A cis-eQTL in NSUN2 promotes esophageal squamous-cell carcinoma progression and radiochemotherapy resistance by mRNA-m5C methylation

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Dear Editor,

Esophageal squamous cell carcinoma (ESCC) has poor prognosis because of the difficulty in early detection and low sensitivity of advanced disease to radiochemotherapy. ESCC presents a high proportion of primary resistance to radiochemotherapy, which may be due to certain individual genetic variations. Expression quantitative trait loci (eQTLs) as proximal and continuous cellular phenotypes have been shown to be helpful to determine how genetic variants may influence phenotype. We examined the ESCC tumor-specific eQTLs based on our previous whole-genome DNA and RNA sequencing data of 94 ESCC samples to discover eQTL-target genes involved in the malignant phenotypes of ESCC. By analyzing the mRNA levels of 376 genes targeted by ESCC-specific eQTLs, we found 10 genes that had significantly higher expression levels in tumor than in normal in all 94 samples, which could be verified in TCGA 90 ESCC samples and Oncomine 51 ESCC samples (Supplementary Fig. 1a and Supplementary Table 1). Among the 10 genes, silencing NSUN2, a gene encoding the 5-methylcytosine (m5C) RNA methyltransferase, had the most inhibitory effect on cell viability than others (Supplementary Fig. 1b).

We found that NSUN2 levels were significantly higher in ESCC than adjacent normal tissues determined by immunohistochemical (IHC) staining and Western blotting (Fig. 1a; Supplementary Fig. 1c and Supplementary Table 2) and high level was correlated with shorter survival time in patients (Fig. 1b). The eQTL analysis indicated 7 candidate SNPs in the adjacent SRSAT and NSUN2 loci that might regulate NSUN2 transcription (Supplementary Table 3) and these SNPs are all in high linkage disequilibrium (Supplementary Fig. 1d). Suggested by the functional annotation, 4 SNPs were selected for electrophoretic mobility shift assays (EMSA) and the results suggested that rs10076470 G > A mutation may create a functional cis-eQTL (Supplementary Fig. 1e). Reporter gene assays showed that the plasmid containing the rs10076470 A allele had significantly higher luciferase expression than the plasmid containing the rs10076470 G counterpart (Supplementary Fig. 1f). RNA-sequencing analysis revealed that ESCC tissues with the AA or AG genotype had significantly higher NSUN2 RNA levels than that with the GG genotype (Fig. 1c). The rs10076470 A allele renders higher NSUN2 expression was further found in ESCC cell lines (Supplementary Fig. 1g).

We then sought for the transcriptional factors (TFs) that might interact with the cis-element formed by rs10076470 G/A SNP using JASPAR and HumanTFDB databases combined with the mRNA levels of these TFs in 170 paired tissue samples. The result suggested STAT1 as the potential candidate TF for the rs10076470 G allele (Fig. 1d and Supplementary Fig. 2a, b). Indeed, STAT1 knockout significantly suppressed NSUN2 expression at both mRNA and protein levels in KYSE450 (GA genotype) and KYSE70 cells (AA genotype) but not in KYSE150 cells (GG genotype) (Supplementary Fig. 2c, d). Reporter gene assays showed that silencing STAT1 significantly reduced the luciferase activity of A allele construct while G allele construct was much less affected (Fig. 1e and Supplementary Fig. 2e). The additional EMSA assays showed that nuclear protein bound to the DNA probe was rs10076470 A specific and the band could be super-shifted when STAT1 antibody was included in the incubation mixture (Fig. 1f and Supplementary Fig. 2f–h), indicating that the protein bound to the DNA is likely STAT1. ChIP-qPCR detection (Fig. 1g) showed significant STAT1 enrichment in KYSE450 and KYSE70 than in KYSE150. Furthermore, the STAT1 and NSUN2 RNA levels were positively correlated in 170 ESCC samples but this correlation was limited in those with the rs10076470 GA or AA genotype (Supplementary Fig. 2i).

Duplicate RNA sequencing on NSUN2 knockout KYSE150 cells revealed downregulation of 2750 and 2780 genes compared with cells without the knockout (Supplementary Fig. 3a). Combined analysis showed overlap of 1348 genes, of which 509 genes had significant higher expression in 170 ESCC than in normal esophageal tissue samples (Fig. 1h and Supplementary Table 4). Pathway enrichment analysis of the 509 genes indicated that DNA repair, RNA metabolism and cell cycle pathways were the most significantly downregulated (Fig. 1i). We then selected the top 10 genes in these three pathways for verification by RT-qPCR in an independent knockout assay and the significant downregulation of almost all genes (27/30) were confirmed (Supplementary Fig. 3b–d). m5C-RNA immunoprecipitation coupled with qPCR analysis showed that NSUN2 knockout significantly decreased m5C-mRNA levels of almost all these tested genes in cells compared with the levels in control cells (Supplementary Fig. 4a, b). The measurement of the m5C global levels in ESCC cells showed consistent results showing that the m5C levels were substantially increased with NSUN2 overexpression and decreased with NSUN2 knockout (Supplementary Fig. 4c, d).

Since TIGAR, among other genes, was the most significantly downregulated and its mRNA m5C level was significantly decreased upon NSUN2 knockout, it was chosen as an example of NSUN2 affected transcript for further investigation. Disruption of NSUN2 expression had significant impact on TIGAR expression at both mRNA and protein levels (Fig. 1j) and Supplementary Fig. 5a–d). IHC staining of esophageal tissue samples from Nsun2−/− mice receiving 4-NQO showed substantially higher Tigar levels in atypical hyperplasia lesions and ESCC than in normal esophageal epithelium and the Nsun2 levels were significantly correlated with the Tigar levels; in contrast, Nsun2+/− mice showed very low levels of Tigar protein in ESCC samples (Supplementary Fig. 5e, f). IHC staining of NSUN2 and TIGAR in human ESCC tissue arrays showed significantly positive correlation (Fig. 1k and Supplementary Table 5). The mRNA stability assays demonstrated that NSUN2 overexpression significantly increased but knockout significantly decreased the half-life of TIGAR mRNA (Fig. 1l and Supplementary Fig. 5g–i). TIGAR may activate the pentose phosphate pathway generating more
reductants to protect cancer cells from killing by ROS. Consistently, we found that NSUN2 overexpression significantly reduced but knockout significantly increased intracellular ROS levels; however, this effect can be partially rescued by the forced TIGAR expression change (Fig. 1m and Supplementary Fig. 5j–l).

In this context, we hypothesized that NSUN2 overexpression in ESCC may be implicated in radioresistance. Indeed, cells overexpressing NSUN2 were not or much less sensitive to irradiation killing and had higher abilities of colony formation compared with control cells; in contrast, cells with NSUN2 knockout had the
Fig. 1  A cis-eQTL variant in NSUN2 locus promotes esophageal squamous-cell carcinoma progression and radiochemotherapy resistance by mRNA-mC modification of cancer-related genes. a Immunohistochemistry (IHC) analysis of NSUN2 protein levels in paired human esophageal squamous-cell carcinoma (ESCC) and adjacent normal tissues. Left panel shows representative IHC pictures of tissue arrays (scale bar, 100 μm) and right panel shows the statistics of IHC score (IRS) indicating that 85.3% (168/197) of ESCCs had significantly higher NSUN2 level compared with normal tissues. **P < 0.001 of Mann-Whitney test. IRS, immunoreactive score. b Kaplan-Meier survival curves for patients with high (IRS ≥ 6) or low (IRS < 6) NSUN2 level in ESCC. HR (95% CI) was computed with Cox hazard proportion model. c Comparison of NSUN2 mRNA levels in ESCCs as function of the rs10076470 genotype, showing that the A allele (GA and AA genotypes) had significantly higher levels compared with the G allele. **P < 0.01 and ns, not significant of Mann-Whitney test. d The rs10076470 A allele resides within a potential STAT1 binding motif. The effect of STAT1 knockdown on luciferase activity of constructs containing the rs10076470 G or rs10076470 A allele in KYSE150 cells. Data are mean ± S.E.M. from 3 replicate experiments. **P < 0.05 and ****P < 0.0001 of Student’s t-test. e Super-shift EMSA competition assay with STAT1 antibody and KYSE150 cell nuclear extract. I points the rs10076470A -specific band and II points a super-shifted band by STAT1. g Chromatin immunoprecipitation coupled qPCR analysis shows that STAT binds to NSUN2 promoter in an allele-specific manner. Data are mean ± S.E.M. from 3 replicate experiments and Anti-S, anti STAT1 antibody. *** and ###P < 0.0001; **** and ####P < 0.0001 and ns, not significant of Student’s t-test between siSTAT1 and siControl and among different genotypes. h Venn diagram displays 1348 overlapped genes among 2750 and 2780 apparently downregulated genes in cells with NSUN2 knockout (KO) #1 and NSUN2 KO #2, respectively. Among 1348 overlapped genes, 509 differentially expressed genes (DEGs) had higher mRNA levels in ESCCs than adjacent normal tissues (fold change >1.4, FDR <0.05). i Metascape gene enrichment analysis of the 509 DEGs (http://metascape.org/gp/index.html#/main/step1). j Western blot analysis of NSUN2 and TIGAR protein levels in KYSE150 cells with NSUN2 overexpression (OE) or KO. k Spearman correlation between NSUN2 and TIGAR protein levels expressed as IRS in ESCC. l The effect of NSUN2 OE on the TIGAR mRNA stability in KYSE150 cells determined by RT-qPCR at indicated time points after treatment with 6 μM actinomycin D. Data are mean ± S.E.M. In most data points, the error bars are within the symbols. ****P < 0.0001 of Student’s t-test. m NSUN2 OE significantly inhibited ROS production in KYSE150 cells, which could be rescued by silencing TIGAR. Data are mean ± S.E.M. from 3 experiments and each had 3 replications. **P < 0.01 and ****P < 0.0001 of Student’s t-test. n The effect of NSUN2 KO on colony formation ability of KYSE150 cells with or without ionizing radiation (IR, 4 Gy) and TIGAR OE. Shown are mean ± S.E.M. from 3 experiments and each had 3 replications. In some data points, the error bars are within the symbols. **P < 0.05; ***P < 0.01; ****P < 0.0001 of Student’s t-test. o Extreme limiting dilution assays show survival fractions in KYSE150 cells with NSUN2 KO caused by IR (4 Gy) and TIGAR OE. **P < 0.05 and ****P < 0.0001 of ELDA analysis program. p The effect of NSUN2 OE on DNA double-strand breaks detected by γ-H2AX in KYSE150 cells with or without IR (4 Gy) and TIGAR knockdown. q The effect of NSUN2 OE on DNA double-strand breaks detected by comet assays in KYSE150 cells with or without IR (4 Gy) and TIGAR knockdown. Left panels show fluorescence images of comet assays (scale bars, 100 μm) and right panels show the statistics. Data are mean ± S.E.M. from 3 replicates and 3 fields were randomly selected from each experiment. siCtrl, siControl; sit, siTIGAR. r Representative images showing NSUN2 and TIGAR immunohistochemical staining in ESCC establishing PDXs with differential radiosensitivity. Scale bars, 100 μm. s Box and bar plots comparing the NSUN2 protein levels in ESCC biopsy specimens with differential sensitivity to adjuvant radiochemotherapy in patients with differential sensitivity. Scale bars, 100 μm. u Box and bar plots comparing the NSUN2 protein levels in ESCC biopsy specimens with differential sensitivity to adjuvant radiochemotherapy. **P < 0.01 of Mann-Whitney test. v The schematic illustration for the possible mechanisms of aberrant NSUN2 expression and NSUN2-mediated radioresistance in ESCC. Part of the schematic illustration is generated from BioRender (https://biorender.com/).
AUTHOR CONTRIBUTIONS
D.L. and C.W. conceptualized, supported, and supervised this study. X.N. contributed to the design and conducted most experiments. W.T. and J.C. and responded to clinical data, sample collection and preparation. L.P. contributed to bioinformatics and statistical analysis. W.L., C.M., X.C. and X.Y. contributed to animal experiments. D.L., X.N., and C.W. prepared and reviewed the manuscript. All authors have read and approved the article.

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REFERENCES
1. Abnet, C. C., Arnold, M. & Wei, W. Q. Epidemiology of esophageal squamous cell carcinoma. Gastroenterology 154, 360–373 (2018).
2. Chen, Y. et al. Comparing paclitaxel plus fluorouracil versus cisplatin plus fluorouracil in chemoradiotherapy for locally advanced esophageal squamous cell cancer: a randomized, multicenter, phase III clinical trial. J. Clin. Oncol. 37, 1695–1703 (2019).
3. Vosa, U. et al. Large-scale cis- and trans-eQTL analyses identify thousands of genetic loci and polygenic scores that regulate blood gene expression. Nat. Genet. 53, 1300–1310 (2021).
4. Peng, L. et al. CCGD-ESCC: a comprehensive database for genetic variants associated with esophageal squamous cell carcinoma in Chinese population. Genomics Proteom. Bioinforma. 16, 262–268 (2018).
5. Bensaad, K. et al. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell 126, 107–120 (2006).

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