

Targeting Lactate Dehydrogenase A Improves Radiotherapy Efficacy in Non-Small Cell Lung Cancer: From Bedside to Bench

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Research

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Abstract

Background

LDHA is overexpressed in many kinds of cancer, and associated with poor prognosis. In the current study, we evaluated the prognostic value of LDHA expression in non-small cell lung cancer (NSCLC), and tested whether LDHA inhibition might improve radiotherapy efficacy in NSCLC.

Methods

LDHA mRNA expression was investigated in NSCLC patients, using online database and further verified by immunohistochemistry. The prognostic value of LDHA was evaluated using Kaplan-Meier plotter database. In vitro, two NSCLC cell lines were pretreated with oxamate, an inhibitor of LDHA, and colony formation method was performed to determine cellular radiosensitivity. Comet assay was used to detect DNA damage after irradiation. Flow cytometry was applied to test cell cycle progression and apoptosis, and autophagy was examined by monodansylcadaverin (MDC) staining.

Results

Both mRNA and protein levels of LDHA expression were up-regulated in NSCLC tissues. High LDHA expression was a poor prognostic factor and associated with radioresistance in NSCLC patients. LDHA inhibition by oxamate remarkably increased radiosensitivity in both A549 and H1975 cancer cells, and enhanced ionizing radiation (IR)-induced apoptosis and autophagy, accompanied by cell cycle distribution alternations. Furthermore, LDHA inhibition induced reactive oxygen species (ROS) accumulation and cellular ATP depletion, which might increase DNA injury and hinder DNA repair activity.

Conclusions

Our study suggests inhibition of LDHA may be a potential strategy to improve radiotherapy efficacy in NSCLC patients, which needs to be further tested by clinical trials.

Background

Lung cancer is the most common malignancy, making up almost 25% of all cancer deaths around the world[1]. Radiotherapy plays a vital role in lung cancer treatment, almost three quarters of all lung cancer patients need to receive radiotherapy at some point in their lives, and concurrent chemo-radiotherapy is now the cornerstone of curative treatment for unresectable locally advanced lung cancer[2]. However, radio-resistance often lead to local failure and tumor progression, the outcomes of stage III patients after CRT is still disappointed, with a 5-year survival rate of 15–20% approximately, although immunotherapy has further improved the survival recently[3]. There is still an urgent need to find new strategy to enhance radiotherapy efficiency in NSCLC patients.

High metabolism has been recognized as a new hallmark of cancer, and emerged as a potential target for anti-cancer drugs[4]. LDHA is a key enzyme evolved in glycolysis, and has been reported to be up-regulated in many kinds of cancer, and associated with poor prognosis[5–9]. Accumulating evidence indicates that LDHA inhibition exhibits promising anti-cancer effects through various mechanisms, as well as synergistically enhances the efficacy of other treatments, including chemotherapy, radiotherapy and target drugs[10, 11]. We previously reported that diverse responses were observed in NSCLC cells after LDHA inhibition, including autophagy or apoptosis, accompanied with different cell cycle distribution alternations[12]. However, the role of LDHA in regulating radiosensitivity of NSCLC has not been elucidated.

In this study, we firstly explored the LDHA mRNA expression in NSCLC patients using online database, and further verified by immunohistochemistry. Then, the prognostic value of LDHA in NSCLC was evaluated using Kaplan-Meier plotter database. In the following experiments in vitro, two NSCLC cell lines were pretreated with oxamate, a widely used inhibitor of LDHA as a structural analogue of pyruvate, and then were exposed to different doses of irradiation, colony formation method was performed to determine cellular radiosensitivity and the possible underlying mechanism was also investigated.

Methods

Online Database

Levels of LDHA mRNA expression in NSCLC tumor and normal tissue were compared by GEPIA browser (<http://gepia.cancer-pku.cn/>), a newly developed interactive web server for analyzing the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from the TCGA and the GTEx projects, which provides customizable functions such as tumor/normal differential expression analysis, profiling according to cancer types or pathological stages, patient survival analysis, similar gene detection, correlation analysis and dimensionality reduction analysis[13]. The association among LDHA mRNA expression level and survival of NSCLC patients was investigated by data mining in the Kaplan-Meier plotter database (<http://kmplot.com>), an online database integrating gene expression and survival information simultaneously download from Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA). The detailed GEO information included are as follows: CaArray, GSE14814, GSE19188, GSE29013, GSE30219, GSE31210, GSE3141, GSE31908, GSE37745, GSE4573, and GSE50081. Overall survival (OS) and progression-free survival (PFS) were calculated by the Kaplan-Meier method and compared with log-rank test.

Gene set enrichment analysis (GSEA)

GSEA analyses of lung adenocarcinoma and normal lung tissue samples from the TCGA database were performed to determine the enriched genes. Based on the median expression value, lung adenocarcinoma samples were subdivided into high and low LDHA expression groups, and the functional gene set file “msigdb.v7.1.symbols.gmt” was used to determine the enriched genes. The p value for all the pathways was calculated after 1000 permutations, and pathway enrichment score was obtained in a weighted

manner. The pathways and genes with a P-value < 0.05 and false discovery rate (FDR) < 0.25 were considered significantly enriched.

Cell culture and Reagents

All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, USA), and cultured in DMEM medium (Gibco; Gaithersburg, MD, United States) containing 10% fetal bovine serum, 100 µg/mL penicillin and 100 µg/mL streptomycin at 37°C under 5% CO₂. Oxamate sodium was bought from SigmaAldrich Corp (St. Louis, MO, USA).

Patient samples and immunohistochemical (IHC) assay

All specimens were collected from NSCLC patients who received radical resection from January 2014 to December 2018 without preoperative treatment in Zhejiang Cancer Hospital. Final diagnosis was confirmed by pathological examination. The expression of LDHA in tumor tissues and adjacent normal tissues was determined by IHC analysis. Briefly, paraffin-embedded tissues cut at 4µm were rehydrated, treated with 3% H₂O₂, and blocked with 3% BSA. Subsequently, the tissue sections were incubated with anti-LDHA antibody (1:200, ab101562) or with control IgG overnight at 4°C. The immunoreactivity of LDHA was evaluated by the LDHA histo-score, calculated by adding the score for the percentage of positive cells (0–100%) and the staining intensity score (0: no staining, 1: weak, 2: moderate, and 3: strong). Tissues with immunoreactivity scores of 0–4 were considered as low expression, and those with scores ≥ 4 were designated high expression. All sections were evaluated by two independent pathologists.

Colony formation assay

Cells treated with 20mmol oxamate or negative control were incubated for 48 h and then were subjected to a range doses of irradiation, including 0, 0.5, 1, 3, 6, 9Gy. Next, the cells were incubated at 37°C in 5% CO₂. Two weeks later, colonies were washed twice with PBS, fixed with methanol for 30 min, and stained with crystal violet. Colonies with ≥ 50 cells were counted. Plating efficiency (PE) was calculated as colony number/plating cell number × 100% and the surviving fraction (SF) was equal to colony number/(cells seeded × plating efficiency). The multi-target single-hit model was applied to fit survival curves using the formula: $SF = 1 - (1 - e^{-D/D_0})^N$, in which D = radiation dose, D₀ = mean death dose; N = extrapolate number, D_q = quasi-threshold dose. The sensitization enhancing ratio (SER) was calculated as a ratio of D₀ between treatment and control groups.

Cell cycle analysis and apoptotic assay

Cells were collected and fixed with 70% pre cooled ethanol overnight, after staining with propidium iodide (10 µg/ml; Sigma-Aldrich) in the dark for 30 min. Flow cytometry was performed on the FACS Calibursystem (Becton Dickinson, San Jose, CA, USA) and cell cycle distribution was analyzed by means of ModFit LT software (Becton Dickinson, CA, USA). AnnexinV-FITC apoptosis kit (BD Biosciences, San Jose, CA, USA) was employed to test apoptosis. Cells were harvested after 24h irradiation, then stained

with AnnexinV/PI for 30 min. The results were analyzed by the FACS Calibur system with ModFit's LT software.

Comet assay

Single cell gel electrophoresis (Neutral) was performed with Comet Assay kit (cat.4250-050-K; Trevigen) according to manufacturer's instructions. Briefly, A549 cells were irradiated at 6 Gy and were collected at 0, 2 and 24 hr after irradiation, followed by washing and resuspension in 4°C PBS. $1-2 \times 10^4$ cells were mixed in 1% low fusion point agarose and placed on a slide. The cells were then treated with lysis solution for 30 minutes, and rinsed in EDTA unwinding solution, and then subjected to alkaline electrophoresis for 30 minutes (25V, 300mA). The samples were then analyzed under fluorescence microscopy (Olympus FV1000). The length of tail, the percentage of DNA in the tail of each comet were measured using CASP software (Version1.2.3; available at <http://casp.sourceforge.net>).

ATP and ROS detection

Intracellular ATP and ROS levels were detected using commercial assay kits (Beyotime, Haimen, China), according to the manufacturer's instructions. Results were expressed as a percentage of the control group.

Statistical analysis

All results were presented as the mean \pm SD (standard deviation of the mean) from three independent experiments. Differences between two groups were examined by the Student's t-test, and ANOVA test was utilized to assess differences between multiple groups. The survival rates were calculated by the Kaplan-Meier method and compared with log-rank test. P-value < 0.05 was considered statistically significant. Statistical analysis was performed using the GraphPad Prism 5.0 and the SPSS 22.0 (SPSS Inc., Chicago, IL, USA) packages.

Results

LDHA is up-regulated in NSCLC tissue and associated with high glycolysis level.

Firstly, GEPIA browser (<http://gepia.cancer-pku.cn/>), an online tool for estimating mRNA expression based on The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) projects, was employed to compare mRNA expression level of LDHA in NSCLC tumor and normal tissues. Box and stage plotting analyses were processed on this database. The cutoff p value was defined as 0.01. As showed in Fig. 1A, LDHA mRNA expression levels were significantly elevated in both squamous and adenoma of lung cancer. Further, the mRNA levels displayed significant correlation with the tumor stage in patients with lung adenocarcinoma (LUAD) but not lung squamous carcinoma (LUSC) (Fig. 1B-C). To confirm the findings from online database, 35 NSCLC and 20 normal adjacent tissues in our biological sample bank were collected and ICH was performed, the results demonstrated the protein levels of LDHA were also increased in both LUAD and LUSC tumor tissues, compared to adjacent normal tissues (Fig. 1D-E). Then, we used GEPIA to explore the gene expression correlation between LDHA and glycolysis

markers, including GLUT-1(also named SLC2A1) and HIF1A, which play critical roles in glycolysis and tumor microenvironment. The results showed that LDHA expression was positively associated with both the expressions of HIF1A and SLC2A1 (Fig. 1F-G). Accordingly, using GSEA analysis, high LDHA expression in lung adenocarcinoma tissues was also positively correlated with hallmark gene sets of glycolysis and hypoxia (Fig. 1H-I), indicating the reliability of the results.

High LDHA expression is associated with radioresistance and worse survival in NSCLC

To explore the association between mRNA expression of LDHA and survival in NSCLC patients, Kaplan-Meier plotter database was utilized, which generated gene expression and clinical data from a total of 1928 NSCLC patients, from the following databases: CaArray, GSE14814, GSE19188, GSE29013, GSE30219, GSE31210, GSE3141, GSE31908, GSE37745, GSE4573, and GSE50081. The cutoff point was automatically selected by the database, to reduce false discovery rate. Overall survival (OS) and progression-free survival (PFS) were calculated using the Kaplan-Meier method and compared with log-rank test. As shown in Fig. 2, the number-at-risk cases, hazard ratios (HRs) with 95% confident intervals (CIs), and P-values were presented accordingly. The results showed that higher levels of LDHA mRNA expression were associated with worse OS and PFS in the overall population. Multivariate analysis further verified the inverse association between LDHA expression and overall survival of NSCLC, with a HR of 2.36 (95% CI: 1.37–4.05, $p < 0.05$). Especially, in the subgroup analysis, we found that LDHA was also associated with inferior PFS in patients who received radiotherapy, with an HR of 2.19(1.21–3.95, $p < 0.05$), even in a relatively small sample. Our results preliminarily indicated that high LDHA expression was a poor prognostic factor for NSCLC patients and might be associated with radioresistance.

LDHA inhibition increased sensitivity of NSCLC cells to irradiation

In vitro study, colony formation assay was used to examine the impact of LDHA inhibition on the radiosensitivity of NSCLC cell lines. According to our previous study[12], A549 and H1975 cell lines were pre-treated with or without a low concentration of 20mmol/L oxamate for 24h, which exerted slight influence on cell viability, and then were irradiated at 0, 0.5, 1, 3, 6 and 9 Gy. After further incubation for 2 weeks, the multi-target single-hit model was adopted to fit the survival curves. As shown in Fig. 3, the survival rates were significantly lower in the combination groups than those in the irradiation groups alone, and the survival curves decreased in a dose-dependent manner in both A549 and H1975 cells. However, the radiosensitive effect of LDHA inhibition was more obvious in H1975 cells, the sensitivity enhancement ratios (SERs) were 1.10 and 1.43 in A549 and H1975, respectively.

LDHA inhibition altered cell cycle distribution after irradiation

To further investigate the underlying mechanism of the radiosensitive effect induced by LDHA inhibition, cells were pretreated with or without 20mmol oxamate and irradiated with 6Gy X-ray, after 24h incubation, flow cytometry was performed to detect alternations in cell cycle distribution. As shown in Fig. 4, irradiation induced G2/M cell arrest significantly while LDHA inhibition alone led to a substantial increase of cells at G0/G1 phase, and a reduction of cells at S phase. When combined with irradiation, LDHA inhibition reduced the ratios of cells entering G2/M cycle after irradiation significantly. The ratios of cells in G2/M cycle were $43.65 \pm 2.80\%$ and $30.25 \pm 2.27\%$ in A549 and H1975 respectively after irradiation alone, and the ratios dropped to $25.35 \pm 0.22\%$ and $16.90 \pm 1.66\%$ ($p < 0.05$) respectively, when combined with 20mmol oxamate.

LDHA inhibition and IR combination induced both apoptosis and autophagy

Apoptosis is one of the main biological effects triggered by both LDHA inhibition and irradiation[14, 15]. Subsequently, we examined whether LDHA inhibition influenced the IR-induced cell apoptosis. Cells were pretreated with 20mmol oxamate for 24h and then received 6Gy irradiation of X-ray, AnnexinV/ PI double staining assay was employed to detect apoptosis. As shown in Fig. 5(A-C), significant apoptosis was observed in H1975 cells after oxamate treatment, while little cells underwent apoptosis in A549 cells, consistent with our previous findings[12]. Oxamate pretreatment enhanced apoptosis in both A549 and H1975 cells after irradiation. To explore other potential mechanism in A549 cells, MD/PI double staining was used to detect autophagy in A549 cells after oxamate treatment. We observed that oxamate stimulated autophagic vacuoles and interacted with apoptosis induced by irradiation in A549 cells (Fig. 5D). Interestingly, similar phenomenon was also found when targeting pyruvate kinase M2 combined with irradiation in A549 cells[16].

LDHA inhibition increased DNA injury through ROS production and hindered DNA repair by ATP depletion.

It is well known that ionizing radiation causes nuclear DNA damage, largely mediated by ROS (reactive oxygen species), and relevant DNA repair mechanisms will also be initiated responding to irradiation, all these factors contribute to the modulation of cell radiosensitivity [17]. We next explored whether LDHA inhibition influence DNA injury using a single cell gel electrophoresis COMET assay, which provided a qualitative method to measure DNA damage by testing tails of fragmented DNA behind cell nuclei [18]. As shown in Fig. 6A-C, oxamate increased both comet tail length and percentage of DNA in the tail significantly after irradiation, indicating that LDHA inhibition slowed the kinetics of X-ray-induced DSB repair. GSEA analysis also supported that LDHA expression was associated with DNA repair capability, as well as G1/S DNA damage checkpoints signaling pathway (Fig. 6F-G). Furthermore, we found that the combination of oxamate and IR decreased intracellular ATP concentration significantly (Fig. 6D). As reported previously [14], LDHA inhibition induced accumulation of reactive oxygen species (ROS) by mitochondrial oxidative phosphorylation pathway, which might increase DNA damage. In our study, N-acetylcysteine(NAC), a thiol antioxidant, was used to pretreat A549 cells with oxamate together. As shown

in Fig. 6E, NAC partly reversed the inhibitive effect of oxamate combined with irradiation, suggesting that ROS induced by LDHA inhibition was involved in the synergistic effect with irradiation.

Discussion

Quite a number of lung cancer patients undergo tumor recurrence and metastasis after radiotherapy, and almost one half of loco-regional failure occur within the radiation field, indicating that radioresistance is widely existed in NSCLC patients[19]. Many factors might be involved in the poor response to radiotherapy, including hypoxia, cancer stem cell phenotype, cell cycle redistribution and activated DNA repair ability[20, 21]. Cancer cells exhibited higher glucose intake and enhanced glycolysis, which providing energy and nutrients rapidly, known as Warburg effect[22]. LDHA also known as LDH5, is a key enzyme involving in the glycolysis, and play a vital role in tumor initiation, maintenance, progression, and metastasis[10]. LDHA is reported to be up-regulated in many kinds of cancer, and associated with poor prognosis, including breast cancer[23], colorectal cancer[7], liver cancer[24], bladder cancer[8] and prostate cancer[5, 9], etc,. Especially, in the era of immune therapy treatment, serum LDH levels seem also to be associated with the efficiency of PD1/PDL1 efficiency [25].

In our study, we found that LDHA were up-regulated in NSCLC cells using online data, verified by IHC in tumor tissues. Then, we further confirmed that LDHA overexpression was associated with worse survival in NSCLC patients and those received radiotherapy. Using GSEA analysis, LDHA expression is positively related to enhanced glucose intake and glycolysis, which promote cancer cell growth and progression. In fact, cancer cell mitochondria and metabolism, also take part in mediating the response to irradiation in tumor, as they regulate multiple processes involved in DNA damage and repair[26]. LDHA overexpression also remodels tumor microenvironment with activated HIF-1 signaling pathways, which is associated with resistance to radiotherapy and resulted in poorer clinical outcomes [21, 27]. Therefore, LDHA up-regulation in NSCLC leads to increased levels of glycolysis and hypoxic microenvironment, which further contribute to development of resistance to radiotherapy.

The mechanism underlying the radiosensitive effect of LDHA inhibition might involve multiple biological processes, for example, reactive oxygen species (ROS) accumulation, cell cycle redistribution, increased DNA damage and DNA repair repression. As we know, LDHA inhibition drives cancer cells metabolism from glycolysis to mitochondrial respiration, which results in enhanced oxygen consumption and increased mitochondrial ROS production[14]. High levels of ROS not only induce cell apoptosis or autophagy, but also significantly lead to DNA injury and influence DNA damage response[17]. In our study, the synergistic effect of LDHA inhibition and irradiation are compensated to some extent after NAC treatment, indicating that ROS played a pivotal role in the effect.

Generally, DNA damage repair efficiency is considered as a major determinant of cell radiosensitivity. Especially, double-strand breaks (DSBs) tend to trigger genomic instability and always induce cell-killing effects[28]. In our study, reduced capacity of DNA repair was observed by COMET assay when LDHA expression was inhibited, and further theoretically verified using GSEA analysis. According to previous

studies[29, 30], the process might be mediated by PI3K-Akt signaling pathway, which both involves in metabolic reprogramming and tumor cell responsiveness to radiation. Consequently, up-regulation of PI3K-Akt signaling pathway results in acquired radioresistance by enhancing aerobic glycolysis[31]. In fact, PI3K-Akt signaling pathway was indeed down-regulated in A549 cells treated with oxamate in our earlier study[32]. In addition, LDHA controls a most potent pathway of rapid ATP production in cancer cells and its blockage deprives cancer cells from a major energy pathway, which may shift cell metabolism from glycolysis to mitochondrial oxidative phosphorylation(OXPHOS) and generate more ROS[33]. Combination of oxamate with radiation becomes more efficient in suppressing ATP formation and reducing DNA repair ability[34, 35]. Of particular interest, one recent study revealed that tumor metabolites, including 2-hydroxyglutarate, fumarate hydratase, and α -ketoglutarate, hindered DNA repair by disrupting local chromatin signaling[36], demonstrating the importance of metabolism in DNA repair activity. Yet, the molecular crosstalk between glycolysis and DNA repair still needs to be further explored.

As for cell cycle distribution, we found that in both A549 and H1975 cells, after oxamate pretreatment, more cells entered G0/G1 cell cycle and fewer cells were found at S phase, while the ratios of cells at G2/M phase were not significantly influenced. As reported by our previous study, G0/G1 arrest was dependent on the activation of GSK-3 β , accompanied by the inhibition of PI3K-Akt pathway[12]. Generally speaking, cells at S phase are the most resistant to radiation, while cells at G2/M phase are the most radiosensitive, and G0/G1 redistribution usually favors radioresistance [37]. Thus, the reduction of cells at radiation-resistant S phase might contribute to increased radiosensitivity in our study. Moreover, using GSEA analysis, we found that LDHA inhibition was associated with inactivation of G1/S DNA Damage Checkpoints signaling pathway[38], which further validated our hypothesis. Similarly, glycolysis inhibitor dichloroacetate was also reported to induce G0/G1 arrest and increase cell sensitivity to the X-ray irradiation in A549 cells [39, 40]. Of note, G2/M arrest was also observed in other cancer cell lines exposed to glycolysis inhibitors and reported to be associated with increased cell radiosensitivity[12, 41]. Therefore, the role of cell cycle re-distribution induced by glycolysis inhibition in modulation of radiosensitivity is still unclear, it's might be an accompanying consequence, instead of upstream signaling initiation, and less critical than the sensitizing effects of energy deprivation[42].

In summary, LDHA inhibition by oxamate suppressed glycolysis and reduced cellular ATP levels, shifted cell metabolism from glycolysis to mitochondrial oxidative phosphorylation (OXPHOS) and generated more ROS, which might increase DNA injury and hinder DNA repair activity. LDHA inhibition also induced cell apoptosis and autophagy accompanied by cell cycle alternations. All these factors contributed to enhanced radiosensitivity in NSCLC cells (Fig. 7).

Of note, although inhibition of LDHA mainly induces cell apoptosis by generating ROS, different responses to glycolysis inhibition were observed in different cancer cells[12, 43, 44]. Many efforts are being made to identify biomarkers to predict the sensitivity of glycolysis inhibitors(including LDHA inhibitors), several genetic mutations have been reported to be associated with the efficiency of glycolysis inhibitors, including Kras[45], PI3K[46], AMPK-mTOR[47],and mitochondrial DNA (mtDNA) mutations[48]. Our results showed the A549 cells with Kras mutation was less sensitive to the combination of LDHA

inhibition and radiotherapy than H1975, reflecting the heterogeneity of lung cancer. In addition, it's recently reported that only a part of NSCLC cell lines may benefit from the combination of radiotherapy and metabolic inhibition[49]. Thus, the radiosensitive effects of LDHA inhibition seems to be different in different cancer cells, efforts are still needed to identify predictive biomarkers and tested by clinical trials.

Conclusions

In this translational study, we found LDHA is up-regulated in both lung adenoma and squamous cells in both mRNA and protein levels. Higher LDHA expression is associated with enhanced glycolysis, hypoxia microenvironment, radioresistance and worse survival in NSCLC. Targeting LDHA might increase radiosensitivity by enhancing DNA damage through ROS and hinder DNA repair by energy deprivation, which give implications for further clinical study and drug development.

Abbreviations

NSCLC, non-small cell lung cancer; LDHA, lactate dehydrogenase A; RT radiotherapy; ROS, reactive oxygen species; GSEA, gene set enrichment analysis; LUAD, lung adenocarcinoma; LUSC, lung squamous carcinoma; HR, hazard ratios; OS, overall survival; PFS, progression-free survival; OXPHOS, oxidative phosphorylation.

Declarations

Ethics approval and consent to participate

The ethics committee of Zhejiang Cancer Hospital approved this work. All participating patients provided their written informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Competing interests

The authors disclose no conflicts.

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Contributions

Yang Y and DU XH conceived the ideas. Yang Y, Chong Y, Chen MY and Dai WM designed and the experiments. Yang Y, Zhou X and Ji YL analyzed and interpreted the data. Qiu GQ provided critical materials. Yang Y wrote the manuscript. All authors commented on drafts of the paper and approved the final manuscript.

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Figures

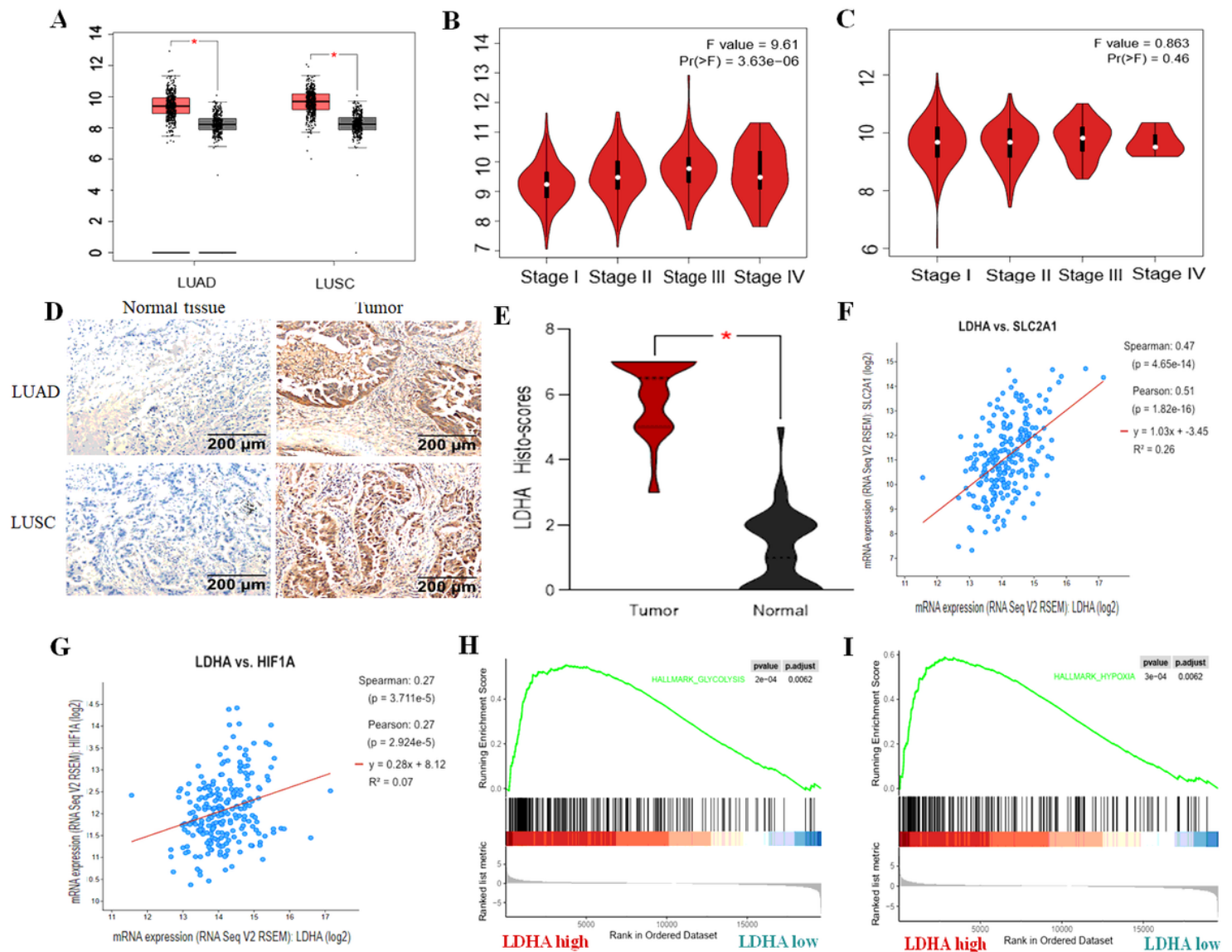


Figure 1

LDHA is up-regulated in NSCLC tissues correlated with enhanced glycolysis and tumor microenvironment. (A) Using GEPIA browser online, LDHA mRNA levels are overexpressed in both lung adenocarcinoma (LUAD) and lung squamous carcinoma (LUSC) ($p < 0.05$). (B) LDHA mRNA level is positively correlated with tumor stage in patients with LUAD ($p < 0.01$). (C) LDHA mRNA level is not significantly correlated with tumor stage in patients with LUSC ($p = 0.46$). (D-E) LDHA protein levels are overexpressed in both LUAD and LUSC tissues, verified by IHC ($p < 0.05$). (F-G) Using GEPIA browser online, LDHA expression is positively associated with HIF1A and GLUT-1 (also named SLC2A1) ($p < 0.05$). (H-I) Using GSEA analysis, high LDHA expression in lung cancer tissues is positively correlated with hallmark gene sets of glycolysis and hypoxia ($p < 0.05$).

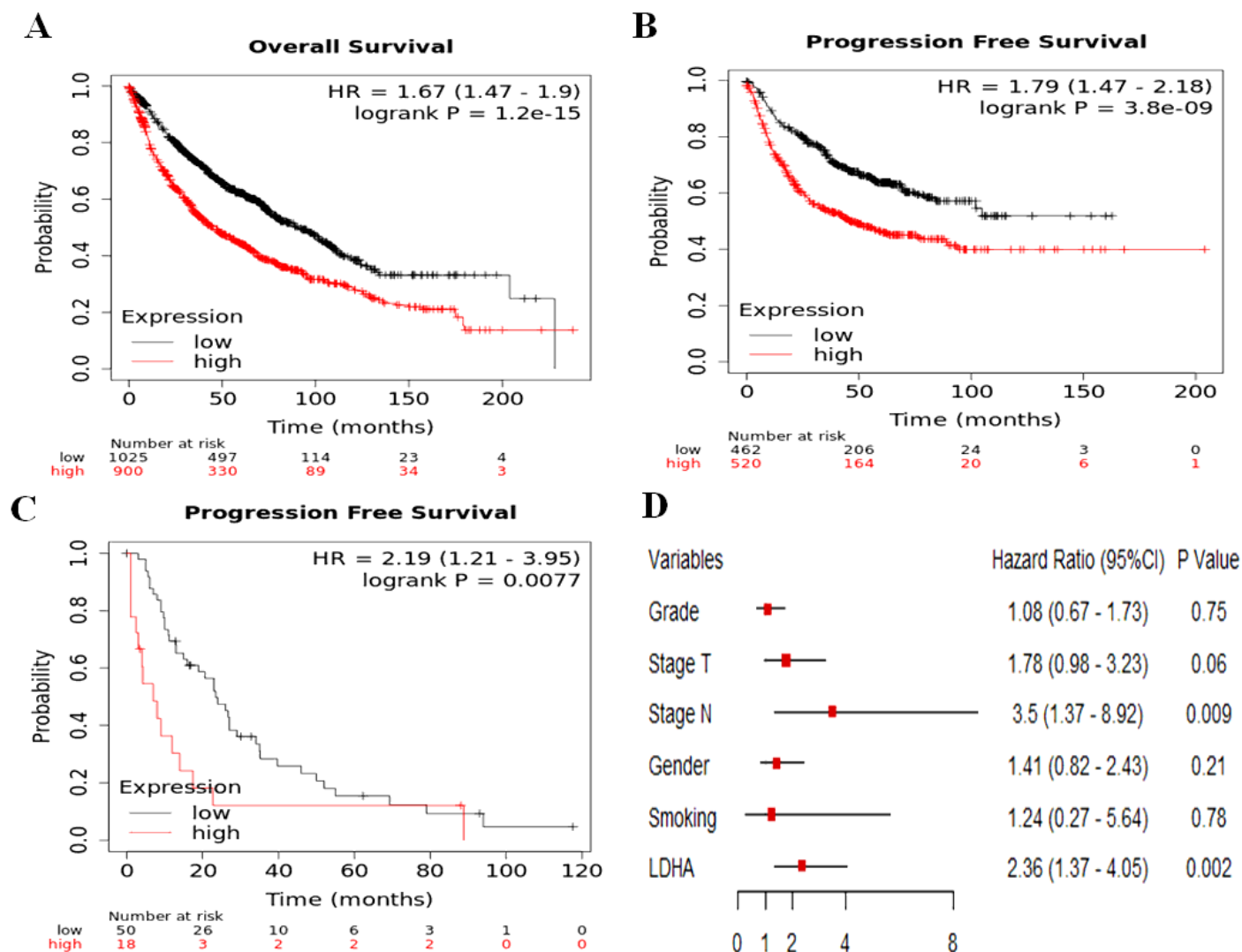


Figure 2

High LDHA expression is associated with worse survival in NSCLC. Using Kaplan-Meier plotter database online including 1928 NSCLC patients, the association between LDHA expression and lung cancer prognosis is explored. (A) Overall survival (OS); (B) Progression Free survival (PFS); (C) PFS in NSCLC patients received radiotherapy; (D) Forest plots displaying multivariate Cox analysis of OS in NSCLC patients. The control groups were patients with the characteristics of grade 1, stage T1, stage N0, female, no smoking, and low LDHA expression, respectively.

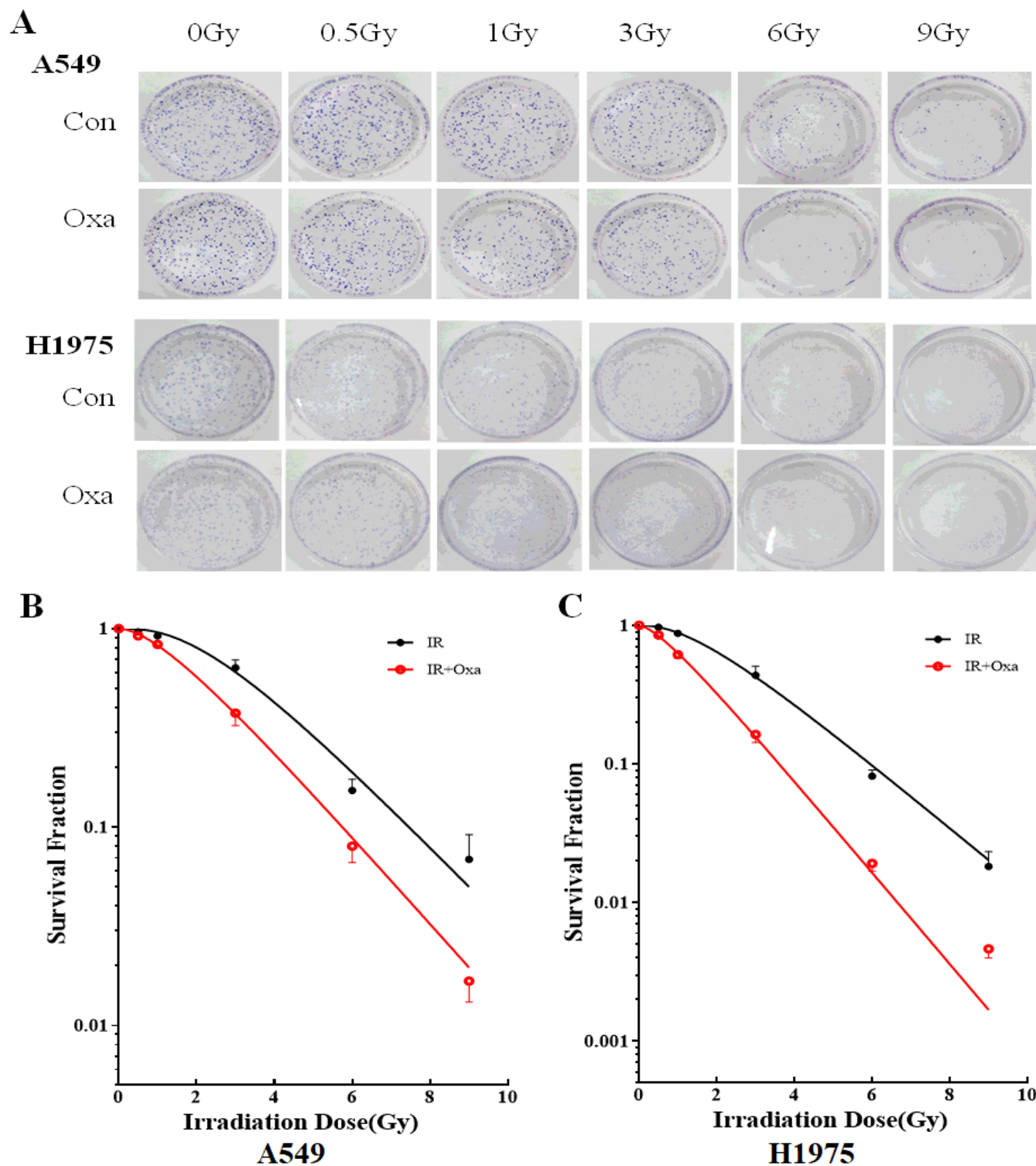


Figure 3

LDHA inhibition by oxamate enhanced the radiosensitivity of A549 and H1975 cells in vitro. (A) Radiation sensitivity was tested by colony formation assays. (B-C) Survival curves were calculated using a multitarget single-hit model. Data represent three independent experiments. Con, control; Oxa, oxamate; IR, irradiation.

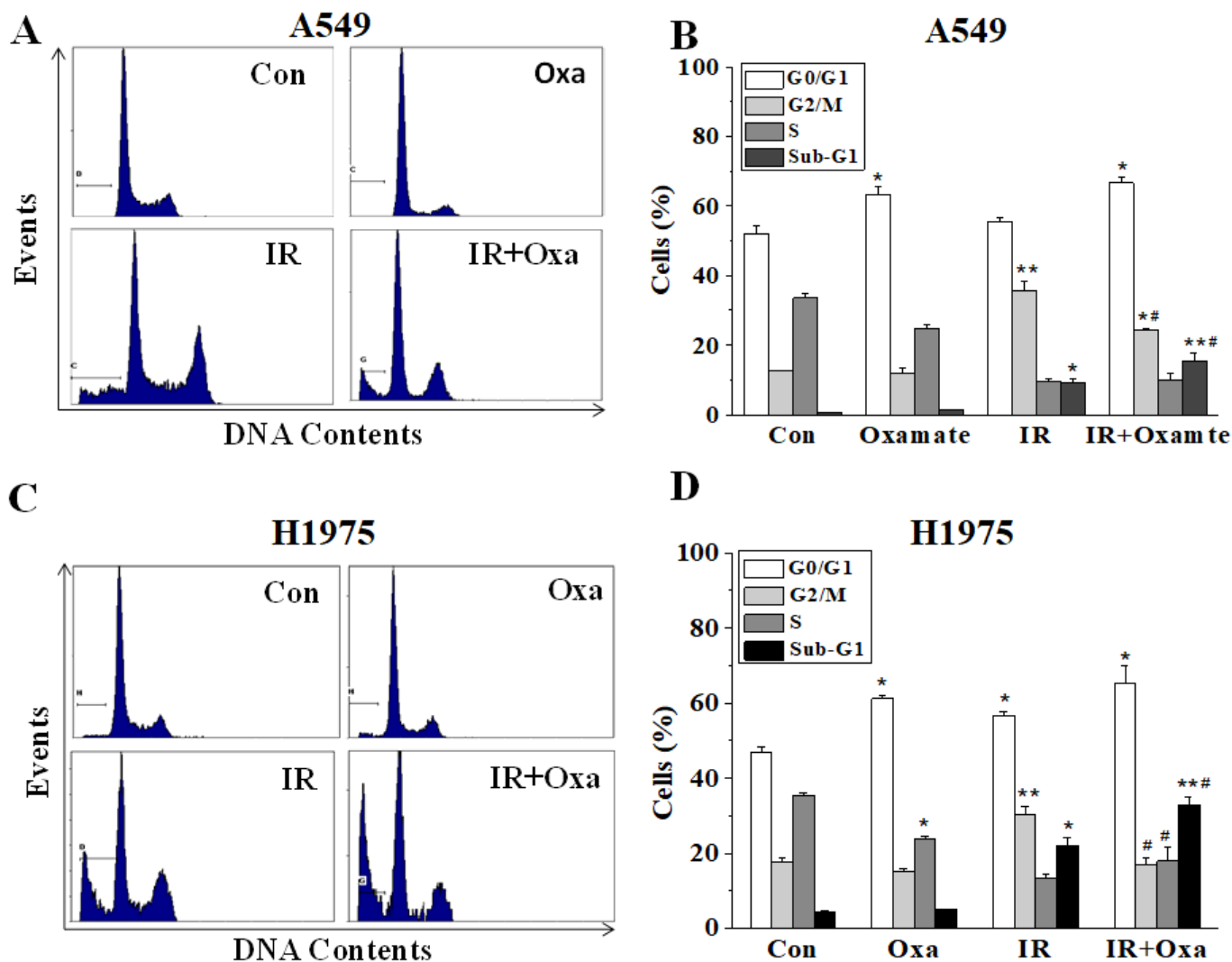


Figure 4

LDHA inhibition alters cell cycle distribution after irradiation. LDHA inhibition induces cell arrest at G0/G1 phase, and a reduction of cells at S phase. When combined with irradiation, LDHA inhibition reduces the ratios of cells entering G2/M cycle after irradiation. (A-B) A549 cells; (C-D) H1975 cells. Con, control; Oxa, oxamate; IR, irradiation. * $p < 0.05$, compared to the control group; # $p < 0.05$, compared to the irradiation group.

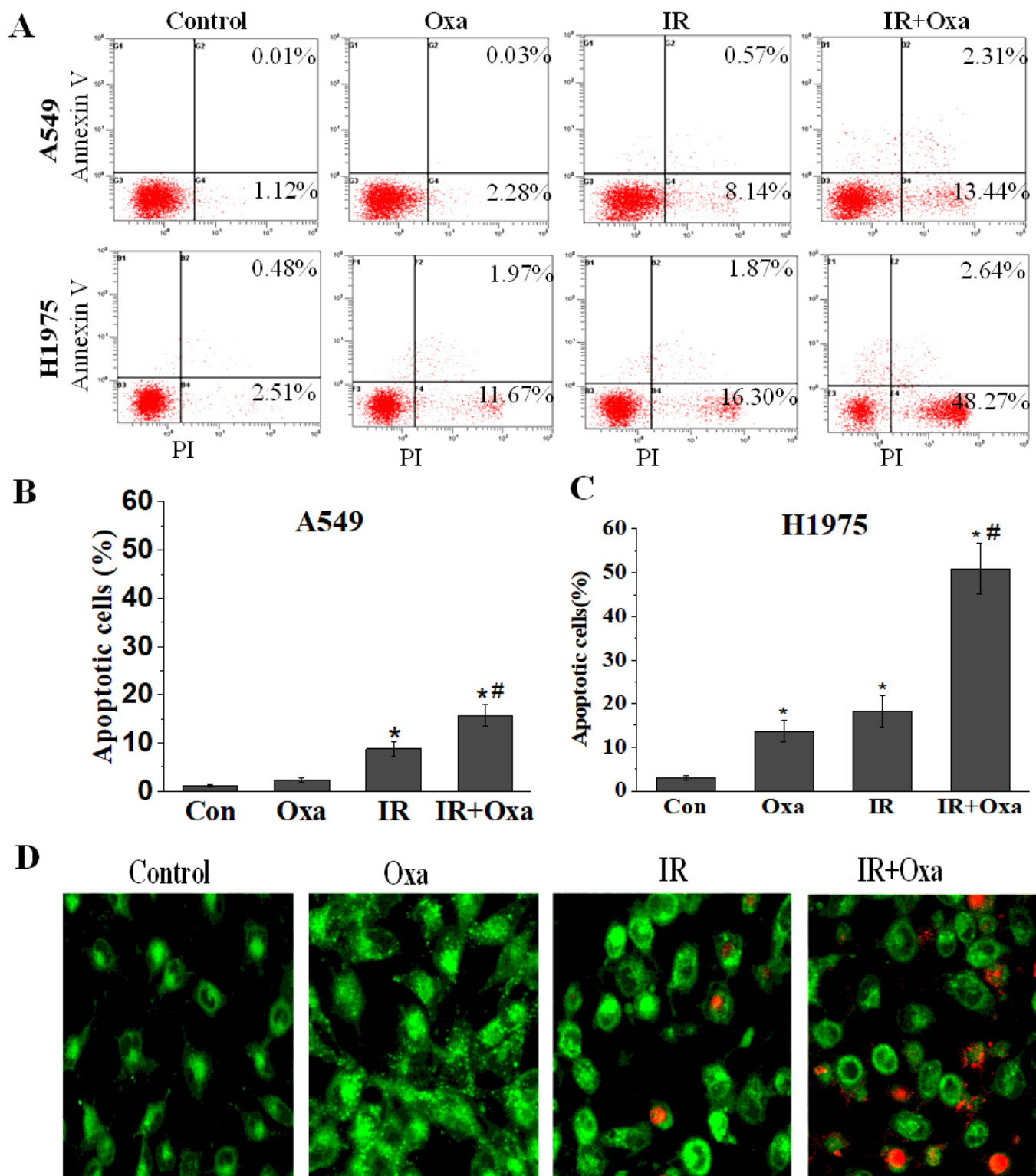


Figure 5

LDHA inhibition and IR combination induce both apoptosis and autophagy. (A-C) LDHA inhibition significantly promotes cell apoptosis after 6Gy X-ray irradiation treatment for 24h.(D) Using MD/PI double staining, autophagy was detected in A549 cells after oxamate treatment, and interacted with apoptosis induced by irradiation. Autophagosomes appear bright green fluorescence and apoptotic

nucleus appear red fluorescence. * p<0.05,compared to the control group;# p<0.05, compared to the irradiation group.

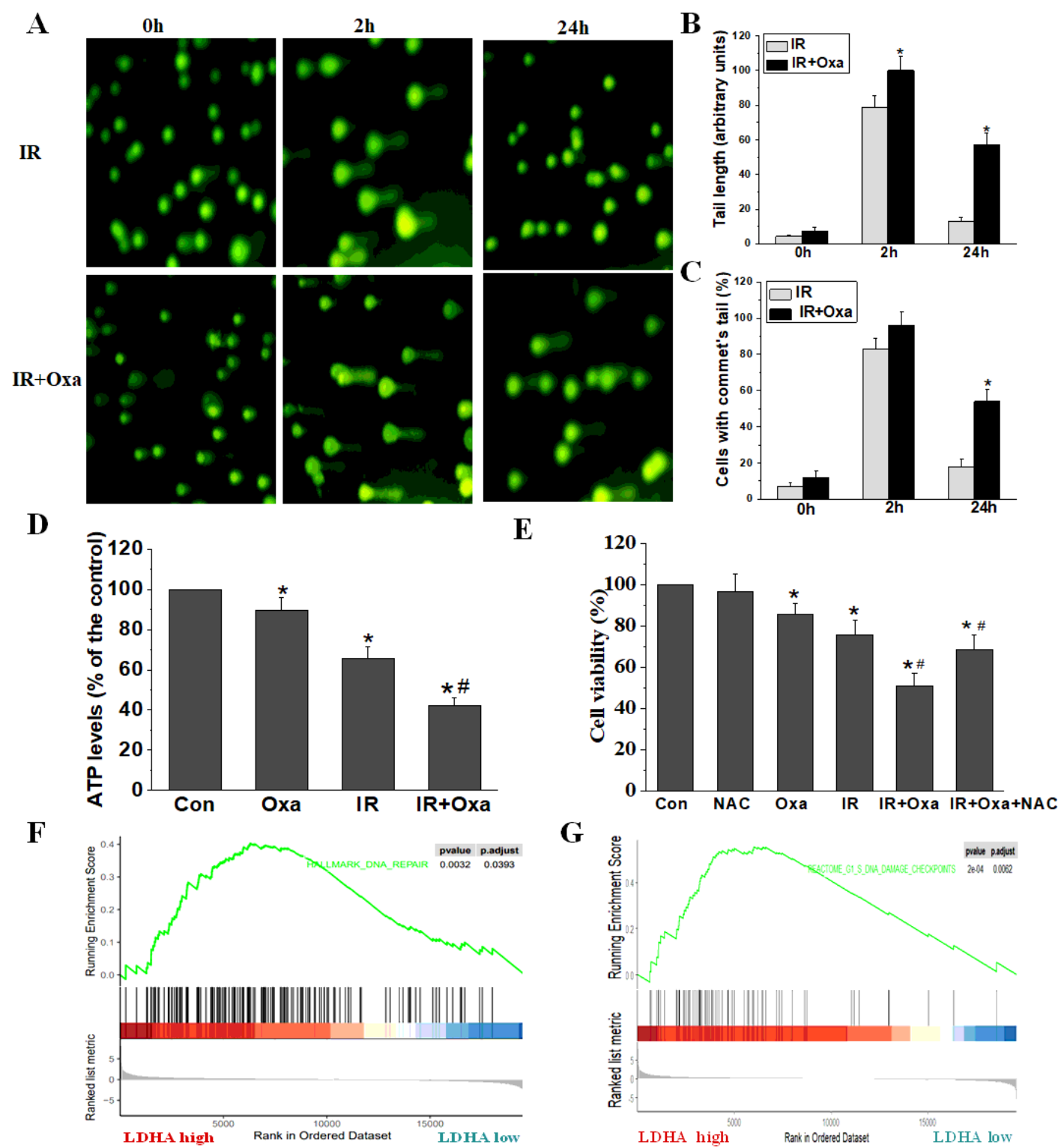


Figure 6

LDHA inhibition increases DNA injury through ROS production. (A)DNA damage detected by the comet assay.(B-C). Oxamate increased both comet tail length and percentage of DNA in the tail significantly after irradiation. (D) Intracellular ATP contents were detected in 549 cells. Cells were incubated with

oxamate (20 μ M) for 24h followed by 6Gy irradiation, after 24h, ATP levels were assayed. (E) N-acetylcysteine(NAC) partly reversed the inhibitive effect of oxamate combined with irradiation.(F-G) LDHA expression was associated with DNA repair capability, as well as G1/S DNA Damage Checkpoints signaling pathway. * $p < 0.05$, compared to the control group; # $p < 0.05$, compared to the irradiation group.

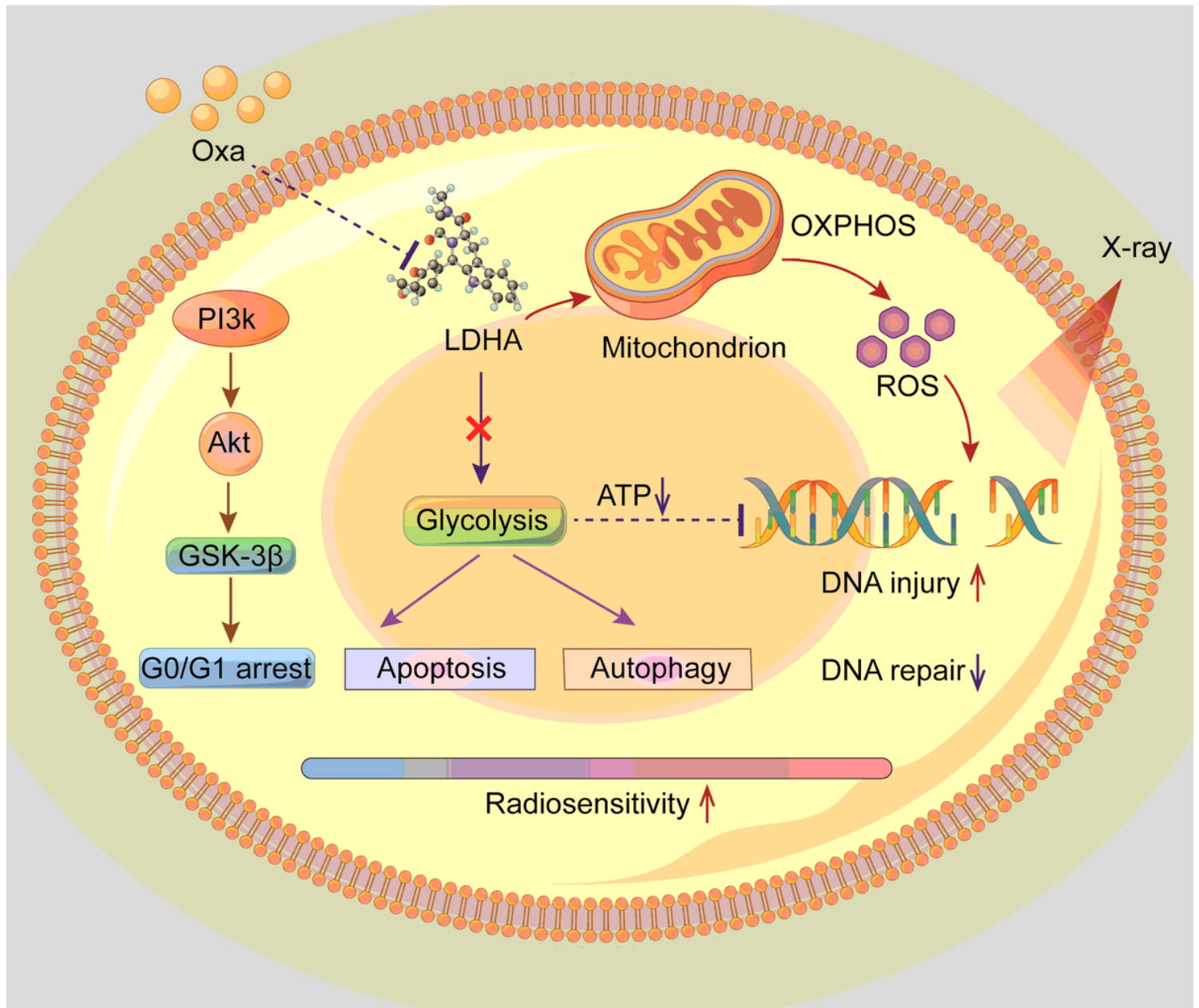


Figure 7

Schematic illustration of the effects observed in this study. LDHA inhibition by oxamate suppressed glycolysis and reduced cellular ATP levels, shifted cell metabolism from glycolysis to mitochondrial oxidative phosphorylation (OXPHOS) and generated more ROS, which might increase DNA injury and hinder DNA repair activity. LDHA inhibition also induced cell apoptosis and autophagy accompanied by cell cycle alternations. All these factors contributed to enhanced radiosensitivity in NSCLC cells.