A miRNA signature suggestive of nodal metastases from laryngeal carcinoma

F. RICCIARDIELLO1, R. CAPASSO2, H. KAWASAKI2,3, T. ABATE4, F. OLIVA1, A. LOMBARDI1, G. MISSO2, D. INGROSSO2, C.A. LEONE5, M. IENGO1, M. CARAGLIA2

1 Ear Nose and Throat Unit, Cardarelli Hospital, Naples, Italy; 2 Department of Biochemistry, Biophysics and General Pathology, Second University of Naples, Naples, Italy; 3 Drug Discovery Laboratory, Wakunaga Pharmaceutical Co., Ltd., Akitakata, Hiroshima, Japan; 4 Ear Nose and Throat Unit, University of Naples Federico II, Naples, Italy; 5 Ear Nose and Throat Unit and Neck Surgery, Monaldi Hospital, Naples, Italy

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 stabilire 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 patognomonico per N+ laryngeal cancer and for N- tissues. 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

Introduction

Laryngeal squamouscellular carcinoma (LSCC) accounts for approximately 2% of all tumours 1, 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) 2. 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) 2. Specific survival for larynx tumour is conditioned by many prognostic factors; the presence of cervical nodal metastasis represents the single most important prognostic factor 3,4. The 2-year overall survival of pN+ patients is reduced by 40-50% (88.01% in the pN0 vs. 41.54% in pN+) 4.

At present, there are no valid prognostic factors that can systematically drive the choice of nodal treatment in laryngeal carcinoma 3. It is a consensus opinion in the literature that biomolecular markers can fill this deficiency 5,6, 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)\textsuperscript{10,11}.

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\textsuperscript{12,14}.

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\textsuperscript{15}.

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” surgery of the larynx (2 OSL, 3 CHEPs, 19 total laryngectomies).

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 $2^{-\Delta \Delta C_{T}}$ 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-
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

| 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 | G2      | 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              |
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 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 from 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.

| miRNA        | 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 |

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-450-5p | 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.      |                |             |

470
Expression profile of miRNAs suggestive for nodal metastasis in laryngeal cancer

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. It was previously correlated with neck nodal metastasis, poor differentiation and advanced stage when overexpressed.

- **miR 27a**: it has been shown that miR27a promotes proliferation and suppresses apoptosis.

- **miR 155**: the expression of tissue and plasma miR155 is significantly upregulated in patients with LSCC, and it was previously reported in precancerous lesions of laryngeal mucosa.

- **miR 21**: some authors have described its overexpression in laryngeal cancer tissues, and its ratio with miR375 (miR21/miR375) has been related with worse prognosis if high, and its high expression in serum is associated with nodal metastasis in LSCC. Recently, miR21 was shown to be deregulated by acidic bile and implicated in precancerous lesion of laryngeal mucosa.

- **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.

- **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. In our study, it was downregulated in tumour tissues in agreement with Hu.

- **miR 708**: it is upregulated in tumour tissues as is miR21 and miR205 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.

- **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.

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. Recently, it has been shown to correlate with local disease recurrence after radiotherapy in a series of patients with laryngeal cancer.

- **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.

- **miR 24**: its upregulation, similar to miR27a, leads to promotion of proliferation and early apoptosis inhibition in LSCC; 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.

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, miR511, miR494, miR25, miR20, and miR10b, 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).

- **miR 542-5p**: overexpressed in tumour tissues in our series of N+ patients, it was previously reported in rhabdomyosarcoma and osteosarcoma.

- **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.

- **miR 135a**: 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.

- **miR 20b**: overexpressed in tumour tissues of N+...
patients, it was associated with laryngeal cancer in 2010 49; its upregulation promotes proliferation, migration and invasiveness in oesophageal tumours 50.

- **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 51; 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 52.

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 53.
- **miR 339-5p**: downregulated in tumour tissues of pN- patients, it has been described as a regulator of breast cancer progression 54.
- **miR 485-3p**: overexpressed in the diseased tissue of patients without lymph node metastasis, it is described as a suppressor of breast cancer metastasis 55.
- **miR 518f**: downregulated in tumour tissues of pN- patients compared to control tissue, it is related to endometrial cancer in which it is downregulated specific of N+ laryngeal cancer cases compared to N- cases and healthy tissues. Furthermore, the authors have detected a miRNA pattern specific in laryngeal cancer tissues (and not in healthy tissues), one expressed exclusively in laryngeal cancer, 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.

**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 at an early stage 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 57. 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.

**Acknowledgments**

M. C. received funding from MIUR for a project (FIRB-PROGRAM AGREEMENTS 2011) entitled: “Application of high-throughput technology platforms for the characterization of new biomarkers and molecular targets in nanovectors for the diagnosis and treatment of human cancer.” M. C. has also received funding from the Campania Region with a project entitled “Public Laboratories Project Hauteville”.

**References**

1. Succo G, Crosetti E, Bertolin A, et al. Benefits and drawbacks of open partial horizontal laryngectomies. Part A: early-intermediate stage glottic carcinoma. Head Neck 2016;38 Suppl 1:E333-40.

2. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer 2013;49:1374-403.

3. Spriano G, Manciocco V, Marchesi P, et al. Il trattamento dell’N nel carcinoma della laringe. Attualità in Oncologia Laringea 2010;553-74.

4. Barroso Ribeiro R, Ribeiro Breda E, Fernandes Monteiro E. Prognostic significance of nodal metastasis in advanced tumours of the larynx and hypopharynx. Acta Otorrinolaringol Esp 2012;63:292-8.

5. Spriano G, Piantanida R, Pellini R, et al. Elective treatment of the neck in squamous cell carcinoma of the larynx: clinical experience. Head Neck 2003;25:97-102.

6. Paludetti G, Almadori G, Bussu F, et al. Prognoesi del cancro della laringe. Identificazione dei Marcatori prognostici nel Tumore della Laringe; 2009. p. 63-68.
Expression profile of miRNAs suggestive for nodal metastasis in laryngeal cancer

7 Albera R, Martone T, Cortesina G. Fattori prognostici clinici e molecolari dei carcinomi squamosi del distretto testa-collo (HNSSC). Identificazione dei Marcatori prognostici nel Tumore della Laringe; 2009. p. 81-96.
8 Almadori G, Bussu F, Galli J, et al. Diminished expression of S100A2, a putative tumour suppressor, is an independent predictive factor of neck node relapse in laryngeal squamous cell carcinoma. J Otolaryngol Head Neck Surg 2009;38:16-22.
9 Bolzoni Villaret A, Barbieri D, Peretti G, et al. Angiogenesis and lymphangiogenesis in early-stage laryngeal carcinoma: prognostic implications. Head Neck 2013;35:1132-7.
10 Bartel DP. miRNAs: target recognition and regulatory functions. Cell 2009;136:215-33.
11 Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. Cell 2009;136:642-55.
12 Pencheva N, Tavazoie SF. Control of metastatic progression by miRNA regulatory networks. Nat Cell Biol 2013;15:546-54.
13 Li Y, Ahmad A, Kong D, et al. Targeting miRNAs for personalized cancer therapy. Med Princ Pract 2013;22:415-7.
14 Liu C, Kelkar K, Liu B, et al. The miRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. Nat Med 2011;17:211-5.
15 Calin GA, Croce CM. MiRNA signatures in human cancers. Nat Rev Cancer 2006;6:857-66.
16 Marioni G, Agostini M, Cappellesso R, et al. miR-19 and SOCS-1 expression in the differential diagnosis of laryngeal (glottic) verrucous squamous cell carcinoma. J Clin Pathol 2016;69:415-21.
17 Wu T, Zhang T, Qu L, et al. MiR-19a is correlated with prognosis and apoptosis of laryngeal squamous cell carcinoma by regulating TIMP-2 expression. Int J Clin Exp Pathol 2013;6:56-63.
18 Wang Y, Zhang ZX, Chen S, et al. Methyltheresis of Sp1 sites within miR-23a-27a-24-2 promoter region influences laryngeal cancer cell proliferation and apoptosis. Biomed Res Int 2016;2016:2061248.
19 Tian Y, Fu S, Qui GB, et al. miRNA-27a promotes proliferation and supresses apoptosis by targeting PIK3R3. Tumour Biol 2015;36:4453-9.
20 Wang JL, Wang X, Yang D, et al. The expression of MicroRNA-155 in plasma and tissue is matched in human laryngeal squamous cell carcinoma. Yonsei Med J 2016;57:298-305.
21 Zhao XD, Zhang W, Liang H, et al. Overexpression of miR-155 promotes proliferation and invasion of human laryngeal squamous cell carcinoma via targeting SOCS1 and STAF3. PLoS One 2013;8:e56395.
22 Cybula M, Wieteska L, Józefowicz-Korczyńska M, et al. New miRNA expression abnormalities in laryngeal squamous cell carcinoma. Cancer Biomark 2016;16:559-68.
23 Zhou P, Zeng F, Liu J,et al. Correlation between mir-21 expression and laryngeal carcinoma risks. J Evid Based Med 2015 Dec 12. doi: 10.1111/jebm.12184 [Epub ahead of print].
24 Hu A, Huang J,Xu WH, et al. MiR-21/miR-375 ratio is an independent prognostic factor in patients with laryngeal squamous cell carcinoma. Am J Cancer Res 2015;5:1775-85.
25 Hu A, Huang J,Xu WH, et al. miR-21 and miR-375 microRNAs as candidate diagnostic biomarkers in squamous cell carcinoma of the larynx: association with patient survival. Am J Transl Res 2014;6:604-13.
26 Wang J, Zhou Y, Lu J,et al. Combined detection of serum exosomal miR-21 and HOTAIR as diagnostic and prognostic biomarkers for laryngeal squamous cell carcinoma. Med Oncol 2014;31:148.
27 Sasaki CT, Vageli DP. miR-21, miR-155, miR-192, and miR-375 deregulations related to NF-kappa B activation in gastrointestinal fluid-induced early preneoplastic lesions of laryngeal mucosa in vivo. Neoplasia 2016;18:329-38.
28 Yu X, Wu Y, Liu Y, et al. miR-21-106b and miR-375 as novel potential biomarkers for laryngeal squamous cell carcinoma. Curr Pharm Biotechnol 2014;15:503-8.
29 Wu Y, Yu J, Ma Y, et al. MiR-148a and miR-375 may serve as predictive biomarkers for early diagnosis of laryngeal carcinoma. Oncol Lett 2016;12:871-8.
30 Cao P, Zhou L, Zhang J, et al. Comprehensive expression profiling of microRNAs in laryngeal squamous cell carcinoma. Head Neck 2013;35:720-8.
31 Zhong G, Xiong X. miR-205 promotes proliferation and invasion of squamous cell carcinoma by suppressing CDK2AP1 expression. Biol Res 2015;48:60.
32 Yilmaz SS, Guzel E, Karatas OF, et al. MiR-221 as a pre- and postoperative plasma biomarker for larynx cancer patients. Laryngoscope 2015;125:E377-81.
33 Tian L, Li M, Ge J, et al. MiR-203 is downregulated in laryngeal squamous cell carcinoma and can suppress proliferation and induce apoptosis of tumours. Tumor Biol 2014;35:5953-63.
34 De Jong MC, Ten Hoeve JJ, Grenman R, et al. Pretreatment microRNA expression impact on epithelial-to-mesenchymal transition predicts intrinsic radiosensitivity in head and neck cancer cell lines and patients. Clin Cancer Res 2015;21:5630-8.
35 Song Y, Tian Y, Bai W, et al. Expression and clinical significance of miRNA-152 in supraglottic laryngeal carcinoma. Tumor Biol 2014;35:11075-9.
36 Xu L, Chen Z, Xue F, et al. MicroRNA-24 inhibits growth, induces apoptosis, and reverses radioresistance in laryngeal squamous cell carcinoma by targeting X-linked inhibitor of apoptosis protein. Cancer Cell Int 2015;15:61.
37 Minor J, Wang X, Zhang F, et al. Methylation of miRNA-9 is a specific and sensitive biomarker for oral and oropharyngeal squamous cell carcinomas. Oral Oncol 2012;48:73-8.
38 Cao G, Dong W, Meng X, et al. MiR-511 inhibits growth and metastasis of human hepatocellular carcinoma cells by targeting PIK3R3. Tumour Biol 2015;36:4453-9.
39 Yang YK, Xi WY, Xi RX, et al. MiRNA494 promotes cervical cancer proliferation through the regulation of PTEN. Oncol Rep 2015;33:2393-401.
40 Zhao Z, Liu J, Wang C, et al. MiRNA-25 regulates small cell lung cancer cell development and cell cycle through cyclin E2. Int J Clin Exp Pathol 2014;7:7726-34.
41 Zhang GJ, Li Y, Zhou H, et al. miR20a is an independent prognostic factor in colorectal cancer and is involved in cell metastasis. Mol Med Rep 2014;10:283-91.
42 Wang YF, Li Z, Zhao XH, et al. MiRNA-10b is upregulated and has an invasive role in colorectal cancer through enhanced Rhoc expression. Oncol Rep 2015;33:1275-83.
43 Hui L, Wu H, Yang N, et al. Identification of prognostic microRNA candidates for head and neck squamous cell carcinoma. Oncol Rep 2016;35:3321-30.

44 Yi L, Yuan Y, et al. MicroRNA-618 modulates cell growth via targeting PI3K/Akt pathway in human thyroid carcinomas. Indian J Cancer 2015;52 Suppl 3:E186-9.

45 Yang Z, Tien P. MiR373 and miR542-5p regulate the replication of enterovirus 71 in rhabdomyosarcoma cells. Sheng Wu Gong Cheng Xue Bao 2014;30:943-53.

46 Cheng DD, Yu T, Hu T, et al. MiR-542-5p is a negative prognostic factor and promotes osteosarcoma tumorigenesis by targeting HUWE1. Oncotarget 2015;6:42761-72.

47 Ye H, Yu X, Xia J, et al. MiR-486-3p targeting ECM1 represses cell proliferation and metastasis in cervical cancer. Biomed Pharmacother 2016;80:109-14.

48 Yan LH, Chen ZN, Li-Li, et al. MiR-135a promotes gastric cancer progression and resistance to oxaliplatin. Oncotarget 2016;7:70699-714.

49 Wang P, Fu T, Wang X, et al. Primary, study of miRNA expression patterns in laryngeal carcinoma by microarray. Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi 2010;24:535-8.

50 Wang B, Yang J, Xiao B. MicroRNA-20b (miR-20b) promotes the proliferation, migration, invasion, and tumorigenicity in esophageal cancer cells via the regulation of phosphatase and tensin homologue expression. PLoS One 2016;11:E0164105.

51 Gao X, Wang Y, Zhao H, et al. Plasma miR-342-3p and miR-1285 as diagnostic and prognostic biomarkers for early stage lung squamous cell carcinoma. Oncotarget 2016;7:59664-75.

52 Xu J, Ai Q, Cao H, et al. MiR-185-3p and miR-324-3p predict radiosensitivity of nasopharyngeal carcinoma and modulate cancer cell growth and apoptosis by taergeting SMAD7. Med Sci Monit 2015;2:2828-36.

53 Mirzaei H, Gholamin S, Shahidseales S, et al. MicroRNAs as potential diagnostic and prognostic biomarkers in melanoma. Eur J Cancer 2016;53:25-32.

54 Yan H, Zhao M, Huang S, et al. Prolactin inhibits BCL6 expression in breast cancer cells through a microRNA-339-5p-dependent pathway. J Breast Cancer 2016;19:26-33.

55 Lou C, Xiao M, Cheng S, et al. MiR-485-3p and miR-485-5p suppress breast cancer cell metastasis by inhibiting PGC-1a expression. Cell Death Dis 2016;7:e2159.

56 Dong P, Ihira K, Xiong Y, et al. Reactivation of epigenetically silenced miR-124 reverses the epithelial-to-mesenchymal transition and inhibits invasion in endometrial cancer cells via the direct repression of IQGAP1 expression. Oncotarget 2016;7:20260-70.

57 Mitchell PS, Parkin RK, Kroh EM, et al. Circulating miRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 2008;105:10513-8.