Plasma miR-151-3p as a Candidate Diagnostic Biomarker for Head and Neck Cancer: A Cross-sectional Study within the INHANCE Consortium

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ABSTRACT

Background: Identification of screening tests for the detection of head and neck cancer (HNC) at an early stage is an important strategy to improving prognosis. Our objective was to identify plasma circulating miRNAs for the diagnosis of HNC (oral and laryngeal subsites), within a multicenter International Head and Neck Cancer Epidemiology consortium.

Methods: A high-throughput screening phase with 754 miRNAs was performed in plasma samples of 88 cases and 88 controls, followed by a validation phase of the differentially expressed miRNAs, identified in the screening, in samples of 396 cases and 396 controls. Comparison of the fold changes (FC) was carried out using the Wilcoxon rank-sum test and the Dunn multiple comparison test.

Results: We identified miR-151-3p (FC = 1.73, $P = 0.007$) as differentially expressed miRNAs in the screening and validation phase. The miR-151-3p was the only overexpressed miRNA in validation sample of patients with HNC with early stage at diagnosis (FC = 1.81, $P = 0.008$) and it was confirmed upregulated both in smoker early-stage cases (FC = 3.52, $P = 0.024$) and in nonsmoker early-stage cases (FC = 1.60, $P = 0.025$) compared with controls.

Conclusions: We identified miR-151-3p as an early marker of HNC. This miRNA was the only upregulated in patients at early stages of the disease, independently of the smoking status.

Impact: The prognosis for HNC is still poor. The discovery of a new diagnostic biomarker could lead to an earlier tumor discovery and therefore to an improvement in patient prognosis.

Introduction

Head and neck cancer (HNC) represents the sixth most common type of cancer worldwide, with more than 930,000 cases and 460,000 deaths annually (1). These cancers originate from several sites of the head and neck region: pharynx (oropharynx, nasopharynx, and hypopharynx), larynx or the oral cavity, and are strongly associated with environmental and lifestyle risk factors such as tobacco and alcohol consumption (2–4). Other known risk factors are human papillomavirus infection, diet, lack of physical activity, and/or family history (5–9).

The prognosis of HNCs is still poor, despite the combined treatment involving surgery, radiotherapy, and chemotherapy (10). The 5-year disease-specific survival rate in patients with HNC in Europe ranges between 25.5% for hypopharyngeal cancer and 63.1% for laryngeal cancer (11). The main reasons for the poor prognosis are frequent locoregional recurrence and metastasis (12, 13). A total of 50% of patients with HNC, in fact, develop recurrent disease within the first 2 years of treatment (14), and it is currently well established that HNC survivors also have an increased risk of developing second primary cancers (SPC) compared with the overall population, with frequent SPC of the head and neck, esophagus, and lung (15–18).

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As in all cancer types, also for HNC an early detection is expected to improve the prognosis: clinical and/or instrumental tools coupled with the identification of novel sensitive and reliable biomarkers would be crucial to this purpose. Although several studies have been performed to depict the molecular landscape of HNC over the last years, the identification of reliable genomic biomarkers is still lacking (19); subclassifications based on genomic profiling might be useful to better understand the biological mechanisms responsible for carcinogenesis (20). The level of molecular markers, including miRNAs, in biofluids is considered a promising noninvasive approach for the early detection of cancer, including HNC (21).

miRNAs are short noncoding RNA molecules of 19–25 nucleotides which play a crucial role in the regulation of gene expression, including oncogenes or tumor suppressor genes, and are emerging as a new molecular tool for noninvasive cancer diagnosis and prognosis (22). Several miRNAs involved in the pathogenesis of HNC have now been identified, and some are currently being evaluated for their diagnostic and/or prognostic performance (23). A recently published meta-analysis revealed that miR-21 and miR-93 are mostly upregulated in HNC, whereas miR-9, miR-203, miR-218, and miR-375 are down-regulated (24). Although the meta-analysis demonstrated that these molecules are promising diagnostic tool with moderate accuracy, to our knowledge, none of these has been broadly used as a HNC diagnostic biomarker in a clinical setting due to the limited accuracy in discriminating cases and controls.

The aim of the current study was to identify specific diagnostic miRNA signatures for oral and laryngeal HNC, within a multicenter International Head and Neck Cancer Epidemiology (INHANCE) consortium (25).

Materials and Methods

Study participants

Participants were selected from the three studies of the INHANCE Consortium (25): Rome (Italy), ARCAGE study (with 10 Western European centers), and Aichi (Japan). We included, as study cases, patients ≥18 years of age with histologically confirmed primary HNC arising in the anatomical sites of the oral cavity or larynx.

The controls were selected among hospital visitors without a history of cancer, with a case–control ratio of 1:1. Participants completed a questionnaire, including information about age, gender, tobacco smoking at the diagnosis, and donated a sample of peripheral blood. Cases and controls were matched by age, ethnicity, gender, and cigarette smoke exposure. A total of 692 Caucasian and 276 Japanese subjects were included. The characteristics of all subjects are listed in Supplementary Table S1.

The study was approved by the local Ethical Committee of each participating center and written informed consents were obtained from all study subjects.

Variables

Different study centres used different cancer codes, which were converted into International Classification of Diseases for Oncology (ICD-O-2) when included into this study. HNCs were classified according to the following anatomic sites using the ICD-10 codes: oral cavity (C00.3–C00.9, C02.0–C02.3, C03.0, C03.1, C03.9, C04.0, C04.1, C04.8, C04.9, C05.0, C06.0–C06.2, C06.8, and C06.9) and larynx (codes C10.1, C32.0–C32.3, and C32.8–C32.9. Cancers were staged according to the TNM Staging System, 7th edition, and categorized as early (stage I and II) and advanced (stage III and IV) stage at diagnosis (26). Individuals were categorized according to smoking status as current smokers or non-current smokers.

Blood samples

Peripheral blood was collected in ethylene diamine tetracetic acid (EDTA) tubes at the time of interview and processed as rapidly as possible (generally within 2 hours). For cases, blood draw was performed at the diagnosis before surgery or any other treatment. Plasma samples were isolated by centrifugation of whole blood at 2,000 × *g* for 10 minutes at room temperature. Samples were stored at −80°C.

Molecular analyses and study design

Cell-free RNA (cfRNA) was isolated from 300 μL of plasma using the NucleoSpin miRNA Plasma kit (Macherey-Nagel). All plasma samples were spiked-in with 10 pmol of Arabidopsis thaliana synthetic miR-159a (synthesized by Eurofins MWG Operon) as a control of RNA extraction.

To identify HNC diagnostic miRNA signatures, a two-stage approach was chosen: a high-throughput screening phase performed in plasma samples of 88 HNC cases and 88 controls, followed by a validation phase of the differentially expressed miRNAs, identified in the screening phase, in samples of 396 HNC cases and 396 controls (Supplementary Table S2).

For the screening phase, the expression levels of 754 miRNAs (those annotated in miRBase v14 at the time of the study design) were quantified using the TaqMan Human MicroRNA Array A + B Card Set v3.0 and the ABI 7900HT instrument (Applied Biosystems), according to manufacturer's instructions (including the preamplification step). qPCR data were collected by the SDS software v2.4 (Applied Biosystems). Threshold cycle (C_t) values were determined using the Expression Suite software (Applied Biosystems), setting automatic baseline and threshold. Only miRNAs with C_t values <35 in at least one group were evaluated in comparative analyses. The relative levels were calculated according to the C_t method and expressed as fold change (FC) compared with the median of controls. Only miRNAs with multiple testing corrected $P < 0.05$ were considered as differentially expressed and selected for the validation phase.

For the second stage of the study (validation phase), custom Low-density Arrays (Life Technologies) were designed with spotted primers and probes, specific for the significant miRNAs selected in the screening phase. Amplification was performed using the TaqMan Universal Master Mix II, no UNG (Life Technologies) and the ABI 7900HT instrument (Applied Biosystems), according to manufacturer's instructions (including the preamplification step). The C_t method was used to determine the relative expression levels, compared with the median level of controls. miRNA 159 was used as the endogenous control.

Statistical analysis

For each miRNA, C_t or ΔC_t values (C_t target − C_t reference gene), and FC were reported as median and interquartile range (IQR). Subgroup analyses were performed by considering current smokers compared with those who were not current smokers, and by restricting the main analyses to the cases with an early stage of disease at diagnosis (stage I and II).

Comparison of the FCs was carried out using the Wilcoxon rank-sum test and the Dunn multiple comparison test. Results with a $P < 0.05$ after multiple comparison correction were considered significant.

In addition, we investigated the significance of the interaction between miRNA expression levels and smoking status by including an interaction term in logistic regression models.

Table 1. miRNAs differentially expressed between cases and controls in the screening phase.

miRNA	Mean C_t, cases	Mean C_t, controls	FC	*P*
miR-145	27.2	26.5	0.62	**0.0003**
miR-151-3p	25.2	26.0	1.72	**0.0030**
miR-191	32.7	34.5	3.48	**0.0031**
miR-409-3p	27.3	28.6	2.60	**0.0080**
miR-425	29.2	31.1	3.64	**0.0002**
miR-628-3p	30.0	31.0	2.04	**0.0026**
miR-766	26.0	27.3	2.28	**0.0052**

Note: *P* values refer to multiple testing-corrected *P* values.
Abbreviations: C_t, threshold cycle; FC, fold change.

Analysis was performed using R 3.5.1 (https://www.r-project.org/) and Stata softwares (StataCorp. 2016. Stata Statistical Software: Release 16. StataCorp LP).

Data availability

The data generated in this study are available upon request from the corresponding author.

Results

Screening phase: miR-145, miR-151-3p, miR-191, miR-409-3p, miR-425, miR-628-3p, and miR-766 are differentially expressed in HNC

In the screening phase, we detected a total of seven differentially expressed miRNAs between cases and controls, including six upregulated (miR-425, miR-151-3p, miR-191, miR-409-3p, miR-628-3p, miR-766) and one downregulated miRNA (miR-145), that were selected for validation (**Table 1**).

Our data indicate that about 1% of the total miRNAs were deregulated in cases. Most miRNAs were not detectable in plasma.

Validation phase: miR-145 and miR-151-3p are upregulated in HNC

In the validation phase, two of the seven miRNAs from the screening were confirmed to be differentially expressed between cases and controls (**Table 2**).

While miR-151-3p was confirmed to be upregulated (replication sample: FC = 1.73, $P = 0.007$; discovery sample: FC = 1.72, $P = 0.003$), the behavior of miR-145 was opposite in the two phases of our study (replication sample: FC = 2.35, $P = 0.008$; discovery sample: 0.62, $P = 0.0003$). The other miRNAs (miR-191, -409-3p, -425, -628-3p, and miR-766) were not differentially expressed, although the direction of the change was the same in the discovery and replication sample In the analysis restricted to the early-stage cases, miR-151-3p was the only overexpressed miRNA in patients with HNC (FC = 1.81, $P = 0.008$; **Table 2**).

Smoker patients display a wider miRNA dysregulation

When comparing the expression of the selected miRNAs between smoker ($n = 199$) and nonsmoker ($n = 197$) cases (**Table 3**), miR-191 was not differentially expressed at the significance level ($P = 0.17$), although the FC indicates overexpression in smokers. All other tested miRNAs showed overexpression in current smokers compared with non-current smokers (miR-145: FC = 1.80, $P = 0.045$; miR-151–3p: FC = 2.41, $P < 0.001$; miR-409–3p: FC = 2.67, $P = 0.007$; miR-628-3p: FC = 2.49, $P = 0.002$; miR-766: FC = 3.01, $P = 0.008$). These differences were not related to cigarette smoke *per se*, but rather to the HNC, because there were no significant differences according to smoking status among controls (smokers $n = 199$; nonsmokers $n = 197$; **Table 3**). Indeed, no significant interactions between miRNA expression levels and smoking status were found (**Table 4**). In addition, among early-stage cases, no differences were observed between current smokers and nonsmokers ($n = 38$ and 85 for smokers and nonsmokers, respectively; **Table 3**).

Besides the differences between smoker and nonsmoker cases, the differential expression of all miRNAs was particularly evident when comparing current smokers with controls (**Table 5**).

None of the seven miRNAs was differentially expressed in the analysis of non-current smokers versus controls. Finally, only miR-151-3p was upregulated both in smoker early-stage cases (FC = 3.52, $P = 0.024$) and nonsmoker early-stage cases (FC = 1.60, $P = 0.025$) compared with controls (**Table 6**).

Discussion

In the current study, we identified miR-151-3p as a candidate diagnostic biomarker for HNC. We firstly used a high-throughput approach to identify candidate deregulated biomarkers in plasma of patients compared with controls. In this first phase of the study, we found that less than 1% of miRNA are differentially expressed in HNC, suggesting that the effect of the background noise of global miRNA release in cfRNA is limited, and confirming the robustness of miRNA dosage as disease biomarkers in general, as widely demonstrated also in other studies. Our study has several strengths,

Table 2. Comparison of miRNA expression between cases and controls in the validation phase.

miRNA	ΔC_t[a] cases, median [IQR]	ΔC_t early stage cases, median [IQR]	ΔC_t controls, median [IQR]	FC, cases vs. controls	FC, early-stage cases vs. controls	*P*, cases vs. controls	*P*, early-stage cases vs. controls
miR-145	9.60 [7.75–11.76]	10.18 [8.91–12.66]	10.83 [8.54–13.24]	2.35	1.58	**0.008**	0.517
miR-151-3p	6.89 [4.90–8.38]	6.82 [4.59–8.26]	7.68 [5.75–9.64]	1.73	1.81	**0.007**	**0.008**
miR-191	16.54 [11.64–18.52]	16.01 [11.67–18.24]	17.43 [11.68–18.56]	1.86	2.68	0.400	0.352
miR-409-3p	8.74 [6.56–10.82]	8.98 [7.42–11.67]	9.30 [6.99–11.82]	1.48	1.25	0.117	0.887
miR-425	12.15 [9.66–17.81]	12.65 [9.17–17.94]	13.26 [9.68–17.98]	2.16	1.52	0.182	0.550
miR-628-3p	11.43 [9.14–16.20]	11.79 [9.18–16.01]	11.46 [9.26–16.94]	1.02	0.79	0.799	0.964
miR-766	9.37 [6.98–11.90]	9.93 [7.41–15.00]	9.98 [7.38–13.28]	1.53	1.04	0.151	0.901

Note: Bold values denote statistical significance at the $P < 0.05$ level.
Abbreviations: C_t, threshold cycle; ΔC_t values (C_t target − C_t reference gene); FC, fold change; IQR, interquartile range.
[a]Difference between C_t value of each miRNA and that of miR-159.

Table 3. Comparison of miRNA expression between smokers and nonsmokers among all cases, early-stage cases, and controls separately.

Group	miRNA	*n* smokers	ΔC_t smokers, median [IQR]	*n* nonsmokers	ΔC_t nonsmokers, median [IQR]	FC	*P*
Cases	miR-145	199	9.46 [6.94–11.41]	197	10.31 [8.86–12.37]	1.80	**0.045**
	miR-151-3p		6.14 [4.44–8.00]		7.40 [6.00–8.82]	2.41	**<0.001**
	miR-191		13.87 [10.98–18.85]		17.03 [12.37–18.52]	8.91	0.178
	miR-409-3p		7.70 [5.89–10.36]		9.12 [7.66–11.68]	2.67	**0.007**
	miR-425		11.61 [9.01–17.80]		13.14 [10.05–17.94]	2.89	0.224
	miR-628-3p		10.77 [8.01–13.37]		12.09 [10.13–17.03]	2.49	**0.002**
	miR-766		8.65 [6.11–11.04]		10.23 [7.87–13.37]	3.01	**0.008**
Early-stage cases	miR-145	38	10.16 [9.45–11.68]	85	10.46 [8.65–13.37]	1.23	0.763
	miR-151-3p		5.86 [5.03–6.86]		7.00 [4.52–8.34]	2.19	0.248
	miR-191		17.80 [12.62–19.25]		15.51 [11.47–18.00]	0.20	0.451
	miR-409-3p		7.99 [6.20–9.47]		9.54 [7.59–12.14]	2.93	0.183
	miR-425		11.90 [9.01–17.80]		14.12 [9.47–18.26]	4.68	0.393
	miR-628-3p		10.99 [9.18–14.22]		12.36 [9.03–16.30]	2.58	0.436
	miR-766		10.77 [7.66–17.94]		9.92 [7.18–14.82]	0.55	0.688
Controls	miR-145	199	10.16 [8.10–11.99]	197	10.69 [8.36–12.52]	1.45	0.652
	miR-151-3p		7.58 [5.78–9.16]		8.14 [5.76–10.36]	1.47	0.560
	miR-191		17.04 [11.96–18.48]		17.50 [11.21–18.73]	1.38	0.802
	miR-409-3p		8.64 [6.91–10.68]		9.35 [6.83–11.28]	1.64	0.919
	miR-425		12.23 [9.75–17.72]		13.81 [9.55–18.44]	2.99	0.655
	miR-628-3p		11.33 [8.74–15.53]		12.23 [9.89–17.90]	1.86	0.350
	miR-766		9.62 [7.08–11.14]		10.38 [7.54–14.80]	1.69	0.430

Note: Bold values denote statistical significance at the $P < 0.05$ level.
Abbreviations: C_t, threshold cycle; ΔC_t values (C_t target − C_t reference gene); FC, fold change; IQR, interquartile range.

including the availability of a large validation sample that has been analyzed independently from the first screening group, the different geographic origin of cases and controls enrolled for the validation phase, the availability of both early and advanced stages of HNC. The main limitations are the paucity of available genomic data of patients and, more importantly, the cross-sectional nature of the study with the consequent lack of longitudinal samples, especially after surgical removal of the HNC. Furthermore, at the time of the study design, next-generation sequencing miRNome analysis was not available and we adopted the microarray method in the screening phase. However in the validation phase, we performed a targeted analysis of the differentially expressed miRNAs identified during the screening. Finally, our conclusions refer to tumors of the oral cavity and larynx and we do not know whether they are generalizable to all subsites of HNC.

Table 4. Results of the interaction analysis between miRNA levels and smoking status.

Group	miRNA	$P_{interaction}$
All cases vs. controls	miR-145	0.139
	miR-151-3p	0.082
	miR-191	0.142
	miR-409-3p	0.106
	miR-425	0.087
	miR-628-3p	0.386
	miR-766	0.061
Early-stage cases vs. controls	miR-145	0.787
	miR-151-3p	0.657
	miR-191	0.467
	miR-409-3p	0.777
	miR-425	0.665
	miR-628-3p	0.577
	miR-766	0.284

As schematized in **Fig. 1**, we identified miR-151-3p as an early marker of HNC. This miRNA was the only upregulated in patients at early stages of the disease, independently to the smoking status. With disease progression, the increase in miR-151-3p is more prominent in smokers compared with nonsmokers. Differently from other miRNAs, miR-151-3p regulation has never been related with smoking *per se* in previous studies. Also, in our group of controls, no differences in miRNA levels were found on the basis of the smoking status. This indicates that the upregulation of this miRNA is HNC related. miR-151-3p has been involved also in other neoplasms, playing a pivotal role in colorectal cancer (27), osteosarcoma (28), cholangiocarcinoma (29), and some others. Thus, miR-151-3p has a potential clinical application as an early diagnostic marker of HNC; conceivably, miRNA levels could be monitored over time, as a marker of progression, especially in smoker patients.

With the progression of the disease, the differences between smokers and nonsmokers are more pronounced (**Fig. 1**): only miR-151-3p and miR-145 are upregulated in both groups.

This latter miRNA provided different results in the screening compared with the validation phase: it was downregulated in the first part of the study, and then upregulated. The explanation of this discrepancy is unknown but it could be related to the different representation of smokers in the two cohorts, which is statistically significant ($X2 = 7.13$; $P = 0.008$). Indeed, in the validation cohort, miR-145 is significantly upregulated in smoker patients, compared with nonsmokers. On the basis of the data of the literature, miR-145 acts as tumor suppressor miRNA via different signaling pathways in some cancer models (30, 31). In addition, in a recent meta-analysis, this miRNA has been reported as downregulated in saliva of patients

Table 5. *Post hoc* analysis comparing miRNA expression between cases (according to smoking status) and controls.

miRNA	ΔC_t smoker cases, median [IQR]	ΔC_t nonsmoker cases, median [IQR]	ΔC_t controls, median [IQR]	FC, smoker cases vs. controls	*P*, smoker cases vs. controls	FC, nonsmoker cases vs. controls	*P*, nonsmoker cases vs. controls
miR-145	9.46 [6.94–11.41]	10.31 [8.86–12.37]	10.83 [8.54–13.24]	2.59	**<0.001**	1.44	0.100
miR-151-3p	6.14 [4.44–8.00]	7.40 [6.00–8.82]	7.68 [5.75–9.64]	2.91	**<0.001**	1.21	0.100
miR-191	13.87 [10.98–18.85]	17.03 [12.37–18.52]	17.43 [11.68–18.56]	11.80	0.227	1.32	0.279
miR-409-3p	7.70 [5.89–10.36]	9.12 [7.66–11.68]	9.30 [6.99–11.82]	3.03	**0.003**	1.13	0.484
miR-425	11.61 [9.01–17.80]	13.14 [10.05–17.94]	13.26 [9.68–17.98]	3.13	**0.048**	1.08	0.246
miR-628-3p	10.77 [8.01–13.37]	12.09 [10.13–17.03]	11.46 [9.26–16.94]	1.61	**0.046**	0.65	0.181
miR-766	8.65 [6.11–11.04]	10.23 [7.87–13.37]	9.98 [7.38–13.28]	2.53	**0.004**	0.84	0.487

Note: Bold values denote statistical significance at the $P < 0.05$ level.
Abbreviations: C_t, threshold cycle; ΔC_t values (C_t target − C_t reference gene); FC, fold change; IQR, interquartile range.

Table 6. *Post hoc* analysis comparing miRNA expression between early-stage cases (according to smoking status) and controls.

miRNA	ΔC_t smoker cases, median [IQR]	ΔC_t nonsmoker cases, median [IQR]	ΔC_t controls, median [IQR]	FC, smoker cases vs. controls	*P*, smoker cases vs. controls	FC, nonsmoker cases vs. controls	*P*, nonsmoker cases vs. controls
miR-145	10.16 [9.45–11.68]	10.46 [8.65–13.37]	10.83 [8.54–13.24]	1.59	0.117	1.29	0.282
miR-151-3p	5.86 [5.03–6.86]	7.00 [4.52–8.34]	7.68 [5.75–9.64]	3.52	**0.024**	1.60	**0.025**
miR-191	17.80 [12.62–19.25]	15.51 [11.47–18.00]	17.43 [11.68–18.56]	0.77	0.344	3.80	0.160
miR-409-3p	7.99 [6.20–9.47]	9.54 [7.59–12.14]	9.30 [6.99–11.82]	2.48	0.088	0.85	0.372
miR-425	11.90 [9.01–17.80]	14.12 [9.47–18.26]	13.26 [9.68–17.98]	2.58	0.195	0.55	0.375
miR-628-3p	10.99 [9.18–14.22]	12.36 [9.03–16.30]	11.46 [9.26–16.94]	1.38	0.361	0.54	0.411
miR-766	10.77 [7.66–17.94]	9.92 [7.18–14.82]	9.98 [7.38–13.28]	0.58	0.423	1,05	0.392

Note: Bold values denote statistical significance at the $P < 0.05$ level.
Abbreviations: C_t, threshold cycle; ΔC_t values (C_t target − C_t reference gene); FC, fold change; IQR, interquartile range.

with squamous HNC (32). Further studies are thus necessary to better elucidate our findings.

The other differentially expressed miRNAs were specific of smoker patients. To our knowledge, very few information is available regarding the role of miR-409-3p in cancer (33), while, no association has been identified with HNC. Several studies have reported the upregulation of miR-425 in different cancer types, such as non–small cell lung, colorectal, or breast cancers: to our knowledge, this is instead the first report for HNC (34, 35). The role of miR-628 is still debated in the literature: while acting as a tumor suppressor in some models (36), the overexpression of this miRNA has been linked with higher aggressivity in some cancer types (37). Similarly, also miR-766 promotes cancer

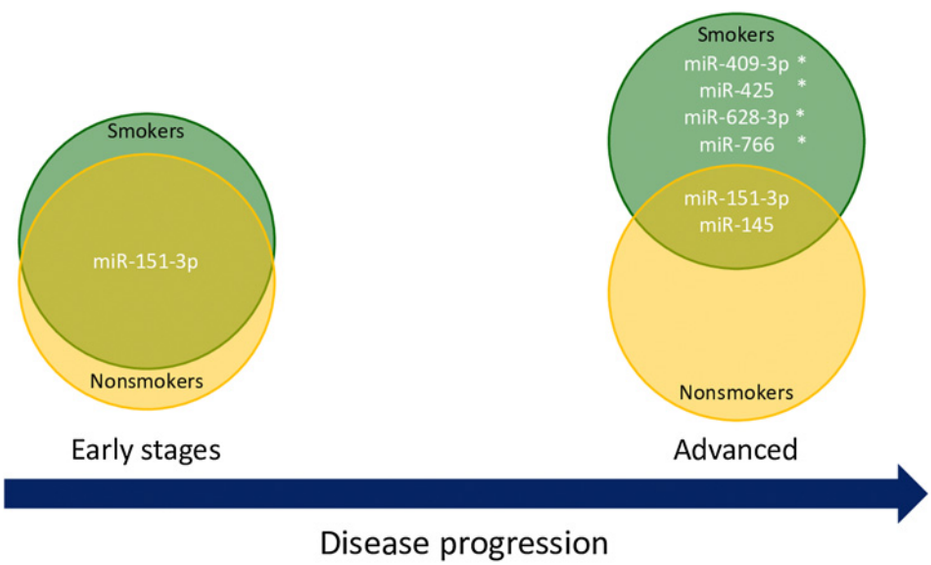

Figure 1.
miRNAs and disease progression. Distribution of identified miRNAs among early-stage and advanced cancer cases. *, Significant in the screening cohort only.

progression in several models, likely by the accumulation of P53 (38). To the best of our knowledge, for the last two miRNAs there is no evidence on their association with HNC.

In conclusion, we report novel miRNAs as potential biomarkers for HNC. In particular, the most promising finding is miR-151-3p, which appears to be an early marker of disease; additionally, the longitudinal evaluation for prognostic purposes of the other miRNAs could be more advisable for smoker patients (miR-145, miR-409-3p, miR-425, miR-628, miR-766).

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

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Note

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