An Integrated Approach Identifies Mediators of Local Recurrence in Head and Neck Squamous Carcinoma

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Abstract

Purpose: Head and neck squamous cell carcinomas (HNSCCs) cause more than 300,000 deaths worldwide each year. Locoregional and distant recurrences represent worse prognostic events and accepted surrogate markers of patients' overall survival. No valid biomarker and salvage therapy exist to identify and treat patients at high-risk of recurrence. We aimed to verify if selected miRNAs could be used as biomarkers of recurrence in HNSCC.

Experimental Design: A NanoString array was used to identify miRNAs associated with locoregional recurrence in 44 patients with HNSCC. Bioinformatic approaches validated the signature and identified potential miRNA targets. Validation experiments were performed using an independent cohort of primary HNSCC samples and a panel of HNSCC cell lines. In vivo experiments validated the in vitro results.

Results: Our data identified a four-miRNA signature that classified HNSCC patients at high- or low-risk of recurrence. These miRNAs collectively impinge on the epithelial–mesenchymal transition process. In silico and wet lab approaches showed that miR-9, expressed at high levels in recurrent HNSCC, targets SASH1 and KRT13, whereas miR-1, miR-133, and miR-150, expressed at low levels in recurrent HNSCC, collectively target SP1 and TGFβ pathways. A six-gene signature comprising these targets identified patients at high risk of recurrences, as well. Combined pharmacological inhibition of SP1 and TGFβ pathways induced HNSCC cell death and, when timely administered, prevented recurrence formation in a preclinical model of HNSCC recurrence.

Conclusions: By integrating different experimental approaches and competences, we identified critical mediators of recurrence formation in HNSCC that may merit to be considered for future clinical development. Clin Cancer Res; 1–12. ©2017 AACR.

Introduction

Head and neck squamous cell carcinomas (HNSCCs) comprehend a relatively common group of neoplasms, with about 550,000 new cases/year worldwide (1). Most patients are diagnosed with a locally advanced potentially curable cancer, but 40% to 60% of these patients eventually recur (2, 3). A recent meta-analysis demonstrated that the combination of chemo- and radiotherapy is a valid, although highly toxic, therapeutic option (4). Despite this aggressive schedule, the 5-year survival of patients with HNSCC ranges from 35% to 55% (4).

Local and distant recurrences represent valid surrogate endpoints to estimate the efficacy of radiotherapy and chemotherapy on patients' survival (5). This observation implies that identifying patients that will recur could be extremely beneficial for the management of patients with HNSCC to avoid unnecessary toxicity and improve patients' survival. To date, no validated biomarkers exist to identify patients with HNSCC with higher risk of recurrence.
Most patients with HNSCC are diagnosed with a locally advanced disease and are treated with the combination of surgery, radiotherapy, and chemotherapy. This highly toxic approach is curative in about half of the cases, but recurrent patients do not have effective salvage therapies. Therefore, there is the urgency to identify and validate solid biomarkers able to classify patients at high risk that may benefit for specific targeted approaches. Our work tackled these two unmet clinical needs and identified a microRNAs signature of locoregional recurrence in patients with HNSCC. Starting from this signature, we identified two druggable pathways (i.e., SP1 and TGFβ) that when timely and concomitantly targeted efficiently prevented recurrence formation in a preclinical model. Both SP1 and TGFβ inhibitors have been already used to treat human patients; thus, our work is of potential immediate translational relevance.

Translational Relevance

Most patients with HNSCC are diagnosed with a locally advanced disease and are treated with the combination of surgery, radiotherapy, and chemotherapy. This highly toxic approach is curative in about half of the cases, but recurrent patients do not have effective salvage therapies. Therefore, there is the urgency to identify and validate solid biomarkers able to classify patients at high risk that may benefit for specific targeted approaches. Our work tackled these two unmet clinical needs and identified a microRNAs signature of locoregional recurrence in patients with HNSCC. Starting from this signature, we identified two druggable pathways (i.e., SP1 and TGFβ) that when timely and concomitantly targeted efficiently prevented recurrence formation in a preclinical model. Both SP1 and TGFβ inhibitors have been already used to treat human patients; thus, our work is of potential immediate translational relevance.

Materials and Methods

Patient samples

Specimens from primary HNSCC were collected from patients who underwent surgery at our institution and at Santa Maria degli Angeli Hospital, Pordenone, Italy. HNSCC specimens were immediately frozen and stored at −80 °C. The study was approved by the Internal Review Board of the Centro di Riferimento Oncologico (CRO) of Aviano (#IRB-08/2013), and all patients provided written informed consent.

Bioinformatic analyses

Computational analysis. Univariate significance test by the permutation test (19) was used to calculate the statistical significance of each of the four miRNAs individually. Testing of sample classification includes building and testing a computational prediction model to predict recurrence based on the miRNAs’ expressions using the Weka software (20). We adopt the undersampling technique described in (21), to counter the effects of class imbalance and the potential of overfitting due to a limited and small minority class data (only 11 recurrent samples). Both methods are further described in the Supplementary Materials and Methods.

Network analysis

After downloading predicted miRNA–gene interactions for the genes in our network from the mirDIP portal ver. 1 (http://ophid.utoronto.ca/mirDIP; ref. 22), which integrates 12 miRNA prediction datasets, we kept only those interactions that were identified in at least three independent datasets. We then integrated the analysis using genes up- or downregulated in head neck recurrences from CDIP, the Cancer Data Integration Portal ver. 1 (http://ophid.utoronto.ca/cdip), a collection of gene expression data from published studies. We also used a list of genes associated with recurrence formation in HNSCC from a published cohort (23).

Next, we uploaded this list of gene IDs into NAViGaTOR 3 as our network visualization tool (http://ophid.utoronto.ca/naviga tor (24) and retrieved known, publicly available human physical protein interaction using the 12D 2.2 portal (http://ophid.utor onto.ca/12d (25). Our goal was to explore the relationships between our four miRNAs and genes known to regulate recurrence formation in HNSCC. Network nodes represent miRNAs and proteins respectively, whereas edges represent physical protein–protein interactions (PPi) and miRNA–gene regulation. We downloaded the list of the four miRNAs targets from the mirDIP database ver. 1 (http://ophid.utoronto.ca/mirDIP/) and then we looked at the intersection among genes that control recurrence formation (23), genes associated to a prosurvival signature (23) and the four miRNAs targets.

PathDIP analyses. We analyzed 56 genes, involved in recurrence formation (23), as potential miR-9 targets. These pathway analyses were conducted using pathDIP Ver. 2.4.3.12 (http://ophid. utoronto.ca/pathDIP; ref. 26).

Analysis of the TCGA dataset. Correlation analysis using HNSCC TCGA RNAseq and microRNA-seq data for SASHJ, KRT13, and hsa-miR-9 were performed using Spearman correlation (27). All statistical analyses were performed using [R] (https://www.r-proj)
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KRT13/SASH1 correlation analysis using HNSCC TCGA RNAseq data were performed using cBioPortal for Cancer Genomics (http://www.cbioportal.org/; ref. 28).

Clustering of RNAseq values was performed using Ward linkage. This approach identified cluster 1 with high expression of KRT13/SASH1 and low expression of TGFβR1/2, SMAD3, and SPI, whereas cluster 2 is characterized by high expression of TGFβR1/2, SMAD3, SPI, and low expression of SASH1/KRT13. The associations of the two clusters with survival was evaluated with the log-rank test using the survival package in R. HNSCC KRT13/SASH1 and low expression of TGFβ/SP1 mics (http://www.cbioportal.org/; ref. 28).

RNAseq data were performed using cBioPortal for Cancer Genomics, below median of mRNA expression, respectively) or by high mRNA expression of KRT13 and low SMAD3 (above median of mRNA expression, below median of mRNA expression, respectively) or by high mRNA expression of SMAD3 and low mRNA expression of KRT13/SASH1. Cox proportional hazards regression analysis based on the KMsurv package in R was used to access the hazard ratios. All HNSCC TCGA data were downloaded using the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/) and the cBioPortal for Cancer Genomics (http://www.cbioportal.org/).

To validate prognostic properties of the four miRNAs signature, we used SurvMicro v0.9 (http://bioinformatics.mty.itesm.mx:8080/Biomatet/Survmicro.jsp; ref. 29). Signature was validated on TCGA LUAD Illumina HiSeq dataset, comprising 311 patient samples. All settings were used as default.

Wet lab analyses

All wet lab analyses were performed according to procedures commonly used in our lab (30, 31) and are described in detail in the Supplementary Materials and Methods.

Molecular biology experiments. miRNA expression profile was performed using the NanoString technology (NanoString nCounter Human miRNA assay (v1.1) that allowed to evaluate the expression of 746 miRNAs (664 Human 82 Viral) along with the one of housekeeping genes (GeneBank GSE89000). NanoString technology, DNA and RNA extraction, quantification and analysis, evaluation of TP53 and HPV status, protein extraction, and Western blotting are described in the Supplementary Materials and Methods.

Cell culture, transfection, and transduction. All cell lines were authenticated by BMR Genomics srl Padova, Italia according to Cell ID System (Promega) protocol and using Genemapper ID Ver 3.2.1, to identify DNA STR profiles. UMSCC74b and UMSCC1 cells were kindly provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). All other head and neck squamous cell lines were obtained from ATCC (LGC Standards).

Xenograft growth in mouse flanks and treatment. Animal experimentation was approved by the Italian Ministry of Health and by a local ethic committee for animal welfare (OPBA) and experiments performed according to committee’s guidelines. Athymic nude mice (Harlan, Foxn1nu, females, 6 weeks old) were injected with 2 × 10^6 FaDu cells bilaterally in the flanks. The evaluation of local relapse and the administration on Myrthramycin A and SB-525334 are provided in the Supplementary Materials and Methods.

Statistical analyses

All graphs and statistical analyses were performed using PRISM (version 6, GraphPad, Inc.) and R, SAS Software 9.2 and R for statistical analyses. In all experiments, differences were considered significant when P was <0.05. Statistical analyses included paired and unpaired t tests, Mann–Whitney unpaired t test and Spearman correlation test, used as appropriate and as specified in each figure. Differences in miRNA expression between patients’ groups were evaluated by nonparametric Wilcoxon test (two groups) or Kruskal–Wallis test (three groups). Correlation between Array and PCR quantification of miRNAs was evaluated through Spearman correlation coefficient.

Results

Identification of miRNAs differentially expressed in recurrent and nonrecurrent HNSCC

Primary HNSCC fresh-frozen surgical samples collected from 44 patients (Table 1) who experienced (n = 11) or not (n = 33) local recurrence within 2 years from the first surgery were analyzed by NanoString technology for the expression of 746 human and 82 viral miRNAs. Statistical analyses of normalized miRNA expression demonstrated that seven miRNAs were significantly different between the two groups (data not shown). Validation of these data by qRT-PCR analyses confirmed significant differences for miR-1, miR-133a, miR-150, and miR-9 between recurrent and nonrecurrent HNSCC. In particular, univarient significance testing confirmed that miR-9 was upregulated, whereas miR-1, miR-133a, and miR-150 were downregulated in tumors from recurrent patients (Supplementary Fig. S1). No significant associations were found between expression of these miRNAs and other clinical and biological variables of the tumors, including the presence of TP53 mutation and the positivity for HPV infection (Table 1; Supplementary Table S1). Accordingly, miR-9 upregulation and miR-1, miR-133a, and miR-150 downregulation in recurrent tumors was also confirmed when only TP53 mutant or HPV-negative cases were considered (Supplementary Fig. S1). Bioinformatic validation by data reiteration confirmed the significance of this interaction (Fig. 1A and B; Supplementary Fig. S2). Using the classifier testing, we calculated the AUC to estimate the ability of the four miRNAs, each one alone or in combination, to predict recurrence. Models 1–4, built using a Naive Bayes or a logistic regression models, in which the classifier combines miR-133a with miR-150, with or without miR-9, achieved a high AUC (80–81%), high sensitivity (82–88%), and low false-positive rates (29–35%), which translated into 65% to 71% specificity (Fig. 1A and B; Supplementary Fig. S2). Our analyses also suggested that the addition of miR-1 does little to improve the classification accuracy (Supplementary Fig. S2C).

These computational analyses are in accord with the notion that miR-1 and miR-133a belongs to the same cluster (32) and, consequently, their expression highly correlates, as we observed in our samples set (Spearman correlation value r = 0.9621; P < 0.0001). Correlation analyses also indicated that miR-133a expression directly correlates with miR-150 (Spearman correlation value r = 0.2984; P = 0.049) and that miR-9 expression inversely correlates with both miR-133a (Spearman correlation value r = −0.1715; P = n.s.) and miR-150 (Spearman correlation value r = −0.4757 and P = 0.0111).

Accordingly, using the HNSCC TCGA dataset (27) we confirmed that (i) miR-1 and -133a expression strongly correlate (R = 0.79; P < 0.0001); (ii) miR-9 inversely correlates with both miR-1 (R = −0.27; P < 0.0001) and miR-133a (R = −0.34; P < 0.0001); and (iii) the expression of miR-1, -133a, -150, and -9 classify patients at high risk of relapse (Fig. 1C).
Bioinformatic analyses identified miRNA targets involved in the regulation of cell plasticity

Confirmed targets of the identified four miRs impinge on EMT process. Specifically, miR-9 promotes EMT by targeting β-catenin (33) and miR-1, miR-133, and miR-150, act as EMT suppressors by targeting SLUG (34), SNAIL (35), and ZEB1 (36), respectively.

Because EMT plays a pivotal role in HNSCC progression and recurrence formation (3, 12), we applied the mirDIP (22) and NAVIGaTOR (24) bioinformatic tools to integrate miRNA-target predictions with experimentally determined PPIs from I2D as described (25, 37), focusing on genes regulating EMT.

We first identified 16 possible common targets of miR-1, -133a, and -150 (Fig. 1G). Further refinement of these results, using two other available HNSCC datasets of coding gene signatures (23) and NAVIGaTOR (24) bioinformatic tools to integrate miRNA targets involved in recurrent HNSCC (Fig. 1G).

Pathway enrichment analyses using pathDIP (26), to integrate miR-1, -133a, and -150 with miR-9 networks, identified significantly enriched pathways (Supplementary Table S2). The most frequently occurring genes belonged to signal transduction (30 genes; $P < 0.05$), EGFR1 (29 genes; $P = 0.01$), immune system (29 genes; $P < 0.02$), integrin 66θ4 (26 genes; $P = 0.001$), TNFα (23 genes; $P = 0.05$), developmental biology (22 genes; $P = 0.02$), and TGFβ signaling (22 genes; $P = 0.04$) pathways. Interestingly, four prognostic genes from the work of Reiss and colleagues (38) are predicted to be regulated by miR-9-5p and miR-150-5p (using an updated mirDIP 3.0.1; http://opid.utoronto.ca/mirDIP; ref. 22).

Experimental validation of identified miRNAs targets involved in the regulation of EMT

Overall, these analyses suggested that the relative expression of miR-1, -133, and -150 could play a functional role in HNSCC progression possibly by regulating EMT.

To experimentally validate miR-9 targets identified in silico, we screened a panel of HNSCC-derived cell lines for endogenous miRNA levels to choose the most appropriate in vitro model. Generally, all cell lines expressed low levels of miR-1, -133a, and -150 and higher levels of miR-9 (Supplementary Fig. S3A). First, we focused on the four most probable miR-9 targets, namely JUP1, SASH1, KRT13, and FLG (Fig. 1G). FaDu and SCC9 cells expressed highest level of miR-9 and low levels of SASH1, KRT13, and of the epithelial marker E-cadherin (Supplementary Fig. S3A and S3B). In both cell lines, miR-9 knockdown resulted in the upregulation of SASH1 and KRT13, but not of JUP1 and FLG mRNAs (Fig. 2A and Supplementary Fig. S3C and S3D). The increased SASH1 and KRT13 expression was also confirmed at protein level (Fig. 2B). In CAL27 and UMSCC1 cells, expressing low endogenous miR-9, its

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**Table 1. Distribution of relapsed/not relapsed patients and mean level of miRNA expression according to selected covariates**

| Sex          | Relapsed | Not relapsed | miR-9   | miR-1   | miR-133a | miR-150 |
|--------------|----------|--------------|---------|---------|----------|---------|
| Men          | 11 (100.0) | 24 (72.7)    | 319.0   | 2193.8  | 396.7    | 537.7   |
| Women        | 0 (0.0)   | 9 (27.3)     | 180.9   | 186.5   | 132.0    | 1752.8  |
| Age          |           |              |         |         |          |         |
| <60 years    | 8 (72.7)  | 15 (46.9)    | 310.8   | 512.3   | 86.2     | 542.4   |
| ≥60 years    | 3 (27.3)  | 17 (53.1)    | 288.2   | 377.7   | 721.0    | 896.1   |
| Cancer site  |           |              |         |         |          |         |
| Tongue/oral cavity | 5 (45.5) | 23 (69.7)    | 149.9   | 724.8   | 662.3    | 499.5   |
| Oropharynx/Hypopharynx | 5 (45.5) | 6 (18.2)     | 329.9   | 3425.2  | 626.3    | 499.5   |
| Larynx       | 1 (9.0)   | 4 (12.1)     | 648.3   | 78.7    | 18.4     | 1452.2  |
| cT           |           |              |         |         |          |         |
| T1–T2        | 3 (27.3)  | 21 (65.6)    | 209.0   | 941.5   | 169.5    | 584.8   |
| T3–T4        | 8 (72.7)  | 11 (34.7)    | 341.2   | 2365.4  | 446.6    | 744.9   |
| cN           |           |              |         |         |          |         |
| 0            | 3 (27.3)  | 13 (40.6)    | 321.0   | 5360.7  | 1024.5   | 626.5   |
| 1–2          | 8 (72.7)  | 19 (59.4)    | 292.2   | 432.0   | 72.5     | 726.6   |
| Adjunct radio/chemotherapy |           |              |         |         |          |         |
| No           | 6 (54.6)  | 26 (78.8)    | 160.0   | 638.9   | 126.2    | 489.1   |
| Yes          | 5 (45.4)  | 7 (21.2)     | 391.6   | 2763.3  | 513.9    | 829.3   |

The significant differences are shown in bold. Abbreviations: cN, clinical evaluation of node status; cT, clinical evaluation of tumor size.
Figure 1.
Identification of miR-1, -133a, -150, and -9 potential targets in HNSCC. ROC curve predicting recurrence formation, using miR-9, -133a, and -150 data iteration, applying the Naive Bayes (A) or the logistic regression (B) models. The AUC is 81.3% (sensitivity 87% and specificity 75%) in A and 80.3% (sensitivity 82% and specificity 71%) in B. C, Kaplan–Meier curve evaluating progression-free survival of patients with HNSCC clustered on the basis of the expression of miR-1, -9, -133a, and -150. D, Venn diagram showing the number of miR-1, -133a, and -150 common potential targets, among the EMT genes, altered in HNSCC. E, Visualization of miR-1, -133a, and -150 and SP1 network in HNSCC. Direct interactions are shown by edges. Red-border triangles identify genes upregulated in recurrent HNSCC and black-border triangles identify genes upregulated in HNSCC primary tumors. F, List of miR-1, -133a, and -150 targets belonging to the TGFβ and WNT pathways. G, Venn diagram showing the number of miR-9 potential targets, among the anti-EMT genes and among the genes altered in HNSCC.
overexpression decreased mRNA and protein expression of SASH1 and KRT13 (Fig. 2C and D). As a proof of principle, we also tested whether miR-9 could directly regulate SASH1 expression acting on its 3’-UTR, which contains three seed sites for miR-9 (Supplementary Fig. S4A). Luciferase assay in FaDu cells demonstrated that miR-9 knockdown significantly increased the luciferase activity when the first seed site (position 217–223) was tested (Supplementary Fig. S4B). Overall,
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miRNAs targets predict prognosis in the TCGA HNSCC dataset

We hypothesized that the expression of SASH1, KRT13, SP1, and members of TGFβ pathway could be used in HNSCC as readout of miRNA activity and tested the expression and the correlation of these genes with miR-9 and miR-1, -133a, and -150, in the TCGA dataset.

In accord with the data obtained using our discovery- and validation-cohorts, correlation analyses confirmed that SP1 positively correlated with miR-9 and inversely with miR-133a (Supplementary Fig. S7A and S7B). Similar results were observed for SMAD2 and SMAD3 expression, used as readouts of SP1 and TGFβ pathway activity (Supplementary Fig. S7C–S7H). Moreover, SASH1 expression directly correlated with KRT13 (Supplementary Fig. S7I) and both were higher in tumor-free cohort (Supplementary Fig. S7L).

Cluster analyses, using the expression of SASH1, KRT13, SP1, TGFβR1, TGFβR2, SMAD2, and SMAD3, divided the patients with HNSCC included in the TCGA dataset in two groups: one with low expression of SP1 and members of TGFβ pathway and high expression of KRT13 and SASH1 (Cluster 1) and the other with the opposite gene expression profile (Cluster 2; Fig. 4A). This clustering had prediction power, because patients included in Cluster 2 displayed worse relapse-free survival than patients included in Cluster 1 (HR 2.01; 95% CI, 1.1–3.8; P = 0.01; Fig. 4B).

Perisurgical treatment with SP1 and TGFβR1 inhibitors prevents local relapse in a xenograft model of HNSCC

The above data suggested that SP1 and TGFβ pathway could act together in the establishment of recurrence in patients with HNSCC. To evaluate this possibility, we first tested the in vitro efficacy of Mithramycin A (MTA), a validated SP1 inhibitor, and of two different TGFβR1 inhibitors (SB525334, SB52 and SB431542, SB43) on HNSCC cell survival. Although MTA was highly active in decreasing HNSCC cell survival in the nanomolar range, both SB52 and SB43 did not significantly affect cell survival, when used up to 100 μmol/L (Fig. 4C). Yet, in all tested cell lines SB52, used at the ineffective dose of 40 μmol/L, reduced by the half the IC50 of MTA (Fig. 4C), suggesting that they could have synergistic effects. Accordingly, MTA used at the concentration of 20 nmol/L, effectively induced PARP1 cleavage (marker of apoptosis) only in combination with SB52 or SB43 (40 μmol/L) in FaDu cells (Fig. 4D).

On the basis of these results, we tested if combined pharmacological inhibition of SP1 and TGFβ pathways could restrain the formation of local recurrences in vitro, using a model of HNSCC recurrences and a perisurgical treatment schedule (Fig. 5A). Mice were subcutaneously injected with FaDu cells and tumor mass allowed to grow up to approximately 1 cm3. Mice were then randomly divided in four groups: (i) sham-treated, (ii) treated with MTA 1 mg/kg, (iii) SB52 15 mg/kg, and (iv) the combination of MTA and SB52. All groups were treated for three consecutive days (day −1, day 0, and day +1, with respect to surgery) and two more doses (day +3 and +6) only for SB52 treatment. Tumors were surgically removed at day 0 and appearance of local recurrence was monitored over the subsequent 8 weeks of follow-up (Fig. 5A). Pathologic analyses of explanted tumors, evaluating the presence and the width of resection margins, demonstrated that no significant differences existed among the four groups in the extent of radical surgery (Fig. 5B).

In vivo validation of identified miRNAs targets involved in the regulation of EMT

To verify if the predicted targets of the four miRNAs were effectivly differently regulated in HNSCC samples, we tested by qRT-PCR the expression SP1, TGFβ-R1, TGFβ-R2, and TGFβ-R3, WNT4 and WNT5A, CTNN1B, SASH1, and KRT13 in the 44 samples described in Supplementary Table S1 and analyzed for the expression of miRNAs.

In line with in vitro results, we observed that miR-9 targets, SASH1 and KRT13, were both significantly downregulated in patients who experienced recurrence (Fig. 3A) and the expression of SASH1 correlated directly with KRT13 and inversely with miR-9 in primary HNSCC (Fig. 3B and C). Among miR-1, -133a, and -150, only SP1 and TGFβ-R1 were significantly upregulated in HNSCC samples from recurrent patients (Fig. 3D) and their expression directly correlated (Fig. 3E). No differential expression was observed for TGFβ-R2 and TGFβ-R3, WNT4, WNT5A, and CTNN1B between patients with or without recurrence (data not shown).

To validate our findings in an independent cohort, we collected 78 HNSCC samples in our Institutes (Supplementary Table S3) and evaluated the expression of the four miRNAs, SASH1, KRT13, SP1, and TGFβR1. In accord with previous results, miRNAs expression did not correlate with any biological variable of patients with HNSCC, including sex, age, cancer site, T stage, and N stage (Supplementary Table S3).

The expression of SASH1 inversely correlated with miR-9 and directly correlated with KRT13 (Supplementary Fig. S6A and S6B). An inverse, although not significant, correlation was noticeable between miR-1 or miR-133a and SP1 expression, as expected from the in vitro results (Supplementary Fig. S6C and S6D).
Explanted tumors (six from controls and eight per group of treatment, respectively) were analyzed for the expression of phosphorylated SMAD2 (pSMAD2; Fig. 5C and D) and SP1 levels (Fig. 5E), as readouts of SB52 and MTA activity, respectively. A single administration of SB52 and/or MTA (administered the day prior to surgery) significantly inhibited SMAD2 phosphorylation and SP1 expression in the tumors (Fig. 5C–E), confirming the in vivo efficacy of these drugs.

Control mice developed local recurrence in 50% of injected sites and MTA used alone did not significantly alter the rate of recurrence formation (4/8, 50%; Fig. 5F). Interestingly, treatment with TGFβR1 inhibitor, SB52, as a single agent considerably increased local recurrence formation, with recurrence occurring in almost all cases (7/8, 90%; Fig. 5F). However, the combination of SB52 + MTA completely prevented recurrence formation (0/8, 0%; \(P = 0.005\) in log-rank test; HR 10.01; by comparing control-
and MTA+SB-treated mice; Fig. 5F), suggesting that perisurgical treatment with MTA and SB52 was sufficient to efficiently suppress HNSCC recurrence in vivo.

**Discussion**

Here, we show that four miRNAs can identify in patients with HNSCC those at high risk of developing recurrence. Using multiple approaches, we have linked the activity of these miRNAs to the regulation of EMT, a process considered an critical step in the progression of HNSCC from a pre-invasive to a frankly invasive stage (3). We identified two principal pathways regulated by these miRNAs that likely play a role in the regulation of HNSCC recurrence via the modulation of EMT, the SP1 and the TGFβ pathways. On one side, miR-1, miR-133a, and miR-150 acted together to regulate SP1 and, possibly, TGFβ pathway members, two pathways largely involved in cancer progression and EMT (39, 40). However, miR-9 acted by lowering the expression of SASH1 and KRT13, two known tumor suppressors with potential anti-EMT roles in HNSCC progression (41–43). Using two independent HNSCC samples cohorts and the TCGA datasets, we confirmed the inverse correlation between the identified miRNAs and their targets’ expression. This finding supports the possibility that the activity of miR-1, miR-9, miR-133a, and miR-150 could be estimated in primary HNSCC by evaluating the expression of SP1, members of the TGFβ pathway, SASH1 and KRT13 (our six-gene signature) and that this balanced activity, more than the sole miRNAs expression, could be used as a marker for identifying patients at high risk of developing recurrence.

From a biological point of view, it is interesting to note that we describe here two new targets of miR-9, SASH1 and KRT13, both already linked to the regulation of cell motility and invasion (41, 44). Although the aim of this study was limited to the assessment of their role as potential readouts of high miR-9 activity in vivo, it will be worth testing if SASH1 and KRT13 may partially mediate the miR-9-induced local relapse in HNSCC, also in light of the recently proposed role for miR9 in promoting EMT and a cancer stem cell phenotype in squamous cell carcinomas (45).

Importantly, our data point to the simultaneous inhibition of SP1 and TGFβ pathway by the concerted action of three miRNAs, miR-1, miR-133a, and miR-150, as a possible mechanism to prevent disease relapse. At a first glance, our data do not fit in the current literature showing that, in mouse models, the inhibition of TGFβ pathway combined with K-Ras activation is linked to the onset of SCC in the skin and in the oral mucosa (45, 46). However, increasing evidences clearly show that, depending on the stages of tumor progression, TGFβ pathway can exert either pro- or antitumorigenic effects (40). For instance, increased TGFβ signaling, in benign tumors or during the course of cancer...
induction, selects for more aggressive cells and contributes to metastasis formation, in different models of HNSCC (47–49). Our data in the mouse model, showing that TGFβR1 inhibition could result in different outcomes, depending on the simultaneous inhibition of SP1 by MTA or not (Fig. 5F), confirm the hypothesis that TGFβ pathway could act as tumor suppressor or tumor promoter in a context-dependent manner (40, 47). In strict accord with our data, SP1 is required for TGFβ-induced EMT in pancreatic cancer (50), and SP1 and SMAD2 proteins have been reported to directly interact in different models (39), supporting the possibility that SP1 and TGFβ pathway may act together to drive EMT, local invasion and, eventually, recurrence formation in HNSCC. The experimental and in silico analyses performed on our and on TCGA samples support this possibility and point to the expression of miR-1, miR-133a, and miR-150 as possible switches of the TGFβ activity.

We are aware that our study has limitations that should be taken into account. The cohort of patients used as a discovery set contains a relatively small number of heterogeneous patients that received different postsurgery treatments. These variables could have an impact on the prognostic value of the miRNA signature and also prevented the possible evaluation of its independent prognostic role in multivariate analyses. Although we experimentally validated the correlation between miRNAs expression and their target, we could not test the potential prognostic value of these miRNAs in a second independent cohort of samples for the absence of a precise follow-up in this group of patients.

Furthermore, it is important to point out that we focused our bioinformatic analyses on the relation between miRNAs expression and EMT regulators, and this represents a possible limitation of our study, since other biological pathways are also significantly altered by the same miRNAs in HNSCC.

In perspective, it will be important to verify if these four miRNAs and/or six genes signatures could be prospectively validated to identify patients at high risk of recurrence who may merit to be treated specific targeted therapies. Because both SP1 and
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TGFβ inhibitors have been already tested in cancer patients, our data are of potential immediate translational relevance.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: F. Citron, J. Armenia, G. Franchin, R. Talamini, A. Vecchione, L. Barzan, G. Baldassarre

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