Supplementary Information

An integrated approach identifies mediators of local recurrence in Head & Neck Squamous Carcinoma

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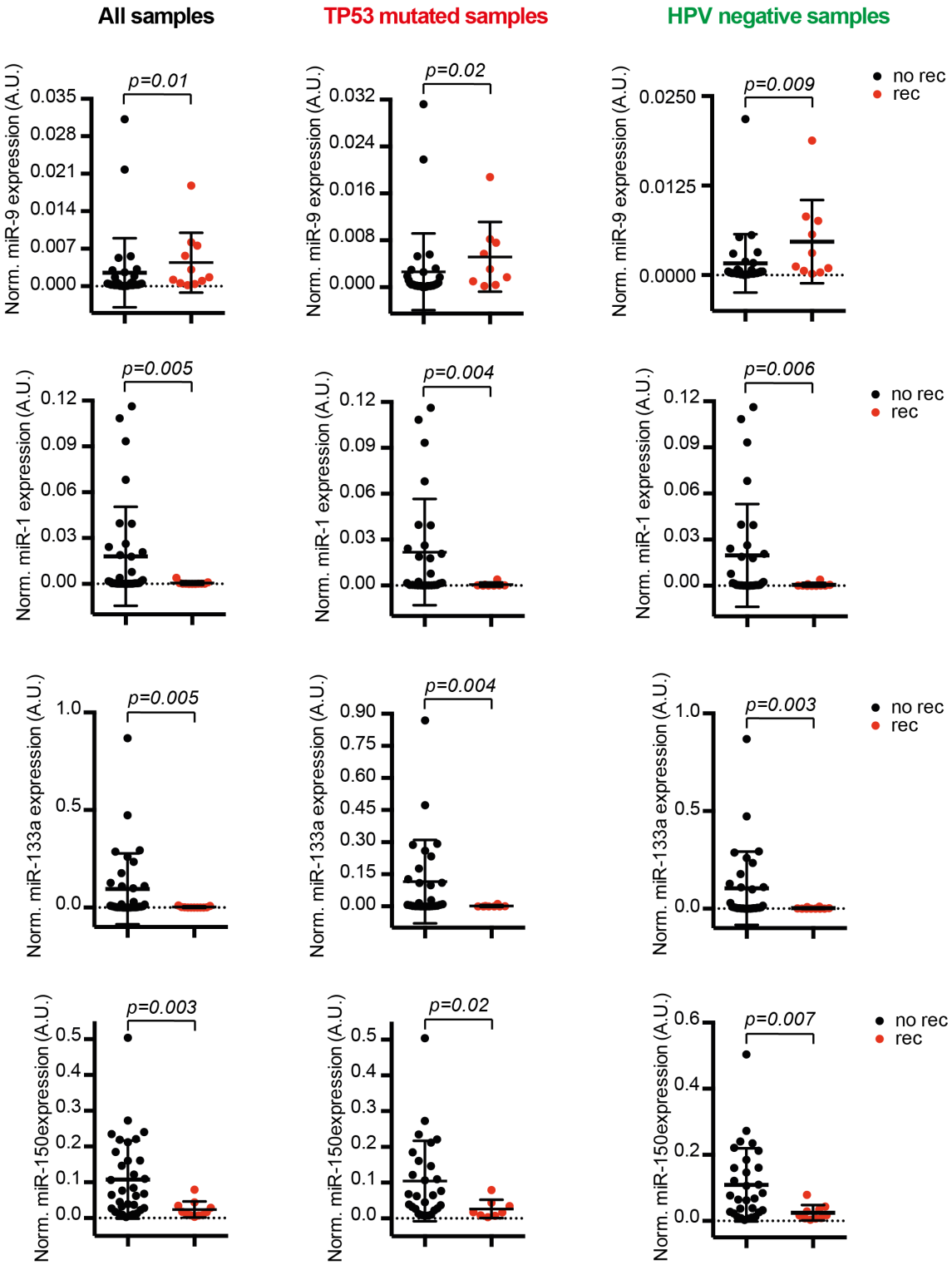
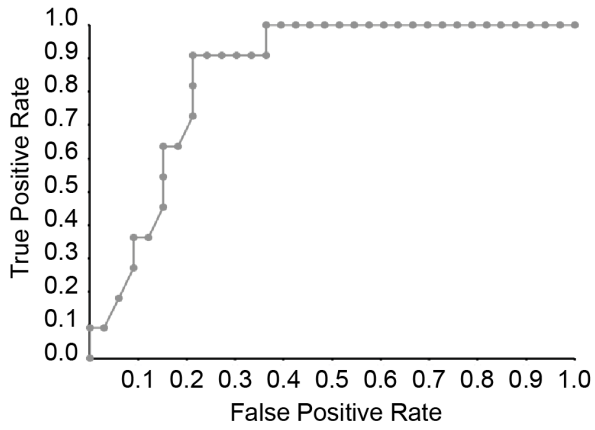
Content



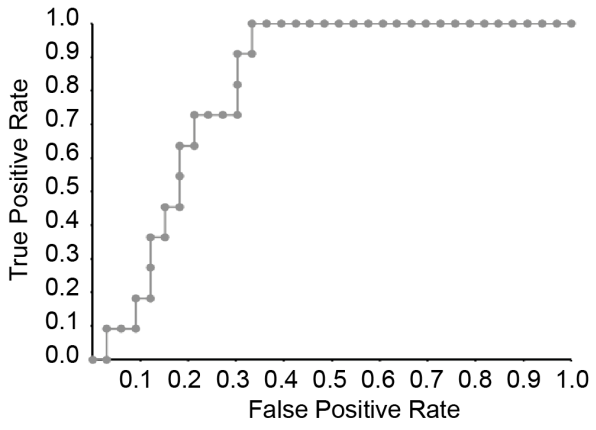
Supplementary Figure S1. *Validation of differentially expressed miRs in the discovery dataset.*

Dot plots showing the normalized expression of miR-1, -9, 133a and 150 in samples from primary HNSCC from recurrent (red) and non-recurrent patients (black). Bars indicate the mean and the 95% confidence interval.
In the left column dot plots show the normalized expression of in all 44 HNSCC samples reported in Table 1. In the central column only TP53 mutated samples were included and in the left column only HPV negative samples were included in the analyses.
Statistical significance is reported in each graph and was calculated using Mann-Whitney test.

A



B



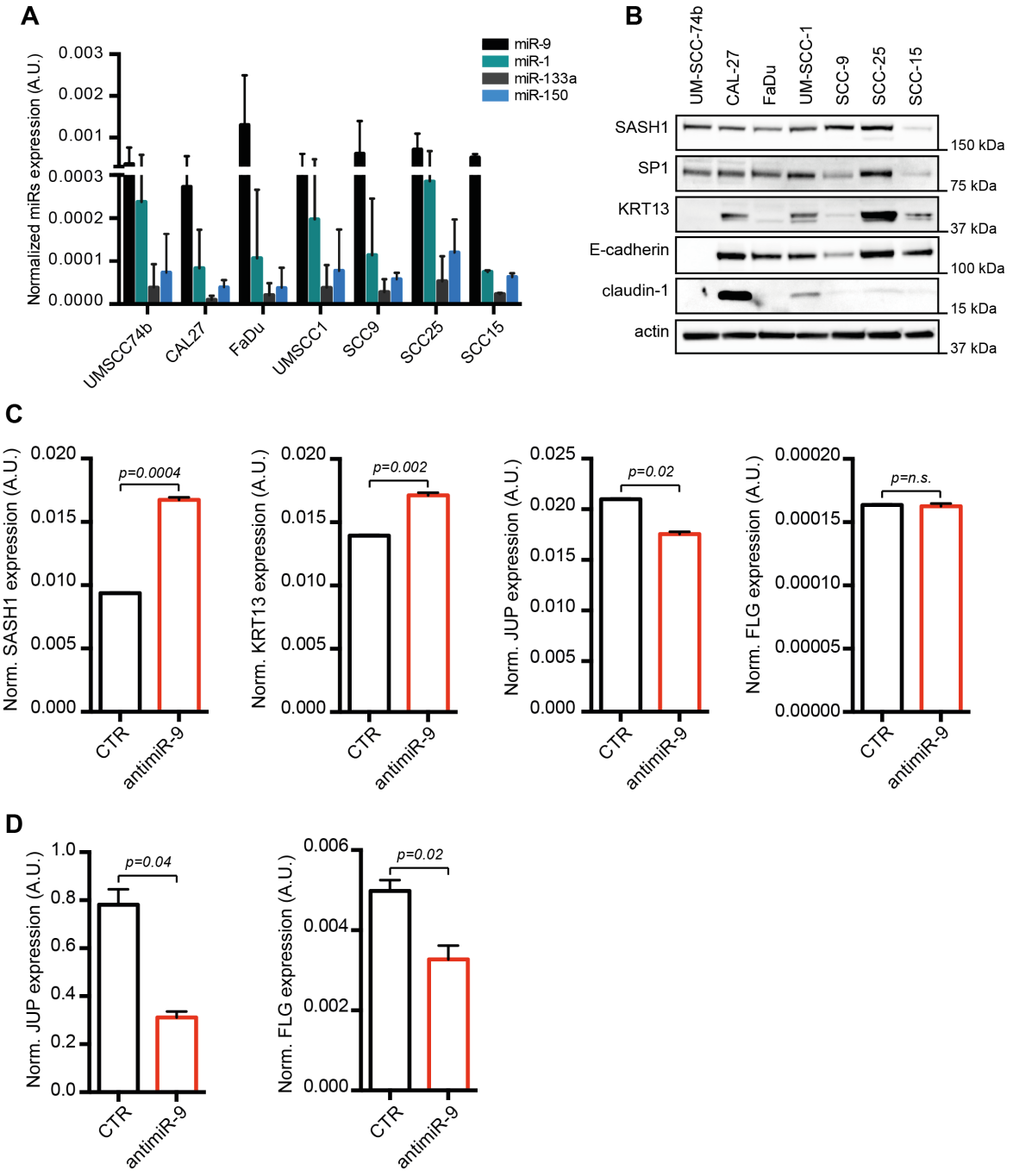
C

Classification		Signature				TPR	FPR	AUC
	Model	miR-1	miR-9	miR-133a	miR-150	(Thr = 0.5)	(Thr = 0.5)	(All Thr)
#1	**Naïve Bayes**	-	x	x	x	0.87±0.07	0.35±0.03	**0.81±0.04**
#2	**Naïve Bayes**	-	-	x	x	0.88±0.04	0.35±0.02	**0.81±0.03**
#3	**Logistic Regression**	-	-	x	x	0.83±0.07	0.31±0.03	**0.81±0.03**
#4	**Logistic Regression**	-	x	x	x	0.82±0.10	0.29±0.04	**0.80±0.04**
#5	Naïve Bayes	x	x	x	x	0.87±0.08	0.38±0.03	0.80±0.04
#6	Logistic Regression	x	x	-	x	0.80±0.10	0.29±0.05	0.79±0.05
#7	Naïve Bayes	x	-	x	x	0.89±0.06	0.38±0.02	0.79±0.03
#8	Naïve Bayes	x	-	-	x	0.82±0.01	0.37±0.03	0.79±0.03
#9	Naïve Bayes	x	x	-	x	0.83±0.08	0.37±0.03	0.79±0.04
#10	Logistic Regression	x	-	-	-	0.83±0.04	0.32±0.04	0.79±0.04
#11	Logistic Regression	x	x	x	x	0.76±0.1	0.32±0.05	0.76±0.05
#12	Logistic Regression	x	-	x	x	0,76±0.06	0.34±0.05	0.76±0.05
#13	Logistic Regression	-	x	x	-	0.77±0.10	0.40±0.06	0.74±0.05
#14	Logistic Regression	-	x	-	x	0.77±0.10	0.37±0.04	0.74±0.04
#15	Naïve Bayes	-	x	-	x	0.77±0.10	0.40±0.05	0.73±0.05
#16	Logistic Regression	x	x	-	-	0.75±0.10	0.41±0.07	0.73±0.05
#17	Naïve Bayes	-	x	x	-	0.92±0.07	0.61±0.04	0.71±0.06
#18	Logistic Regression	x	x	x	-	0.72±0.10	0.40±0.06	0.71±0.06
#19	Logistic Regression	x	-	x	-	0.78±0.06	0.46±0.05	0.70±0.04
#20	Naïve Bayes	x	x	x	-	0.89±0.07	0.61±0.03	0.69±0.06
#21	Naïve Bayes	x	x	-	-	0.88±0.07	0.63±0.04	0.68±0.07
#22	Naïve Bayes	x	-	x	-	0.91±0.02	0.61±0.03	0.65±0.03

Supplementary Figure S2. *Design of a recurrence prediction model in HNSCC based on miRs expression using a bioinformatic approach.*

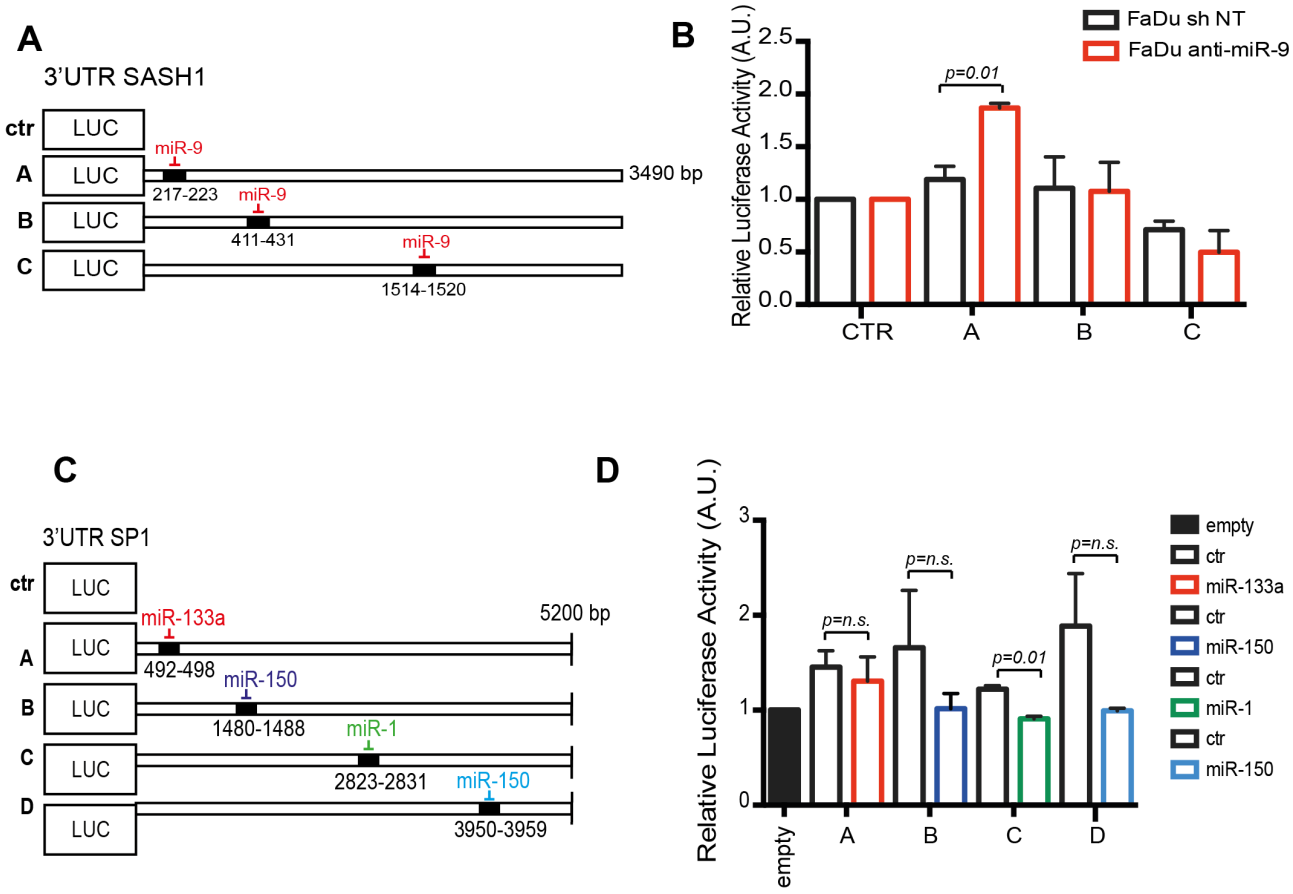
A/B. ROC curve predicting recurrences formation using miR-133a and -150 data iteration applying the Naïve Bayes (**A**) or the Logistic Regression (**B**) models. The AUC is 81% (Sensitivity 88% and Specificity 65%) in **A** and 81% (Sensitivity 83% and Specificity 69%) in **B.**

C. Table reporting the predictive value of miRs combination tested using Logistic Regression or the Naïve Bayes models. TPR = True Positive Value (Sensitivity); FPR = False Positive Ratio (Sensitivity); AUC = Area Under the Curve. Thr = Threshold.



Supplementary Figure S3. *In vitro validation of miR-9 putative targets*

A. Graph showing the normalized expression of miR-1, -9, 133a and 150 in a panel of HNSCC cell lines. Data represent the mean (±S.D.) of 3 independent experiments performed in duplicate.
B. Western blot analysis of the indicated proteins in a panel of HNSCC cells. Actin was used as loading control.
C. Graphs showing (from left to right) the normalized expression of SASH1, KRT13, JUP and FLG mRNA in control and miR-9 knock-down FaDu cells.
D. Graphs showing (from left to right) the normalized expression of JUP and FLG mRNA in control and miR-9 knock-down SCC9 cells.
Data report the median value (±S.D.) of 3 independent experiments performed in duplicate.
Statistical significance is reported in each graph and was calculated using unpaired two-tailed Student's t-test.



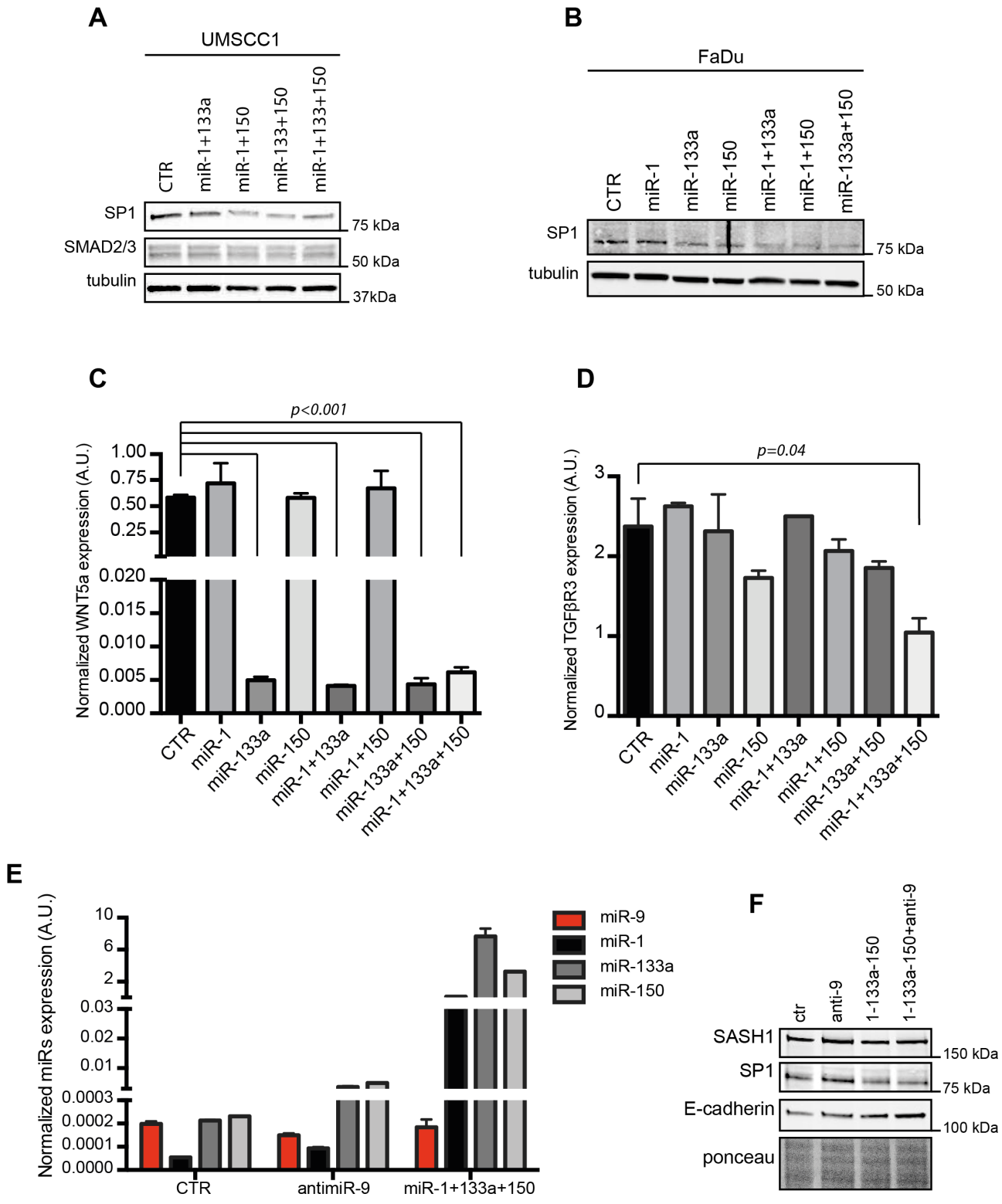
Supplementary Figure S4. *Validation of putative miRs seed sites in the 3'-UTR of SASH1 and SP1 genes*

A. Schematic design of the pGL3-vectors used to test the potential miR-9 seed sites in the SASH1 3'-UTR regulatory region. A, B and C, indicates the 3 regions tested in the luciferase experiments shown in B.

B. Graphs showing the normalized luciferase activity of pGL3-vectors shown in **A** transfected in control or miR-9 knock-down cells. Data represent the median value (±S.D.) of three different experiments. Statistical significance is report in the graph and was calculated using the Mann-Whitney test.

C. Schematic design of the pGL3-vectors used to test the potential miR-1, -133a and -150 seed sites in the SP1 3'-UTR regulatory region. A, B, C and D indicate the 4 regions tested in the luciferase experiments shown in D.

D. Normalize luciferase activity of pGL3-vectors shown in C. 293T17 cells were transfected with control, miR-1, -133a or -150 microRNA and the indicated pGL3-constructs. Data represent the median value (±S.D.) of three different experiments. Statistical significance reported in the graph was calculated using the Mann-Whitney test.



Supplementary Figure S5. *The combined expression of miRs-1, 133a and -150 is necessary to downregulate SP1 mRNA and protein levels.*

A. Western blot analysis of SP1 and SMAD2/3 protein expressions in UMSCC1 cells transfected with miR-1, -133a and -150 as indicated. Tubulin was used as loading control.

B. Western blot analysis of SP1 protein expression in FaDu cells transfected with miR-1, -133a and -150 alone or in combination, as indicated. Tubulin was used as loading control.

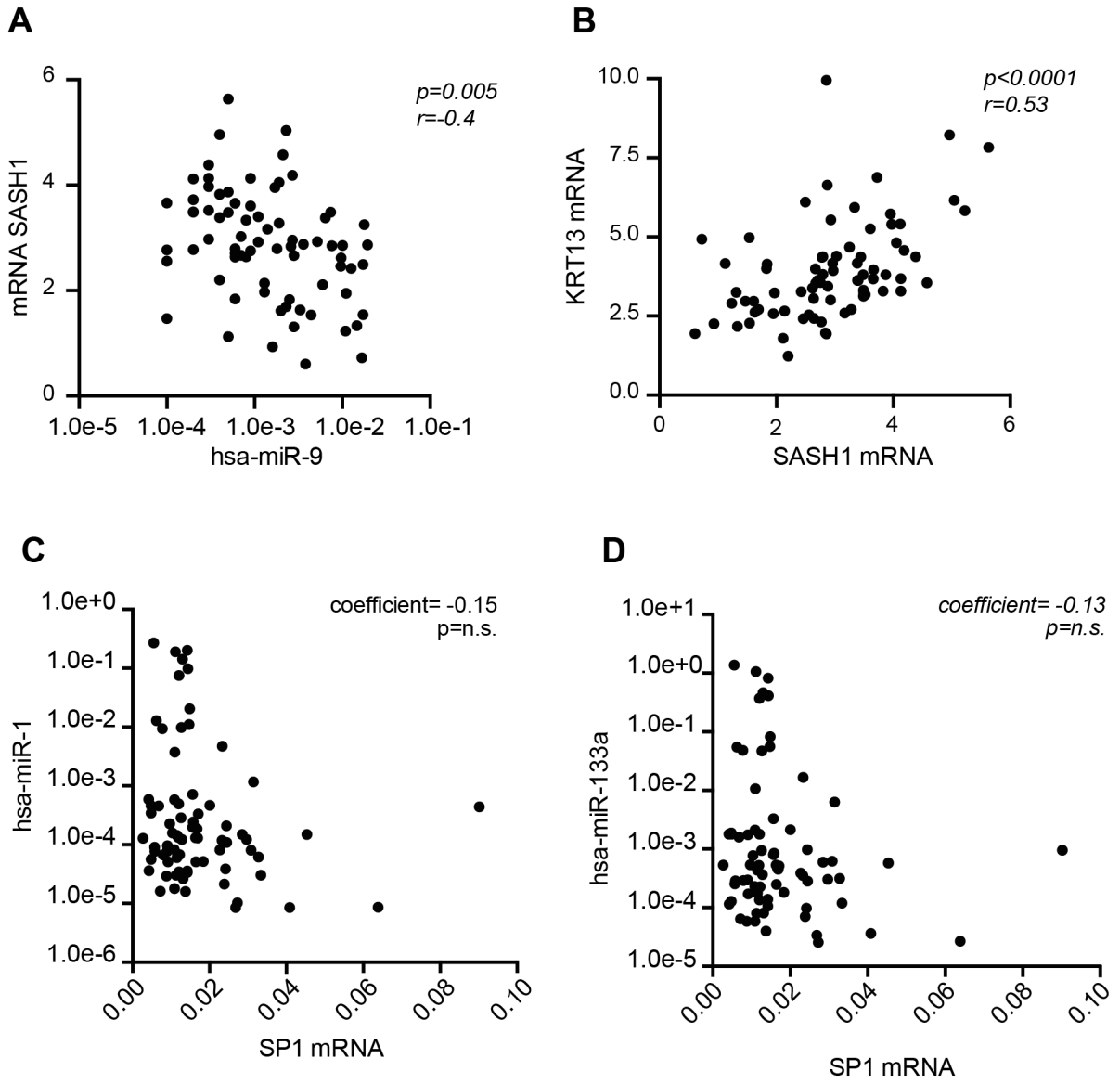
C. Graph showing the normalized expression of WNT5a in CAL27 cells expressing miR-1, -133a and -150 alone or in combination.

D. Graph showing the normalized expression of TGFβ-R3 mRNA in CAL27 cells expressing miR-1, -133a and -150 alone or in combination.

Data report the median value (±S.D.) of 3 independent experiments performed in duplicate. Statistical significance is reported in each graph and was calculated using unpaired two-tailed Student's t-test.

E. Graph reporting the normalized expression of miR-9, -1, -133a, -150 in FaDu cell line transfected with anti-miR-9 or with miR-1, -133a and 150 or their combination, as indicated. Data represent the median value (±SEM) of one experiment performed in triplicate.

F Western Blot analysis of E-cadherin, SP1 and SASH1 protein expressions in FaDu cell line treated as in E. Ponceau staining of the membrane was used as loading control.

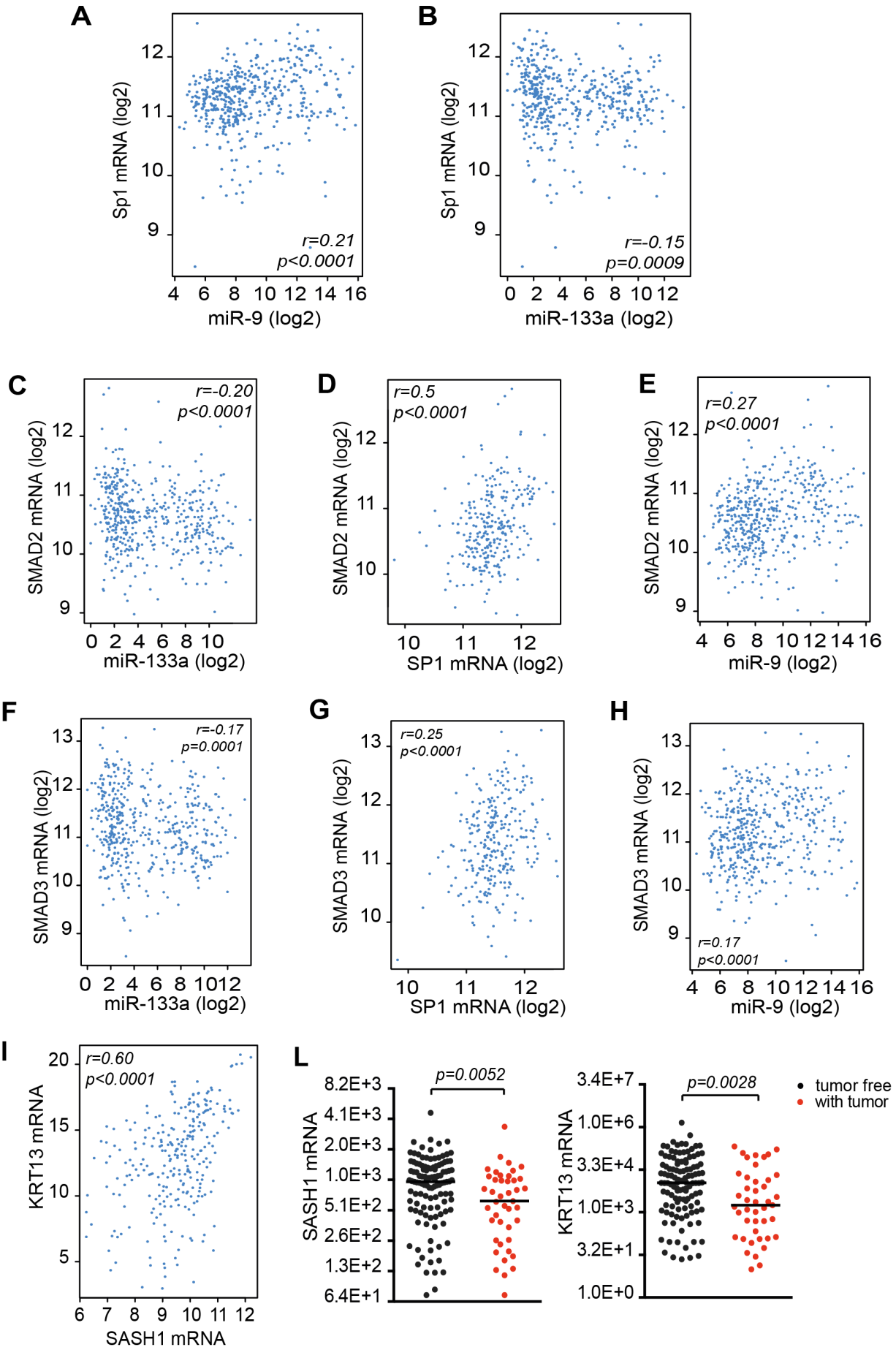


Supplementary Figure S6. *Correlation analyses of miRs putative targets in primary HNSCC collected in our Institute and described in Supplementary table S2 as validation set.*

A/B. Correlation analysis between SASH1 and miR-9 (A) or KRT13 (B) expressions in HNSCC samples included in the validation set.

C/D. Correlation analysis between SP1 and miR-1 (C) or miR-133a (D) or miR-9 (E) expressions in HNSCC samples included in the validation set.

All graphs report correlation value *(r)* and statistical significance *(p)* both calculated with non-parametric two-tailed Spearman correlation test.


A
Sp1 mRNA (log2)
miR-9 (log2)
r=0.21
p<0.0001
B
Sp1 mRNA (log2)
miR-133a (log2)
r=-0.15
p=0.0009
C
SMAD2 mRNA (log2)
miR-133a (log2)
r=-0.20
p<0.0001
D
SMAD2 mRNA (log2)
SP1 mRNA (log2)
r=0.5
p<0.0001
E
SMAD2 mRNA (log2)
miR-9 (log2)
r=0.27
p<0.0001
F
SMAD3 mRNA (log2)
miR-133a (log2)
r=-0.17
p=0.0001
G
SMAD3 mRNA (log2)
SP1 mRNA (log2)
r=0.25
p<0.0001
H
SMAD3 mRNA (log2)
miR-9 (log2)
r=0.17
p<0.0001
I
KRT13 mRNA
SASH1 mRNA
r=0.60
p<0.0001
L
SASH1 mRNA
p=0.0052
KRT13 mRNA
p=0.0028
tumor free
with tumor

Supplementary Figure S7. *Prognostic significance of 4 miRs-targets in the TCGA HNSCC dataset.*

A/B. Dot plots showing the mRNA expression of SP1 and miR-9 (A) or SP1 and miR-133a (B) in the HNSCC TCGA dataset. Correlation value *(r)* and statistical significance *(p)* were calculated with the Spearman correlation test.

C/E. Correlation analyses between SMAD2 and miR-133a (A), SP1 (B) and miR-9 (C) mRNA expression in HNSCC samples included in the TCGA dataset.

F/H. Correlation analyses between SMAD3 and miR-133a (D), SP1 (E) and miR-9 (F) mRNA expression in HNSCC samples included in the TCGA dataset. Correlation and statistical analyses were calculated with nonparametric two-tailed Spearman correlation (*r*). Statistical significance (*p*) is reported in each plot.

I. Correlation analyses between SASH1 and KRT13 mRNA expressions in HNSCC samples included in the TCGA dataset. Correlation and statistical analyses were calculated with nonparametric two-tailed Spearman correlation (*r*). Statistical significance (*p*) is reported in each plot.

L. Dot plot showing the expression of SASH1 (left panel) and KRT13 (right panel) mRNA in HNSCC samples included in the TCGA dataset and divided based on the presence (red dots) or absence (black dots) of tumor.

Supplementary Table S3.

Median miRs' expression (qRT-PCR) according to selected characteristics

Characteristics	n	miR-1	miR-9	miR-133a	miR-150
Sex					
Men	40	1.688	11.671	5.809	354.52
Woman	21	1.446	18.657	5.223	609.28
Wilcoxon test		*p=0.7226*	*p=0.5410*	*p=0.9154*	*p=0.5023*
Age					
<60 years	18	1.235	10.037	4.153	497.13
≥60 years	41	1.793	18.657	5.520	418.95
Wilcoxon test		*p=0.4569*	*p=0.3821*	*p=0.3821*	*p=0.5834*
Cancer site					
Oral cavity/Tongue	38	1.688	7.783	5.353	462.50
Oro-/Hypo-/Pharynx	14	1.973	17.535	7.110	236.50
Larynx	6	1.692	35.955	4.595	677.85
Other	3	0.825	20.530	3.921	299.20
Kruskall-Wallis test		*p=0.8006*	*p=0.0561*	*p=0.7513*	*p=0.5434*
cT					
T1-T2	37	1.582	9.675	5.482	466.43
T3-T4	24	1.674	18.125	5.456	317.56
Wilcoxon test		*p=0.8247*	*p=0.6157*	*p=0.9529*	*p=0.3226*
cN					
N0	32	1.916	12.702	5.353	462.50
N1-N2	29	1.292	11.845	5.520	352.91
Wilcoxon test		*p=0.5251*	*p=0.8852*	*p=0.2724*	*p=0.9080*
Adjuvant radiotherapy					
No	34	1.467	11.671	5.809	387.54
Yes	27	1.793	15.720	5.346	458.57
Wilcoxon test		*p=0.5614*	*p=0.8617*	*p=0.6843*	*p=0.6318*

cT = Clinical Evaluation of Tumor size

cN = Clinical Evaluation of Node status

Extended Experimental Procedures

Primary tumors collection

We collected primary HNSCC specimens from patients underwent surgery at CRO National Cancer Institute, Aviano and at Santa Maria degli Angeli Hospital, Pordenone, Italy. HNSCC specimens were immediately frozen and stored at -80°C. The Internal Review Board of the Centro di Riferimento Oncologico (CRO), Aviano, approved this study (#IRB-08/2013) and each enrolled patient signed an informed consent.

Bioinformatic analyses

Computational analysis includes two parts: A) Uni-Variant Statistical Significance Testing of miR-1, miR-9, miR-133a, and miR-150 (one miRNA at a time). B) Testing Sample Classification based on molecular signatures using models constructed from combinations of miRNAs. More specifically, we test the above 4 miRNAs together, and every combination of 2 or 3 miRNAs listed above.

Uni-variant Significance Test by The Permutation Test (Corcoran *et al*, 2005) was used to calculate the statistical significance of each of the four miRNAs (miR-1, miR-9, miR-133a, and miR-150) individually. A low p-value for the miRNA indicates higher confidence in how well it separates recurrence from non-recurrence patients. For this, we used the permutation test to estimate the p-values for each miRNA. The permutation test works by first measuring the observed difference between the two patient groups (recurrence vs. non-recurrence) for the given gene, then, it randomly shuffles the samples across the two groups k times and it counts how often it encounters a difference greater or equal to the observed difference. When K is large (we used 10000000), the p-value is the ratio between how often a difference greater or equal to the observed difference is

measured randomly (due to chance) out of K. The permutation test is advantageous because it is parametric, it makes no assumptions, and it can be used with small sample size.

Testing of Sample Classification was used to build and test a computational prediction model that predicts recurrence based on the miRNAs expressions. However, the accuracy of prediction relies on the process of classifier construction. In this process, we address challenges mentioned earlier to improve prediction accuracy. Therefore, we adopt the under-sampling technique described in (Klement *et al*, 2011), to counter the effects of class imbalance and the potential over-fitting. The idea is based on selecting equal portions from both patient groups used to train the classifier. For illustration, if the training data has 9 recurrence cases and 29 non-recurrence cases, we select all 9 recurrence cases, plus randomly selected (without replacement) 9 non-recurrence cases to a total of 18 cases for use in training the classifier. In other words, the under-sampling policy is to include the entire minority class cases and randomly subsample an equal proportion of cases in the majority class. This process is then repeated and the average performance is determined. The repeated training and testing demonstrates the reliability and robustness of predictions. For method of classification, we report our results using two very different methods: the Naïve Bayes and the Logistic Regression methods. While the former is a simple probabilistic classifier based on applying Bayes' theorem, the latter constructs a regression model for binary classification. Classifier testing policy is based on repeated 10-fold cross-validation. This strategy follows: we first partition the data randomly into 10 stratified folds. The stratification ensures that every fold i of the 10 folds has approximately the same proportions of positive (recurrence) samples and negative (non-recurrence) samples. For every fold i, we use the remaining 9 folds for training a classifier, and then, we test the resulting classifier on samples in fold i. This process is repeated for all 10 folds to produce predictions for all samples. Then, we calculate the performance metrics for evaluation (they are described below). This entire testing strategy (starting with the random data partitioning) is repeated 10000 times and the average and standard deviations are calculated. The reason for the

repetitions is to eliminate the potential systematic overlap of 8 folds in testing two consecutive folds, a consequence of 10-fold cross validation strategy.

Cell Biology Experiments

Cell culture. FaDu, Cal27, UMSCC1 and UMSCC74b cells were cultured in Dulbecco modified Eagle medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco). SCC9, SCC15 and SCC25 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (Sigma) and Ham's F12 medium (Sigma) containing 1.2 g/L sodium bicarbonate (Sigma), 2.5 mM L-glutamine (Sigma), 15 mM HEPES (Sigma) and 0.5 mM sodium pyruvate (Sigma) supplemented with 400 ng/ml hydrocortisone (Sigma) and FBS 10%. All cell lines were authenticated by BMR Genomics srl Padova, Italia, according to Cell ID ™ System (Promega, USA) 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).

All *in vitro* studies were performed in triplicate, unless otherwise specified.

Lentiviral trasduction of FaDu and SCC9. FaDu and SCC9 cells were transduced with Lentiviruses expressing anti-miR-9-5p (MISSION® Lenti miRNA inhibitor human has-miR-9-5p, HLTUD0946, Sigma) according to the manufacturer protocol. Cells were transduced with anti-miR-9 (or control) lentivirus and selected in 1.0-1.5 μg/ml puromycin.

miR-mimic or inhibitor transfection. Anti-miR-9 (MISSION® Synthetic miRNA inhibitor Sigma) were used to transiently knockdown miR-9 in FaDu and SCC9 cells. Hsa-miR-1, -133a, -150 (mission® miRNA mimic) were used to overexpress alone or in combination these microRNAs. miR inhibitor, mimic or control were transfected into different HNSCC cells using Oligofectamine Transfection Reagent (LifeTechnologies) according to manufacturer protocol.

Cell viability and IC50 drugs calculation. HNSCC cells were seeded in 96-well culture plates (2-4x10^3 cells/well) and after 24 hours treated with increasing doses of MTA, SB43 or SB52 for 72 hours. Combination treatments using MTA plus SB52 were performed as above using the MTA at the IC50 concentration, calculated for each cell line after 72 hours of treatment. Cell viability was determined at the end of treatment using the CellTiter 96 AQueous cell proliferation assay kit (Promega).

Luciferase assay. Luciferase assay was used to validate miR-9, miR-1, miR-133a and miR-150 putative target sites on SP1 and SASH1 3' UTR. We amplified sequences surrounding the putative miR-1, -133a, -150 and -9 binding sites from FaDu cells genomic DNA using specific primers carrying restriction endonuclease sequence XbaI. PCR products were cloned in pGL3 control vector (Promega) digested with XbaI (Promega), at the 3' of the luciferase gene, which is under the regulation of SV40 promoter. Primers used to amplified SASH1 and SP1 sequences are reported below:

Primer	***Sequence 5'- 3'***	***GeneBank Accession***
3' UTR SASH1 A FW	TCTAGATGGACAAGAGCCACCCTTTC	NM_015278
3' UTR SASH1 A Rev	TCTAGAAGACAGAAGAGCAAGGGCAC	NM_015278
3' UTR SASH1 B FW	TCTAGATGGCTGCGAATGCTCTATCT	NM_015278
3' UTR SASH1 B Rev	TCTAGACTGGTCGAGCCAGATGTTCTA	NM_015278
3' UTR SASH1 C FW	TCTAGATGTTTTACAACTGATTTCAGCACA	NM_015278
3' UTR SASH1 C Rev	TCTAGATCAAGGGCTTGTGGTCAAGG	NM_015278
3' UTR SP1 A FW	TCTAGATTCTCTTCTCAGCTCTTCCATGA	NM_138473
3' UTR SP1 A Rev	TCTAGAAGTCGAAGAAGCTGATCCCAA	NM_138473
3' UTR SP1 B FW	TCTAGAGTTCTTGGGTGGTTCCTAAGGG	NM_138473
3' UTR SP1 B Rev	TCTAGAACAATGCTTTTATGGCTGGGC	NM_138473
3' UTR SP1 C FW	TCTAGATGCTGGTTTACCCTCAACCC	NM_138473
3' UTR SP1 C Rev	TCTAGACTGCCCTCGAAGTTTTGGTC	NM_138473
3' UTR SP1 D FW	TCTAGACATAGCTCTCCTTCCCCCTCA	NM_138473
3' UTR SP1 D Rev	TCTAGATTGGGAAGGCAGACAAAGAGA	NM_138473

For the luciferase assays, 293T17, CAL27 or FaDu were cotransfected with 500 ng of the reporter construct and 50 ng of pRL-TK vector (internal control) in 24-well plate using FuGENE® HD Transfection Reagent (Promega) according to manufacturer's recommendations. After transfection cell lysates were assayed for luciferase activity using the Dual-Luciferase reporter assay system (Promega). Values were normalized using Renilla luciferase.

Reagents Mithramycin A (MTA), an aureolic acid compound that specifically inhibits SP1 activity, SB-525334 (SB52) and SB431542 (SB43), two potent and selective inhibitors of TGFβR1. All compounds were purchased from Sigma and used for *in vitro* and/or *in vivo* experiments.

Molecular Biology Experiments

DNA extraction and evaluation of TP53 mutational status and HPV DNA status Total DNA was isolated from patient-derived primary tumors using QIAamp DNA Mini Kit (QIAGEN, Germany) according to manufacturer protocol. Total DNA was quantified using QuantiFluor® dsDNA System (Promega).

HPV genotyping was determined in all samples using the INNO-LiPA HPV Genotyping Extra assay (Innogenetics, Belgium) and following the manufacturer's instructions. The INNO-LiPA HPV assay is a PCR-based hybridization assay that includes a cocktail of biotinylated consensus primers (SPF 10) to amplify a 65 bp region within the L1 ORF (open reading frame) of multiple HPV types. After DNA extraction, all specimens were subjected to PCR amplification (40 cycles) using the Inno-LiPA HPV Genotyping Extra Amp. The PCR product was then denatured, and a 10-µl aliquot was hybridized onto nitrocellulose strips where the HPV type-specific oligonucleotides were already bound. After 60 min at 49°C, the PCR product bound to a specific probe was detected by an alkaline phosphatase-streptavidin conjugate and colorimetric detection.

TP53 mutational status was assessed by next generation sequencing (NGS) with an amplicon-based

strategy to cover with at least a 1000X coverage and 5% of sensitivity the whole *TP53* sequence. *TP53* NGS primer list is available upon request. PCR products were generated using a high fidelity Taq polymerase (Phusion High-Fidelity DNA Polymerase; Thermo Scientific, Waltham, MA) and subjected to NGS on a MiSeq sequencer (Illumina, San Diego, CA). Data were analyzed with MiSeq reporter (Illumina) and IGV software against human genome assembly hg19.

RNA extraction and qRT-PCR analyses. Total RNA for miR microarray and qRT-PCR analyses was isolated from patient-derived primary tumors or cell cultures using Trizol solution (Roche Applied Science Mannheim, Germany) according to manufacturer protocol. Total RNA was quantified using NanoDrop (Thermo Fisher Scientific Inc., USA). Mirs expression profile was performed using the Nanostring™ technology that allowed to evaluate the expression of 746 miRs (664 Human 82 Viral) along with the one of Housekeeping genes.

Differentially expressed miRs were validated using the TaqMan single tube MicroRNA Assays. All reagents, primers and probes were obtained from Applied Biosystems and Reverse Transcriptase (RT) reactions and qRT-PCR were performed according to the manufacturer instructions (Applied Biosystems, Life Technologies). Normalization of the validation set was performed on the U6 RNA. All RT reactions were run in an Opticon qRT-PCR Thermocycler (Bio-Rad). Comparative qRT-PCR was performed in triplicate, including no-template controls. miR levels were quantified using the MyiQ2 (Bio-Rad). Relative expression was calculated using the comparative Ct method.

For gene expression analysis, RNA was retro-transcribed with AMV Reverse Transcriptase to obtain cDNAs, according to provider's instruction (Promega). Absolute quantification of targets was evaluated by qRT-PCR, using SYBR Green dye-containing reaction buffer (Real SG Master Mix 5x, Experteam). Standard curves (10-fold dilution from 10^{1} to 10^{-4} attomoles) were prepared for all the analyzed genes (TGFβR1, TGFβR2, TGFβR3, SASH1, SP1, KRT13, β-catenin, WNT4, WNT5a, JUP, FLG, HPV-E6 and -E7) and housekeeping genes (GAPDH, SDHA and POL2a). The

incorporation of the SYBR Green dye into the PCR products was monitored in real time PCR, using the MyiQ2 Two Color Real-time PCR Detection System (Bio-Rad). Ct values were converted into attomoles and normalized expression was evaluated by using GAPDH, SDHA or POL2a as housekeeping genes.

Primer	***Sequence 5' – 3'***	***Gene Bank Accession***
Human TGFβR1 FW	CATTTTTCCCAAGTGCCAGT	NM_001306210.1
Human TGFβR1 Rev	ACACCCCTAAGCATGTGGAG	"
Human TGFβR2 FW	CCATGTCTCACAGCCAGCTA	NM_001024847.2
Human TGFβR2 Rev	CCAGGAGAAATAAGGGCACA	"
Human TGFβR3 FW	CCAAGATGAATGGCACACAC	NM_003243.4
Human TGFβR3 Rev	CCATCTGGCCAACCACTACT	"
Human SASH1 FW	CTGTCACCCCCTCAGTGTTT	NM_015278.3
Human SASH1 Rev	GAACAGGGTGGAGTCCGTTA	"
Human Sp1 FW	GGTGCCTTTTCACAGGCTC	NM_138473.2
Human Sp1 Rev	CATTGGGTGACTCAATTCTGCT	"
Human KRT13 FW	GTCTTCAGCACCCAGAGGAG	NM_153490.2
Human KRT13 Rev	TTCGAGAAAGGCAGGAAACT	"
Human β-catenin FW	GAAACGGCTTTCAGTTGAGC	NM_001904.3
Human β-catenin Rev	CTGGCCATATCCACCAGAGT	"
Human WNT4 FW	GCTGTGACAGGACAGTCGAT	NM_030761.4
Human WNT4 Rev	GCCTCATTGTTGTGGAGGTT	"
Human WNT5A FW	GGGTGGGAACCAAGAAAAAT	NM_003392.4
Human WNT5A Rev	TGGAACCTACCCATCCCATA	"
Human JUP FW	GAAAAGCTGCTCTGGACCAC	NM_002230.2
Human JUP Rev	GACGTTGACGTCATCCACAC	"
Human FLG FW	GGCAAATCCTGAAGAATCCA	NM_002016.1
Human FLG Rev	TGCTTTCTGTGCTTGTGTCC	"
Human GAPDH FW	GAAGGTGAAGGTCGGAGTC	NM_001289746.1
Human GAPDH Rev	GAAGATGGTGATGGGATTTC	"
Human SDHA FW	AGAAGCCCTTTGAGGAGCA	NM_004168.3
Human SDHA Rev	CGATTACGGGTCTATATTC	"
Human POL2a FW	GCAAATTCACCAAGAGAGACG	NM_000937.4
Human POL2a FW	CACGTCGACAGGAACATCAG	"
HPV16 E6 1 FW	ACAAACCGTTGTGTGATTTGTT	
HPV16 E6 1 Rev	CAGTGGCTTTTGACAGTTAATACA	
HPV16 E6 2 FW	GCAAAGACATCTGGACAAAAAG	
HPV16 E6 2 Rev	ACCGACCCCTTATATTATGGAATC	
HPV16 E7 1 FW	GAACCGGACAGAGCCCATTA	
HPV16 E7 1 Rev	ACACTTCGAACAAAAGGTTACA	
HPV16 E7 2 FW	TAACCTTTTGTTCGAAGTGTGA	
HPV16 E7 2 Rev	TTTGTACGCACAACCGAAGC	

Protein extraction and Western Blot Analyses. For cellular protein lysates, cells were scraped on ice using cold Ripa lysis buffer (150 nM NaCl, 50 mM Tris HCl pH 8, 1% Igepal, 0.5% sodium

deoxycholate, 0.1% SDS) supplemented with a protease inhibitor cocktail (CompleteTM, Roche), 1 mM Na_3VO_4 (Sigma), 100 mM NaF (Sigma) and 1 mM DTT (Sigma). Proteins were separated in 4-20% SDS-PAGE (Criterion Precast Gel, Bio-Rad) and transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked with 5% dried milk in TBS-0·1% Tween 20 or in Odyssey Blocking Buffer (LI-COR, Biosciences) and incubated at 4°C overnight with primary antibodies.

Primary antibodies were from Cell Signaling (rabbit polyclonal Claudin-1 #4933, rabbit polyclonal SMAD2/3 #5678, rabbit monoclonal pS465-7 SMAD2 #3108, rabbit monoclonal β-Actin #8457), Abcam (mouse monoclonal SP1 #ab51513), Bethyl (rabbit polyclonal SASH1 #A302-265), Sigma (rabbit polyclonal KRT13 #SAB2104755, mouse monoclonal Tubulin #T8203), Transduction Lab (mouse monoclonal E-cadherin #610404), Santa Cruz (mouse monoclonal PARP-1 #sc8007, goat polyclonal Vinculin N19 #sc7649), Millipore (mouse monoclonal GRB2, MABS89). Membranes were washed in TBS-0·1% Tween20 and incubated 1 hour at RT with IR-conjugated (AlexaFluor680, Invitrogen or IRDye 800, Rockland) secondary antibodies for infrared detection (Odyssey Infrared Detection System, LI- COR) or with the appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) for ECL detection (Clarity Western ECL Substrate, BioRad). Band quantification was performed using the Odyssey v1.2 software (LI- COR) or the QuantiONE software (Bio-Rad Laboratories). The Re-Blot Plus Strong Solution (Millipore) was used to strip the membranes, when reblotting was needed.

Xenograft growth in mouse flanks and treatment. Primary tumors were established by subcutaneous injection of $2x10^6$ FaDu cells bilaterally in the flanks of female athymic nude mice (Harlan, 6 weeks old). Growth of primary tumors was monitored by measuring tumor width (W) and length (L) and calculating tumor volume based on the formula ($W^2xL/2$). When tumors reached a volume of 1000-1200 mm^3, mice pre-anesthetized underwent surgery to remove the primary tumors. An expert pathologist evaluated the presence and the width of surgical resection margins in explanted tumors.

Margins widths were measured with an optical microscope equipped with an ocular micrometer. To evaluate the effect of SB-525334 (Sigma) and Mithramycin A (Sigma) on the formation of recurrences, the day before surgery, the animals were randomly divided into groups according to experimental design (3 mice/control group, 4 mice/treatment). The administration of the drugs was performed following a "peri-operative schedule", i.e. mice received the treatments the day before, the same day and the day after surgery. Vehicle or Mithramycin A 1 mg/kg, resuspended in DMSO, were administered intraperitoneally for three consecutive days, following the "peri-operative schedule". Vehicle or SB525334, resuspended in PBS, were administered 15 mg/kg with oral gavages following the "peri-operative schedule", and also three and six days after surgery. The appearance of local recurrence was monitored by macroscopic examination of mice over a period of 8 weeks. Unless tumor burden was incompatible with the well-being of the animals, mice were sacrificed at the end of the experiment and pathologically examined for the presence of microscopic recurrences. *In vivo* experiment was carried out in strict accordance with the recommendations contained in the Guide for the Care and Use of Laboratory Animals of the CRO Aviano, National Cancer Institute, Italy. The project was reviewed and approved by the CRO Institutional Animal Care and Use Committee and conducted according to that committee's guidelines. All mice were monitored twice a week and euthanized when required in accordance to the "AVMA guidelines on Euthanasia". All efforts were made to minimize suffering.