Milan, Italy) as follows: 5 µL 2x SsoAdvanced Universal SYBR Green Supermix, 1 µL Primer forward, 1 µL Primer reverse, 1 µL cDNA, 2 µL RNase-free water. cDNA was diluted at the ratio of 1:10. The primer sequences are as follows: IncRNA AF147447: forward 5′-TCTTCTAATGCGTCTTGTCTCC-3′, reverse 5′-CCCTATAAACAACCTTAAACAC-3′; MUC2: forward 5′-AGAGGTGGAGCGGGCAAA-3′, reverse 5′-GCAGGGTTCTCTCCGC-3′; GAPDH: forward 5′-TGTTGTTGGCGTACAGG-3′, reverse 5′-GGAAATCGGTCCGTGATGAAG-3′. Cycling program consisted in incubation for 30 s at 95 °C and 2-step cycling of denaturation (15 s at 95 °C) and combined annealing/extension (15 s at 60 °C) for 35 cycles. The analyses were performed using the Rotor-Gene Q thermal cycler (Qiagen). Each sample was tested in triplicate. GAPDH was selected as reference miRNA due to its stability across samples. The calculation of relative expression was performed using 2–ΔΔCt and expressed as log2.

**MUC2 immunohistochemistry and clinico-pathological classification**

Immunohistochemistry for MUC2 was carried out on histological sections, 5 µm thick, of formalin-fixed, paraffin-embedded ITAC samples provided by the Pathological unit. Sections were rehydrated, treated with citrate buffer at 90 °C for 1 h to retrieve antigen, then with 0.3% (v/v) H2O2 in 60% (v/v) methanol to quench endogenous peroxidase. Aspecific sites saturation was carried out by incubation at room temperature for 15 min with 1% BSA and 0.1% TRITON X in PBS. The sections were then incubated overnight with rabbit polyclonal anti-MUC2 antibody at final dilution of 1:50 (sc-15,334 Santa Cruz Biotech). Immunoo reaction was revealed by biotinylated anti-rabbit secondary antibody at final dilution of 1:200 and then by immunoperoxidase method (Vectastain Elite kit, Vector, Burlingame, CA, USA), using 3,3′-diaminobenzidine as chromogen. As negative controls, sections incubated with only the primary or the secondary antisera were used. Positivity for MUC2 immunoreaction was carried out by two independent observers in blind fashion by using a Reichert–Jung Microstar IV light microscope (Cambridge Instruments, Buffalo, NY, USA) at ×200 final magnification (test area: 72 346 µm2).

AJCC VIII Edition TNM classification was used for clinical staging [20], while all ITAC cases were reviewed by an expert head and neck oncologist.
pathologist and Barnes’s classification was implemented for defining each subtype [21].

miR-34c-3p gene target prediction and pathway functional analysis

Putative miR-34c-3p targets were predicted using miRTarBase database that provides information on experimentally validated miRNA-target interactions or by using Target Scan (https://www.targetscan.org/vert_80/) [22]. Pathway analysis was then performed in order to generate hypotheses about the relevant biological functions controlled by the miR-34c-3p-target interactions by using the GO-Elite pathway analysis tool (freely available at http://www.genmapp.org/go_elite/) and the list of target genes as input file.

Magnetic resonance imaging (MRI)

MRI were assessed in a subset of patients (n = 10) in which the pre-surgical MRI was carried out at the Careggi University Hospital, Florence, Italy. MRI scans in ITAC patients were performed via 1.5 T Magnetom Aera (Siemens Healthcare, Erlangen, Germany) with a devoted head and neck coil. The MR acquisition protocol (Supplementary Table 1) included an axial fat saturated echo-planar imaging-based Diffusion Weighted Imaging (DWI) with two different b-values (b 50–800 s/mm²). Apparent Diffusion Coefficient (ADC) maps were reconstructed from DWI images. ADC values were calculated by positioning three regions of interest (ROI) with an average intratumoral area of 0.30–0.40 cm² each on three contiguous axial sections. ADC value of the trapezius muscle on the same side of the tumor were also obtained and ADC ratio was calculated as ADC tumor/ADC muscle. ADC values were obtained by two independent observers in a blinded fashion.

DWI is a MR imaging technique based upon measuring the random Brownian motion of water molecules within a voxel of tissue. The extent of tissue cellularity and/or high cellular crowding determine the impedance of water molecule diffusion that can be quantitatively assessed using ADC values, displayed as a parametric map reflecting the degree of diffusion of water molecules through different tissues.

Statistical analysis

Categorical variables were described using proportions and compared by Fisher’s exact test. Continuous data, including ADC values, were expressed as mean ± SD, or median value and interquartile range (IQR), and compared by Student’s t-test or by Mann–Whitney U test, as requested by data distribution. Wilcoxon matched-pairs signed-ranks test was used to compare the expression of mir-34c, IncRNA AF147447 and MUC2 mRNA in tumor tissue vs. normal mucosa. The correlation between ADC ratio and miR-34c and between mir-34c and IncRNA AF147447 in plasma samples was assessed by linear regression analysis. A p<0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Characteristics of ITAC patients

The main clinicopathological features of ITAC patients are summarized in Table 1. All patients were males, 9 smokers and 8 never-smokers, with a mean age of 73.5 years, and all were professionally exposed to wood (53%) or leather (47%). The mean latency period from the last known exposure and ITAC diagnosis was 23.2 (range 20–41) years. At the time of diagnosis, three patients had already distant metastases, while no nodal metastases were present. Overall, we had stage II-III (41%), stage IVA/B (41%), stage IV/C (18%) cases. The distribution by histological subtype was as follows: 7 patients (41%) had a mucinous subtype, 4 patient (24%) had a well differentiated tumor, and 6 patients (35%) had a moderately or poorly differentiated tumor. Orbital extension was documented in 6 (35%) and intracranial extension in 8 (47%) patients. Within a median time of 27 months, 3 patients died of the disease, and none was lost at follow-up.

MiR-34c, IncRNA AF147447 and MUC2 mRNA expression in paired samples of tumor tissue and normal mucosa

Higher tumor expression of miR-34c was found when we compared ITAC tissues to their respective paired normal mucosa, independently of histological subtypes (Fig. 1, panel A; p = 0.0215). However, when different subtypes were analyzed separately and compared to their paired normal mucosa, miR-34c was overexpressed only in differentiated tumors and not in the mucinous ones (Fig. 1, panel B; p = 0.0039). In addition, mir-34c was significantly lower in tumor tissue from mucinous ITACs compared to differentiated ones (p = 0.0256).

No differences in the expression of IncRNA AF147447 or MUC2 mRNA in paired samples of tumor tissue and normal mucosa were observed (Fig. 1, panel C and D). Immunohistochemical positivity for MUC2 was detected in 30.76% of our cases: in 40% of mucinous tumors and in 25% on non-mucinous ITACs.

MiR-34c and MUC2 expression in ITAC tissues: correlation with pathological features

Although not statistically significant, when miR-34c tumor expression was dichotomized according to the median value of the group, high tumor levels of miR-34c were associated to lower stage: 62% of patients with TNM II-III and no patient with stage IVC had high miR-34c tumor expression (Fig. 2, panel A; p = 0.11). MUC2 mRNA expression in tumor specimens was significantly associated with orbital extension (p = 0.0103) (Fig. 2, panel B).

MiR-34c and IncRNA AF147447 expression in the plasma and in paired samples of nasal washings from ITAC and from the unaffected sinusonal cavity

Both mir-34c and IncRNA AF147447 were detectable in the plasma and nasal washings from ITAC patients. In nasal washings, mir-34c was

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Table 1

Overview of the clinical and pathological characteristics of ITAC patients.

| Characteristic                  | Value |
|--------------------------------|-------|
| N                              | 17    |
| Age, mean±SE                   | 73.47±2.4 |
| Sex, male                      | 17 (100%) |
| Smokers                        | 9 (53%) |
| No                             | 8 (47%) |
| Yes                            | 9 (53%) |
| Professional exposure          |       |
| wood                           | 9 (53%) |
| leather                        | 8 (47%) |
| Histotype                      |       |
| mucinous                       | 7 (41%) |
| well differentiated            | 4 (24%) |
| moderately-poorly differentiated| 6 (35%) |
| TNM                            |       |
| II-III                         | 7 (41%) |
| IV A/B                         | 7 (41%) |
| IVC                            | 3 (18%) |
| Orbital extension              |       |
| no                             | 11 (65%) |
| yes                            | 6 (35%) |
| Intracranial extension         |       |
| no                             | 9 (53%) |
| yes                            | 8 (47%) |
| Survival                       |       |
| alive                          | 14 (82%) |
| died of disease                | 3 (18%) |
more abundant than lncRNA AF147447 but both showed similar levels in samples collected from the affected and from the non-affected sinonasal cavity (data not shown). In plasma samples, on the contrary, the lncRNA AF147447 was more abundant. A borderline significant decrease in miR-34c plasma levels in patients with TNM stage IV compared to those with stage II-III (\( p = 0.0721 \)) was also observed (Supplementary Fig. 1). Linear regression showed that mir-34c and lncRNA AF147447 plasma levels were positively correlated (\( r = 0.7483; \ p = 0.005 \)), (Supplementary Fig. 2).

**Mir-34c and lncRNA AF147447 expression in nasal washings: correlations with clinicopathological features of ITAC patients**

Low expression of miR-34c in nasal washings was significantly associated with both orbital and intracranial extension (\( p = 0.009 \) and \( p = 0.0315 \), respectively; Fig. 3, panel A and B). A borderline association with advanced TNM stage and low expression of miR-34c in nasal washings was also found (\( p = 0.054 \)) while high levels of lncRNA AF147447 were significantly associated to orbital extension (\( p = 0.0071 \)) (Fig. 3, panel C and D). Within our follow-up period, three deaths were registered, all patients displaying miR-34c nasal washings levels below the median level; however, this association was not statistically significant (\( p = 0.25 \)) (Supplementary Fig. 3).

The horizontal mark indicates the mean±SD.

**Magnetic resonance imaging of ITAC tissue and correlations with mir-34c**

A significant inverse correlation between ADC ratio and miR-34c expression in ITAC tissue was observed (\( r = 0.87; \ p<0.05 \)) (Fig. 4, panel A). Moreover, patients with differentiated ITAC subtypes showed an ADC ratio (ADC ITAC/ADC muscle) significantly lower than those with mucinous ones (Fig. 4, panel B).
Target gene prediction of miR-34c and functional analysis

To elucidate the biological functions and signaling pathways associated with miR-34c, target gene prediction was performed using mirTarBase algorithm and a total of 1386 genes were identified (accessed on March 10, 2022). These target genes are enriched in 6 KEGG and 13 Wiki pathways linked to tumorigenesis and inflammation (Wnt, Focal adhesion and MAPK pathway, mTOR, phagocytosis, IL-1, T-Cell Receptor and Co-stimulatory Signaling and Interferon type I) as shown in Table 2.

Discussion

Several studies demonstrated that miRNAs are involved in the development and progression of head and neck cancers either behaving as tumor-promoting miRNAs (oncomiRNAs) or as tumor suppressor miRNAs [11, 12], but so far, only two studies addressed their relevance in ITACs [15,16].

MiR-34c is a recognized tumor suppressor able to inhibit cell migration and invasion in vitro and to prevent tumor growth and metastasis in an in vivo xenograft model of nasopharyngeal carcinoma [23]. Accordingly, mir-34c is down regulated in nasopharyngeal, laryngeal and lung cancers [24-29]. In addition, low mir-34c expression correlates with worse disease-free and overall survival in laryngeal and lung cancer patients [30-32] and with radioresistance [29].

Re et al. (2021) reported no differences in the expression of miR-34c in ITAC samples compared to adjacent normal mucosa but high mir-34c tumor expression correlated with poor disease-free and overall survival [16].

Our results add new translational insights on the clinical and biological significance of mir-34c in ITAC patients since its expression varies across different subtypes, being downregulated in mucinous ITAC compared to differentiated tumors. Since the mucinous subtypes carry a poor prognosis [33], it can be hypothesized that the downregulation of mir-34c may contribute to tumor aggressiveness. This is consistent with the tumor suppressive role of mir-34c against cancer cell growth, invasion, and metastasis by targeting Notch2, c-Met and CDK1 [24,27,32].

MRI is part of the ITAC diagnostic workup and a miRNA–MRI integrated analysis has been proposed as an innovative tool in prostate tumors [34], but has never been explored in ITAC. As previously reported in mucinous lung cancers [35], our mucinous ITAC cases had higher ADC values than differentiated carcinomas likely because mucinous subtypes present lower cellular crowding and mucous content; moreover, ours is the first study to report an inverse correlation between miR-34c and ADC values in ITAC, despite being a preliminary finding that warrants further validations, it suggests that ADC values may vary according to tumor molecular features; accordingly, radiomic studies performed in breast, prostate, and ovarian cancers, demonstrated that ADC values may vary according to gene and receptor expression and p53 mutational status [36-38]; on this basis, a multidisciplinary approach integrating radiological findings with gene and miRNA expression holds promise to impact the characterization of these rare cancers.

Our group previously reported that the frequency of immunopositivity for MUC2 was higher in mucinous than non-mucinous subtypes [19] and the results of the present study confirm these findings. A functional link among MUC2, mir-34c and IncRNA AF147447 was reported in gastric cancer where IncRNA AF147447 repressed MUC2 expression by increasing mir-34c [18]; the lack of correlations among these biomarkers in ITAC tissues, suggests that these interactions have a marginal biological role in ITAC tumorigenesis and that other signalings, are likely involved. Accordingly, our functional analysis showed that miR-34c targets are associated with critical cancer pathways (e.g. focal adhesion, MAPK, Wnt, and mTOR) and with inflammatory signalings (e.g. phagocytosis, IL1, Interferon 1 and T cell receptor signaling). Wnt and MAPK have been reported among the most frequently mutated pathways in ITAC [39,40] and Wnt activation has been associated with worse
The role of inflammation in ITAC is supported by several findings: wood dust and leather exposure, the main etiological factors for ITAC, may cause chronic inflammation and oxidative stress resulting in the activation of NFκB, COX2 and Wnt pathway [42]. Moreover, chronic inflammation and oxidative stress generated by wood dust and leather exposure, are supposed to be responsible for the high frequency of G→A transitions inflammatory signature in TP53 and KRAS genes [2,42,43]; notably, miR-34c down-regulation has been observed in ITAC patients occupationally exposed to wood and leather [16] and it was linked to epigenetic events such as CpG island methylation or loss of transcriptional induction of p53 [44,45].

Moreover, among miR-34c gene targets, there is the Eukaryotic translation initiation factor EIF2S1 that is upregulated in ITAC [46] and that, together with EIF4B and EIF4E, is involved in mTOR pathway and Interferon type I signaling, central players in tumor-immune system interactions.

In our attempt to identify circulating biomarkers for ITAC, nasal washings were selected because of their direct contact with the tumor and not invasive collection method. MiRNA detection in nasal washings has been proposed as a diagnostic and prognostic tool for nasopharyngeal carcinoma [47] but has been never explored in ITACs. Notably, we demonstrated for the first time, that low nasal washings expression of mir-34c-3p correlates with orbital and intracranial disease extension; interestingly, miR-34c-3p is predicted to target Protein Tyrosine Phosphatase PTP4A1 and PTP4A2, two closely related family members of PTP4A3, whose gains were more frequently reported in ITACs with advanced stages and intracranial invasion [33]. Gains of PTPN1 were also more frequently associated to intracranial extension [33] and the interaction between PTPN1, also known as PTP1B, and miR-34c, experimentally demonstrated in glioma [48], has been associated with increased proliferation and metastasis in colon and lung cancer [49,50]. Since intracranial invasion and local recurrence are the most common causes of death in ITAC [39], low mir-34c levels in nasal washings appears of potential prognostic relevance in these patients. Accordingly, miR-34c-3p in serum exosomes of lung cancer patients was significantly lower than in healthy subjects and showed a negative association with disease-free survival [28]. Notably, there may be also a potential therapeutic application of mir-34c since the delivery of exosomes containing low levels of miR-34c-3p accelerated invasion and metastasis of lung cancer cells by upregulating integrin α2β1 [28]. Accordingly, in nasopharyngeal cancer cell lines, exosome containing miR-34c inhibited epithelial-mesenchymal transition and ameliorated radio resistance by targeting β-catenin/Wnt signaling [29].

Fig. 4. MRI of ITAC tissue: correlations with mir-34c and histological subtypes. ADC values were expressed as the ratio between ADC of ITAC tissue and ADC of trapezius muscle on the same side. Panel A: correlation between ADC ratio and miR-34c expression in tumor samples; panel B: ADC ratio in differentiated and mucinous ITAC histological subtypes. *** p<0.001. Axial ADC representative images: panel C) ITAC with low ADC ratio; panel D) ITAC with high ADC ratio. ITAC tumours are highlighted with red dashed lines and three exemplificative ROIs for each ITAC are reported.
Conclusion

In conclusion, the present study provides first evidence that miR-34c is detectable in nasal washings from ITAC patients showing promising associations with disease severity and extension. This is an exploratory, hypothesis generating study certainly limited by the small sample size however: 1) ITAC is a rare cancer 2) most of the studies conducted so far were retrospective other than prospective and 3) we measured miR-34c expression in different matrices such as tissues, nasal washings, and blood providing indications for further research in the field of biomarkers discovery and in the identification of potential targetable pathways involved in ITAC tumorigenesis.

Larger studies are needed to validate our results but the analysis of miR-34c in nasal washings may be considered as a potential new tool in screening and monitoring programs of ITAC patients and of professionally exposed wood and leather workers. Moreover, our findings suggest to further explore the involvement of pathways epigenetically regulated by miR-34c in ITAC tumorigenesis, for the development of new therapeutic approaches for this rare cancer.

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CRediT authorship contribution statement

Elisabetta Bigagli: Conceptualization, Investigation, Methodology, Validation, Supervision, Writing – original draft, Writing – review & editing. Giandomenico Maggiore: Conceptualization, Data curation, Resources, Writing – review & editing. Lorenzo Cinci: Data curation, Investigation, Visualization, Writing – review & editing. Mario D’Ambrosio: Methodology, Investigation, Writing – review & editing. Luca Giovanni Locatello: Data curation, Resources, Writing – review & editing. Cosimo Nardi: Data curation, Visualization, Writing – review & editing. Annarita Palomba: Data curation, Resources, Formal analysis, Writing – review & editing. Gianluca Leopardi: Data curation, Resources, Writing – review & editing. Pietro Orlandi: Data curation, Resources, Writing – review & editing. Giuseppe Lici: Data curation, Resources, Writing – review & editing. Oreste Gallo: Data curation, Resources, Writing – review & editing. Cristina Luceri: Conceptualization, Methodology, Formal analysis, Validation, Visualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

None.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tran.2022.101507.

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