Supplementary Appendix

miR-9 modulates and predicts the response to radiotherapy and EGFR-inhibition in HNSCC

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Appendix Figure S1. miR-9 expression is associated with higher in vivo growth of HNSCC cells.

A. qRT-PCR analyses of normalized miR-9 expression in FaDu, SCC9, CAL27 and UMSCC1 cancer cells and NHBE normal epithelial cells. Data are the mean (±SD) of three independent experiments each performed in duplicate. A.U. = Arbitrary Units.

B. Western Blot (WB) analyses of the p53 protein expression in FaDu, SCC9, CAL27 and UMSCC1 cells. Tubulin was used as loading control.

C. Graph reporting the mean value (±SD) of tumor volume in nude mice injected in both flanks with 1x10^6 FaDu (n=4 mice) or 5x10^6 CAL27 cells (n=3 mice) followed for up to 8 weeks.

D. Typical images of control (shCTR) and antimiR-9 FaDu cells not stimulated (untreated) or treated with TGFβ 10ng/mL for 48 hours.

E. Scatter plot reporting the distance covered by control (shCTR) and antimiR-9 FaDu cells as indicated in a typical analysis.

F. Graphs reporting the velocity and the distance covered by cells as in E. Data are the mean (±SD) of two independent experiments, performed in triplicate, in which at least 8 cells were tracked.

In A, C and F, unpaired t-test was used to verify the statistical significance. *p<0.05; **p<0.01; ***p<0.001
Appendix Figure S2. MiR-9 expression sustains the tumor initiating properties of HNSCC cells.

A. qRT-PCR analyses of normalized miR-9 expression in control (shCTR) and antimiR-9 SCC9 cells used in the experiment reported in B-E.

B. On the left, WB analyses of the indicated protein expression in control and antimiR-9 SCC9 cells described in B. Actin was used as loading control. Right graph shows the quantification of SASH1, KRT13 and Zo-1 protein expression. Data represent the mean (±SD) of three biological replicates.

C. Graph reporting the growth of SCC9 cells described in A-B followed up for 5 days using MTS assay and expressed as fold over day 1. Data represent the mean (±SD) of three independent experiments, performed in sextuplicate.
D. Colony formation assay of the cells described in A-B. On the left, representative images of the clones are shown, and on the right, the graph reports the number of clones per well.

E. Sphere forming assay with cells described in A-B. On the left, representative images of the spheres are shown, on the right, graph reports the percentage of sphere forming efficiency.

F. qRT-PCR analyses of normalized miR-9 expression in control and miR-9 overexpressing UMSCC1 cells used in the experiment reported in H-I.

G. On the left, WB analyses of the indicated protein expression in control and miR-9 overexpressing UMSCC1 cells described in F. Actin was used as loading control. Right graph shows the quantification of SASH1, KRT13 and Zo-1 protein expression. Data represent the mean (±SD) of three biological replicates.

H. Graph reporting the growth of UMSCC1 cells described in F-G followed up for 5 days using MTS assay and expressed as fold over day 1. Data represent the mean (±SD) of three independent experiments, performed in sextuplicate.

I. Colony formation assay of the cells described in F-G. On the left, representative images of the colonies are shown, and the graph on the right reports the number of colonies per well.

J. qRT-PCR analyses of normalized miR-9 expression in control (shCTR) and antimiR-9 CAL27 cells used in the experiment reported in K-L.

K. On the left, WB analyses of the indicated protein expression in control and antimiR-9 CAL27 cells described in J. Actin was used as loading control. Right graph shows the quantification of SASH1, KRT13 and Zo-1 protein expression. Data represent the mean (±SD) of three biological replicates.

L. Graph reporting tumor volume (mean ±SD) in NSG mice injected in both flanks with 1x10^6 control (shCTR) or antimiR-9 CAL27 cells followed for up to 25 days (n=3 mice/group). In the figure A.U. = Arbitrary Units; t-test was used to verify the statistical significance. *p<0.05; **p<0.01; ***p<0.001.
Appendix Figure S3. miR-9 protects HNSCC cells from cell death induced by anti-EGFR therapies.

A. Graphs report the cell viability of control (shCTR) and antimiR-9 SCC9 cells treated with increasing concentration of Gefitinib (left) or Cetuximab (CTX) for 72 hours and analyzed using MTS cell viability assay.

B/C. Graphs report cell viability of control and miR-9 UMSCC1 (B) or CAL27 (C) cells treated with increasing concentration of Cetuximab (CTX) for 72 hours and analyzed using MTS cell viability assay.

In the figure data represent the mean (±SD) of three independent experiments each performed in sextuplicate. Unpaired t-test was used to verify the statistical significance at each dose. *p<0.05; ***p<0.001.
Appendix Figure S4. SASH1 expression does not impact on proliferation, survival and motility of HNSCC cells.

A. WB analyses of the indicated protein expression in control and SASH1 overexpressing FaDu (top panel) and CAL27 (bottom panel) cells. Tubulin was used as loading control.
B/C. Graph reporting the growth of control and SASH1 overexpressing FaDu (B) or CAL27 (C) cells followed up for 5 days using MTS assay. Data are expressed as fold over day 1, and represent the mean value (±SD) of three independent experiments, performed in sextuplicate.

D. Colony formation assay of the CAL27 cells described in A. On the left, representative images of the clones are shown, and on the right, graph reporting number of clones per well.

E. Sphere forming assay using FaDu cells described in A. On the left, representative images of the spheres are shown, on the right, graphs report the area of the sphere and percentage of sphere forming efficiency, as indicated.

F. Matrigel evasion assay of CAL27 cells described in A. On the right, representative phase-contrast images are reported. Left graph reports the distance covered by the individual cells from the edge of the drop.

G. WB analyses of SASH1 protein expression in control (shCTR) and SASH1 silenced (shSASH1#2 and #4) CAL27 cells. Actin was used as loading control.

H. Graph reporting the growth of CAL27 described in G and followed up for 5 days using MTS assay. Data are expressed as fold over day 1 and represent the mean (±SD) of three independent experiments, performed in sextuplicate. Two-way ANOVA with Sidak’s multiple comparison test was used to verify the statistical significance.

I. Colony formation assay of the CAL27 cells described in G. On the left, representative images of the clones are shown, and on the right, graph reporting number of clones per well.

J. Cell migration assay of CAL27 cells described in G. On the left, representative images of CAL27 cells allowed to migrate for 2 hours in transwell assay and then allowed to attach for 8 hours. Right graph shows the percentage of migrated cells per well.

In the figure n.s = not significant; *p<0.05; **p<0.01.
Appendix Figure S5. Genetic or pharmacological inhibition of Sp1 phenocopies the miR-9 loss in HNSCC cells.

A. Graph reporting the growth of FaDu cells silenced for Sp1 (shSP1#3 and #5) or not (shCTR) and followed up for 5 days using MTS assay. Data are expressed as fold over day 1 and represent the mean value (±SD) of three independent experiments, performed in sextuplicate. Two-way ANOVA with Sidak’s multiple comparison test was used to verify the statistical significance.

B. WB analyses of Sp1 protein expression in control and Sp1 silenced CAL27 cells. Histone H3 was used as loading control.

C. Graph reporting the growth of CAL27 cells described in followed for 5 days using MTS assay. Data are expressed as fold over day 1 and represent the mean (±SD) of three independent experiments, performed in sextuplicate. Two-way ANOVA with Sidak’s multiple comparison test was used to verify the statistical significance.

D. Sphere forming assay with cells described in B. On the left, representative images of the spheres are shown. The graphs report the percentage of sphere forming efficiency and the spheres area as indicated. Data are expressed as mean value (±SD) and two-way ANOVA with Sidak’s multiple comparison test was used to verify the statistical significance.
Sphere forming assay with FaDu cells treated with 5 nMol/L (E) or CAL27 cells treated with 2 nMol/L of Myramycin A (MTA) (F) or not (untreated). On the left, representative images of the spheres are shown. On the right, the graph reports the percentage of sphere forming efficiency in the first and second generation of spheres. Data are expressed as mean value (±SD) of three independent experiments performed in duplicate and two-way ANOVA test was used to calculate the statistical significance. **p<0.01; ***p<0.001.
Appendix Figure S6. Overexpression of miR-9 leads to radio-resistance in HNSCC cells.

A. Graph reporting cell viability of control (shCTR) and miR-9 overexpressing CAL27 cells treated with increasing concentration of Cisplatin, 5-FluorUracil, Paclitaxel or Bleomycin for 72 hours and analyzed using MTS cell viability assay. Data represent the mean (±SD) of three independent experiments each performed in sextuplicate. Unpaired t-test was used to verify the statistical significance.
B. Clonogenic assay of CAL27 cells as described in A and treated with 2 Gy RT. The left panel shows typical images of the clones. Right graph reports the percentage (±SD) of survived cells respect to not irradiate cells (NIR). Unpaired t-test was used to verify the statistical significance.

C. WB analyses of the γH2AX expression in control (shCTR) and miR-9 overexpressing CAL27 (top panel) and UMSCC1 (bottom panel) cells not irradiated (NIR) and collected 1, 8 or 24 hours after 2Gy IR (F). Actin and tubulin were used as loading control.

D. Left panels show representative immunofluorescence staining of γH2AX (green), pS10-H3 (red) and nuclei (blue) performed in control (shCTR) and miR-9 overexpressing CAL27 cells, not irradiated (NIR) or irradiated with 2Gy, and collected at different time points (1h, 8h and 24 h post radiation). On the right, graphs reporting the percentage of γH2AX (left) and pS10-H3 (right) positive cells described in A evaluated in not irradiated (NIR) and 1, 8 or 24 hours after 2Gy IR using immunofluorescence analyses. Data represent the mean (±SD) of three independent experiments, in which at least 10 randomly selected fields were evaluated for the presence of γH2AX or pS10-H3 positive cells. Unpaired t-test was used to verify the statistical significance at each time point.

E. Graph reporting cell viability of control (shCTR) and antimiR-9 CAL27 cells, overexpressing or not SP1 and treated with increasing concentration of Bleomycin for 72 hours and analyzed using MTS cell viability assay. Data represent the mean (±SD) of three independent experiments each performed in sextuplicate. Two-way ANOVA with Sidak’s multiple comparison test was used to verify the statistical significance.

F. WB analyses of the γH2AX expression in control and miR-9 overexpressing CAL27 cells treated with Cisplatin (CDDP) as indicated. Actin was used as loading control.

G. qRT-PCR analyses of normalized miR-9 expression in CAL27 cells treated with increasing concentration of CDDP, as indicated. Data represent the mean (±SD) of three independent experiments each performed in duplicate. One-way ANOVA test was used to verify the statistical significance.

H. Graph reporting the normalized Luciferase activity of miR-9 reporter vector in FaDu cells treated with Bleomycin (BLEO) for 16 hours (0) and then allowed to repair for 1 or 2 hours. Data represent the mean (±SD) of three independent experiments each performed in duplicate. One-way ANOVA test was used to verify the statistical significance.

In the figure *p<0.05; **p<0.01; ***p<0.001.
| Head and Neck Cancer Samples |
|------------------------------|
| **Characteristic** | **Value** |
| **Age - Year** | |
| Median | 64 |
| Range | 33 - 92 |
| **Cancer site** - no. (%) | |
| Oral cavity / Tongue | 58 (38.7) |
| Tongue | 5 (3.3) |
| Oro-Pharynx | 46 (30.7) |
| Hypo-Pharynx | 11 (7.3) |
| Larynx | 26 (17.3) |
| Tonsil | 3 (2) |
| Nasal Cavity | 1 (0.7) |
| **Tumor Grade** - no. (%) | |
| G1 | 10 (6.7) |
| G2 | 78 (52.0) |
| G3 | 53 (35.3) |
| Not Available or Specified | 9 (6.0) |
| **Histology** - no. (%) | |
| SCC* | 150 (100) |
| **cT** - no. (%) | |
| T1 | 33 (22) |
| T2 | 56 (37.3) |
| T3 | 27 (18) |
| T4 | 29 (19.4) |
| Not Available or Specified | 5 (3.3) |
| **cN** - no. (%) | |
| N0 | 73 (48.67) |
| N1 | 28 (18.67) |
| N2 | 37 (24.67) |
| N3 | 2 (1.33) |
| Not Available or Specified | 10 (6.67) |

**Appendix Table S1. Patients and tumor features of the CRO-Aviano collection**

Appendix Table S1 summarizes the pathological and histological tumor status in HNSCC cohort collected at the CRO of Aviano. * SCC = Squamous Cells Carcinoma; ** cT = Clinical Tumor size; *** cN = Clinical Node Status
**Appendix Table S2.** *HNSCC patients treated with Radiotherapy and Cetuximab*

Appendix Table S2 summarizes the pathological and histological tumor status, including TP53 mutations and the presence of HPV infections in HNSCC cohort collected at CRO Aviano NCI and Gemelli Hospital.

**Legend to Table S2**

| Acronym | Description |
|---------|-------------|
| N/A     | Not Available |
| SCC     | Squamous Cells Carcinoma |
| cT      | Clinical Tumor size |
| cN      | Clinical Node Status |
| cM      | Clinical Metastasis Status |
| CT      | Chemotherapy |
| RT      | Radiotherapy |
| CTX     | Cetuximab |
| PD      | Progression Disease |
| SD      | Stable Disease |
| CR      | Complete Response |
| PR      | Partial Response |
Appendix Table S3. Primers used to clone Luciferase reporter vectors.

Appendix Table S3 summarizes the sequences of the primers used to clone the different regions of the KLF5 3’-UTR and Sp1 promoter.

| Primer                        | Sequence 5’- 3’                                                                 | GeneBank  |
|-------------------------------|-------------------------------------------------------------------------------|-----------|
| 3’UTR KLF5 A For              | TGGGCTCCCTCAAATGACAG                                                         | NM_001730 |
| 3’UTR KLF5 B Rev             | GACCCCTTTTGGCATTTTG                                                         | NM_001730 |
| 3’UTR KLF5 mut A             | GGGAAATACATTGTATTAATACCGGAGTGTGGGTATTTAAA                                   |           |
| 3’UTR KLF5 mut B             | GCTTATTTTTCTGCCCCTCCGTATAACAGCATCAGCATC                                     |           |
| Sp1 promoter -146 For         | gctagcGGGCTTTGTGGCGCGCGTGCTGCTC                                              | NM_138473 |
| Sp1 promoter -281 For         | gctagcGCAACTTAGTCTACACGCGTCTGCTGCTGCTC                                     | NM_138473 |
| Sp1 promoter -443 For         | gctagcCTATCAAAGCTTTGCTATCC                                                   | NM_138473 |
| Sp1 promoter -1612 For        | gctagcGGCACCCTAACACGGTGAGGCGAG                                              | NM_138473 |
| Sp1 promoter -20 Rev          | ctgagGCTCAAGGGGGGTCTGAGGGGTGAG                                             | NM_138473 |
| Gene   | Primer Forward 5’ – 3’ | Primer Reverse 5’ – 3’ | GeneBank ID   |
|--------|------------------------|------------------------|---------------|
| ACTB   | CCAGAGGCGTACAGGGGATAG  | CCAACCGCGAGAGATGA      | NM_001101     |
| ALOXE3 | CCCATGAAAATTGACATCC    | CATCCAGCTTCTTCAGGGG    | NM_021628     |
| AMMECRI| GCAGAGATGCTGGCTTGG     | AGCTCATTCCCTTGATG       | NM_015365     |
| ATP10B | AGAAGCCAAAAAGTGCTCA    | ACAAGGTTGACACCAACAGG    | NM_025153     |
| CD24   | AACTAATGCCACACCGAGG    | CCTCTTTTCCTTGGCCAT     | NM_013230     |
| EGFR   | TGCGTCTCTGGTGCTGAAT    | GGTCTTACCTCCAAGCTT     | NM_002211.3   |
| ELOVL4 | TTACACTGACTGCCCTTCC    | GTCACACCTTGGTAAAGAAA   | NM_005228     |
| FAXDC2 | ACAGCTTTCATCTGCTGCC    | AAAGTAGCCAGAGACCC       | NM_032385     |
| FLG    | GGCAAATCTGAGAATCCA     | TGCCTTTGCTGCTTGTCCT    | NM_002016     |
| JUP    | GAAAGCTGCTGCTGGACCAC   | GACGTTGACGTACCTCACAC    | NM_001352773  |
| KLF5   | CCCCTTCACATACACATGC    | AGTTAATGCGAGGTGATG     | NM_001730     |
| KLHL18 | CTTGCTGCTGGCAAAGACAG   | TGGACTTGCAAAGAAATG     | NM_025010     |
| LDLRAP1| TTTCCTTCTCTCCCTTTG     | TCCAAGGCTCTCCGCTAAG    | NM_015627     |
| NCOA1  | CATGGTCAGGCAAAAACCTT   | GCTTGCCGATTTTTGCTGTAT  | NM_001362952  |
| RASGRP1| GAGTTGATTGGCGGCTGAT    | GTGAGGTCGGTACGATCT     | NM_005739     |
| RCOR1  | TTCAAGGGAATTCTCAGAGT   | CTTCGGGACTTTCAGGAA     | NM_015156     |
| SASH1  | CTGTCAACCCCTCAGTTT     | GAACAGGAGTGAGTCCTGTA   | NM_015278.3   |
| SCYL3  | TCAGTTGCTGTTTGCAGAC    | TCGAGCTGCTTGGAGATGA    | NM_181093     |
| SDHA   | AGAAGCCCTTGGAGGAGCA    | CGATTACGGGCTTATATTCC   | NM_004168.3   |
| SERPINB8| CCACAATTCACACACAGC     | TCACAGCCAACAGCTGGAATA  | NM_198833     |
| SPI    | GTTGCGCTTTCACACGCTC    | CATTTGGGTGACTCAATCTGCT | NM_138473     |

**Appendix Table S4.** Primers used in qRT-PCR analyses.

Appendix Table S4 summarizes the sequences of the primers (forward and reverse) used for qRT-PCR analyses.
| Antibody                  | Catalog Number | Vendor       | Application and Dilution |
|--------------------------|----------------|--------------|--------------------------|
| SASH1                    | #A302-265A     | Bethyl       | WB (1:500)               |
| pY1068-EGFR              | #3777          | Cell Signaling | WB (1:1000)           |
| β-Actin                  | #8457          | Cell Signaling | WB (1:2000)           |
| pT202-Y204-ERK1/2        | #9101          | Cell Signaling | WB (1:1000)           |
| ZO-1                     | #8193          | Cell Signaling | WB (1:500)             |
| Histone H3               | #4499          | Cell Signaling | WB (1:1000)           |
| KRT13                    | #SAB2104755    | Millipore-Sigma | WB (1:500)           |
| α-Tubulin                | #T8203         | Millipore-Sigma | WB (1:2000)           |
| Sp1                      | #SAB140397     | Millipore-Sigma | WB (1:500)           |
| p53                      | #OP43L         | Millipore-Sigma | WB (1:1000)           |
| pS139-H2AX (γH2AX)       | #05-636        | Millipore-Sigma | WB (1:1000), IF (1:500) |
| pS10-H3                  | #06-570        | Millipore-Sigma | WB (1:1000), IF (1:500) |
| KLF5                     | sc-398470      | Santa Cruz   | WB (1:500)               |
| ERK1                     | sc-271269      | Santa Cruz   | WB (1:1000)               |
| EGFR                     | sc-03          | Santa Cruz   | WB (1:1000)               |
| Vinculin                 | sc-73614       | Santa Cruz   | WB (1:1000)               |
| Ki67 clone 30-9          | #70-4286       | Ventana      | IHC (1:500)               |
| Sp1                      | ab227383       | Abcam        | IHC (1:300)               |

**Appendix Table S5. Primary antibodies.**

Appendix Table S5 summarizes the primary antibodies (catalog number and vendor) and the dilution used in the different experiments. **WB:** Western Blot, **IF:** Immunofluorescence, **IHC:** Immunohistochemistry.
| Amplified Region | Forward (5’ – 3’) | Reverse (5’ – 3’) | Reference (PMID) |
|------------------|------------------|------------------|------------------|
| SP1 -253/+7      | GCAAGCGAGTCTTGGCATTGG | CGCTCATGTTGGCGAGCTGAGG | This Manuscript |
| SP1 -480/-230    | ATATCCCCGATTCTGTGGTGGC | ATCCAATGGGAAGACTCGCT | This Manuscript |
| SP1 -673/-486    | GCCCTCGTTAATTCGGCAGT | GCAAAATCCTAGTGCGGCAGGA | This Manuscript |
| SP1 -891/-674    | CGCTAAAGCGTCCCCACTCAA | GAAACTTTGGAGTGGCAGAGGA | This Manuscript |
| SP1-1602/-1402   | CGTGGAGCAGTCGAATACCA | CCGGCCTTAATAGCTTGTCGA | This Manuscript |
| Neg Ctr 1        | TGGTACAACACACAGCTCAGTG | AAGCTGGACATGGTTGTGTG | 32805052 |
| Neg Ctr 2        | CATCGAATGGAATGAAAGGATC | ACCATTGAGTATTGAGCAGTA | 19593370 |
| Neg Ctr 3        | CCTATTGTGGTGGAACACCA | TGGTTTGCTAGCCTACTTCTTA | 20875108 |
| EPPK1            | TGGGGGCTGTTGAGGGGAAAG | GGCGGGCCCCCTCTGACACCA | 33827480 |
| LAMC2            | TCCCTAGCTGCTTTCTTTCG | AAGGTTGAGATGGCAGTGCAG | 20875108 |
| SERPINE1         | CCAAAGACTCTCACCATTGACTTT | GCTATGCTGACAGTGAACACAG | 20875108 |
| EPHA2            | AACAGTTAAGTGTCAGCAGAAAGG | GTACAGTGGGCAGGCGCA | 20875108 |
| INPP4B           | AGCTGTAGGCGACCTTTGTT | CTCTCGTGGCTGCTGTAAGG | 20875108 |
| SOX17            | ATTAACCTGCGGGGTCTCATT | CGGGAGCAGTTACTTCTTG | 24770696 |

Appendix Table S6. Primers used in ChIP analyses.

Appendix Table S6 summarizes the sequences of the primers (forward and reverse) used for the amplification of different regions of SP1 promoter in the ChIP experiments. Negative (Neg Ctr 1, Neg Ctr 2, Neg Ctr 3) and positive (promoter of EPPK1, LAMC2, SERPINE1, EPHA2, INPP4B) were included to assess the specificity of the anti-KLF5 antibody.