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Expression of *TP53* mutation-associated microRNAs predicts clinical outcome in head and neck squamous cell carcinoma patients

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Background: *TP53* mutation is associated with decreased survival rate in head and neck squamous cell carcinoma (HNSCC) patients. We set out to identify microRNAs (miRNAs) whose expression associates with *TP53* mutation and survival in HNSCC.

Patients and methods: We analyzed *TP53* status by direct sequencing of exons 2 through 11 of a prospective series of 121 HNSCC samples and assessed its association with outcome in 109 followed-up patients. We carried out miRNA expression profiling on 121 HNSCC samples and 66 normal counterparts. miRNA associations with *TP53* mutations and outcome were evaluated.

Results: A *TP53* mutation was present in 58% of the tumors and *TP53* mutations were significantly associated with a shorter recurrence-free survival. This association was stronger in the clinical subgroup of patients subjected to adjuvant therapy after surgery.The expression of 49 miRNAs was significantly associated with *TP53* status. Among these 49, we identified a group of 12 miRNAs whose expression correlates with recurrence-free survival and a group of 4 miRNAs that correlates with cancer-specific survival. The two groups share three miRNAs. Importantly, miRNAs that correlate with survival are independent prognostic factors either when considered individually or as signatures.

Conclusions: miRNAs expression associates with *TP53* status and with reduced survival after surgical treatment of squamous cell carcinoma of the head and neck.

Key words: TP53 mutation, microRNA, HNSCC, clinical outcome

introduction

Head and neck squamous cell carcinomas (HNSCCs) comprise 5.5% of all incidence of cancers and represent the sixth leading cancer worldwide with ~600 000 cases reported annually. Local recurrence affects about 60% of patients and metastases develop in 15%–20% of cases [1]. Advances in the surgical and medical treatments for HNSCC over the past two decades have not improved overall disease outcomes [2].

TP53 is the most frequent target of genetic alterations in human cancers, with a prevalence of 20%–80% in HNSCC [3] (http://www-p53.iarc.fr). The majority of *TP53* mutations resides in the exons 5–8, and they are missense mutations [4], some of which have been reported to have gain-of-function properties, thereby positively contributing to cancer progression [5–7]. A number of studies reported that *TP53* mutations are generally associated with shorter recurrence-free and/or overall survival in HNSCC [8–15].

MicroRNAs (miRNAs) are 22 nucleotides-long double stranded small RNAs, able to modulate gene expression at post-transcriptional level. According to several studies miRNAs expression profile is emerging among the best markers for diagnosis, staging and treatment of cancer, including HNSCC [16, 17]. A correlation between the expression of specific miRNAs, such as miR-375 and -210, and outcome of HNSCC patients was indeed reported [18, 19].

Several studies have demonstrated the implication of wild-type p53 in the regulation of maturation and expression of

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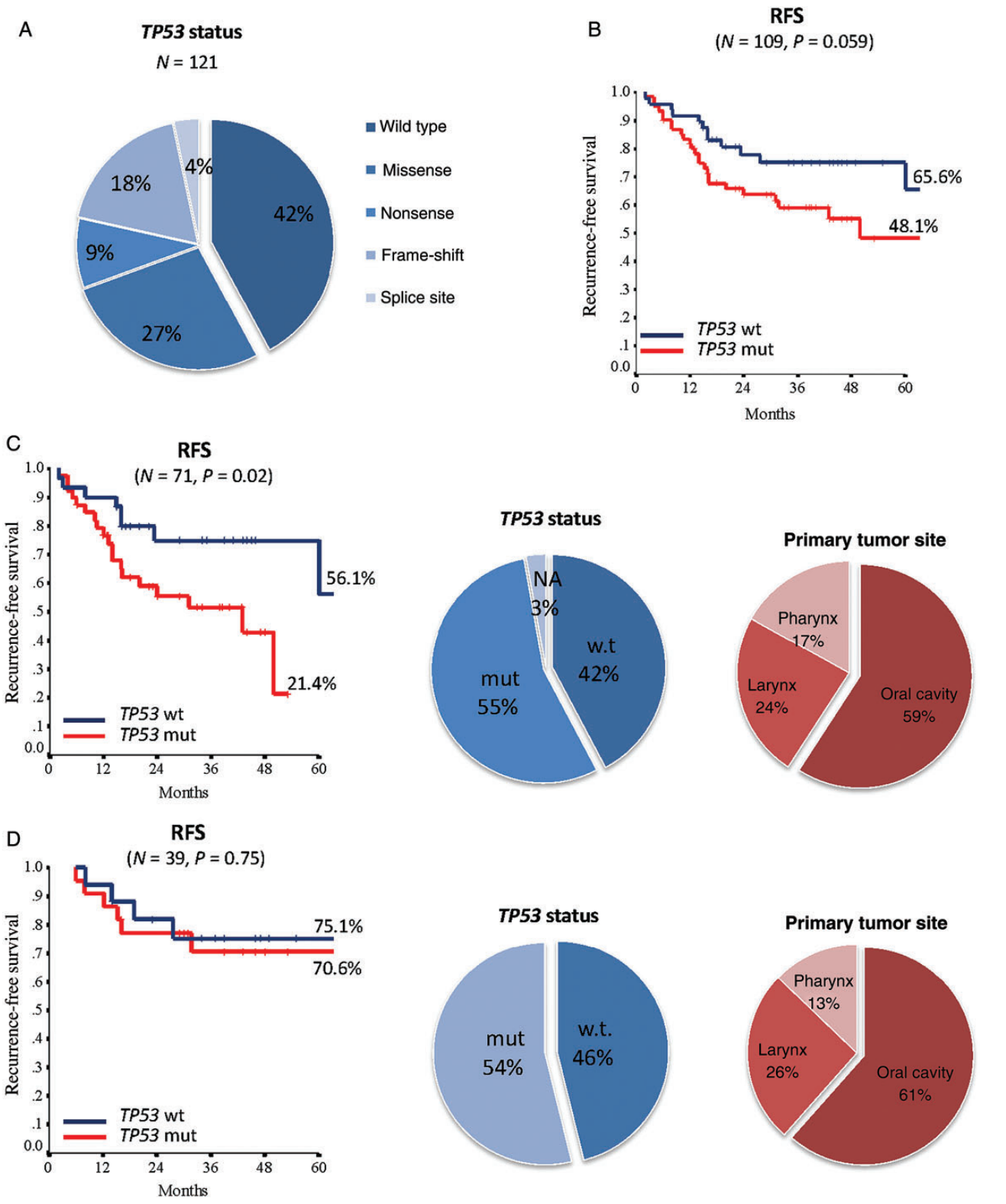


Figure 1. TP53 status correlates with recurrence-free survival. (A) Pie chart representing the distribution of the different TP53 mutation types identified in 121 HNSCC patients. (B) Kaplan–Meier analysis showing the correlation between TP53 status and recurrence-free survival in 109 HNSCC patients. (C and D) On the left, Kaplan–Meier analyses showing the correlation between TP53 status and recurrence-free survival in the clinical subgroups of HNSCC patients subjected (C) or not (D) to adjuvant treatment with radiation or chemoradiation therapy after the surgery. On the right, Pie chart representing the frequency of TP53 mutations and the distribution of primary tumor sites in two clinical subgroups subjected or not to adjuvant therapy.

miRNAs in cancer [20], and we recently reported that also mutant p53 controls the expression of miRNAs, such as miR-128-2 and miR-223 [21, 22]. Moreover, miRNAs were recently found differentially expressed between wild-type and mutant p53 breast tumors [23].

Here, we report that *TP53* mutations are associated with shorter recurrence-free survival in a cohort of 109 HNSCC patients, as expected. We identified 49 miRNAs whose expression discriminates tumors with wild-type from those with mutated *TP53* gene. In particular, 44 and 5 miRNAs are, respectively, up- and down-regulated in the *TP53*-mutated group compared with the wild-type group. Moreover, we assessed that 12 and 4 of the 49 *TP53*-associated miRNAs, are able to predict, respectively, recurrence-free survival and cancer-specific survival, independently from other clinical variables, either individually or as groups.

materials and methods

study population and clinical samples

The study, approved by the scientific ethic committee of the Italian National Cancer Institute 'Regina Elena' (Rome) (protocol CE/379/08), involved 123 prospectively enrolled patients with histologically confirmed primary HNSCC undergoing curative treatment at the Otolaryngology Head and Neck Surgery Department. Only patients who did not receive any anticancer therapy before surgery were included in the study. Only HNSCC patients who developed local recurrence after 1 month from the surgery and with a follow-up ≥12 months were considered for the prognostic study.

TP53 mutational analysis

See supplementary material, available at *Annals of Oncology* online.

RNA extraction, labeling and microRNA microarray hybridization and analysis

See supplementary material, available at *Annals of Oncology* online.

statistical methods

The association between categorical variables was tested by the Pearson χ^2 test, χ^2 test for trend or Fisher Exact tests, when appropriate.

The hazard risk was estimated for each variable using the Cox univariate model. A multivariable Cox proportional hazard model was also developed using stepwise regression (forward selection), enter limit and remove limit were $P = 0.10$ and $P = 0.15$, respectively. Survival curves were calculated by the Kaplan–Meier product-limit, the log-rank test was used to assess differences between subgroups. Significance was defined at the $P < 0.05$ level. The Odds Ratio was estimated for each variable. The SPSS (version 18.0, SPSS Institute, Chicago, IL) statistical software was used for all analyses.

results

TP53 mutation predicts development of local recurrence in HNSCC

Demographic and clinical characteristics of the HNSCC patients included in the study are reported in supplementary Material and Table S1, available at *Annals of Oncology* online. The incidence of *TP53* mutation in the HNSCC cohort was determined by direct sequencing of *TP53* exons 2 through 11. Seventy of 121 patients (58%) presented single or multiple *TP53* mutations in the tumor tissue (Figure 1A and supplementary Table S1, available at *Annals of Oncology* online). Only one normal tissue counterpart had a *TP53* mutation and was not included in subsequent analyses. We identified only five HPV-positive cases (supplementary Material and Table S1, available at *Annals of Oncology* online).

Many studies have shown that *TP53* gene mutations are associated with an increased risk for loco-regional recurrence and poor outcome [24]. In our HNSCC series, *TP53* mutation was associated with a higher probability to develop local recurrence after diagnosis (Figure 1B). In multivariable Cox proportional hazards regression model, *TP53* mutation, as well as primary tumor site in the pharynx and adjuvant therapy, result as independent negative prognostic factors for RFS (Table 1). Primary tumor site and adjuvant therapy were also prognostic factors for CSS (Table 1).

As *TP53*-mutated proteins have been extensively characterized for their ability to confer chemo- and radio-resistance to cancer cells, including HNSCC cells [13, 25], we examined the association of *TP53* mutation with RFS in the two clinical subgroups of patients subjected or not to adjuvant therapy. Interestingly, we observed by Kaplan–Meier analysis that *TP53* mutation, despite occurring with similar frequency in the two subgroups (Figure 1C and D, right), was significantly associated with lower RFS, compared with wild-type *TP53*, only in HNSCC patients subjected to adjuvant therapy after surgery (Figure 1C) and not in patients who did not receive adjuvant therapy (Figure 1D). Of note, the distribution of primary tumor sites was similar in the two analyzed subgroups (Figure 1C and D, right). In multivariable analysis, *TP53* mutation (HR 2.42, CI

Table 1. Results of multivariable analysis of prognostic factors

Factors	Recurrence-free survival		Cancer-specific survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Primary tumor site				
Pharynx versus larynx and oral cavity	3.58 (1.69–7.57)	0.001	4.49 (2.17–9.29)	<0.0001
Adjuvant therapy				
Yes versus no	2.09 (0.97–4.50)	0.06	4.03 (1.63–10.0)	0.003
TP53 status				
mut versus wt	1.92 (0.94–3.94)	0.07	/	/

$P < 0.1$ was considered significant.

Mut, mutant; wt, wild type; HR, hazard risk; CI, confidence interval; / = not evaluable.

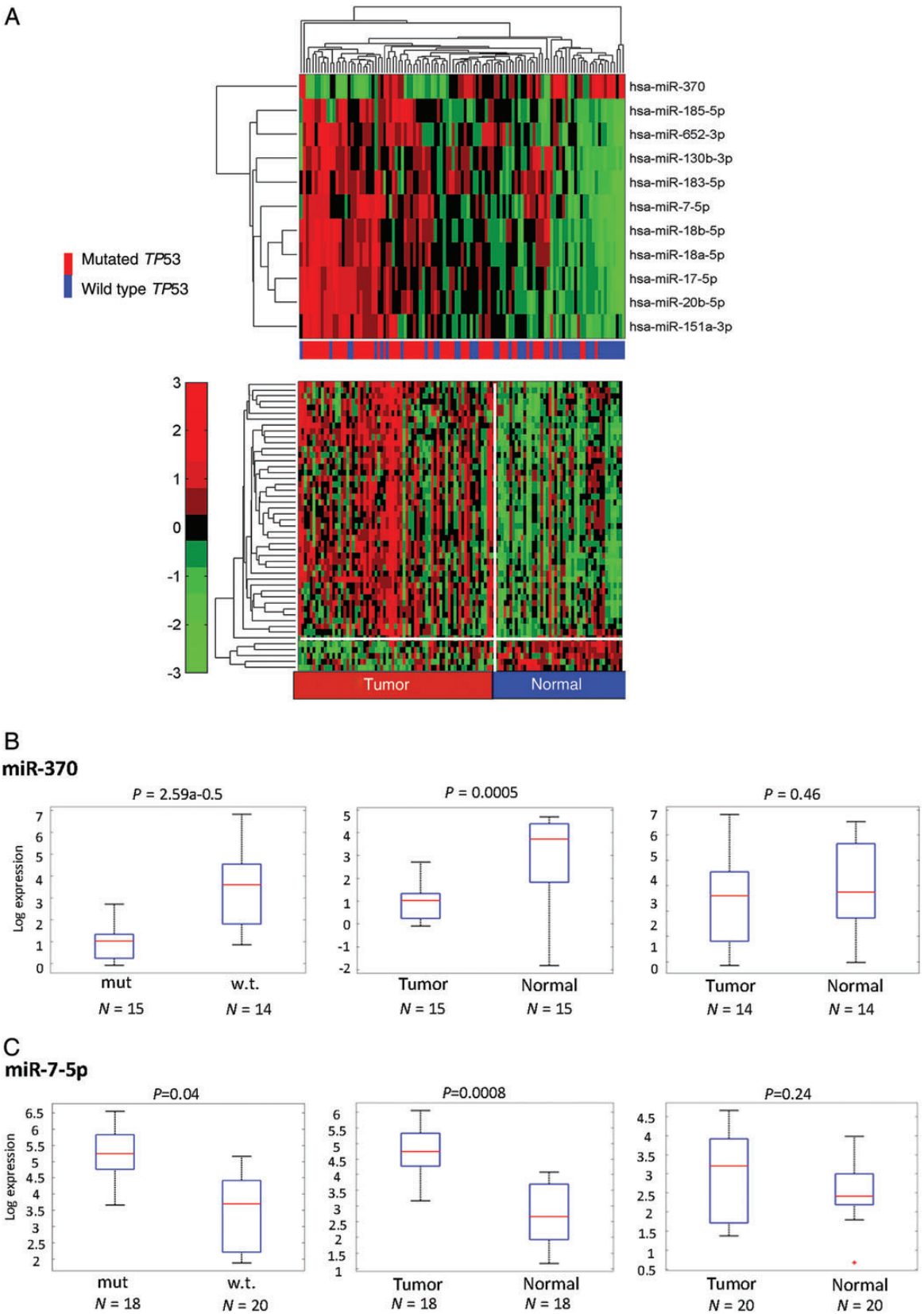


Figure 2. microRNAs are differentially expressed between TP53 wild-type and mutated HNSCC samples. (A) Heatmap relative to the unsupervised clustering analysis of the best 11 of 49 miRNAs distinguishing TP53-mutated and wild-type tumors. (B) Heatmap relative to the semi-unsupervised clustering analysis representing the deregulation of 49 TP53-related miRNAs in TP53-mutated tumors versus normal samples. (C) Validation of two representative TP53-related miRNAs (miR-7-5p and miR-370, up- and down- regulated, respectively, in the mutant TP53 group) was carried out by RT-qPCR in a subgroup of HNSCC patients. Comparison of miRNAs expression between TP53-mutated and wild-type tumors is shown in the left plots. Comparisons of miRNAs expression in tumor versus normal samples, in the subsets of TP53-mutated tumors (middle plot) and TP53-wt tumors (right plot), are also shown. wt, wild type; mut. mutation.

Table 2. Results of Kaplan–Meier

microRNA	KM analysis[a]	*t*-Test[a]	Multivariable analysis[a]	
	Log-rank *P*	*P*	HR (95% CI)	*P*
Recurrence-free survival				
'hsa-miR-205-5p'	0.003	1.05E−14	4.98 (1.67–14.9)	0.004
'hsa-miR-429'	0.007	1.69E−21	4.45 (1.59–12.45)	0.004
'hsa-miR-21-3p'	0.007	3.50E−17	3.12 (1.28–7.6)	0.01
'hsa-miR-331-3p'	0.008	2.49E−22	3.45 (1.24–9.64)	0.01
'hsa-miR-200a-3p'	0.039	1.45E−18	3.1 (1.18–7.9)	0.02
'hsa-miR-19a-3p'	0.055	2.89E−22	2.86 (1.1–7.7)	0.03
'hsa-miR-21-5p'	0.055	1.86E−18	2.77 (1.04–7.38)	0.04
'hsa-miR-151a-3p'	0.03	2.01E−30	3 (1–8.97)	0.04
'hsa-miR-17-3p'	0.01	9.99E−22	2.82 (0.98–8.14)	0.05
'hsa-miR-18b-5p'	0.009	1.49E−27	2.54 (0.97–6.69)	0.06
'hsa-miR-324-5p'	0.03	1.09E−21	2.62 (0.85–8)	0.09
'hsa-miR-96-5p'	0.018	1.40E−22	2.19 (0.87–5.53)	0.1
Cancer-specific survival				
'hsa-miR-139-3p'	0.004	1.08E−25	0.33 (0.12–0.87)	0.02
'hsa-miR-21-5p'	0.04	4.29E−05	2.41 (1.1–5.53)	0.04
'hsa-miR-21-3p'	0.03	5.20E−08	2.17 (0.98–4.83)	0.06
'hsa-miR-17-3p'	0.02	3.33E−07	2.1 (0.91–4.71)	0.08

t-Test and multivariable analyses of prognostic microRNAs.

[a]The analyses were carried out considering high versus low expression groups excluding the patients with an intermediate signal. Multivariable model adjusted for primary tumor site (RFS, CSS), adjuvant therapy (RFS, CSS) and *TP53* status (RFS).

KM, Kaplan–Meier; HR, hazard risk; CI, confidence interval.

1.01–5.78, $P = 0.047$) and site (HR 3.13, CI 1.28–7.62, $P = 0.01$) remained independent prognostic factors for recurrence-free survival in the group of patients who received adjuvant therapy.

TP53 mutation associates with the expression of specific microRNAs in HNSCC

miRNAs expression profiling was carried out for 121 HNSCC samples and 66 normal counterparts. After filtering miRNAs that were expressed at very low levels or not expressed in most of the cohort, 209 miRNAs were considered for further analysis. We evaluated whether the expression levels of miRNAs in tumor tissues were different by the presence or absence of *TP53* mutations. Considering the whole tumor series, we found 49 miRNAs that were able to distinguish wild-type from mutated *TP53*-carrying tumors (supplementary Table S2, available at *Annals of Oncology* online). A subgroup of the 49 *TP53*-related miRNAs was also associated with other clinical variables; we then confirmed the associations miR-*TP53* by constructing multivariable regression models adjusting for these potentially confounding clinical variables (supplementary Table S2, available at *Annals of Oncology* online).

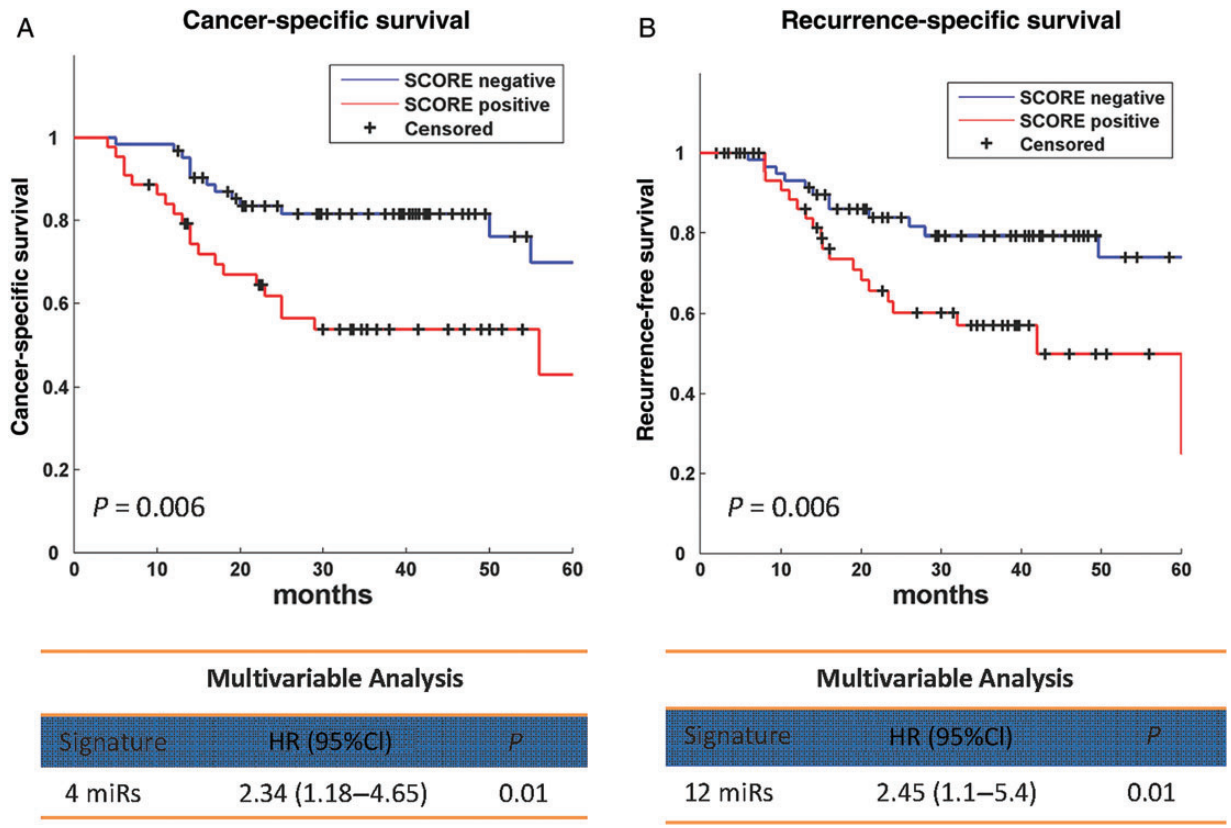
In particular, we found 44 miRNAs that were up- and 5 miRNAs that were down-regulated in the *TP53*-mutated group compared with the wild-type group (supplementary Table S2, available at *Annals of Oncology* online); all except one of these miRNAs were also respectively up- and down-regulated in the tumor tissues compared with normal tissues, specifically in the *TP53*-mutated group (supplementary Table S3, available at *Annals of Oncology* online). On the contrary, only 10 of these 49 miRNAs were significantly altered in tumor versus normal tissues in the wild-type *TP53* group and also showed lower statistical significance (supplementary Table S3, available at *Annals of Oncology* online).

Heatmaps obtained from the unsupervised clustering of the 11 miRNAs with best performance in distinguishing groups with mutated and wild-type *TP53* (Figure 2A) and from the semi-unsupervised clustering of the 49 *TP53*-related miRNAs distinguishing tumor and normal samples (Figure 2B) are shown.

We validated by RT-qPCR the up- and down-regulation of, respectively, miR-7-5p and miR-370, in tumors with mutated *TP53* versus tumors with wild-type *TP53* (Figure 2C, left). We also confirmed that these miRNAs are modulated between tumor and normal tissues specifically in the group of tumors with mutated *TP53* (Figure 2C, middle), while no significant changes in expression are observed in patients with wild-type *TP53* (Figure 2C, right).

expression of TP53 mutation-related microRNAs associates with survival in HNSCC

We next evaluated if the expression of the 49 *TP53*-associated miRNAs predicts clinical outcome. We observed, through Kaplan–Meier analysis, that the expression of 12 miRNAs (e.g. miR-17-3p, miR-18b-5p, miR-324-5p, miR-19a-3p, miR-200a-3p, miR-331-3p, miR-21-3p, miR-21-5p, miR-205-5p, miR-151a-3p, miR-96-5p and miR-429) was associated with shorter RFS (Table 2), while the expression of 4 miRNAs (e.g. miR-17-3p, miR-21-3p, miR-21-5p and miR-139-3p) was associated with shorter CSS (Table 2). Three of such miRNAs (e.g. miR-17-3p, miR-21-3p, miR-21-5p) associated with both RFS and CSS.



Multivariable Analysis

Signature	HR (95%CI)	*P*
4 miRs	2.34 (1.18–4.65)	0.01

Multivariable Analysis

Signature	HR (95%CI)	*P*
12 miRs	2.45 (1.1–5.4)	0.01

Figure 3. miRNAs expression predicts clinical outcome of HNSCC patients. (A and B) Kaplan–Meier analyses representing the correlation between the expression of the groups of 12mG and 4mG and clinical outcome in the whole series of HNSCC patients. Kaplan–Meier analyses were carried out dividing the patients in two subsets: patients with positive score versus patients with negative score according to the expression level of the groups of 12 (A) and 4 (B) prognostic miRNAs. See supplementary Material, available at *Annals of Oncology* online, for score calculation details. The multivariable analyses relative to cancer-specific survival (A) and recurrence-free survival (B) are shown at the bottom.

In multivariable Cox proportional hazards regression model, adjusting for other significant prognostic indicators, we determined that each of these miRNAs predicts RFS and/or CSS independently from the other variables (Table 2 and supplementary Table S4, available at *Annals of Oncology* online).

We next investigated whether these 12 and 4 miRNAs groups (which will be indicated hereafter with the terms 12mG and 4mG) predict, respectively, RFS and CSS when they are considered as signatures. We assigned a score to each patient based on the expression levels of the 12mG or of the 4mG (see supplementary Material, available at *Annals of Oncology* online), and Kaplan–Meier analyses were carried out comparing patients with positive and negative scores. As shown in Figure 3A, patients with positive score for the 12mG showed reduced RFS compared with patients with negative score. Moreover, patients with positive score for the 4mG showed reduced CSS compared with patients with negative score (Figure 3B). When the 12mG and 4mG scores were tested in multivariable analyses, we could assess that the identified groups of miRNAs were able to predict outcome independently from the other clinical variables (e.g. tumor site, adjuvant treatment and *TP53* status) (Figure 3A and B, bottom, and supplementary Table S4, available at *Annals of Oncology* online). Of note, adjusting for the 12mG score, *TP53* status did not remain an independent prognostic factor for RFS (supplementary Table S4, available at *Annals of Oncology* online). Finally, the association between miRNAs groups and outcome remained significant also in χ^2 test (supplementary Table S5, available at *Annals of Oncology* online).

discussion

The main finding of our study is that *TP53* mutations are associated with the up- or down-regulation of specific miRNAs in squamous cell carcinomas of the head and neck. Some of these miRNAs are able to predict RFS and CSS. Of note, these prognostic miRNAs are strong predictors of survival also when considered as signatures.

The presence of a *TP53* mutation was previously associated with decreased survival in HNSCC [9], as compared with wild-type *TP53*. We detected single or multiple *TP53* mutations in 58% of the analyzed tumors and this frequency is in line with what recently reported [26].

We explored the possibility that *TP53* mutations are associated with miRNA expression alteration in HNSCC. We identified 49 miRNAs significantly associated with *TP53* status. Interestingly, 5 of the 49 miRNAs discriminating wild-type and mutant *TP53* (miR-135b-5p, miR-18a-5p, miR-18b-5p, miR-224-5p and miR-452-5p) were previously identified as expressed at higher levels in mutant *TP53* versus wild-type *TP53* in a similar study in breast cancer [23].

We examined the expression levels of *TP53*-associated miRNAs in tumor versus normal samples separately on the *TP53*-mutated and wild-type groups. Of note, only in the *TP53*-mutated group, all miRNAs except one were also deregulated in the tumor tissue compared with the normal counterparts. Several of these miRNAs have been previously reported to be altered in head and neck tumors with same kind of deregulation (up- or down-regulation) [27]. Of note, when we evaluated the group of miRNAs predicting RFS (12 miRNAs) or CSS (4 miRNAs) in multivariable analyses we observed that they predict outcome independently from the other relevant clinical variables (e.g. *TP53* status, tumor site and adjuvant therapy). On the contrary *TP53* mutations are no more significant adjusting for miRNAs signatures expression, indicating that miRNAs overcome *TP53* status in recurrence prediction.

Some of the *TP53*-associated miRNAs predicting clinical outcome was also previously reported to be prognostic in HNSCC, as miR-151a-3p, miR-324-5p and miR-21 [28, 29].

In conclusion, we have demonstrated that the expression of signatures of *TP53* mutation-associated miRNAs, composed of 12 and 4 miRNAs, predicts, respectively, the risk of local recurrence insurgence and poor outcome, independently from other relevant prognostic indicators.

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disclosure

The authors have declared no conflicts of interest.

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