

HEAD AND NECK

# A miRNA signature suggestive of nodal metastases from laryngeal carcinoma

## *miRNA signature predittivo di metastasi linfonodali da carcinoma della laringe*

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### SUMMARY

The discovery that miRNAs are frequently deregulated in tumours offers the opportunity to identify them as prognostic and diagnostic markers. The aim of this multicentric study is to identify a miRNA expression profile specific for laryngeal cancer. The secondary endpoint was to identify specific deregulated miRNAs with potential as prognostic biomarkers for tumour spread and nodal involvement, and specifically to search for a miRNA pattern pathognomonic for N+ laryngeal cancer and for N- tissues. We identified 20 miRNAs specific for laryngeal cancer and a tissue-specific miRNA signature that is predictive of lymph node metastases in laryngeal carcinoma characterised by 11 miRNAs, seven of which are overexpressed (upregulated) and four downregulated. These results allow the identification of a group of potential specific tumour biomarkers for laryngeal carcinoma that can be used to improve its diagnosis, particularly in early stages, as well as its prognosis.

KEY WORDS: Laryngeal cancer • miRNA • Nodal metastasis • Expression profile of miRNA • Prognostic factor

### RIASSUNTO

*La scoperta che i microRNA sono frequentemente deregolati nei tumori consente di utilizzarli come marker prognostici e diagnostici. Lo scopo di questo studio multicentrico è stato stilare un profilo di espressione di miRNA specifico per il carcinoma della laringe. L'obiettivo secondario è stato identificare particolari miRNA deregolati da usare come potenziali biomarker predittivi di diffusione tumorale e di coinvolgimento linfonodale, nello specifico è stato ricercare un pattern di miRNA patognomonic per tessuto di carcinoma della laringe N+ e per N- rispettivamente. Gli Autori hanno identificato venti miRNA specifici per carcinoma della laringe ed inoltre una miRNA signature tessuto-specifica predittiva di metastasi linfonodali da carcinoma della laringe caratterizzata da 11 miRNA, sette dei quali over-espressi (up-regolati) e quattro down-regolati. Questi risultati permettono l'identificazione di un gruppo di potenziali biomarker tumore-specifici per il carcinoma della laringe che potrebbe essere usata per migliorare la sua diagnosi, in particolare negli stadi iniziali, e soprattutto per la sua prognosi.*

PAROLE CHIAVE: Carcinoma della laringe • miRNA • Metastasi linfonodali • Profilo di espressione dei miRNA • Fattori prognostici

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### Introduction

Laryngeal squamocellular carcinoma (LSCC) accounts for approximately 2% of all tumours<sup>1</sup>, with an incidence of 39,900 new cases per 100,000 people in 2012 and a male-female ratio of 8.8:0.8 (9:0.7 in Italy)<sup>2</sup>. It is considered the second most frequent neoplasia of the respiratory system after lung cancer.

Mortality estimated for LSCC was 19,800 cases per 100,000 in 2012 in Europe, with a male-female ratio of 4.3:0.3 (3.3:0.3 in Italy)<sup>2</sup>.

Specific survival for larynx tumour is conditioned by many prognostic factors; the presence of cervical nodal

metastasis represents the single most important prognostic factor<sup>3,4</sup>. The 2-year overall survival of pN+s patients is reduced by 40-50% (88.01% in the pN0 vs. 41.54% in pN+)<sup>4</sup>.

At present, there are no valid prognostic factors that can systematically drive the choice of nodal treatment in laryngeal carcinoma<sup>5</sup>.

It is a consensus opinion in the literature that biomolecular markers can fill this deficiency<sup>6,9</sup>, especially considering the high potential of the studies on miRNAs.

miRNAs are small non-coding RNAs that regulate post-transcriptional gene expression through mechanisms of degradation of the messenger (only in vegetables and

bugs) or simple sequestration with inhibition of translation (the mechanism present in humans)<sup>10,11</sup>.

An important feature of miRNAs is their ability to take part simultaneously in different pathways through the contemporary interaction with multiple messenger targets. Currently there are many studies that show the key role of miRNAs in the genesis, progression and metastatic ability of tumours<sup>12,14</sup>.

Different miRNAs are implicated in tumorigenesis by acting through oncogenes or through tumor-suppressor genes, therefore their expression in tumoural tissues, in comparison to healthy tissue, can reveal under- or over-expression<sup>15</sup>.

The discovery that miRNAs are frequently deregulated in tumours offers the opportunity to identify them as prognostic and diagnostic markers.

The aim of this multicentric study was to identify a miRNA expression profile specific for laryngeal cancer. The secondary endpoint was to identify specific deregulated miRNAs with potential as prognostic biomarkers of tumour spread and nodal involvement, and specifically a miRNA pattern pathognomonic for N+ laryngeal cancer and for N- tissues.

## Materials and methods

### *Patient enrollment*

This study included 24 patients suffering from laryngeal carcinoma, 22 males and 2 females, with an average age of 60 years (39-77). All the patients came from Campania and were treated for a laryngeal tumour at the Complex Operative Unit (COU) of Otorhinolaryngology of the University Hospital Policlinico “Federico II”, at the COU of Otorhinolaryngology and Cervico-facial Surgery of the Hills Specialist Hospital Monaldi-Cotugno-CTO and at the COU of Otorhinolaryngology of the “A. Cardarelli” National Relief Hospital from January to June 2014. All patients underwent the following diagnostic procedures:

1. Laryngeal endoscopy.
2. Computerised tomography (CT) of the neck and chest with and without contrast.
3. Multiple laryngeal biopsies with histological examination.

The TNM classification was applied in all cases according to the 2010 AJCC criteria. All patients were submitted to “open” laryngeal surgery and bilateral nodal cervical emptying. In all cases a sample of approximately 1 cm x 0.5 cm was withdrawn both from healthy tissue and macroscopically tumoral tissue from the removed larynx, which was immediately introduced into RNA later<sup>®</sup> tubes. The same patients were submitted to blood draw, and serum was subsequently cryopreserved at -80°C.

The study was approved by the respective Ethics Committees.

### *Extraction of miRNAs*

The RNA was drawn out using the mirVana PARIS kit (Ambion) according to the protocol described by the supplier. A 0.5 mg sample of tumour tissue and the same quantity of healthy tissue were used. The concentration of the RNA was determined using a Nano Drop spectrophotometer by nanodrop reading.

### *Expression profile of miRNAs*

The miRNA expression profile was determined using the TaqMan Array Card Type A (Life Technologies) according to the protocol Megaplex pool A. Experiments were performed on a thermocycler Viia7 (Life Technologies, Inc.), and the relative expression was computed by using the  $2^{-\Delta\Delta CT}$  formula and normalised using the endogenous U6. For the determination of miRNAs, we used standard cards that allow assessment of 382 different miRNAs of known function. The cards were provided by the manufacturer and used following the manufacturer’s instructions (Life Technologies, Inc.).

### *Analysis of miRNA expression profile in laryngeal tumoural tissues*

The RNA extracted from patients’ samples was assembled into two pools, the first including patients with stage T3 and T4 tumours and nodal involvement (N+) and the second comprising patients with stage T3 and T4 tumours without nodal involvement (N-). The control pool consisted of RNA extracted from healthy biopsy tissue taken from the same patients enrolled for pools N+ and N-.

## Results

All patients were submitted to “open” surgery of the larynx (2 OSL, 3 CHEPs, 19 total laryngectomies).

In all patients, histological examination led to a diagnosis of squamous cell carcinoma. After histological examination, patients were classified according to both the TNM and histological grading as detailed in Table I.

The pTNM scores of the 24 treated patients and grading were as follow:

- 17 pT3, 7 pT4;
- 12 pN0, 3 pN1, 7 pN2, 2 pN3;
- 11 G2, 12 G3, 1 G4.

All patients included in the study of miRNA expression were selected with homogeneous characteristics regarding both T (pT3 and pT4) and grading. These 24 patients were then divided into two homogenous groups with respect to age, T stage and histological grade on the basis of lymph node involvement found in histologi-

cal examination: 12 patients were pN+ and 12 patients pN-, respectively.

The characteristics of patients based upon the degree of T and presence of lymph node involvement in selected patients were:

- 9 patients pT3N0;
- 8 patients pT3N+;
- 3 patients pT4N0;
- 4 patients pT4N+.

The miRNAs extracted from the 24 selected patients were analysed, and the results of differential expression of miRNAs are described below and shown in Tables II to VI.

Expression analysis showed that normal tissues expressed 180/382 miRNAs, the N- pool expressed 207/382 miRNAs and the N+ pool expressed 200/382 miRNAs.

Comparative analysis between the N+ and N- pools and the control pool showed that in both groups of patients, 89 miRNAs were overexpressed compared to normal tissue counterparts, and are collected in three groups in Table II on the basis of their relative expression; 17 miRNA were downregulated, and are shown in Table III.

Analyzing the N+ and N- pools separately and comparing them to healthy control tissues from the same patient, it is

**Table II.** miRNAs overexpressed in tumour tissues in comparison with healthy tissues in patients with LSCC.

miRNAs with fold change >10	miRNAs with 5 < fold change < 10	miRNAs with 2 < fold change < 5
hsa-miR-106b	hsa-let-7d	hsa-let-7a-
hsa-miR-10b	hsa-miR-101	hsa-let-7e-
hsa-miR-130b	hsa-miR-103	hsa-let-7 g-
hsa-miR-15b	hsa-miR-106a	hsa-miR-10a-
hsa-miR-185	hsa-miR-135b	hsa-miR-125b-
hsa-miR-19a	hsa-miR-141	hsa-miR-127-
hsa-miR-205	hsa-miR-142-5p	hsa-miR-130a-
hsa-miR-20a	hsa-miR-15a	hsa-miR-132-
hsa-miR-21	hsa-miR-17	hsa-miR-138
hsa-miR-221	hsa-miR-181a	hsa-miR-148a
hsa-miR-25	hsa-miR-182	hsa-miR-149
hsa-miR-299-5p	hsa-miR-193a-5p	hsa-miR-152
hsa-miR-455	hsa-miR-19b	hsa-miR-155
hsa-miR-494	hsa-miR-210	hsa-miR-192
hsa-miR-511	hsa-miR-223	hsa-miR-194
hsa-miR-598	hsa-miR-23b	hsa-miR-199a-3p
hsa-miR-708	hsa-miR-27a	hsa-miR-200a
hsa-miR-9	hsa-miR-27b	hsa-miR-200b
	hsa-miR-340	hsa-miR-203
	hsa-miR-34a	hsa-miR-24
	hsa-miR-429	hsa-miR-26b
	hsa-miR-532	hsa-miR-28-3p
	hsa-miR-655	hsa-miR-29a
	hsa-miR-660	hsa-miR-29b
	hsa-miR-886-3p	hsa-miR-29c
	hsa-miR-92a	hsa-miR-301b
	hsa-miR-99b	hsa-miR-30b
		hsa-miR-30c
		hsa-miR-32
		hsa-miR-324-5p
		hsa-miR-331
		hsa-miR-335
		hsa-miR-337-5p
		hsa-miR-374
		hsa-miR-422a
		hsa-miR-425-5p
		hsa-miR-454
		hsa-miR-483-5p
		hsa-miR-508
		hsa-miR-532-3p
		hsa-miR-590-5p
		hsa-miR-744
		hsa-miR-758
		hsa-miR-99a

**Table I.** p TNM, Grading and laryngectomy type of the 24 patients enrolled in the study.

Number	pTNM	Grading	Laryngectomy type
1	T3N2	G3	Total
2	T4N2	G2	Total
3	T3N3	G2	Total
4	T4N2	G3	Total
5	T3N0	G2	Total
6	T4N0	G2	Total
7	T3N0	G2	Total
8	T3N2	G3	Osl
9	T3N0	G3	Chep
10	T3N1	G3	Total
11	T4N0	G3	Total
12	T4N1	G2	Total
13	T3N0	G3	Total
14	T4N3	G3	Total
15	T3N1	G4	Total
16	T3N0	G3	Total
17	T3N2	G2	Chep
18	T3N2	G3	Osl
19	T3N0	G2	Total
20	T3N2	G2	Total
21	T3N0	G3	Total
22	T4N0	G3	Total
23	T3N0	G2	Total
24	T3N0	G2	Chep

**Table III.** miRNAs downregulated in tumor tissues in comparison with healthy tissues of patients with LSCC.

miRNA	N- fold change	N+ fold change
hsa-miR-1	0.072	0.040
hsa-miR-126	0.528	0.592
hsa-miR-133a	0.016	0.009
hsa-miR-133b	0.118	0.046
hsa-miR-139-5p	0.210	0.354
hsa-miR-140-3p	0.378	0.333
hsa-miR-186	0.857	0.497
hsa-miR-204	0.514	0.507
hsa-miR-375	0.175	0.742
hsa-miR-449	0.125	0.013
hsa-miR-449b	0.445	0.139
hsa-miR-486	0.403	0.450
hsa-miR-489	0.588	0.605
hsa-miR-539	0.195	0.154
hsa-miR-574-3p	0.705	0.385
hsa-miR-628-5p	0.549	0.440
hsa-miR-885-5p	0.222	0.130

**Table IV.** Twelve miRNAs with different expression between the two groups of patients (N-, N+). miRNAs overexpressed are in red, miRNAs downregulated compared to healthy control tissue of patients with LSCC are in blue.

miRNA	N- fold change	N+ fold change
hsa-let-7b	1.391	2.437
hsa-miR-135a	0.631	3.538
hsa-miR-20b	1.467	3.981
hsa-miR-212	0.147	0.756
hsa-miR-324-3p	1.375	2.476
hsa-miR-328	1.482	0.519
hsa-miR-365	3.353	1.352
hsa-miR-376a	1.338	0.586
hsa-miR-493	1.986	0.539
hsa-miR-500	2.771	1.297
hsa-miR-642	0.452	1.375
hsa-miR-886-5p	1.221	3.049

**Table V.** miRNAs expressed only in pathological tissue and not in control healthy tissue from the same patients.

hsa-miR-181c	hsa-miR-509 5p
hsa-miR-183	hsa-miR-512 3p
hsa-miR-18a	hsa-miR-517a
hsa-miR-22	hsa-miR-517c
hsa-miR-331 5p	hsa-miR-523
hsa-miR-362 3p	hsa-miR-548c 5p
hsa-miR-363	hsa-miR-570
hsa-miR-424	hsa-miR-576 3p
hsa-miR-455 3p	hsa-miR-579
hsa-miR-502 3p	hsa-miR-583 3p

evident that 12 miRNAs were differentially expressed in the two groups of patients and compared to healthy control tissue (Table IV). In particular, 4 miRNAs were overexpressed in N+ patients with respect to both the N- and healthy tissues, 3 miRNAs were downregulated in N+ patients compared to both the N- and healthy tissues, 2 were overexpressed in N- patients with respect to both the N+ and healthy tissues, 2 miRNAs were downregulated in N- patients compared to both N+ and healthy tissues, 1 was overexpressed in N+ patients compared to healthy tissues and downregulated in N- patients compared to the healthy tissue of 24 selected patients with LSCC.

Twenty miRNAs were expressed only in the two groups of patients (N+ and N-) and not in healthy control tissues from the same patients (Table V). Therefore, these miRNAs are expressed only in tumour tissues.

Fifteen miRNAs were expressed only in the N+ group, and 23 miRNAs were expressed only in the N- group (Table VI).

**Table VI.** Twenty-three miRNAs expressed only in the N- group, 15 miRNAs expressed only in the N+ group. Red: overexpression with respect to healthy control tissue from the patients with LSCC; blue: downregulation with respect to healthy control tissues from the patients with LSCC; n.e.c.: no expression change.

N-	Fold change	N+	Fold change
hsa-miR-146b-3p	1879	hsa-miR-190	0787
hsa-miR-148b	2455	hsa-miR-486-3p	0047
hsa-miR-338-3p	1043	hsa-miR-542-5p	2795
hsa-miR-339-5p	0359	hsa-miR-618	13980
hsa-miR-485-3p	2172	hsa-miR-198	n.e.c.
hsa-miR-518b	0829	hsa-miR-342 5p	n.e.c.
hsa-miR-518f	0509	hsa-miR-369 3p	n.e.c.
hsa-miR-627	0827	hsa-miR-373	n.e.c.
hsa-miR-216b	n.e.c.	hsa-miR-433	n.e.c.
hsa-miR-296	n.e.c.	hsa-miR-450b 5p	n.e.c.
hsa-miR-323 3p	n.e.c.	hsa-miR-487b	n.e.c.
hsa-miR-372	n.e.c.	hsa-miR-545	n.e.c.
hsa-miR-382	n.e.c.	hsa-miR-597	n.e.c.
hsa-miR-503	n.e.c.	hsa-miR-876 3p	n.e.c.
hsa-miR-518c	n.e.c.	hsa-miR-876 5p	n.e.c.
hsa-miR-529a	n.e.c.		
hsa-miR-522	n.e.c.		
hsa-miR-548d	n.e.c.		
hsa-miR-582 5p	n.e.c.		
hsa-miR-636	n.e.c.		
hsa-miR-651	n.e.c.		
hsa-miR-873	n.e.c.		
hsa-miR-137	n.e.c.		

## Discussion

Laryngeal tumours identical in site, subsite and clinical stage, and subjected to the same treatment may have different clinical outcomes and prognosis, especially when considering nodal spreading.

In this study, we analysed the expression of miRNAs in tissues resulting from carcinomas of the larynx to identify a tissue-specific miRNA signature predictive of unfavourable development toward lymph node metastases.

Even if the population under study is very limited in number, the results are of considerable interest. The comparative data show that the miRNA expression profiles in pathological tissues compared to healthy tissues exhibit a clear majority of overexpressed miRNAs with only a few hypoexpressed miRNAs.

Some of the miRNAs overexpressed in the diseased tissues have already been described in the literature, also in relation to cancer of the larynx:

- **miR19a:** Marioni et al., recently, have demonstrated its higher expression in malignant glottis lesions than in benign conditions<sup>16</sup>. It was previously correlated with neck nodal metastasis, poor differentiation and advanced stage when overexpressed<sup>17</sup>.
- **miR 27a:** it has been shown that miR27a promotes proliferation and suppresses apoptosis<sup>18 19</sup>.
- **miR 155:** the expression of tissue and plasma miR155 is significantly upregulated in patients with LSCC<sup>20</sup>; furthermore, it seems to play a role in development of LSCC in terms of promotion of proliferation and invasion<sup>21</sup>.
- **miR 21:** some authors have described its overexpression in laryngeal cancer tissues<sup>22 23</sup>, and its ratio with miR375 (miR21/miR375) has been related with worse prognosis if high<sup>24 25</sup>; and its high expression in serum is associated with nodal metastasis in LSCC<sup>26</sup>. Recently, miR21 was shown to be deregulated by acidic bile and implicated in precancerous lesion of laryngeal mucosa<sup>27</sup>.
- **miR 106b:** it was found to be upregulated in LSCC tissues, together with miR21, and their level were found to be increased in poorly/moderately differentiated (G2-G3) cancer tissues and associated with lymph node metastasis<sup>28</sup>.
- **miR 375:** according to Wu et al., increased expression of miR375 is associated with a more aggressive phenotype of LSCC; moreover, a high-level expression of miR375 and miR148a in patients with laryngeal dysplasia may predict malignant transformation<sup>29</sup>. In our study, it was downregulated in tumour tissues in agreement with Hu<sup>25</sup>.
- **miR 708:** it is upregulated in tumour tissues as is miR21 and miR205<sup>30</sup> according to our data.
- **miR 205:** it is upregulated in tumour tissues (as in our study), and in addition it significantly induces cell proliferation and invasion by suppressing CDK2AP1<sup>31</sup>.
- **miR 221:** Yilmaz demonstrated that it is upregulated in LSCC plasma samples, but was at normal levels in postoperative plasma; he proposed it as a diagnostic marker of LSCC<sup>32</sup>.

Among the overexpressed miRNAs (fold change between 2 and 5) in tumour tissues, some have described to be down-regulated in patients with LSCC.

- **miR 203:** according to Tian et al., its lower expression is related to poor differentiation, advanced clinical stage, lymph node involvement and decreased 5-year overall survival<sup>33</sup>. Recently, it has been shown to correlate with local disease recurrence after radiotherapy in a series of patients with laryngeal cancer<sup>34</sup>.
- **miR 152:** it was described as significantly downregulated in supraglottic laryngeal carcinoma tissues and its expression was correlated with p T and p N stages in patients with supraglottic LSCC<sup>35</sup>.
- **miR 24:** its upregulation, similar to miR27a, leads to promotion of proliferation and early apoptosis inhibition in LSCC<sup>18</sup>; according to Xu et al., miR24 expression is significantly lower in LSCC cell lines and it inhibits growth-related apoptosis and enhances radiosensitivity in LSCC<sup>36</sup>.

Of considerable interest are miRNAs detected in our study and not yet associated with cancer of the larynx, even though they have been previously associated with other tumours. This is the case of six miRNAs: **mir9**<sup>37</sup>, **mir511**<sup>38</sup>, **mir494**<sup>39</sup>, **mir25**<sup>40</sup>, **mir20**<sup>41</sup>, and **mir10b**<sup>42</sup>, which are greatly overexpressed in several tumour tissues compared to healthy control tissues from the same patients.

Interestingly, we identified some miRNAs with specific expression in either N+ or N- cases.

The analysis of the N+ group detected the following miRNAs:

- **miR618:** strongly overexpressed in our study in N+ patients; it is considered by Hui to be a prognostic factor for HNSCC (head and neck squamous cell carcinoma)<sup>43</sup>. It has been also correlated with thyroid cancer<sup>44</sup>.
- **miR 542-5p:** overexpressed in tumour tissues in our series of N+ patients, it was previously reported in rhabdomyosarcoma<sup>45</sup> and osteosarcoma<sup>46</sup>.
- **miR 486-3p:** downregulated in tumour tissues of patients with nodal metastases compared to healthy control tissues from the same patients, its decreased level has been associated with metastasis in cervical cancer patients<sup>47</sup>.
- **miR 135 a:** on the basis of our data, this miRNA is overexpressed in tumour tissues of N+ patients compared to healthy control tissues and downregulated in tumour tissues of patients without lymph node involvement compared to healthy tissue from the same patients. Its high expression in gastric cancer tissues is more likely to have aggressive characteristics, among which lymphatic metastasis<sup>48</sup>.
- **miR 20b:** overexpressed in tumour tissues of N+

patients, it was associated with laryngeal cancer in 2010<sup>49</sup>; its upregulation promotes proliferation, migration and invasiveness in oesophageal tumours<sup>50</sup>.

- **miR 324-3p:** overexpressed in tumour tissues of N+ patients, it is upregulated in plasma of stage I of lung squamous cell carcinoma compared to healthy controls<sup>51</sup>; furthermore, its low expression might be an important marker for prediction of low response to RT/CRT and poor overall survival and recurrence-free survival<sup>52</sup>.

Analyzing the 12 N- patients, the most interesting miRNAs for their biological functions are the following:

- **miR 148b:** overexpressed in the diseased tissue of pN-patients, it has been linked with melanoma<sup>53</sup>.
- **miR 339-5p:** downregulated in tumour tissues of pN-patients, it has been described as a regulator of breast cancer progression<sup>54</sup>.
- **miR 485-3p:** overexpressed in the diseased tissue of patients without lymph node metastasis, it is described as a suppressor of breast cancer metastasis<sup>55</sup>.
- **miR 518f:** downregulated in tumour tissues of pN-patients compared to control tissue, it is related to endometrial cancer in which it is downregulated<sup>56</sup>.

Through analysis of these results, we may define a tissue-specific miRNA signature that is predictive of lymph node metastases in laryngeal carcinoma characterised by 11 miRNAs, seven of which are overexpressed (upregulated) and four downregulated, in particular: miR618, miR542-5p, let 7b, miR135a, miR20b, miR324-3p, and miR886-5p are overexpressed; and miR486-3p, miR328, miR376a and miR493 are downregulated. This signature is suggestive to be predictive of lymph node involvement even if the validation of these results on a wider series of patients is strongly warranted.

## Conclusions

We have identified a group of miRNAs with characteristic expression profiles in diseased tissues compared to matched healthy tissue from the same patients; in addition, we have highlighted a miRNA pattern specific of N+ laryngeal cancer cases compared to N- cases and healthy tissues. Furthermore, the authors have detected a miRNA pattern expressed specifically in laryngeal cancer tissues (and not in healthy tissues), one expressed exclusively in laryngeal cancer with N+ and another one present in N-. These results are largely innovative, at least in our opinion, and allow the identification of a group of potentially specific tumour biomarkers for laryngeal carcinoma that can be used to improve its diagnosis, particularly at early stages, and to detect patients with minimal residual disease or recurrence if the miRNA pattern specific of laryngeal cancer is present; but, overall, they can be useful to predict prognosis atient in early stages on the basis of the identification of the miRNAs signature suggestive for nodal involvement. In this case, the miRNAs could lead to tailored treatment.

The technologies of molecular biology are not yet available in all centres, so that the use of miRNA profiling with microarray techniques on large scale in diagnosis of laryngeal carcinoma is not readily possible. However, the methods of real-time PCR are presently relatively cheap and easy to perform. The bottleneck in this type of study is, in fact, the identification of differentially expressed miRNAs through the use of low-density arrays (as in our case) and their subsequent validation in a large population of patients. Once validated, the miRNA biomarkers are easy to detect in the tissue of patients with cancer and other neoplasms. Another advantage of miRNAs is their presence in all body fluids, and in particular in plasma and serum of patients, in which they can be easily detected and quantified<sup>57</sup>. A further phase of the present study is, in fact, the determination of an array of circulating miRNA in serum from the same patients, which will be determined and cross-referenced with those obtained in tissues of the same patients. In this way, we can outline a limited group of very reliable miRNAs that can be validated (or not) together with a “portfolio of prognostic factors” (clinical and pathological) for routine use in clinical evaluation.

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