**iScience, Volume *25***

## Supplemental information

# A positive feed-forward loop between *Fusobacterium nucleatum* and ethanol metabolism reprogramming drives laryngeal cancer progression and metastasis

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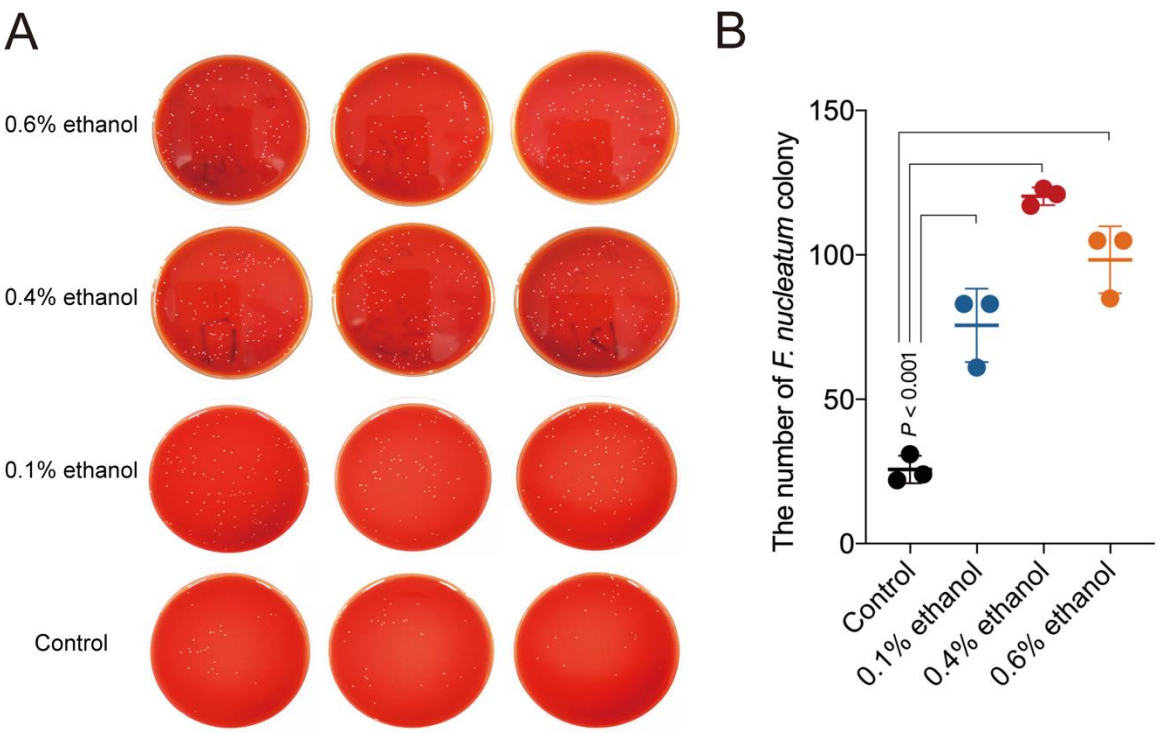


**Figure S1. Ethanol promotes *F. nucleatum* proliferation, related to Figure 2.**

(A) Representative data of *F. nucleatum* colony in 0.1%, 0.4% and 0.6% (v/v) ethanol by colony formation under different experimental conditions (n = 3 for each group).

(B) Statistical analysis of the number of *F. nucleatum* colony to different experimental conditions (n = 3 for each group) as assessed by the ordinary two-way ANOVA. Data are represented as mean ± SD.

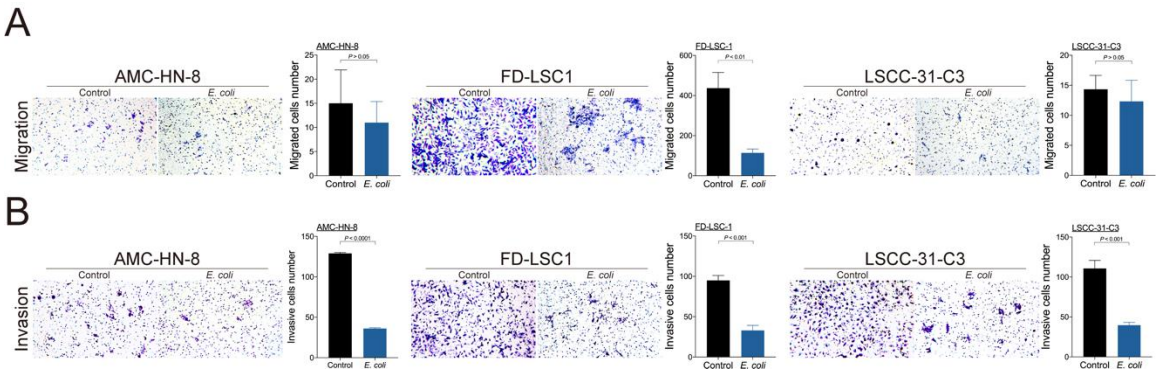


**Figure S2. *E. coli* does not promote cell migration and invasion in LSCC, related to Figure 2.**

(A) Transwell migration assays were performed in 3 LSCC cell lines cultured in the presence or absence of *E. coli strain DH5a* as calculated with the Student's unpaired t test.

(B) Transwell invasion assays were performed in 3 LSCC cell lines cultured in the presence or absence of *E. coli strain DH5a* as calculated with the Student's unpaired t test. Data are represented as mean ± SD.

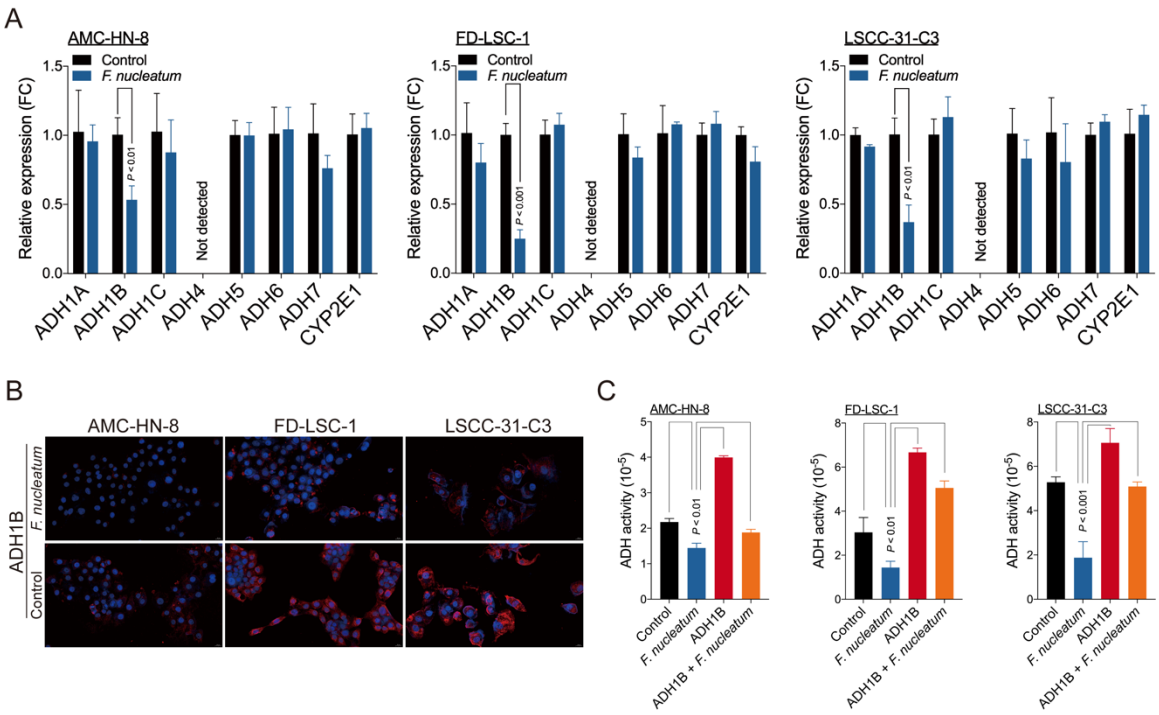


**Figure S3. *F. nucleatum* inhibits ADH1B expression, related to Figure 2.**

(A) Statistical analysis of the relative expression of 7 members of ADH family and CYP2E1 in 3 LSCC cell lines cultured in the presence or absence of *F. nucleatum* as assessed by Students unpaired t-test.

(B) Representative immunofluorescence was showed (40 × magnification) on ADH1B expression in 3 LSCC cell lines in the presence or absence of *F. nucleatum*. Bar scale, 20 μm.

(C) The ADH enzymatic activity was measured by ELISA assay in 3 LSCC cell lines with or without ADH1B-overexpressing lentiviral vector transduction and subsequently cultured with *F. nucleatum* as assessed by the ordinary one-way ANOVA. Data are represented as mean ± SD.

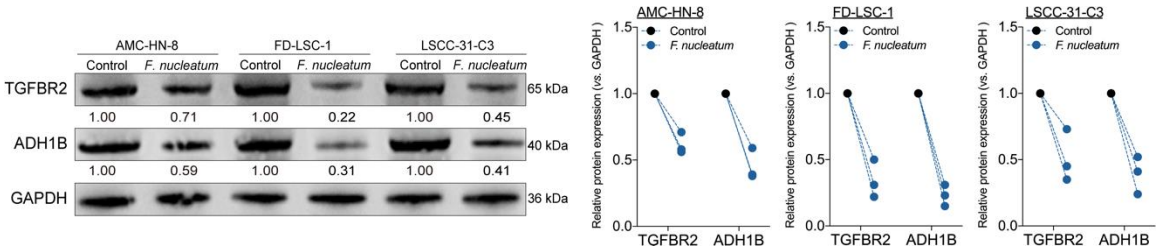


**Figure S4. *F. nucleatum* inhibits ADH1B and TGFBR2 expression, related to Figure 2, 3.**

Western blotting was performed to assess ADH1B and TGFBR2 expression in 3 LSCC cell lines cultured with *F. nucleatum*.

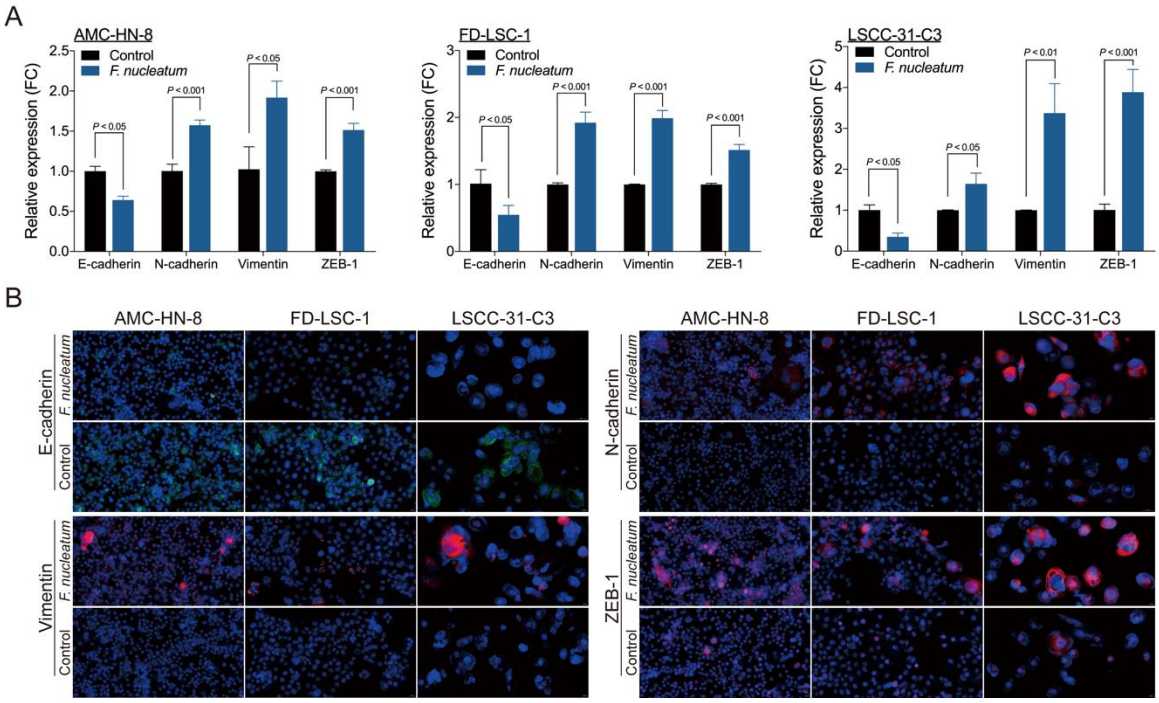


**Figure S5. *F. nucleatum* promotes EMT in LSCC, related to Figure 3.**

(A) Statistical analysis of the relative expression of EMT-related genes (E-cadherin, N-cadherin, Vimentin, and ZEB-1) in 3 LSCC cell lines cultured in the presence or absence of *F. nucleatum* as assessed by Students unpaired t-test.

(B) Representative immunofluorescence was showed (40 × magnification) on E-cadherin, N-cadherin, Vimentin, and ZEB-1 expression in 3 LSCC cell lines in the presence or absence of *F. nucleatum*. Bar scale, 20 μm. Data are represented as mean ± SD.

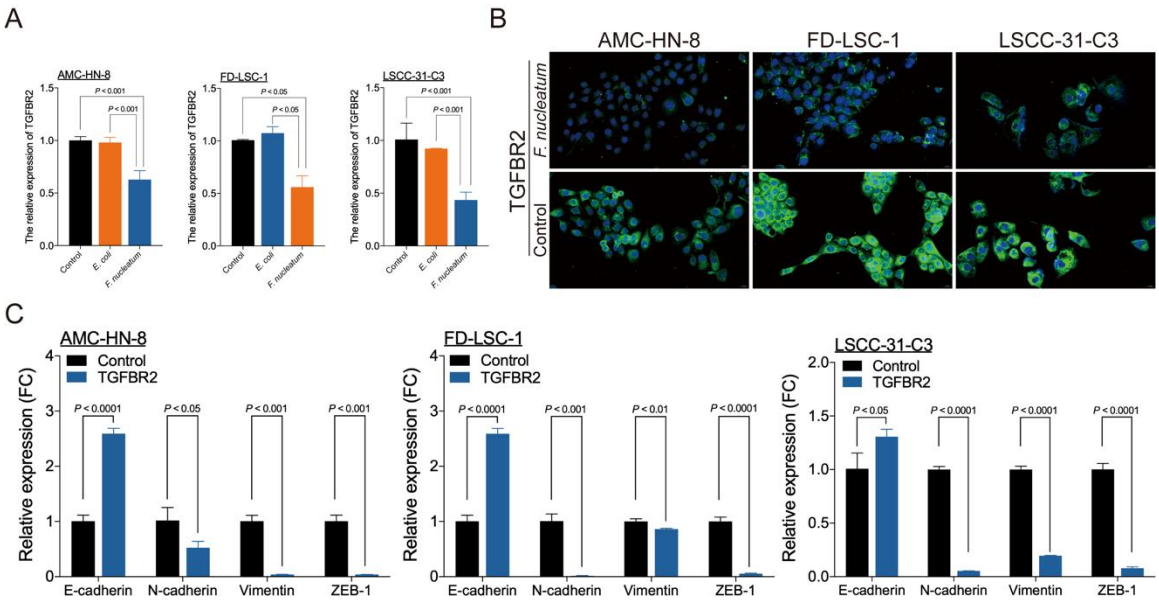


**Figure S6. *F. nucleatum*-induced EMT in LSCC by suppressing TGFBR2 expression, related to Figure 3.**

(A) Statistical analysis of the relative expression of TGFBR2 in 3 LSCC cell lines cultured with *F. nucleatum* or *E. coli* as calculated with Students unpaired t-test.

(B) Representative immunofluorescence was showed (40 × magnification) on TGFBR2 expression in 3 LSCC cell lines in the presence or absence of *F. nucleatum*. Bar scale, 20 μm.

(C) Statistical analysis of the relative expression of EMT-related genes (E-cadherin, N-cadherin, Vimentin, and ZEB-1) in 3 LSCC cell lines with or without TGFBR2 overexpressed lentiviral vector transduction as calculated with Students unpaired t-test. Data are represented as mean ± SD.

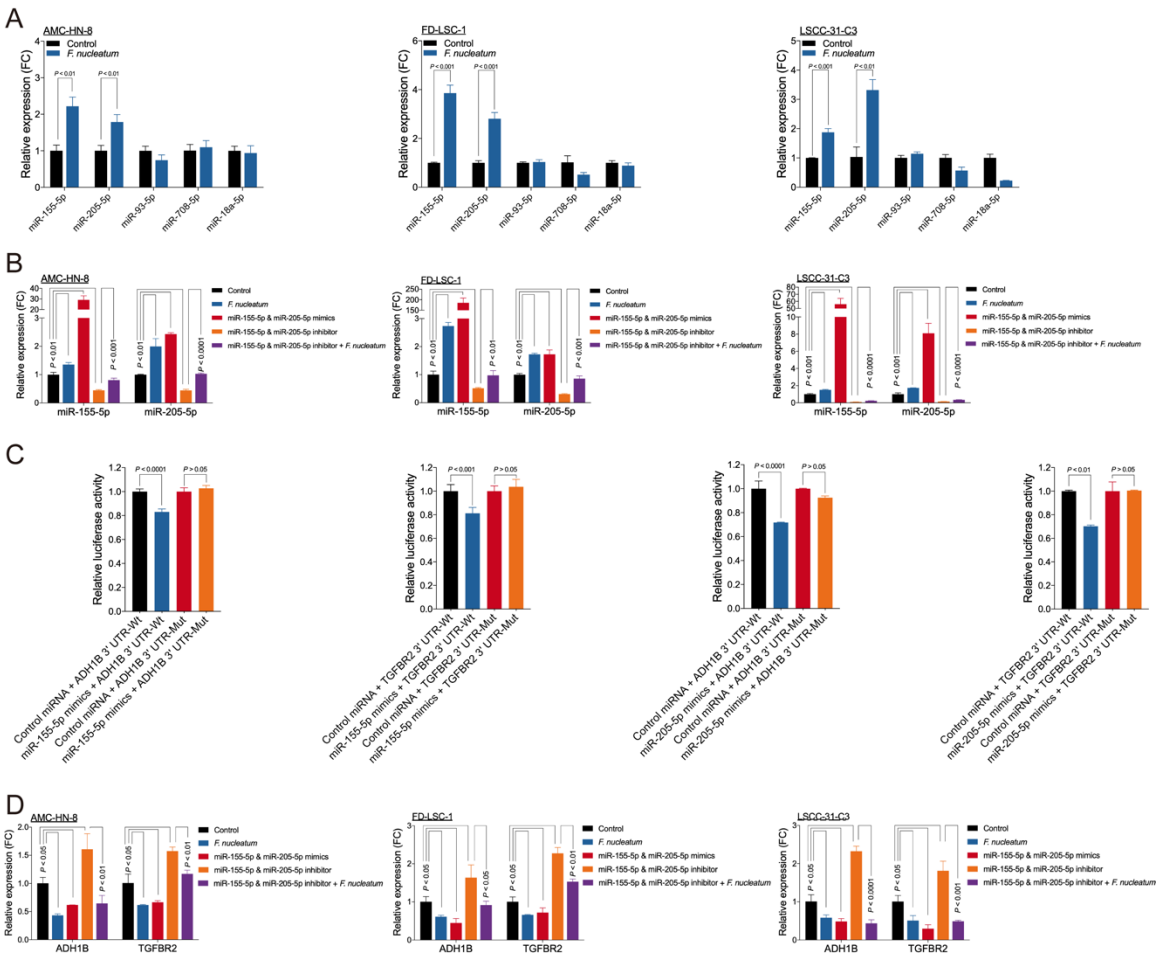


**Figure S7. *F. nucleatum* inhibits ADH1B and TGFBR2 expression by increasing miR-155-5p and miR-205-5p, related to Figure 4.**

(A) Statistical analysis of the relative expression of LSCC-related miRNAs in 3 LSCC cell lines transfected with miR-155-5p and miR-205-5p mimics or inhibitor and cultured in the presence or absence of *F. nucleatum* as calculated with Students unpaired t-test.

(B) Statistical analysis of the relative expression of miR-155-5p and miR-205-5p in 3 LSCC cell lines transfected with miR-155-5p and miR-205-5p mimics or inhibitor and cultured in the presence or absence of *F. nucleatum* as calculated with Students unpaired t-test.

(C) Dual-luciferase activity was measured in HEK293T cell line cultured treated with miR-155-5p & miR-205-5p mimics or control miRNA. Dual-luciferase reporters expressing wild-type (Wt) or mutant (Mut) 3'UTRs from human ADH1B and TGFBR2 mRNA were used.

The dual-luciferase activity was normalized based on the control miRNA transfection as assessed by the ordinary one-way ANOVA.

(D) Statistical analysis of the relative expression of ADH1B and TGFBR2 in 3 LSCC cell lines transfected with miR-155-5p and miR-205-5p mimics or inhibitor and cultured in the presence or absence of *F. nucleatum* as calculated with Students unpaired t-test. Data are represented as mean ± SD.

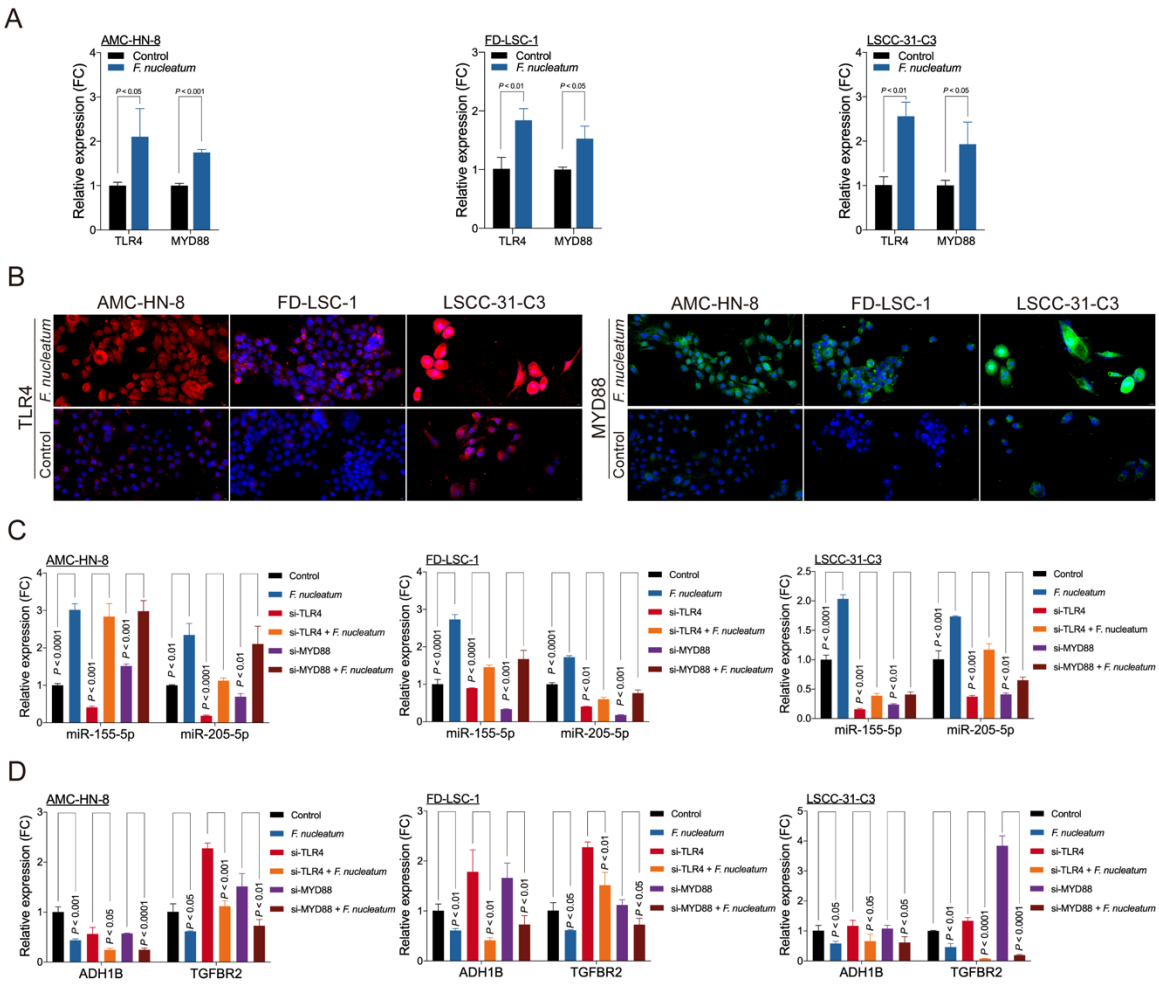


**Figure S8. TLR4 and MYD88 are involved in *F. nucleatum*-mediated ADH1B and TGFBR2 expression, related to Figure 4.**

(A) Statistical analysis of the relative expression of TLR4 and MYD88 in 3 LSCC cell lines cultured in the presence or absence of *F. nucleatum* as assessed by Students unpaired t-test.

(B) Representative immunofluorescence was showed (40 × magnification) on TLR4 and MYD88 expression in 3 LSCC cell lines in the presence or absence of *F. nucleatum*. Bar scale, 20 μm.

(C) Statistical analysis of the relative expression of miR-155-5p and miR-205-5p in 3 LSCC cell lines transfected with TLR4 and MYD88 siRNAs and cultured in the presence or absence of *F. nucleatum* as calculated with Students unpaired t-test.

(D) Statistical analysis of the relative expression of ADH1B and TGFBR2 in 3 LSCC cell lines transfected with TLR4 and MYD88 siRNAs and cultured in the presence or absence of *F. nucleatum* as calculated with Students unpaired t-test. Data are represented as mean ± SD.

**Table S1. The Correlation of *F. nucleatum* content (-ΔCt) and clinicopathologic factors in Cohort 1, related to Figure 1.**

| Characteristics | Total, n | *F. nucleatum* amount (-ΔCt) (mean ± SD) | *P* |
|---|---|---|---|
| Age, years | | | 0.1430 |
| ≥ 60 | 95 | -7.7938 ± 4.8223 | |
| < 60 | 36 | -9.0972 ± 4.3039 | |
| Sex | | | 0.8450 |
| Male | 128 | -8.1428 ± 4.7559 | |
| Female | 3 | -8.5411 ± 1.6662 | |
| Smoking history | | | 0.4429 |
| Yes | 120 | -8.2583 ± 4.6641 | |
| No | 11 | -6.9917 ± 5.2320 | |
| **Drinking history** | | | **0.0421** |
| **Yes** | **79** | **-7.3842 ± 5.1911** | |
| **No** | **52** | **-9.3183 ± 3.5956** | |
| Hypertension | | | 0.3141 |
| Yes | 37 | -7.5016 ± 5.3059 | |
| No | 94 | -8.4078 ± 4.4520 | |
| Diabetes | | | 0.8050 |
| Yes | 15 | -8.6199 ± 5.3399 | |
| No | 116 | -8.0914 ± 4.6398 | |
| Tumor subsite | | | 0.6989 |
| Supraglottic | 52 | -8.1244 ± 4.2122 | |
| Glottic & Subglottic | 79 | -8.1701 ± 5.0302 | |
| **pT stage** | | | **0.0029** |

| | | | |
|---|---|---|---|
| **T1 - T2** | **54** | **-9.4000 ± 5.4448** | |
| **T3 - T4** | **77** | **-7.2767 ± 3.9141** | |
| pN stage | | | 0.3283 |
| N0 | 90 | -8.4682 ± 4.7903 | |
| N+ | 41 | -7.4577 ± 4.4933 | |
| **pTNM stage*** | | | **0.0167** |
| **I - II** | **41** | **-9.7544 ± 5.0619** | |
| **III - IV** | **90** | **-7.4220 ± 4.3711** | |
| Pathological differentiation | | | 0.1051 |
| Moderate & Poor | 91 | -8.5776 ± 4.8884 | |
| Well | 40 | -7.1835 ± 4.1569 | |
| **Tumor diameter** | | | **0.0168** |
| **> 3cm** | **58** | **-7.0433 ± 4.4707** | |
| **≤ 3cm** | **73** | **-9.0328 ± 4.7308** | |

*According to the 8th American Joint Committee on cancer (AJCC) stage system.

**Table S2. Multiple linear regression method with *F. nucleatum* content (-ΔCt) as the dependent variable, related to Figure 1.**

| Linear regression | *B* | *P* value | 95% CI |
|---|---|---|---|
| **Drinking index** | **0.4053** | **< 0.0001** | **0.0004 - 0.0009** |
| **pTNM stage*** | **0.2752** | **0.0011** | **0.5662 - 2.2058** |
| Age | 0.1093 | 0.1859 | -0.0320 - 0.1631 |
| Hypertension | 0.0285 | 0.7291 | -1.398 - 1.9926 |
| Smoking index | -0.0166 | 0.8374 | -0.0009 - 0.0007 |
| Sex | -0.0126 | 0.8795 | -5.3130 - 4.5556 |
| Diabetes | 0.0103 | 0.9003 | -2.2428 - 2.5466 |

Drinking index: the average gram of alcohol intaked per day × years spent drinking; Smoking index: the number of cigarettes smoked per day × years spent smoking. *According to the 8th American Joint Committee on cancer (AJCC) stage system.

**Table S3. The correlation of *F. nucleatum* content (-ΔCt) and clinicopathologic factors in Cohort 2, related to Figure 1.**

| Characteristics | Total, n | *F. nucleatum* amount (-ΔCt) | | *P* |
|---|---|---|---|---|
| | | Low, n, % | High, n, % | |
| All cases, n | 40 | 20 (50.00%) | 20 (50.00%) | |
| Age, years | | | | 0.3332 |
| ≥ 60 | 24 | 14 (70.00%) | 10 (50.00%) | |
| < 60 | 16 | 6 (30.00%) | 10 (50.00%) | |
| Smoking history | | | | > 0.9999 |
| Yes | 27 | 13 (65.00%) | 14 (70.00%) | |
| No | 13 | 7 (35.00%) | 6 (30.00%) | |
| **Drinking history** | | | | **0.0225** |
| **Yes** | **23** | **8 (40.00%)** | **16 (80.00%)** | |
| **No** | **17** | **12 (60.00%)** | **4 (20.00%)** | |
| Hypertension | | | | > 0.9999 |
| Yes | 15 | 8 (40.00%) | 7 (35.00%) | |
| No | 25 | 12 (60.00%) | 13 (65.00%) | |
| Diabetes | | | | 0.4872 |
| Yes | 2 | 2 (10.00%) | 0 (0.00%) | |
| No | 38 | 18 (90.00%) | 20 (100.00%) | |
| Tumor subsite | | | | > 0.9999 |
| Supraglottic | 16 | 8 (40.00%) | 8 (40.00%) | |
| Glottic | 24 | 12 (60.00%) | 12 (60.00%) | |
| pT stage | | | | 0.7512 |
| T1 - T2 | 18 | 10 (50.00%) | 8 (40.00%) | |
| T3 - T4 | 22 | 10 (50.00%) | 12 (60.00%) | |

| | | | | |
|---|---|---|---|---|
| pN stage | | | | > 0.9999 |
| N0 | 23 | 11 (55.00%) | 12 (60.00%) | |
| N+ | 17 | 9 (45.00%) | 8 (40.00%) | |
| pTNM stage* | | | | 0.4801 |
| I - II | 11 | 7 (35.00%) | 4 (20.00%) | |
| III - IV | 29 | 13 (65.00%) | 16 (80.00%) | |
| Pathological differentiation | | | | > 0.9999 |
| Well | 6 | 3 (15.00%) | 3 (15.00%) | |
| Moderate | 34 | 17 (85.00%) | 17 (85.00%) | |
| Tumor diameter | | | | > 0.9999 |
| > 3cm | 13 | 7 (35.00%) | 6 (30.00%) | |
| ≤ 3cm | 27 | 13 (65.00%) | 14 (70.00%) | |
| Recurrence | | | | **0.0011** |
| Yes | 17 | **3 (15.00%)** | **14 (70.00%)** | |
| No | 23 | **17 (85.00%)** | **6 (30.00%)** | |
| Cancer-specific death | | | | |
| Yes | 11 | **2 (10.00%)** | **9 (45.00%)** | **0.0310** |
| No | 29 | **18 (90.00%)** | **11 (55.00%)** | |

*According to the 8th American Joint Committee on cancer (AJCC) stage system.

**Table S4. The correlation of *F. nucleatum* content (-ΔCt) and clinicopathologic factors in Cohort 3, related to Figure 6.**

| Characteristics | Total, n | *F. nucleatum* amount (-ΔCt) | | *P* |
|---|---|---|---|---|
| | | Low, n, % | High, n, % | |
| All cases, n | 74 | 41 (55.41%) | 33 (44.59%) | |
| Age, years | | | | 0.2436 |
| ≥ 60 | 41 | 20 (48.78%) | 21 (63.64%) | |
| < 60 | 33 | 21 (51.22%) | 12 (36.36%) | |
| Sex | | | | 0.5826 |
| Male | 71 | 40 (97.56%) | 31 (93.94%) | |
| Female | 3 | 1 (2.44%) | 2 (6.06%) | |
| Smoking history | | | | 0.6301 |
| Yes | 46 | 24 (58.54%) | 22 (66.67%) | |
| No | 28 | 17 (41.46%) | 11 (33.33%) | |
| **Drinking history** | | | | **0.0182** |
| **Yes** | **42** | **18 (43.90%)** | **24 (72.73%)** | |
| **No** | **32** | **23 (56.10%)** | **9 (27.27%)** | |
| Hypertension | | | | 0.6301 |
| Yes | 28 | 17 (41.46%) | 11 (33.33%) | |
| No | 46 | 24 (58.43) | 22 (66.67%) | |
| Diabetes | | | | 0.7439 |
| Yes | 10 | 5 (12.20%) | 5 (15.15%) | |
| No | 64 | 36 (87.80%) | 28 (84.85%) | |
| Tumor subsite | | | | 0.6194 |
| Supraglottic | 24 | 12 (29.27%) | 12 (36.36%) | |
| Glottic | 50 | 29 (70.73%) | 21 (63.64%) | |

| | | | | |
|---|---|---|---|---|
| **pT stage** | | | | **0.0187** |
| **T1 - T2** | **37** | **26 (63.41%)** | **11 (33.33%)** | |
| **T3 - T4** | **37** | **15 (36.59%)** | **22 (66.67%)** | |
| pN stage | | | | 0.5635 |
| N0 | 59 | 34 (82.93%) | 25 (75.76%) | |
| N+ | 15 | 7 (17.07%) | 8 (24.24%) | |
| **pTNM stage*** | | | | **0.0182** |
| **I - II** | **32** | **23 (56.10%)** | **9 (27.27%)** | |
| **III - IV** | **42** | **18 (43.90%)** | **24 (72.73%)** | |
| Pathological differentiation | | | | 0.7989 |
| Well | 21 | 11 (26.83%) | 10 (30.30%) | |
| Moderate & Poor | 53 | 30 (73.17%) | 23 (69.70%) | |
| Tumor diameter | | | | 0.4597 |
| > 3cm | 25 | 12 (29.27%) | 13 (39.39%) | |
| ≤ 3cm | 49 | 29 (70.73%) | 20 (60.61%) | |
| **Recurrence** | | | | **0.0009** |
| **Yes** | **33** | **11 (26.83%)** | **22 (66.67%)** | |
| **No** | **41** | **30 (73.17%)** | **11 (33.33%)** | |
| **Cancer-specific death** | | | | **0.0255** |
| **Yes** | **25** | **9 (21.95%)** | **16 (48.48%)** | |
| **No** | **49** | **32 (78.05%)** | **17 (51.52%)** | |

*According to the 8th American Joint Committee on cancer (AJCC) stage system.

**Table S5. Primer sequences used for qRT-PCR and FISH, related to STAR Methods.**

| Preimers | Forward primer (5' to 3') | Reverse primer (5' to 3') | Propose |
|---|---|---|---|
| *F. nucleatum* | CAACCATTACTTTAACTCTACCATGTTCA | GTTGACTTTACAGAAGGAGATTATGTAAAAATC | qPCR |
| FUS664 | CTTGTAGTTCCGC(C/T)TACCTC | N/A | FISH |
| EUB338 | GCTGCCTCCCGTAGGAGT | N/A | FISH |
| has-miR-155-5p | AACCCCTATCACGATTAGCATTAA | N/A | FISH |
| has-miR-205-5p | CAGACTCCGGTGGAATGAAGGA | N/A | FISH |
| has-miR-155-5p | CGCTTAATGCTAATCGTGATAGGGGTT | N/A | qPCR |
| has-miR-205-5p | TCCTTCATTCCACCGGAGTCT | N/A | qPCR |
| PGT | ATCCCCAAAGCACCTGGTTT | AGAGGCCAAGATAGTCCTGGTAA | qPCR |
| U6 | GTGCTCGCTTCGGCAGCACAT | N/A | qPCR |
| GAPDH | TGTAGTTGAGGTCAATGAAGGG | ACATCGCTCAGACACCATG | qPCR |
| TGFBR2 | GTAGCTCTGATGAGTGCAATGAC | CAGATATGGCAACTCCCAGTG | qPCR |
| TLR4 | GGATGAGGACTGGGTAAGGAATGA | AGCGGCTCTGGATGAAGTGC | qPCR |

| | | | |
|---|---|---|---|
| MYD88 | GCCGCCGGATGGTGGTGGTTGT | TTGGTGCAGGGGTTGGTGTAGTCG | qPCR |
| E-cadherin | ATTTTTCCCTCGACACCCGAT | TCCCAGGCGTAGACCAAGA | qPCR |
| N-cadherin | TGCGGTACAGTGTAACTGGG | GAAACCGGGCTATCTGCTCG | qPCR |
| Vimentin | AGTCCACTGAGTACCGGAGAC | CATTTCACGCATCTGGCGTTC | qPCR |
| ZEB-1 | GATGATGAATGCGAGTCAGATGC | ACAGCAGTGTCTTGTTGTTGT | qPCR |
| ADH1A | AGTCATCCCACTCGCTATTCC | GTCCCCTGAGGATTGCTTACA | qPCR |
| ADH1B | GCTGGGGAATTGAAGCCAACA | CAGCATGTGTATGTTCAGGGCAAG | qPCR |
| ADH1C | TGCTACTGACTGGACGCACG | CAGCCACAAGTTTGGGGACAGAT | qPCR |
| ADH4 | AGTTCGCATTCAGATCATTGCT | CTGGCCCAATACTTTCCACAA | qPCR |
| ADH5 | ATGGCGAACGAGGTTATCAAG | CATGTCCCAAGATCACTGGAAAA | qPCR |
| ADH6 | ACAGGCCAAGTCATCAGATGC | CCACAACCTTTATGCGAACTTCC | qPCR |
| ADH7 | ACATGAGGCAACTGGGATTGT | CATTGCATTCTCTACATTGTGGC | qPCR |
| CYP2E1 | GTGATGCACGGCTACAAGG | GGGTGGTCAGGGAAAACCG | qPCR |