LncRNA ZFAS1 contributed to irradiation resistance in nasopharyngeal carcinoma by inhibiting hsa-miR-7-5p/ENO2

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Abstract

Background: In our previous research, we found that lncRNA ZFAS1 could promote nasopharyngeal carcinoma by inhibiting its downstream target axis. But we hadn't shown whether ZFAS1 had association with irradiation resistance of NPC. In this study, we aimed to study the role of ZFAS1 in irradiation resistance of nasopharyngeal carcinoma.

Methods: Bioinformatics analysis was first conducted to identify the potentially significant gene that participated in radio resistance of nasopharyngeal carcinoma. qRT-PCR was used to detect the expression of ZFAS1, miR-7-5p and ENO2 mRNA. The location of ZFAS1 was measured in cell fractions. The relationship between ZFAS1, miR-7-5p and ENO2 mRNA was validated by luciferase reporter assay and RNA pull down. Edu assay and flow cytometric apoptosis assay were conducted to observe how ZFAS1, miR-7-5p and ENO2 affected nasopharyngeal carcinoma proliferation and apoptosis under irradiation.

Results: ZFAS1 was upregulated in nasopharyngeal carcinoma, which was associated with radio resistance. ZFAS1 strengthened the ability of irradiation resistance in nasopharyngeal carcinoma. ZFAS1 acted on miR-7-5p to promote ENO2 upregulation, thereby promoting the irradiation resistance.

Conclusion: ZFAS1 sponged miR-7-5p to further affect ENO2 expression to augmenting the irradiation resistance in nasopharyngeal carcinoma.

Background

Nasopharyngeal carcinoma (NPC) is a type of malignant tumor which occurs in nasopharyngeal epithelial cells with high morbidity and mortality in China. [1] NPC usually induces poor prognosis in patients due to the strong ability of migration and early distant metastasis [2, 3]. Epstein-Barr virus is often considered as a risk factor for the development of NPC with other factors containing smoking cigarette, eating nitrosamine dietary, long-term exposure to chemical carcinogens and genetic susceptibility in consideration [4, 5]. At present, radiotherapy is the first choice to be considered for patients' treatment [6, 7]. But there are also a number of patients with radio resistance have poor prognosis [7, 8]. Therefore, it is necessary to discover how to reverse radio resistance in NPC patients.

Long noncoding RNA (lncRNA) is a type of RNA that is more than 200 nucleotides in length with regulating gene expression at multiple levels [9]. Recent studies have shown that lncRNA is involved in many important regulatory processes including X-chromosome silencing, genomic imprinting, chromatin modification, transcriptional activation and transcriptional interference [10–14]. LncRNA ZFAS1 locates at the chromosome 20q13.13 with the exon count of 5, and is first found in breast cancer [15]. There were some researches reporting that the expression of ZFAS1 was aberrant in hepatocellular carcinoma, prostate cancer, colorectal cancer and esophageal squamous cell carcinoma [16–19]. In our previous study, we also reported that lncRNA ZFAS1 participated in the development of NPC as a tumor promoter [20]. However, we hadn't demonstrated whether ZFAS1 played a role in radio resistance in NPC.

MiRNA with a length of 18–25 nt has been proved to play an essential role in the development of many cancers including pancreatic cancer, breast cancer, prostate cancer, colorectal cancer, gastric cancer, lung squamous cell carcinoma and ovarian cancer [21–27]. The involvement of miR-7-5p in human cancer genesis has been reported during the last six years; however, the participation of miR-7-5p in irradiation resistance of human cancers including nasopharyngeal carcinoma has never been studied. Only that Kazuo's team reported in 2019 that miR-7-5p was up-regulated in clinically relevant resistance HeLa (cervical cancer cell line) and SAS (oral squamous cell carcinoma) cell lines [28]. In addition, controversial roles of miR-7-5p in chemo-drug resistance have been reported in human hepatocellular carcinoma [29], cervical cancer [30], breast cancer [31], and small cell lung cancer [32]. Whether in nasopharyngeal miR-7-5p promotes radio resistance remains undiscovered.

ENO2 is one of three enolase isoenzymes that were found in mammals [33]. ENO2 was majorly located in mature neurons and cells for neuronal origin and highly expressed in some cancers including glioblastoma, neuroendocrine prostate cancer and renal cell carcinoma [34–36]. There was a report covering that ENO2 was a responsive gene of HIF, which took part in the development of tumor growth [37]. We identified ENO2 to be a potential downstream effector of miR-7-5p, and predicted it to significantly involve in NPC irradiation resistance. How miR-7-5p-ENO2 axis participates in NPC irradiation resistance was studied in the present study.

In our previous study, we demonstrated that lncRNA ZFAS1 was the promoter of NPC. However, we didn't verify whether ZFAS1 affected radio resistance. In addition to the last research, we identified a novel downstream effector axis, miR-7-5p-ENO2. We then studied the effects of ZFAS1-miR-7-5p-ENO2 interactome on NPC resistance to radiation.

Materials And Methods

Tissues collection

Fifty-five patients who were diagnosed with nasopharyngeal carcinoma in West China Hospital of Sichuan University were enrolled in our study. The ethics committee of West China Hospital of Sichuan University approved our study protocol. NPC tissues and adjacent healthy tissues were frozen until experiments and clinical characteristics were recorded. The collection and the use of tissues followed the ethical standards built by the Helsinki Declaration.

Cell culture

The human NPC cell lines SUNE-1, 5-8F, and C666-1 as well as normal nasal mucosal epithelial cell NP-69 were all bought from BeNa Culture Collection (Beijing, China). SUNE-1, 5-8F, and C666-1 were cultured in RPMI-1640 with 10% FBS.

RNA was detected by real-time quantification PCR.

Total RNA was disintegrated by Trizol (DP501, Tiangen Biochemical, China). The RNA was reverse transcribed into cDNA using a cDNA synthesis kit (KR211, Tiangen Biochemical, China) after checking the purity of RNA. Next, the expression of lncRNA ZFAS1, miR-7-5p and ENO2 mRNA were analyzed by ABI 7500 using a SYBR Green PCR kit (FP411, Tiangen Biochemical, China). U6 acted as a reference gene for miR-7-5p while GAPDH acted as a reference gene for lncRNA ZFAS1 and ENO2 mRNA.

Cell transfection

Si-ZFAS1-1, si-ZFAS1-2, miR-7-5p inhibitor, ENO2 siRNA (si-ENO2), and si-NC were designed and synthesized by Tiangen Biochemical (Beijing, China). Lipofectamine 2000 reagent (ThermoFisher, 11668027, USA) was used to transfect plasmids into target cells (SUNE-1 and C666-1 cell lines) on the basis of the protocol.

Cell fractionation

The Invitrogen PARIS Kit (ThermoFisher, AM1921, USA) was employed for separating and purifying cytoplasmic and nuclear RNA based on specifications. Expression levels of lncRNA ZFAS1, GAPDH (cytoplasmic control) and U6 (nuclear control) in cytoplasm and nucleus were examined using qRT-PCR.

Luciferase reporter assay

We purchased constructed plasmids containing wild type or mutant type of lncRNA ZFAS1 and ENO2 mRNA from Tiangen Biochemical (Beijing, China). Next, we transfected these plasmids into SUNE-1 and C666-1 cell lines by Lipofectamine 2000 reagent (ThermoFisher, 11668027, USA). Then, miR-892b mimics were co-transfected into the same cells by the same method. After 48 hours, we gathered cells to lyse by lysis buffer. The dual-luciferase reporter assay system (GeneCopoeia, LF031, China) was used to analyze the relative luciferase activity.

RNA pull-down assay

RNA pull-down experiment was performed as the instruction of the kit (ThermoFisher, 20164, USA). First, miR-7-5p, antisense oligo, and miR-7-5p mutant were labeled with biotin, which can bind to the streptavidin magnetic beads. Cell lysates were incubated with biotin-labeled miR-7-5p, antisense oligo, and miR-7-5p mutant. Only biotin labeled miR-7-5p could bind to the target in a RISC dependent manner. Then, the incubated lysate samples went through the streptavidin magnetic beads. The elusion was done using non-denaturing Biotin Elution Buffer or SDS-PAGE Loading Buffer. At last, qRT-PCR was undertaken to measure the expression of lncRNA ZFAS1 or miR-7-5p in the elution.

CCK-8 assay

CCK-8 kit was purchased from Dojindo Laboratories (Kumamoto, Japan) to determine the cell viability. In the irradiation dose-dependent assay, cell viability of every group was detected at 0Gy, 4Gy, and 8Gy doses of irradiation respectively. In the irradiation time-dependent assay, cell viability of every group was

detected at 24h, 48h, 72h and 96h, respectively. 10 μL CCK-8 solution was added to every well to incubate with the cells for 2h. The absorbance of cells in every well was read at 450 nm using an ELISA plate reader.

Edu assay

The proliferation of NPC cells under irradiation was measured by Edu assay which was conducted using Edu Apollo DNA in vitro kit bought from RIBOBIO (C10341-3, Guangzhou, China). All manipulates were done following the protocol from the kit. Three random fields under a fluorescence microscope were selected and the Edu positive cells were counted. The Edu positive rate (=Edu positive cell number/DAPI positive cell number) represented the proliferation condition of the cells. Representative images of every group were given.

Flow cytometric apoptosis assay

Flow cytometry was employed for the apoptosis detection. Briefly, cell suspension with 10^4 cells were put in every tube, and went through centrifugation to lose the culture media. The cells were then washed with cold PBS twice. Then, the cold PBS was removed. 100 μl 1× binding buffer was added to the cells and the cells were re-suspended. 5 μl Annexin V and 5 μl PI (Beyotime, Beijing, China) were added to the cells in dark and incubated for 15 min. 300 μl 1× binding buffer was added to the cells and the cells were re-suspended again. The cell suspension was moved to 5 ml flow tubes. Within the next one hour, the cells went through flow cytometry analysis.

Statistical analyses

All the data were analyzed using Graphpad Prism 8.0. All results represented three independent experiments. Values were expressed as mean±SD. Two-tailed t-test was used for analyzing differences between two groups, while one-way ANOVA with Dunnett's post hoc test was used for analyzing differences in multiple groups. P-values less than 0.05 were regarded as statistically significant.

Result

ENO2 and miR-7-5p were identified as the potential downstream interactome of ZFAS1 in NPC

WebGestalt algorithm (figure1.A-B) and Metascape algorithm (figure1.C-D) were used to enrich the pathway and GO terms of GSE48503 differentially expressed genes (DEGs). The results showed that glycolysis and HIF-1 pathway were the primary enriched pathways in NPC irradiation resistance. Glycolysis and HIF-1 pathway were proved to be closely associated with irradiation resistance of cancers [38-41]. We thus hypothesized that the genes that participated in glycolysis and/or HIF-1 pathway could be a critical gene that participated in NCP irradiation resistance. We found the ENO2 and EGLN3 were the two genes with this potential. Thus, we used STRING algorithm to further analyse the interaction strength between the DEGs. We saw that ENO2 showed the most interaction evidence with its neighboring genes

(figure1. E). Therefore, we chose ENO2 as our study subject. Subsequently, we sought for the common miRNAs that were both downstream targets of ZFAS1 and upstream regulators of ENO2. The down stream target miRNAs of ZFAS1 were obtained from ENCORI database (http://starbase.sysu.edu.cn/), and the upstream regulating miRNAs of ENO2 were obtained from TargetScan Human 7.2 (http://targetscan.org/vert_72/). Lastly, the expression patterns of the overlapped six miRNAs were explored on dbDEMC database (figure1. F). They were all shown to be significantly downregulated in NPC; however, we have known that miR-7-5p was a significant tumor suppressor in many human cancers and it has been only limitedly studied in NPC, and its role in NPC under irradiation has not been investigated yet. To fill this gap, we determined miR-7-5p to be our miRNA of interest.

ZFAS1 was upregulated in nasopharyngeal carcinomas and NPC cell lines, and primarily located in cytoplasm.

ZFAS1 had a higher expression level in nasopharyngeal carcinomas compared with adjacent healthy tissues (figure2.A). At the same time, the expression of ZFAS1 was found significantly upregulated in NPC cell lines SUNE-1, 5-8F and C666-1 rather than in normal nasal mucosal epithelial cell NP-69 (figure2.B). Subsequently, total RNA was extracted from SUNE-1 and C666-1 cell lines with nuclear and cytoplasm RNA separated. The result of qRT-PCR showed that ZFAS1 was majorly located in cytoplasm (figure2.C).What's more, both SUNE-1 and C666-1 cells went through irradiation treatment. The survival analysis result suggested that both cell lines were dead completely at 8Gy and the IC50 was somewhere between 2Gy and 4Gy (figure2.D). Before further experiments were conducted, we testified and validated the transfection efficiency of ZFAS1 siRNAs in both cell lines (figure2.E).

Knockdown of ZFAS1 increased the radiation sensitivity of nasopharyngeal carcinoma in vitro.

We wonder whether knockdown of ZFAS1 could increase the irradiation sensitivity of NPC cells, thus we detected the survival outcomes of NPC cells under different doses of irradiation treatment and different timing using CCK-8 assay. We found that along with the increase of irradiation, the knockdown of ZFAS1 showed more decreased cell survival compared with the control group (figure3. A). We found that exposure to 8Gy irradiation gave the lowest survival rate in SUNE-1 and C666-1 cell lines through observing the minimum survival rate. In addition, under 8Gy irradiation treatment, both cell lines demonstrated significantly decreased survival rate in a time dependent manner (figure3. B). Next, we observed that both si-ZFAS1s decreased the proliferation of SUNE-1 and C666-1 cell lines compared with control group by Edu assay. The Edu positive rate in ZFAS1 knockdown groups decreased by approximately ½ in SUNE-1 cell line and 1/3 in C666-1 cell line (figure3. C). Furthermore, knockdown of si-ZFAS1 resulted in more apoptotic cells than control group under 8Gy irradiation treatment. The apoptosis rate in ZFAS1 knockdown groups was more than 2-fold of that in control group (figure3. D).

ZFAS1 was the upstream target gene of miR-7-5p.

The binding sequences of ZFAS1 on miR-7-5p were obtained from ENCORI and were illustrated in figure4. A. To determine the relationship between ZFAS1 and miR-7-5p, we carried out both luciferase reporter

assay and RNA pull-down assay. The luciferase reporter gene assay result suggested that wild type ZFAS1 could bind with miR-7-5p to decrease the fluorescence intensity compared with other groups (figure4.B). RNA pull-down assay results also showed that ZFAS1 was enriched with the presence of miR-7-5p mimics compared with the presence of antisense oligo or miR-7-5p mutant (figure4.C). Moreover, the expression of miR-7-5p was significantly less in NPC tissues than in the adjacent tissues (figure4.D). Then, we detected the miR-7-5p expression in NPC cell lines and normal control cell NP-69. The result showed that miR-7-5p was significantly downregulated in NPC cell lines contrast to NP-69 (figure 4.E). It was also revealed that ZFAS1 had a negative association with miR-7-5p (figure4.F). Again, before we conducted any further experiments, we examined the transfection efficiency of certain molecules. To be precise, after transfection of si-ZFAS1 (si-ZFAS1-1), miR-892b inhibitor, si-NC and co-transfection si-ZFAS1+miR-7-5p inhibitor, we observed that si-ZFAS1 group expressed 1.5-fold more miR-7-5p while miR-7-5p inhibitor group expressed 75% less miR-7-5p compared with control group. In addition, the expression of miR-7-5p showed no significant difference between the co-transfection of si-ZFAS1 and miR-7-5p inhibitor group and the control group (figure4. G).

Knockdown of ZFAS1 promoted the radiation sensitivity of NPC cells by acting on miR-7-5p.

To further discuss how ZFAS1 affects radiation sensitivity of NPC by regulating miR-7-5p, we designed a rescue experiment. CCK-8 assay results suggested that si-ZFAS1 weakened the cell viability of SUNE-1 cell line at 48h, 72h and 96h, and C666-1 cells at 72h and 96h, which was offset by the introduction of miR-7-5p inhibitor at the environment of 8Gy irradiation (figure5.A). Equally, Edu assay results demonstrated a similar result with CCK-8 assay. The proliferating SUNE-1 and C666-1 cells significantly decreased in si-ZFAS1 group but increased in miR-7-5p inhibitor group: the Edu positive rate dropped approximately half in si-ZFAS1 group, and raised by 20% in miR-7-5p inhibitor group. What's more, when we cotransfected miR-7-5p and si-ZFAS1, we found that the proliferating cells increased contrast to si-ZFAS1 group (figure5.B). Besides, we determined that si-ZFAS1 promoted cell apoptosis while miR-7-5p inhibitor inhibited cell apoptosis in SUNE-1 and C666-1 cell lines. The apoptosis rate in the si-ZFAS1 group was more than 2-fold of the control group, whereas that in the miR-7-5p inhibitor group was merely less than half of the control group. The number of apoptotic cells did not differ from the control group when miR-7-5p inhibitor and si-ZFAS1 were co-transfected (figure5.C).

MiR-7-5p was the upstream target gene of ENO2 mRNA.

The binding scheme predicted by TargetScan Human 7.2 (http://www.targetscan.org/vert_72/) showing the targeting relationship between miR-7-5p and ENO2 mRNA was illustrated in figure6.A. The binding relationship was validated using dual luciferase reporter gene assay and RNA pull-down assay. Luciferase reporter assay demonstrated that wide type ENO2 mRNA be targeted by miR-7-5p and led to the fluorescence intensity decrease compared with other groups (figure6.B). RNA pull-down assay results showed that ENO2 was significantly enriched with the addition of miR-7-5p mimics contrast to antisense oligo and miR-7-5p mutant groups in both SUNE-1 cell line and C666-1 cell line (figure6.C). When we detected ENO2 mRNA expression in NPC tissues and NPC cell lines, the result revealed that ENO2 mRNA

was significantly upregulated in NPC tissues and cells (figure6.D-E). The expression of miR-7-5p and ENO2 mRNA was negatively associated (figure6.F). What's more, we built cell models stably transfected with si-NC, si-ENO2, miR-7-5p inhibitor, and si-ENO2+miR-7-5p inhibitor for further experiments (figure6. G).

MiR-7-5p inhibition enhanced the radiation resistance of NPC cells by acting on ENO2 mRNA.

To further explore how miR-7-5p took part in radiation resistance in NPC by regulating ENO2 mRNA, we performed rescue experiments. Cell viability was measured by CCK-8 assay, the results of which suggested that at the environment of 8Gy irradiation, si-ENO2 significantly weakened the cell viability of NPC cells with miR-7-5p inhibitor offsetting it at 72h and 96h in both SUNE-1 and C666-1 cell lines (figure7.A). Equally, Edu assay demonstrated a similar result with CCK-8 assay. The proliferating cells in SUNE-1 and C666-1 cell lines decreased in si-ENO2 group, but increased in miR-7-5p inhibitor group. What's more, when we cotransfected miR-7-5p inhibitor with si-ENO2, we found that the proliferating cells increased contrast to si-ENO2 group (figure7.B). Besides, we determined that si-ENO2 promoted cell apoptosis by almost 50%, while miR-7-5p inhibitor inhibited cell apoptosis by almost 50% in SUNE-1 and C666-1 cell lines. The number of apoptosis cell increased when miR-7-5p inhibitor and si-ENO2 were co-transfection compared with si-ENO2 group (figure7.C). In figure 8, we simply illustrated the hypothesized mechanism in NPC radio resistance involving ZFAS1, miR-7-5p and ENO2. Basically, under the irradiation, the high ZFAS1 and ENO2 levels with low miR-7-5p level resulted in more resistance to irradiation of cancerous cells with less resistance to irradiation, i.e., ZFAS1 promoted NPC cell's resistance to irradiation by downregulating miR-7-5p, thereafter releasing more ENO2 mRNA.

Discussion

In our experiments, we observed that ZFAS1 was significantly upregulated in NPC tissues and cells as well as ENO2 while miR-7-5p was downregulated in NPC tissues and cells. Our results revealed that ZFAS1 knockdown weakened irradiation resistance in NPC in vitro. To further explore the mechanism how ZFAS1 affected the irradiation sensitivity, we introduced miR-7-5p inhibitor and ENO2 siRNA to our cell models, SUNE-1 and C666-1 cell lines. It was found that the knockdown of ZFAS1 and ENO2 led to increased sensitivity to irradiation, whereas the inhibition of miR-7-5p led to the opposite.

In the last two years, lncRNA ZFAS1 has been proved to promote cancer genesis of NPC by some teams. For instance, it was reported to promote NPC genesis by activating Wnt/β-actin pathway [42] and by inhibiting PI3K/AKT pathway [43]. In addition, it was reported to regulate miR-135a to promote NPC genesis [44]. In our previous study, lncRNA ZFAS1 was also found highly expressed in NPC tissues and augmented NPC by regulating miR-893b-LPAR1 interactome [20]. Since 2017, accumulating studies have been conducted to study the mechanism of drug resistance involving ZFAS1 in various human cancers. For instance, ZFAS1 was found to promote resistance to Adriamycin in T-cell acute lymphoblastic leukemia [45]. ZFAS1 knockdown led to significantly impaired resistance to cis-platinum or paclitaxel in gastric cancer [46]. Increased resistance to temozolomide in glioma [47]. Other lncRNAs have been

reported to be associated with the irradiation resistance of NPC. For instance, lncRNA MINCR was overexpressed in NPC with worse prognosis in patients by promoting the radio resistance of NPC. MINCR decreased NPC cell radiation sensitivity through the competitive binding with miR-892b, increasing ZEB1, and activating AKT/PI3K signaling [5]. In addition, lncPVT1 silence promoted the radio sensitivity in NPC cells through inhibiting H3K9 and TIF1β combination. PVT1 was proposed to act as an oncogenic part in radio resistance through activating HIF-1α [48]. However, lnc ZFAS1 has not been studied in the radiation resistance of human cancers including NPC. To fill this gap, we herein further discussed whether ZFAS1 affected radio resistance in NPC. In our study, we found that the knockdown of lncRNA ZFAS1 was able to impair the radio resistance in NPC, shown by the impaired cell survival and proliferation, and enhanced cell apoptosis.

During the last one decade, miR-7-5p has been discovered to be a tumor suppressor in various human cancers. It was found significantly downregulated and played a tumor suppressing role in gastric cancer, presented by inhibited colony formation and invasion [49]; in colorectal cancer, presented by impaired cell viability, proliferation, migration, invasion, and EMT [50]; and breast cancer, demonstrated by suppressed cell proliferation, migration and invasion [51]. Also, in human NPC, it was found to be a tumor suppressor and led to significantly suppressed colony formation [52]. Yet, how miR-7-5p regulates irradiation resistance in human cancers has not been thoroughly studied. Herein, we found that the inhibition of miR-7-5p by miR-7-5p inhibitor significantly enhanced the radio resistance of NPC cells, thus we concluded that miR-7-5p, as a ceRNA of ZFAS1, could enhance the radio sensitivity in NPC.

ENO2 has been reported as a HIF-responsive gene in a xenograft model in renal cell carcinoma [37]. A study also reported that silencing ENO2 by siRNA technique selectively inhibited the growth and survival of ENO1 deleted cells in glioblastoma [34]. In addition, there was a study determining that ENO2 involved in the glycolytic pathway in colorectal cancer [53]. Plus, ENO2 was upregulated in neuroendocrine prostate cancer, leading to epithelial to mesenchymal transition and cancer stem cell phenotype [54]. Combining with our study, we concluded that ENO2 might be an oncogene in NPC, and it was significantly upregulated in NPC. We also hypothesized that ENO2 might take part in glycolytic pathway and HIF-1α pathway to regulate tumor radio resistance.

In our study, we identified that ENO2 could be an important player in hypoxia response via HIF-1 signaling by a bioinformatics method. Hypoxic tumor cells are resistant to radiotherapy. HIF-1 signaling has been regarded as an important signaling in radiotherapy resistance of human cancers [55]. Yet, we haven't been able to design experiments to study the role of ZFAS1 on tumor hypoxia, and we did not research how ENO2 regulated HIF-1α pathway by being regulated by ZFAS1 and miR-7-5p. In addition, animal experiments remain to be conducted to validate the effects of the interactome in vivo.

Conclusion

Taken together, our experiment suggested that lncRNA ZFAS1 acted as a radiation resistance enhancer in NPC. Mechanically, ZFAS1 competitively bound with miR-892b, thereby increasing the expression of

ENO2 to enhance the radiation resistance of NPC.

Declarations

Ethics approval and consent to participate

Ethic Committee of West China Hospital (Sichuan, China) approved the study.

Consent for publication

Informed consent was obtained from all patients.

Availability of Data and Materials

The datasets used during the current study are available from the corresponding author on reasonable request.

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Competing interests

There is no conflict of interest existed among the authors.

Authors' contributions

SXL designed the experiments. JJP and FL conducted the experiments. HZ and QW collected the data and wrote the manuscript.

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Not applicable.

Abbreviations

miRNAs: microRNAs; PBS: phosphate bufered solution; EdU: 5-ethynyl-2'-deoxyuridine; NPC: Nasopharyngeal carcinoma ; qPCR: quantitative real-time PCR.

References

1. Lu, Y., et al., *EVI1 promotes epithelial-to-mesenchymal transition, cancer stem cell features and chemo-/radioresistance in nasopharyngeal carcinoma.* J Exp Clin Cancer Res, 2019. **38**(1): p. 82.
2. Guo, Y., et al., *Improved Radiotherapy Sensitivity of Nasopharyngeal Carcinoma Cells by miR-29-3p Targeting COL1A1 3'-UTR.* Med Sci Monit, 2019. **25**: p. 3161-3169.

3. Gupta, A.K., et al., *Local recurrence in head and neck cancer: relationship to radiation resistance and signal transduction.* Clin Cancer Res, 2002. **8**(3): p. 885-92.
4. Hildesheim, A. and C.P. Wang, *Genetic predisposition factors and nasopharyngeal carcinoma risk: a review of epidemiological association studies, 2000-2011: Rosetta Stone for NPC: genetics, viral infection, and other environmental factors.* Semin Cancer Biol, 2012. **22**(2): p. 107-16.
5. Zhong, Q., Y. Chen, and Z. Chen, *LncRNA MINCR regulates irradiation resistance in nasopharyngeal carcinoma cells via the microRNA-223/ZEB1 axis.* Cell Cycle, 2020. **19**(1): p. 53-66.
6. Liang, Z.G., et al., *The role of autophagy in the radiosensitivity of the radioresistant human nasopharyngeal carcinoma cell line CNE-2R.* Cancer Manag Res, 2018. **10**: p. 4125-4134.
7. Lee, A.W., et al., *Management of Nasopharyngeal Carcinoma: Current Practice and Future Perspective.* J Clin Oncol, 2015. **33**(29): p. 3356-64.
8. Kong, F., et al., *Patterns of local-regional failure after primary intensity modulated radiotherapy for nasopharyngeal carcinoma.* Radiat Oncol, 2014. **9**: p. 60.
9. Sun, W., et al., *Decreased expression of long noncoding RNA AC096655.1-002 in gastric cancer and its clinical significance.* Tumour Biol, 2013. **34**(5): p. 2697-701.
10. Engreitz, J.M., et al., *The Xist lncRNA exploits three-dimensional genome architecture to spread across the X chromosome.* Science, 2013. **341**(6147): p. 1237973.
11. Ard, R., P. Tong, and R.C. Allshire, *Long non-coding RNA-mediated transcriptional interference of a permease gene confers drug tolerance in fission yeast.* Nat Commun, 2014. **5**: p. 5576.
12. Kanduri, C., *Long noncoding RNAs: Lessons from genomic imprinting.* Biochim Biophys Acta, 2016. **1859**(1): p. 102-11.
13. Li, Y., et al., *HBXIP and LSD1 Scaffolded by lncRNA Hotair Mediate Transcriptional Activation by c-Myc.* Cancer Res, 2016. **76**(2): p. 293-304.
14. Sun, M., et al., *LncRNA HOXA11-AS Promotes Proliferation and Invasion of Gastric Cancer by Scaffolding the Chromatin Modification Factors PRC2, LSD1, and DNMT1.* Cancer Res, 2016. **76**(21): p. 6299-6310.
15. Askarian-Amiri, M.E., et al., *SNORD-host RNA Zfas1 is a regulator of mammary development and a potential marker for breast cancer.* RNA, 2011. **17**(5): p. 878-91.
16. Li, T., et al., *Amplification of Long Noncoding RNA ZFAS1 Promotes Metastasis in Hepatocellular Carcinoma.* Cancer Res, 2015. **75**(15): p. 3181-91.
17. Cui, X., et al., *ZNFX1 anti-sense RNA 1 promotes the tumorigenesis of prostate cancer by regulating c-Myc expression via a regulatory network of competing endogenous RNAs.* Cell Mol Life Sci, 2019.
18. Xia, L., et al., *Circular RNA circ-CBFB promotes proliferation and inhibits apoptosis in chronic lymphocytic leukemia through regulating miR-607/FZD3/Wnt/beta-catenin pathway.* Biochem Biophys Res Commun, 2018. **503**(1): p. 385-390.
19. Li, Z., et al., *Exosomal lncRNA ZFAS1 regulates esophageal squamous cell carcinoma cell proliferation, invasion, migration and apoptosis via microRNA-124/STAT3 axis.* J Exp Clin Cancer

Res, 2019. **38**(1): p. 477.

20. Peng, J., et al., *Long noncoding RNA ZFAS1 promotes tumorigenesis and metastasis in nasopharyngeal carcinoma by sponging miR-892b to up-regulate LPAR1 expression.* J Cell Mol Med, 2020. **24**(2): p. 1437-1450.
21. Ko, J., et al., *miRNA Profiling of Magnetic Nanopore-Isolated Extracellular Vesicles for the Diagnosis of Pancreatic Cancer.* Cancer Res, 2018. **78**(13): p. 3688-3697.
22. Telonis, A.G. and I. Rigoutsos, *Race Disparities in the Contribution of miRNA Isoforms and tRNA-Derived Fragments to Triple-Negative Breast Cancer.* Cancer Res, 2018. **78**(5): p. 1140-1154.
23. Nagesh, P.K.B., et al., *miRNA-205 Nanoformulation Sensitizes Prostate Cancer Cells to Chemotherapy.* Cancers (Basel), 2018. **10**(9).
24. Marcuello, M., et al., *Analysis of A 6-Mirna Signature in Serum from Colorectal Cancer Screening Participants as Non-Invasive Biomarkers for Advanced Adenoma and Colorectal Cancer Detection.* Cancers (Basel), 2019. **11**(10).
25. Yu, L., et al., *Clinical Utility of a STAT3-Regulated miRNA-200 Family Signature with Prognostic Potential in Early Gastric Cancer.* Clin Cancer Res, 2018. **24**(6): p. 1459-1472.
26. Galka-Marciniak, P., et al., *Somatic Mutations in miRNA Genes in Lung Cancer-Potential Functional Consequences of Non-Coding Sequence Variants.* Cancers (Basel), 2019. **11**(6).
27. Kanlikilicer, P., et al., *Exosomal miRNA confers chemo resistance via targeting Cav1/p-gp/M2-type macrophage axis in ovarian cancer.* EBioMedicine, 2018. **38**: p. 100-112.
28. Tomita, K., et al., *MiR-7-5p is a key factor that controls radioresistance via intracellular Fe(2+) content in clinically relevant radioresistant cells.* Biochem Biophys Res Commun, 2019. **518**(4): p. 712-718.
29. Kabir, T.D., et al., *A microRNA-7/growth arrest specific 6/TYRO3 axis regulates the growth and invasiveness of sorafenib-resistant cells in human hepatocellular carcinoma.* Hepatology, 2018. **67**(1): p. 216-231.
30. Yang, F., et al., *MicroRNA-7-5p Promotes Cisplatin Resistance of Cervical Cancer Cells and Modulation of Cellular Energy Homeostasis by Regulating the Expression of the PARP-1 and BCL2 Genes.* Med Sci Monit, 2018. **24**: p. 6506-6516.
31. Gao, D., et al., *Screening circular RNA related to chemotherapeutic resistance in breast cancer.* Epigenomics, 2017. **9**(9): p. 1175-1188.
32. Lai, J., et al., *MiR-7-5p-mediated downregulation of PARP1 impacts DNA homologous recombination repair and resistance to doxorubicin in small cell lung cancer.* BMC Cancer, 2019. **19**(1): p. 602.
33. Law, M.L. and F.T. Kao, *Regional mapping of the gene coding for enolase-2 on human chromosome 12.* J Cell Sci, 1982. **53**: p. 245-54.
34. Muller, F.L., et al., *Passenger deletions generate therapeutic vulnerabilities in cancer.* Nature, 2012. **488**(7411): p. 337-42.
35. Kim, J., et al., *FOXA1 inhibits prostate cancer neuroendocrine differentiation.* Oncogene, 2017. **36**(28): p. 4072-4080.

36. Teng, P.N., et al., *Differential proteomic analysis of renal cell carcinoma tissue interstitial fluid.* J Proteome Res, 2011. **10**(3): p. 1333-42.
37. Zhang, T., et al., *The contributions of HIF-target genes to tumor growth in RCC.* PLoS One, 2013. **8**(11): p. e80544.
38. Augoff, K., A. Hryniewicz-Jankowska, and R. Tabola, *Lactate dehydrogenase 5: an old friend and a new hope in the war on cancer.* Cancer Lett, 2015. **358**(1): p. 1-7.
39. Moreno-Sanchez, R., et al., *Energy metabolism in tumor cells.* FEBS J, 2007. **274**(6): p. 1393-418.
40. Bhattarai, D., X. Xu, and K. Lee, *Hypoxia-inducible factor-1 (HIF-1) inhibitors from the last decade (2007 to 2016): A "structure-activity relationship" perspective.* Med Res Rev, 2018. **38**(4): p. 1404-1442.
41. Wigerup, C., S. Pahlman, and D. Bexell, *Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer.* Pharmacol Ther, 2016. **164**: p. 152-69.
42. Chen, X., et al., *Long non-coding RNA ZFAS1 promotes nasopharyngeal carcinoma through activation of Wnt/beta-catenin pathway.* Eur Rev Med Pharmacol Sci, 2018. **22**(11): p. 3423-3429.
43. Wang, X., et al., *LncRNA ZFAS1 promotes proliferation and migration and inhibits apoptosis in nasopharyngeal carcinoma via the PI3K/AKT pathway in vitro.* Cancer Biomark, 2019. **26**(2): p. 171-182.
44. Wang, M., et al., *Knockdown of lncRNA ZFAS1 inhibits progression of nasopharyngeal carcinoma by sponging miR-135a.* Neoplasma, 2019. **66**(6): p. 939-945.
45. Liu, Q., et al., *The regulatory ZFAS1/miR-150/ST6GAL1 crosstalk modulates sialylation of EGFR via PI3K/Akt pathway in T-cell acute lymphoblastic leukemia.* J Exp Clin Cancer Res, 2019. **38**(1): p. 199.
46. Xu, W., et al., *Silencing of lncRNA ZFAS1 inhibits malignancies by blocking Wnt/beta-catenin signaling in gastric cancer cells.* Biosci Biotechnol Biochem, 2018. **82**(3): p. 456-465.
47. Li, X., et al., *The long noncoding RNA ZFAS1 promotes the progression of glioma by regulating the miR-150-5p/PLP2 axis.* J Cell Physiol, 2020. **235**(3): p. 2937-2946.
48. Wang, Y., et al., *The lncRNA PVT1 regulates nasopharyngeal carcinoma cell proliferation via activating the KAT2A acetyltransferase and stabilizing HIF-1alpha.* Cell Death Differ, 2019.
49. Xin, L., et al., *DNA-methylation-mediated silencing of miR-7-5p promotes gastric cancer stem cell invasion via increasing Smo and Hes1.* J Cell Physiol, 2020. **235**(3): p. 2643-2654.
50. Zheng, Y., P. Nie, and S. Xu, *Long noncoding RNA CASC21 exerts an oncogenic role in colorectal cancer through regulating miR-7-5p/YAP1 axis.* Biomed Pharmacother, 2020. **121**: p. 109628.
51. Li, Y.L., et al., *Up-regulated lnc-lung cancer associated transcript 1 enhances cell migration and invasion in breast cancer progression.* Biochem Biophys Res Commun, 2020. **521**(2): p. 271-278.
52. Zhong, Q., et al., *Circular RNA CDR1as sponges miR-7-5p to enhance E2F3 stability and promote the growth of nasopharyngeal carcinoma.* Cancer Cell Int, 2019. **19**: p. 252.
53. Yeh, C.S., et al., *Significance of the glycolytic pathway and glycolysis related-genes in tumorigenesis of human colorectal cancers.* Oncol Rep, 2008. **19**(1): p. 81-91.

54. Lee, E., et al., *Reduction of two histone marks, H3k9me3 and H3k27me3 by epidrug induces neuroendocrine differentiation in prostate cancer.* J Cell Biochem, 2018. **119**(4): p. 3697-3705.
55. Moeller, B.J. and M.W. Dewhirst, *HIF-1 and tumour radiosensitivity.* Br J Cancer, 2006. **95**(1): p. 1-5.

Tables

Table 1 Clinical parameters of patients with nasopharyngeal carcinoma in this study

Pathological characteristics	Case(n)
Gender	
Male	29（52.7%）
Female	26（47.3%）
Age	
≥25	30（54.5%）
<25	25（45.5%）
T classification	
T1+T2	19（34.5%）
T3	20（36.4%）
T4	16（29.1%）
N classification	
N0+N1	20（36.4%）
N2+N3	35（63.6%）
Distant metastasis	
Negative	37（67.3%）
Positive	18（32.7%）

Table 2 The sequences of the primers in this study

Primer	Sequences
lnc ZFAS1	
Forward sequence	5'-ATTGTCCTGCCCGTTAGAGC-3'
Reverse sequence	5'-ACTTCCAACACCCGCATTCA-3'
miR-7-5p	
Forward sequence	5'-AAAACTGCTGCCAAAACCAC-3'
Reverse sequence	5'-GCTGCATTTTACAGCGACCAA-3'
ENO2	
Forward sequence	5'-TCGCTTTGCCGGACATAACT-3'
Reverse sequence	5'-GACACATCGTTCCCCCAAGT-3'
GAPDH	
Forward sequence	5'-GTCAAGGCTGAGAACGGGAA-3'
Reverse sequence	5'-AAATGAGCCCCAGCCTTCTC-3'
U6	
Forward sequence	5'-TGCGGGTGCTCGCTTCGGCAGC-3'
Reverse sequence	5'-CCAGTGCAGGGTCCGAGGT-3'

Figures

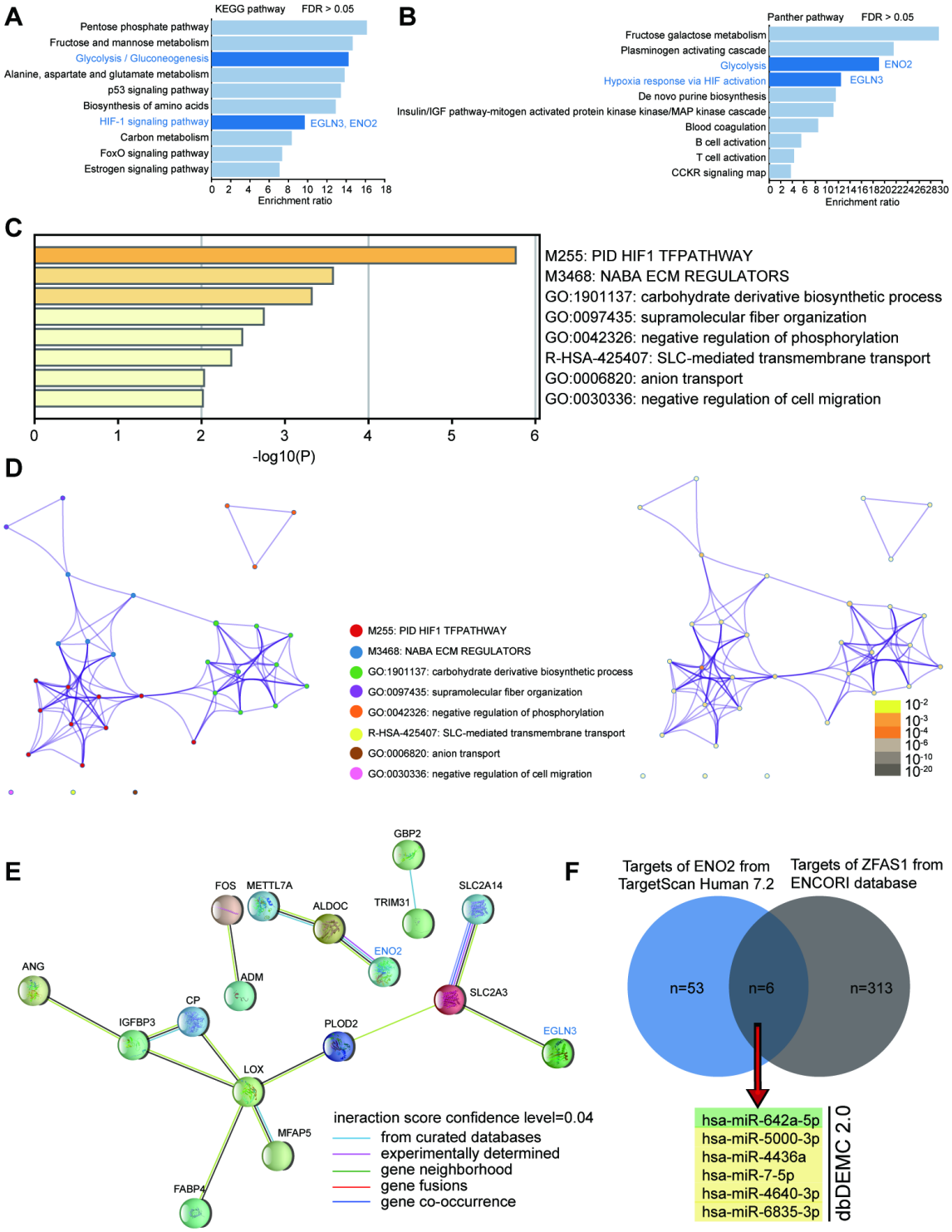

Figure 1

The identification of potential mRNAs and miRNAs that participate in NPC and NPC irradiation resistance. A. KEGG pathway enrichment of GSE48503 DEGs using WebGestalt algorithm (http://www.webgestalt.org/option.php). B. Panther pathway enrichment of GSE48503 DEGs using WebGestalt algorithm. C. The heatmap of Metascape analysis of GSE48053 DEGs. D. The enrichment cluster of Metascape analysis of GSE48053 DEGs. E. STRING analysis of the DEGs showing the

interaction between the DEGs. F. The overlapped miRNAs of the targets of ENO2 predicted by TargetScan Human 7.2 and those of ZFAS1 predicted by ENCORI, and the expression pattern in NPC in dbDEMC 2.0 database.

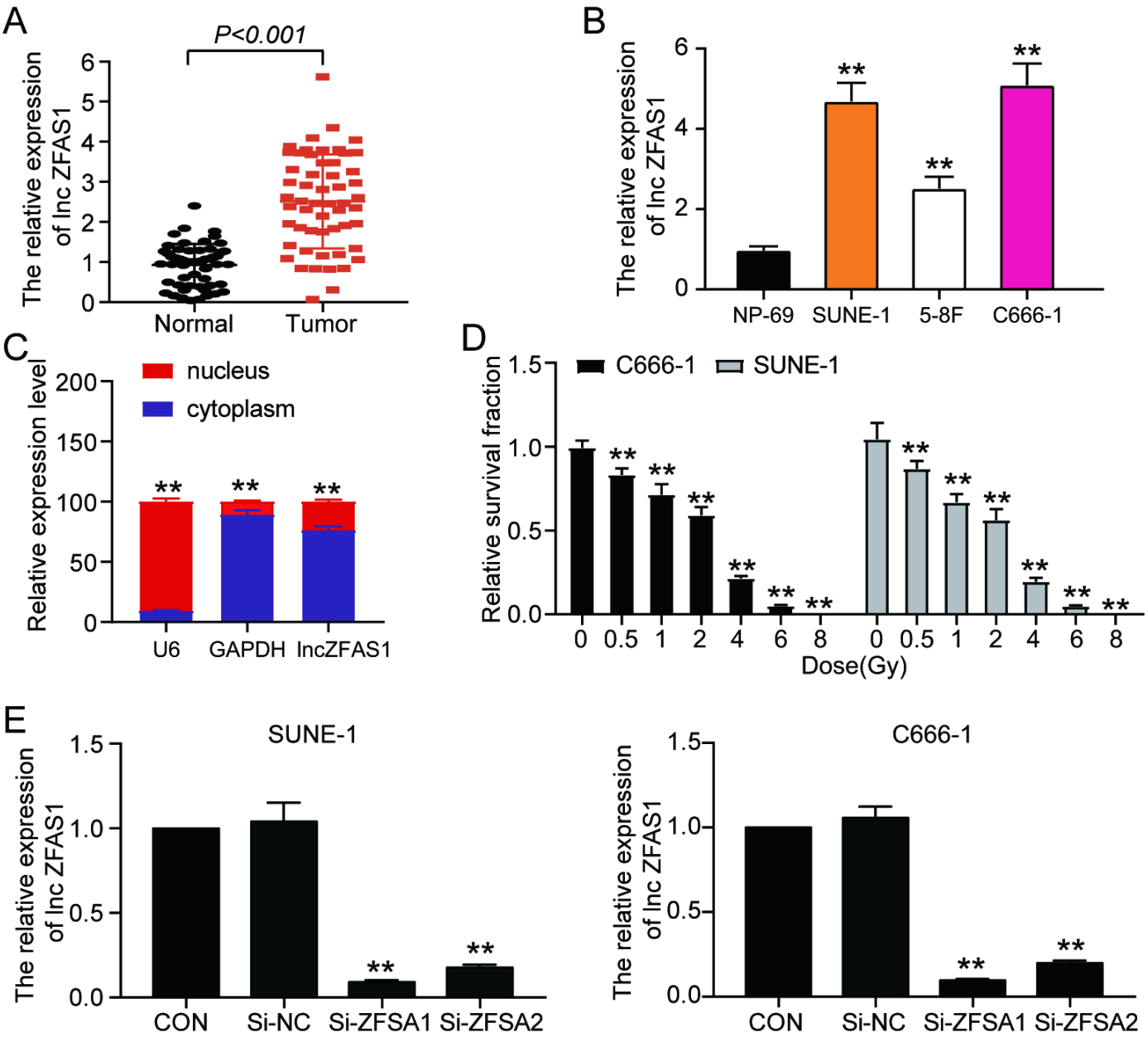

Figure 2

ZFAS1 was upregulated in nasopharyngeal carcinoma and located in cytoplasm. A. The different expression levels of ZFAS1 between nasopharyngeal carcinoma tissues and normal adjacent tissues was measured by qRT-PCR. B. The different expression of ZFAS1 between nasopharyngeal carcinoma cell lines containing SUNE-1,5-8F, and C666-1, and normal nasal mucosal epithelial cell NP-69 was detected by qRT-PCR. **P<0.01, compared with NP-69 cell line. C. The expression of ZFAS1 at nuclear or cytoplasm

in SUNE-1 and C666-1 cell lines was measured by qRT-PCR. **P<0.01, compared with the corresponding cytoplasm. D. The survival rate of SUNE-1 and C666-1 nasopharyngeal carcinoma cell lines after giving different doses of irradiation. **P<0.01, compared with 0 Gy. E. The transfection efficiency validation of si-ZFAS1-1 and si-ZFAS1-2, which are siRNAs that target ZFAS1 using qRT-PCR. **P<0.01, compared with CON (control) group.

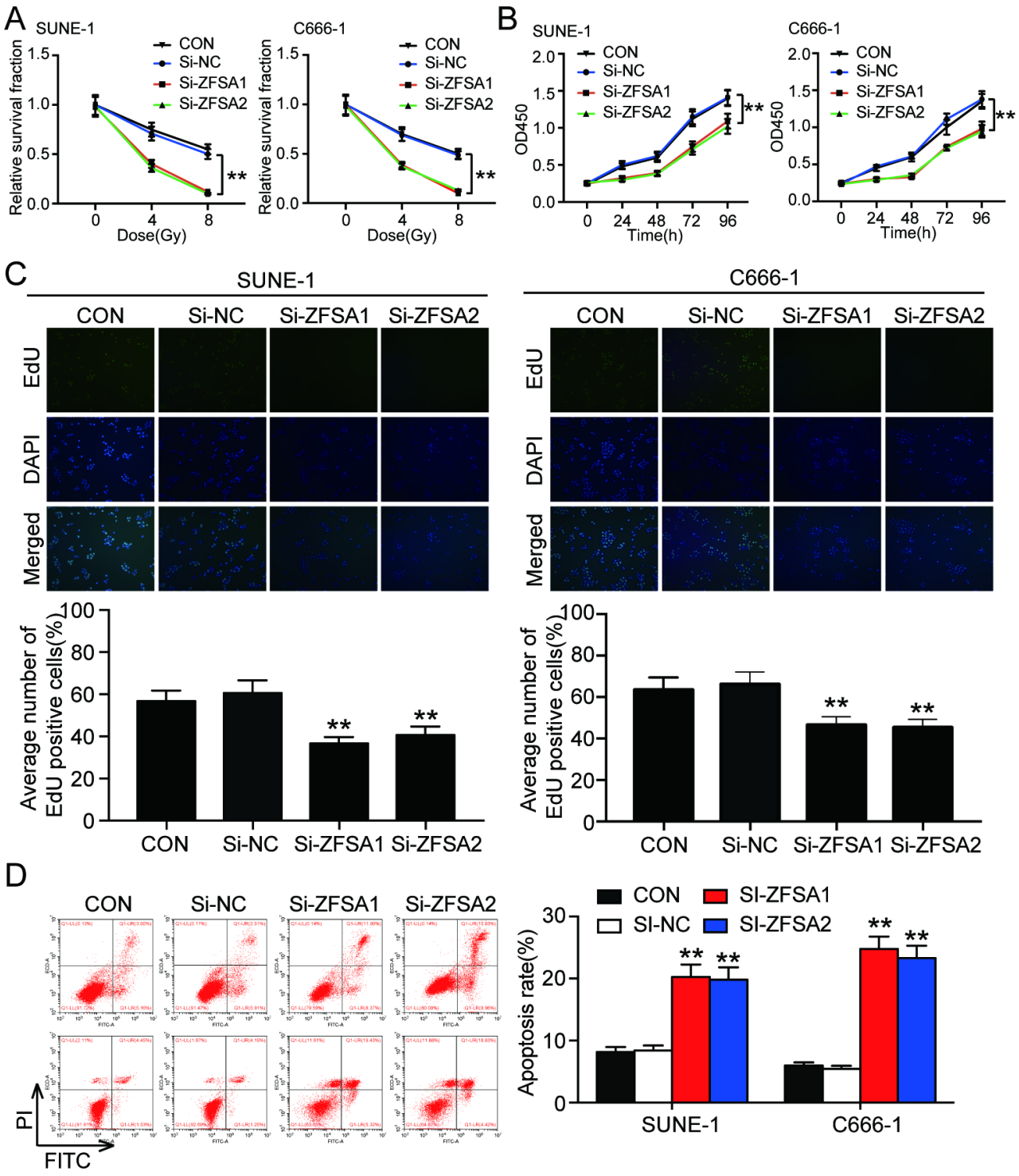

Figure 3

The knockdown of ZFAS1 weakened the radiation resistance of nasopharyngeal carcinoma cells in vitro. A. The viability changes of nasopharyngeal carcinoma cell lines SUNE-1 and C666-1 after giving different doses of irradiation. B. At 8Gy irradiation, the survival rate of cells in every groups was detected at 24h, 48h, 72h, and 96h in SUNE-1 and C666-1 cell lines. C. Average numbers of Edu positive cells in SUNE-1 and C666-1 cell lines with 8Gy irradiation. D. The cell apoptosis of SUNE-1 and C666-1 cell lines with 8Gy irradiation detected by flow cytometry. **P<0.01, compared with CON group.

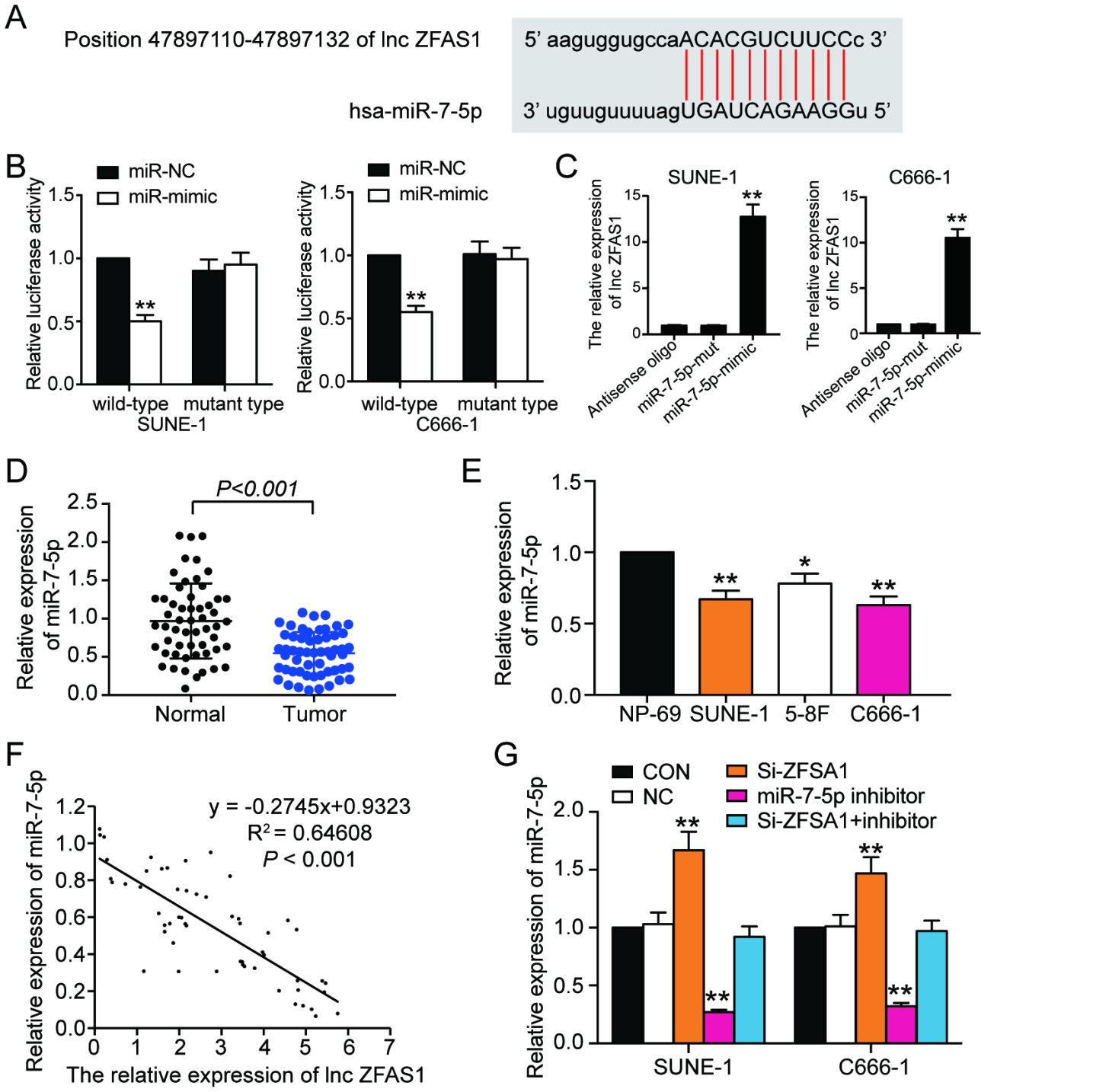

Figure 4

ZFAS1 was the upstream gene of miR-7-5p. A. The predicted binding sequences between ZFAS1 and miR-7-5p obtained from ENCORI algorithm. B. Luciferase reporter assay was used to determine the target relationship between ZFAS1 and miR-7-5p. **P<0.01, compared with miR-NC group. NC: negative control. C. RNA pull down was used to demonstrate the association between ZFAS1 and miR-7-5p. mut: mutant. **P<0.01, compared with antisense oligo group. D. The expression of miR-7-5p in NPC tissues and adjacent tissues. E. The expression of miR-7-5p in NPC cell lines. *P<0.05, **P<0.01, compared with NP-69 cell line. F. ZFAS1 expression showed a negative relationship with miR-7-5p expression. G. The transfection efficiency of miR-7-5p inhibitor. **P<0.01, compared with CON group.

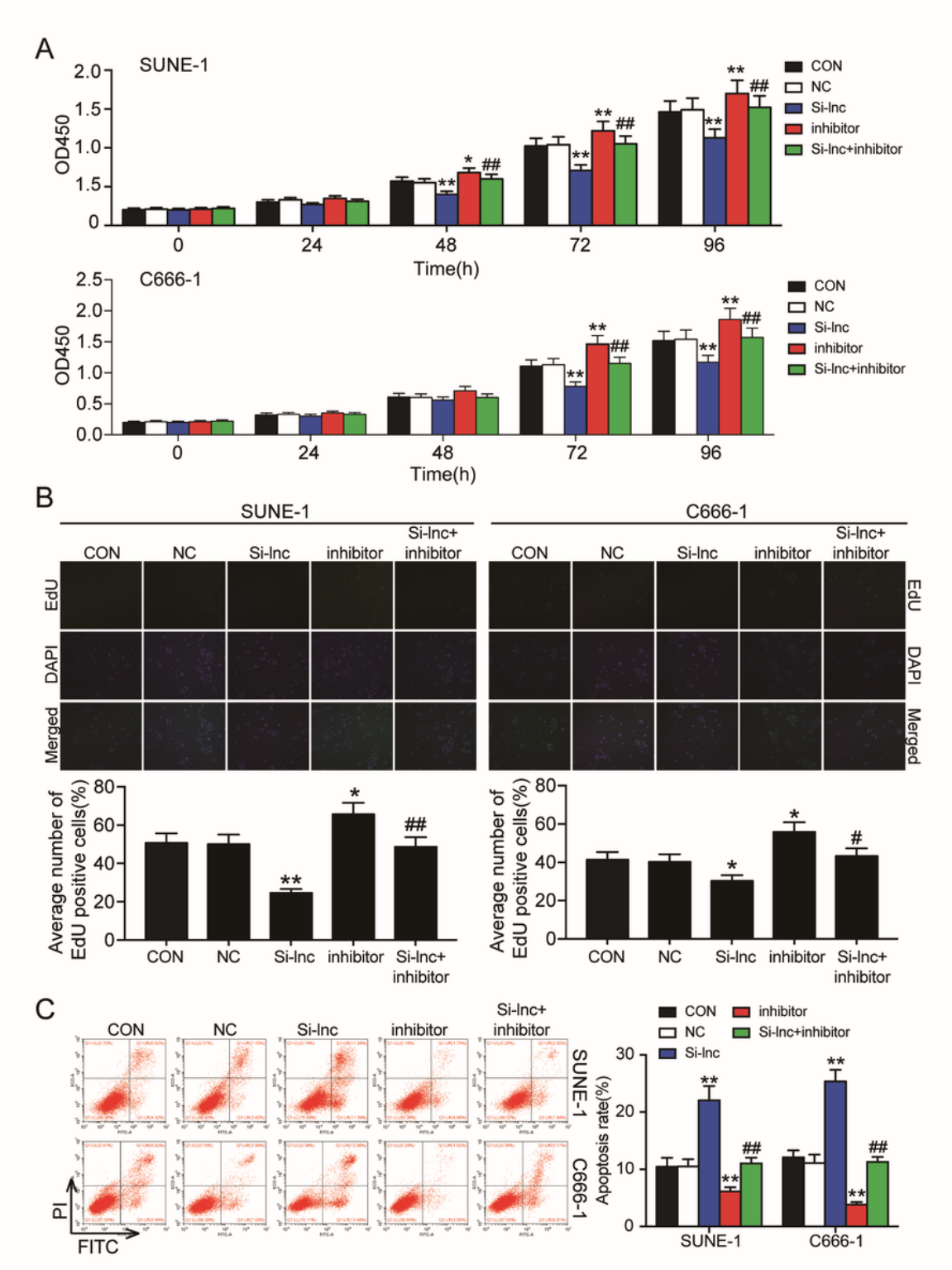

Figure 5

The knockdown of ZFAS1 suppressed the radiation resistance of nasopharyngeal carcinoma cells by acting on miR-7-5p. A. CCK-8 was used to detect the viability of SUNE-1 and C666-1 cells after transfection with si-ZFAS1 or miR-7-5p inhibitor under 8Gy irradiation. B. Edu assay was used to observe the cell proliferation of SUNE-1 and C666-1 cell lines after transfection with si-ZFAS1 or miR-7-5p under 8Gy irradiation. C. The cell apoptosis in SUNE-1 and C666-1 cell lines after transfection with si-ZFAS1 or

miR-7-5p inhibitor with giving 8Gy irradiation by flow cytometry. *P<0.05, **P<0.01, compared with CON group; #P<0.05, ##P<0.01, compared with miR-7-5p inhibitor group.

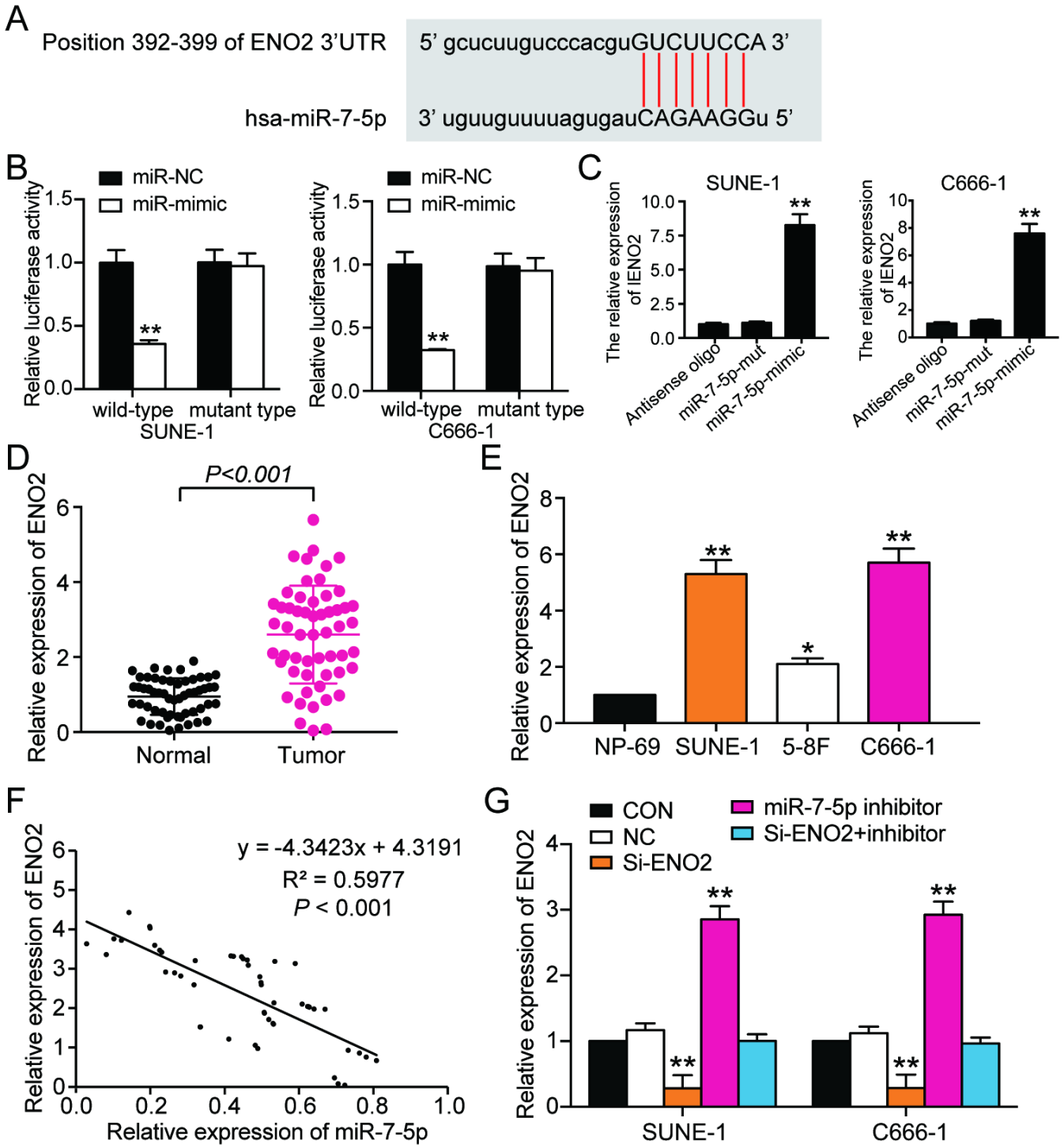

Figure 6

MiR-7-5p was the upstream target gene of ENO2. A. The predicted binding sites of miR-7-5p and ENO2 mRNA was illustrated. B. Luciferase reporter assay results showed that ENO2 was the downstream target gene of miR-7-5p. **P<0.01, compared with miR-NC group C. RNA pull down assay demonstrated the

regulation relationship between ENO2 and miR-7-5p. **P<0.01, compared with antisense oligo group. D. The expression of ENO2 mRNA in NPC tissues and adjacent healthy tissues. E. The expression of ENO2 in NPC cell lines and normal cell NP-69. *P<0.05, **P<0.01, compared with NP-69 cell line. F. ENO2 mRNA expression had a negative relationship with miR-7-5p expression. G. qRT-PCR was used to observe the expression of ENO2 mRNA in SUNE-1 and C666-1 cells after transfection with si-ZFAS1 or miR-7-5p. **P<0.01, compared with CON group.

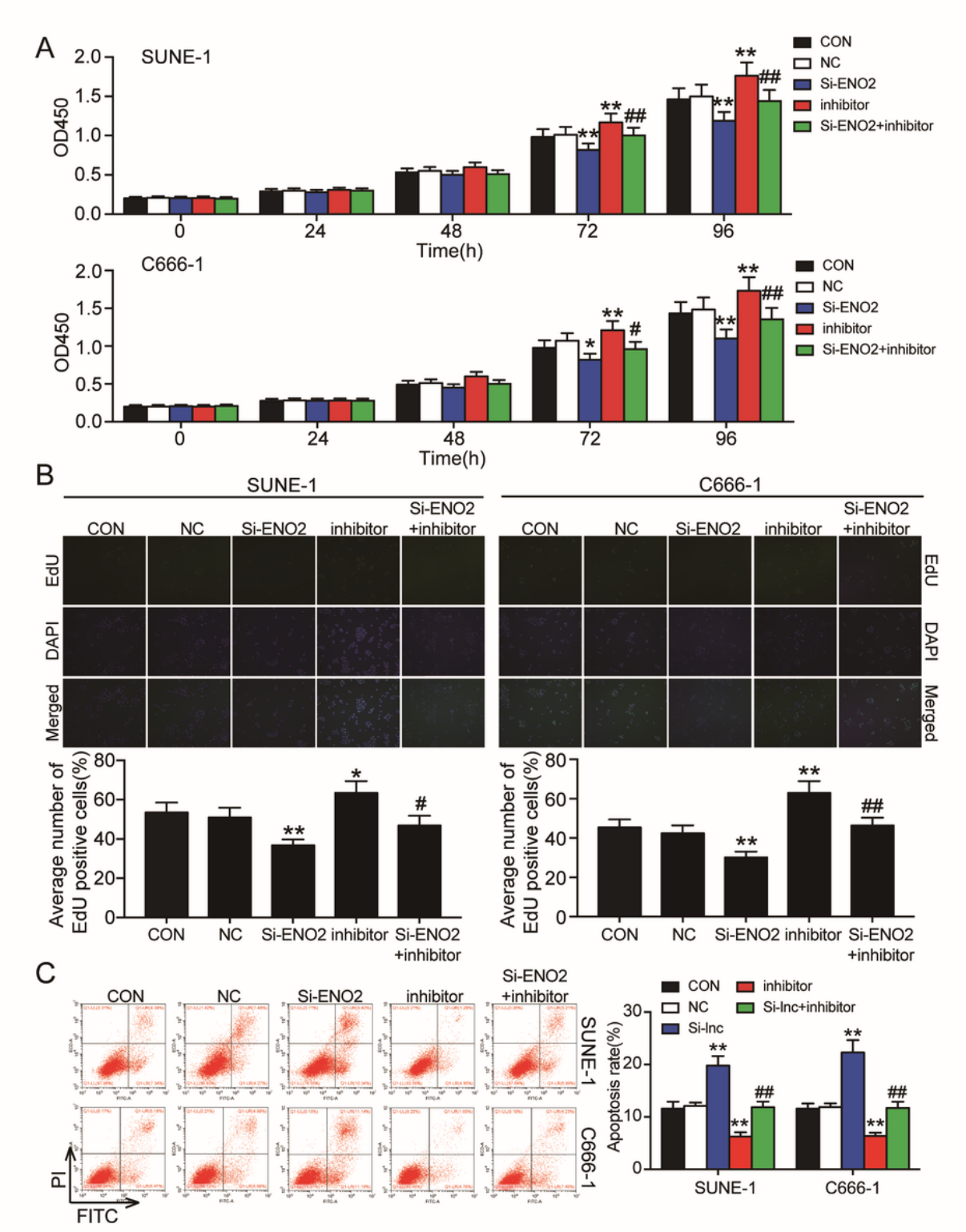

Figure 7

MiR-7-5p inhibition enhanced the radiation resistance of nasopharyngeal carcinoma cells by acting on ENO2. A. CCK-8 assay results showed that si- ENO2 suppressed the cell viability in SUNE-1 and C666-1 cell lines while miR-7-5p inhibition promoted the cell viability at 8 Gy irradiation. B. Edu assay showed that si-ENO2 suppressed the growth of SUNE-1 and C666-1 cell lines while miR-7-5p inhibition enhanced cell viability at 8 Gy irradiation. C. The cell apoptosis in SUNE-1 and C666-1 cell lines after transfection with si-ENO2 and miR-7-5p inhibitor under 8 Gy irradiation.

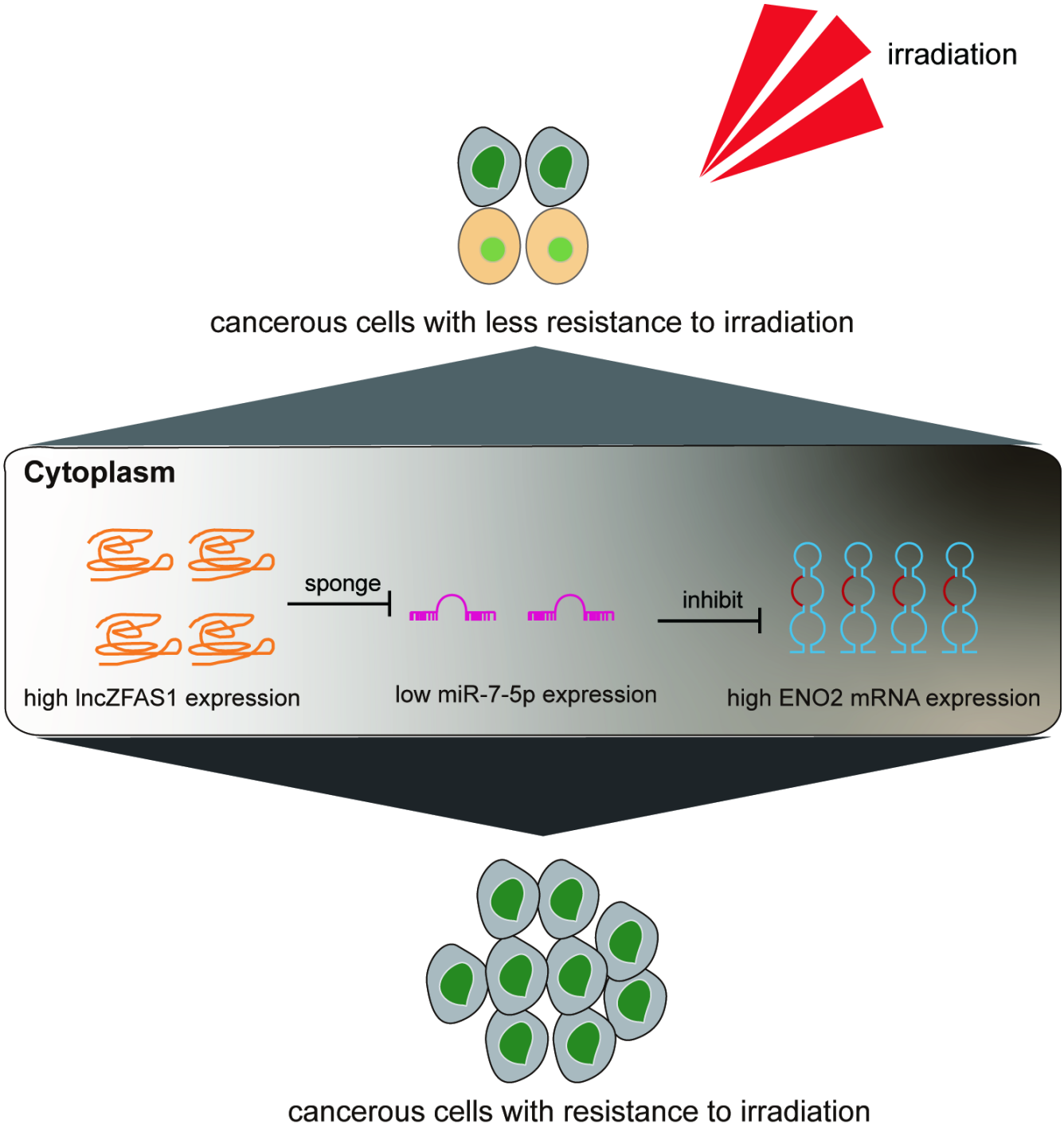

Figure 8

The illustration of the hypothesized mechanism involving ZFAS1, miR-7-5p and ENO2 in NPC resistance to irradiation. Basically, high ZFAS1 level, low miR-7-5p level, and high ENO2 mRNA level in NPC results in enhanced resistance to irradiation.