Bioinformatic Analysis of Transcriptome Changes Underlying Metformin-induced Reversal of Drug Resistance in Head and Neck Squamous Cell Carcinoma Cells

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Abstract

Background: Metformin is the first-line drug for type II diabetes, and recent studies indicate that metformin plays an inhibitory role in multiple cancers. Metformin can also enhance the effect of chemotherapy. Although head and neck squamous cell carcinoma cells are sensitive to metformin, the mechanisms related to the metformin response and the chemosensitization effect have not been fully studied.

Results: In this study, we aimed to elucidate the molecular mechanisms of metformin in HNSCC by transcriptome analysis and to reveal the underlying mechanisms of the sensitizing effects of metformin by combined online dataset analysis. mRNA sequencing and functional analysis of HNSCC samples after metformin treatment and functional analysis of mRNAs with opposite metformin-induced effects in chemosensitive versus chemoresistant cells revealed the molecular pathways, mainly the base excision repair pathway, by which this small molecule drug sensitizes HNSCC cells to treatment.

Conclusions: These findings indicate that metformin exerts a hypersensitization effect by regulating the BER pathway in tumour cells, reducing their self-repair capacity after chemotherapy-induced DNA damage. In addition, the genes identified by transcriptome analysis are candidates for further investigation into the effector targets of metformin in the inhibition of HNSCC and could be applied to improve the treatment in HNSCC patients who develop resistance after advanced chemotherapy.

Background

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide, with approximately 890,000 new cases and 450,000 related deaths worldwide in 2018^{1,2}. Treatment options for HNSCC vary depending on tumour stage and include surgical excision, radiotherapy, chemotherapy, targeted therapy, and immunotherapy³. HNSCC that is diagnosed early tends to respond well to a single treatment modality, but the prognosis of advanced HNSCC is poor. Although traditional chemotherapy based on cisplatin can obviously improve the outcomes of advanced HNSCC patients, it does not affect their overall survival, and patients often experience recurrence and metastasis after undergoing initial treatment⁴. Thus, 5-year survival rate of advanced HNSCC patients is still less than 50%, and one of the main reasons for treatment failure is tumour resistance to chemotherapy, and drug resistance may occur during or after the course of chemotherapy⁵. Therefore, a novel chemotherapeutic approach with low toxicity that can combat tumour resistance is urgently needed to overcome the current challenges. At present, much attention has been given to small molecule targeted agents for combating drug resistance, and chemotherapy with small molecule drugs combined with conventional chemotherapeutic agents has shown better therapeutic effects than conventional regimens⁶.

1,1-Dimethylbiguanide hydrochloride (metformin), a small molecule insulin sensitizer, has played an irreplaceable role in the treatment of type II diabetes. Moreover, its antitumour effects are gradually being explored⁷. Metformin exerts antitumour effects in prostate, colorectal, and breast cancer in vivo or in vitro

by inhibiting tumour cell proliferation and inducing apoptosis^{8–10}. In epidemiological studies of head and neck cancer, diabetic patients treated with metformin had a lower risk of HNSCC than those who were not treated with metformin¹¹. In addition, metformin potentiates the therapeutic effect of cisplatin on non-small-cell lung cancer¹², breast cancer¹³, nasopharyngeal cancer¹⁴ and oral squamous cell carcinoma¹⁵. In addition, metformin holds promise as a novel antineoplastic drug candidate due to its low cost and low toxicity profile.

Multomics technologies, including genomics, transcriptomics and proteomics, have been developed in recent years for high-throughput screening and identification of drug targets¹⁶. However, these traditional methods are expensive and require multidisciplinary collaboration and complex analytical procedures. With the development of bioinformatics, systems biology is emerging as a more comprehensive approach to integrate compound target interactions from the molecular level to the systems level¹⁷. Transcriptomics using microarray mRNA sequencing (mRNA-seq) technology offers the opportunity to simultaneously detect changes in the expression of a large number of genes and thereby gain information about changes in cellular mechanisms induced by small molecule compounds^{18,19}.

Although numerous studies have reported the tumour suppressor role played by metformin in the treatment of HNSCC, most studies lack comprehensive analysis of the profiles and enriched pathways of protein interaction networks. In addition, metformin-induced transcriptome changes in HNSCC have not been previously mapped by RNA-seq. In this study, we used RNA-seq data combined with HNSCC datasets from the Gene Expression Omnibus (GEO) to conduct analysis by bioinformatics methods and techniques. The molecular functions of the integrated mRNAs and the signalling pathways involved may provide further insight into what metformin-induced changes occur in HNSCC and the underlying sensitization mechanism. This study provides a reliable basis for exploring the molecular mechanism by which metformin reverses drug resistance in HNSCC and the molecular targets for clinical combination chemotherapy.

Results

Metformin inhibits growth and induces apoptosis in HNSCC

We used two human-derived HNSCC cell lines, Cal27 and FaDu, to determine the effect of metformin on HNSCC cells. Analysis of CCK-8 experiments revealed a significant dose- and time-dependent decrease in the viability of Cal27 (Fig. 1a) and FaDu (Fig. 1b) cells after metformin treatment. We found that the decrease for both cell lines was more potent at 48 h than at 24 h and 72 h, so we chose 48 h as the incubation time to observe the cell biological changes in subsequent experiments. After 48 h of metformin treatment, the median lethal concentrations of Cal27 and FaDu were 25.67 mM and 20.37 mM, respectively (Fig. 1c). According to the concentration-cell viability curves, we chose 0 mM, 10 mM, 20 mM, 30 mM and 40 mM as the concentrations for both cell lines for subsequent experiments. The migration ability of Cal27 and FaDu cells in the presence of metformin was tested using a cell migration

assay (Fig. 1d). There was a dose-dependent decrease in wound healing with metformin treatment, indicating clear inhibition of cell migration (Fig. 1e). These findings indicated that metformin obviously inhibited the proliferation and migration abilities of Cal27 and FaDu cells. We further investigated the apoptotic effect of metformin on Cal27 and FaDu cells by the annexin V-FITC/PI double staining method. As shown (Fig. 1f), the flow cytometric apoptosis assay indicated that the number of cells undergoing apoptosis increased in a dose-dependent manner with metformin treatment.

Differential transcript analysis

We used Cal27 cells to determine the effects of metformin on the transcriptome of HNSCC cell lines. According to the results of the flow cytometric apoptosis assay, we found that the results for the two types of cells were significantly different but were most similar in the control group after 30 mM metformin treatment, so we chose to use 30 mM metformin as the treatment concentration for whole transcriptome sequencing with three biological replicates in each group. When investigating the differences between the two groups of samples, we focused on the differential transcripts and conducted further studies by using DEseq2 to compare the two groups to obtain a significant P-value, Q-value and fold change between transcripts and filtering the differential transcripts by filtering the conditions as P-value < 0.05 and $|\log_2\text{-fold change}| > 1$, resulting in 4360 differential transcripts for comparison. As shown in (Fig. 2a), clustering of transcripts and samples separately according to their signal values across samples can reveal the correlation profiles within and between sample groups from the clustering results of samples, the cluster of transcripts into a single class can be found from the clustering results of transcripts with similar expression profiles across samples, which may have similar functions. To demonstrate the overall picture of differences between groups, group differences were evaluated in terms of both fold change and significance level and displayed using volcano plots (Fig. 2b).

In addition, we set all human genes as the background to determine potential action targets of metformin by performing GO enrichments and KEGG pathway analysis of downregulated and upregulated genes. GO analysis classifies gene functions into biological processes, cellular components, and molecular functions. Ranked by the degree of enrichment, the top three biological processes that were downregulated in Cal27 cells by metformin were DNA helicase activity, ATP-dependent DNA helicase activity, and NAD binding. The top ranked cellular component categories were condensed chromosome, central region, condensed chromosome kinetochore, and kinetochore. The top ranked molecular functions were DNA replication initiation, mitotic sister chromatid segregation, and sister chromatid segregation (Fig. 2d). As shown in the KEGG enrichment analysis results ($P < 0.05$) in Fig. 2c, multiple cancer-related protein pathways were involved in the mechanism of metformin-induced mRNA downregulation in Cal27 cells, including DNA replication, cell cycle, IL-17 signalling pathway, mismatch repair, rheumatoid arthritis, biosynthesis of amino acids, pyruvate metabolism, carbon metabolism, glycolysis/gluconeogenesis, and citrate cycle (TCA cycle). Among them, DNA replication is closely related to the pharmacological action of metformin (p-value = 4.7758415, 3277188e-12). Similarly, for the upregulated DEGs in Cal27 cells after metformin treatment, the top three ranked by biological processes were testosterone dehydrogenase (NAD⁺) activity, cGMP binding, and growth factor activity. The top ranked cellular components were

cardiac myofibrils, banded collagen fibrils, and fibrillar collagen trimers. The top ranked molecular functions were peptide cross-linking, positive regulation of angiogenesis, response to mechanical stimuli (Fig. 2f). Notably, among the KEGG pathways enriched in upregulated DEGs, cytokine-cytokine receptor interaction was particularly closely related to the pharmacological effects of metformin (p-value = 0.000021506369976113) (Fig. 2e).

Establishment of a resistance network in HNSCC cells reversed by metformin

To predict the pharmacological mechanism by which metformin reverses HNSCC resistance, we extracted DEGs in HNSCC patients before and after chemotherapy from the GSE102787 microarray database and then processed the collected data using the GEO2R online analysis tool to obtain 1418 DEGs ($P < 0.05$, $|\log_2\text{-fold change}| \geq 0.5$). Genes that were upregulated (or downregulated) by metformin causing Cal27 intersected with genes that were downregulated (or upregulated) in the GSE102787 dataset, and Venny analysis showed that in both datasets, the directions of expression changes by metformin and chemotherapy were the opposite, with 95 and 189 DEGs in the metformin and chemotherapy datasets, respectively (Fig. 3a).

To further understand the inter-DEG interactions, we built a protein-protein interaction (PPI) network with the STRING database. After removing the isolated genes without interactions, we obtained and visually analysed the screened DEG-associated PPI network, which had 274 nodes and 500 edges (Fig. 3b). The interacting genes were imported into the cytoHubb plugin, and the top ten genes in the PPI network ranked according to the node value mapping degree size (CDK2, flap endonuclease 1 [FEN1], CDC6, HNRNPA1, BUB3, RAN, BIRC5, HNRNPAB, SNRPF, and FBXO5) were selected as the key genes using the MCC algorithm (Fig. 3c). In addition, 2 significant modules were identified via MCODE; all of the nodes were downregulated DEGs in the module 1 (Fig. 3d) and module 2 (Fig. 3e).

Survival analysis of hub genes

When GEPIA was used to analyse the correlation between the expression of key screened genes and the prognosis of HNSCC, we found that the symptom-free survival of patents with high FEN1 expression was significantly shorter than that of those with low FEN1 expression ($P = 0.04$) (Fig. 4a). However, there was no significant correlation between the expression of the remaining nine key genes and HNSCC prognosis ($P > 0.05$). Thus, FEN1 was identified as a key gene by which metformin reverses drug resistance in HNSCC.

To gain more insight into the screened DEGs, GO and KEGG protein pathway enrichment analyses were performed with the KOBAS database. For the DEGs changed by metformin, the top three biological processes were negative regulation of ubiquitin protein ligase activity induced in the mitotic cell cycle, mRNA splicing and via spliceosome. For the cellular components, the results showed that the DEGs were mainly distributed in the nucleoplasm, nucleus, and spliceosomal complex. In terms of molecular function classification, DEGs were especially involved in protein binding, protein kinase binding, and

sequence-specific DNA binding (Fig. 4b). More importantly, KEGG analysis (Fig. 4c) indicated that the screened DEGs were mainly enriched in pathways related to cancer, such as the spliceosome pathway.

After that, we performed KEGG protein pathway enrichment analysis of FEN1, which showed that FEN1 is mainly involved in DNA replication, nonhomologous end-joining, and base excision repair (BER) (Fig. 5). We speculated that FEN1 might be activated in response to metformin treatment, subsequently affecting the formation of the BER complex and reducing the self-repair ability of HNSCC cells when DNA double-strand breaks (DSBs) occur and reversing HNSCC resistance to chemotherapeutic drugs.

Validation of bioinformatics analysis results

To validate the RNA-seq data and the reliability of the key genes screened by bioinformatics analysis, we examined the mRNA expression levels of related genes after metformin treatment in Cal27 cells by RT-qPCR. The results showed that FEN1, PCNA, POL β , PARP1, LIG1, APEX and XRCC1 were downregulated, and the RT-qPCR results of all evaluated genes were consistent with the RNA-seq results (Fig. 6a).

In addition, we further investigated whether the combination of metformin and cisplatin synergistically inhibited cell proliferation. Compared with treatment with metformin or cisplatin alone, combination treatment reduced cell viability, and the extent of the decrease in survival was significantly greater than that with cisplatin treatment alone in Cal27 (Fig. 6b) and FaDu (Fig. 6c). These results suggest that metformin enhances cisplatin-mediated antiproliferative effects in HNSCC cells.

Discussion

Unlike most previous studies restricted to focusing on single genetic events or the results of single cohort studies, our study utilized bioinformatics techniques to screen potential target genes for the reversal of HNSCC drug resistance by metformin based on HNSCC gene expression data in RNA-seq and GEO datasets. These genes play a role in biological processes such as the negative regulation of ubiquitin-protein ligase activity induced in the mitotic cell cycle, mRNA splicing and spliceosomal processes. They are similarly involved in the spliceosome signalling pathway. After screening ten key genes using STRING 11.0 to analyse the PPI network of these DEGs, FEN1, a gene associated with HNSCC prognosis, was identified through the GEPIA database; metformin was predicted to potentially regulate the BER pathway by inhibiting the formation of BER complexes and then reverse the resistance of HNSCC to chemotherapeutic drugs. The agreement between the RNA-seq results and the GEO analysis results makes our findings more credible, which is also a prominent feature of this study. Finally, RT-qPCR combined with metformin/cisplatin cytotoxicity experiments were performed to confirm our conjecture.

FEN1 is a multifunctional, structure-specific nuclease that plays a crucial role in maintaining human genome stability²⁰. In mice, homozygous knockout of FEN1 is embryonically lethal²¹, and elevated expression of FEN1 similarly correlates with increased malignancy of the tumours²². FEN1 plays a key role in the long patch base excision repair (LP-BER) pathway to maintain genomic stability by regulating DNA replication and repair, which makes it an ideal target for cancer therapy. DNA repair pathways have

been reported to enable tumour cells to survive DNA damage induced by antitumour agents²³. Thus, inhibitors of specific DNA repair pathways might improve therapeutic efficacy when used in combination with DNA-damaging chemotherapeutic agents²⁴. Previous studies have shown that silencing FEN1 results in increased sensitivity of glioma and gastric cancer cells to cisplatin²⁵, suggesting the importance of FEN1 in DNA damage repair.

BER, a key cellular DNA repair pathway activated in response to DNA base damage, repairs DNA base lesions induced by various factors and is considered essential for both the development and treatment of cancer. BER is initiated by DNA glycosylases that recognize and remove damaged bases, leaving an abasic site for the next processing step of short patch repair and long patch repair²⁶. The pathway is a coordinated process involving more than 30 proteins. While insufficient BER activity leads to high cellular mutation rates and tumourigenesis, cancer cells often rely on increased BER activity to tolerate oxidative stress. Many current treatments, including nucleotide analogues incorporated into DNA (e.g., 5-fluorouracil, 5-FU), antifolates (e.g., pemetrexed), demethylating agents (e.g., decitabine, 5-aza 2'-deoxycytidine), platinum drugs (e.g., cisplatin), and alkylating agents that generate DNA damage (e.g., temozolomide, TMZ). DNA damage-inducing agents, designed to induce genotoxicity, are only efficacious in treating tumours when the amount of DNA damage caused by chemotherapy exceeds the repair capacity of cancer cells²⁷. Targeting the BER pathway in combination with chemotherapy will therefore generate damage that extends beyond the BER capacity of cancer cells and could be an effective avenue to improving the efficacy of chemotherapy.

Because our understanding of DNA damage in cancer is still lacking, thus far, we have only vague ideas about how to implement BER-targeting strategies therapeutically. Although we know that cancer is genomically unstable and that BER substrates are prevalent in cancer genomes, we have only a rudimentary understanding of how different lesions lead to a cancer phenotype and how they can be exploited to kill cancer cells. In our study, it was predicted by bioinformatics methods that metformin might have a sensitizing effect as an inhibitor of FEN1 in chemotherapy approaches for HNSCC, and metformin was found to have the same inhibitory effect on LP-BER pathway-related proteins by RT-qPCR. Our results provide a novel mechanism for the study of chemoresistance and metformin as a sensitizer in HNSCC cells and indicate that BER may be a potential therapeutic target for the treatment of HNSCC drug resistance.

Conclusions

In summary, in this study, through comprehensive analysis of transcriptome expression profiles and identification of key genes and pathways associated with the reversal of drug resistance in HNSCC by metformin, the mechanism of sensitization in HNSCC was elucidated from a bioinformatics perspective, which could pave the way for the study of HNSCC and the development of inhibitors targeting HNSCC drug resistance. However, the role of related signalling pathways in reversing HNSCC drug resistance and

their regulatory mechanisms remain unclear and need to be further verified in clinical samples by molecular biology research experiments.

Methods

Reagent

Metformin (LKB1-AMPK activator) was purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China) was dissolved in normal culture medium as a stock solution (1 M) and stored at -20°C. For experimental solutions, the stock solutions were further diluted with normal culture medium to generate the required concentrations.

Cell culture

The human HNSCC cell lines Cal27 and FaDu were purchased from the American Type Culture Collection (ATCC, Manassas, VA). All cell lines were cultured in high glucose DMEM (Gibco) supplemented with 1% penicillin streptomycin (HyClone, Logan, UT, USA) and 10% fetal bovine serum (Gibco) at 37°C in a saturated humidity incubator at 5% CO₂. Cells grown to confluence were digested with 0.25% trypsin containing 0.02% EDTA.

Cytotoxicity experiments

The viability of HNSCC cells after treatment with a range of concentrations of metformin (0–60 mM) was detected using the Cell Counting Kit-8 (CCK-8, Dojindo) following the manufacturer's instructions. The cells were plated at a density of 3×10^4 /ml and resuspended in complete medium; 100 µl of the suspension was added to each well of a 96-well plate and routinely incubated overnight in a 37°C in a 5% CO₂ incubator. For attachment, the cells were washed with phosphate-buffered saline (PBS), and incubation was continued with different concentrations of metformin diluted in advance. The medium in the well plates was changed after 24 h, 48 h, and 72 h, and 10 µL CCK-8 was added to each well. The cells were incubated at 37°C in 5% CO₂ in the dark, and the absorbance at 450 nm of each well was measured after 1.5 h. Cell viability was calculated as follows: cell viability = ([OD] test – [OD] blank)/([OD] control – [OD] blank)×100%. Half-maximal inhibitory concentration (IC₅₀) values were finally calculated using GraphPad Prism 8 for MacOS (version 8.2.1, La Jolla, CA).

Scratch assay

Evenly spaced horizontal lines (1 cm apart) were drawn on the bottom of 6-well plates with a marker for a total of at least 3 lines for each well. HNSCC cells were seeded into the 6-well plates, and cells were incubated until they reached confluence. A 100-µL pipette tip was used to make scratches perpendicular to the horizontal lines on the bottom of the wells, and the detached cells were removed by gently washing the wells three times with PBS. Serum-free medium containing different concentrations of metformin (0 mM, 10 mM, 20 mM) was added for culture. Wound healing was observed under a microscope after 0 h, 12 h, and 24 h and recorded.

Apoptosis assay

After incubating the cells in a 6-well plate for 24 h, they were treated with different concentrations of metformin solution (0–440 mM) for 48 h. The supernatant was collected, and the cells were resuspended and washed twice with precooled PBS. The cells were incubated in the dark for 15 min at room temperature with reagents from the FITC annexin V apoptosis detection kit I (BD Biosciences) and detected according to the manufacturer's protocol. Samples were detected with a flow cytometer (BD Biosciences, San Diego, CA, USA), and data were acquired by FlowJo 10.4 analysis.

Agilent microarray analysis

Cells were incubated with complete medium containing 30 mM metformin for 48 h ($n = 3$). Total RNA was extracted using the miRNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and assessed with a Bioanalyzer 4200 (Agilent, Santa Clara, CA, USA). Then, next-generation libraries were prepared using the VAHTS mRNA-seq v2 Library Prep Kit for Illumina® (Vazyme, Nanjing, China). The library quality was determined by a Bioanalyzer 4200 (Agilent, Santa Clara, CA, USA). Then, the mRNA-seq libraries were sequenced in a HiSeq X10 system (Illumina, San Diego, CA, USA) on a 150-bp paired-end run. The significantly up- or downregulated genes were selected according to thresholds of $p\text{-value} < 0.05$ and $|\log_2(\text{fold change})| > 1$.

Microarray data acquisition

In this study, we compared the expression levels of mRNAs from the NCBI GEO (<https://www.ncbi.nlm.nih.gov/geo/>). The gene expression profiles of HNSCC patients with primary (cisplatin sensitive) or recurrent (cisplatin resistant) disease were obtained by downloading a high-throughput gene expression dataset (GSE102787)²⁸. Based on the GPL6480 platform (Agilent-014850 whole human genome microarray 4x44k g4112f), this dataset genomic data and corresponding clinical information for a total of 7 pairs of primary and recurrent HNSCC cell lines from the University of Michigan. We focused on identifying the main gene expression changes associated with cisplatin resistance in primary cells (UMSCC-14A and UMSCC-17A) compared with the advanced HNSCC cells (UMSCC-14B and UMSCC-17B).

Data processing and differentially expressed gene analysis

The Cal27 transcriptome sequencing results after metformin treatment were background corrected and normalized, and expression values were calculated using the Affy, Impute, and Limma packages of R software (version: 4.0.3). Fold change (FC) and adjusted p-values were used to screen differentially expressed genes (DEGs). $|\log_2\text{-fold change}| > 1$ and $p\text{-value} < 0.05$ were defined as the screening criteria for DEGs. Heatmaps and volcano plots of the DEGs were constructed using Pheatmap, Ggplot2 and other packages. Similarly, we used R software (version: 4.0.3) to screen DEGs in the original GEO dataset GSE102787. The filtering criteria were $|\log_2\text{-fold change}| \geq 0.5$ and $p\text{-values} < 0.05$. Subsequently, we determined the intersecting DEGs of the two datasets through the Venn package in R software.

Functional enrichment analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology Based Annotation System (KOBAS) database 3.0 (<http://kobas.cbi.pku.edu.cn>) is used for genetic function analysis. KOBAS performs statistical tests based on mapping to genes with known annotations to identify significantly enriched protein regulatory pathways and biological functions²⁹. To reveal the biological significance of the screened DEGs in HNSCC progression, we performed gene ontology (GO) functional analysis and KEGG pathway enrichment analysis of DEGs from the Venn diagram using KOBAS, and terms with $P < 0.05$ were considered significantly enriched.

Protein-protein interaction (PPI) network construction

To gain more insight into potential associations between candidate genes, we used the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) 11.0 database (<https://string-db.org>). DEGs were subjected to PPI analysis. The results of the analysis were imported into Cytoscape 3.7.1 to construct a protein interaction network. The cytoHubba plug-in in Cytoscape was applied to screen out the hub genes in the gene expression network, whose connection degrees ranked in the top ten, with the maximum clique centrality (MCC) algorithm.

Survival analysis

To further elucidate the relationship between hub gene expression and the prognosis of HNSCC patients, we used gene expression profiling interactive analysis (GEPIA) (<http://gepia.cancer-pku.cn>) for survival analysis³⁰. In the analysis results, $P < 0.05$ was considered statistically significant, and the hub genes that met the criteria were considered the key genes affecting the prognosis of HNSCC.

Real time quantitative PCR analysis

Total RNA was isolated from cell lines using TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Reverse transcription was performed from 2 μ g RNA to synthesize cDNA. Quantitative RT-PCR was performed in the ABI7500 system using SYBR Green PCR Master Mix (Takara, Dalian, China) and specific primers. The expression level of each gene was normalized to that of GAPDH. The $2^{-\Delta\Delta CT}$ method was used to calculate the fold changes in transcript levels, and each experiment was repeated three times. The corresponding sequences of the forward and reverse primers are provided in the Table 1.

Statistical analysis

All experiments were performed in triplicate. Experimental data are expressed as the mean $\pm$ standard deviation (SD). The significance of differences between samples in any two groups was analysed by t-test using GraphPad Prism 8 for MacOS (version 8.2.1, La Jolla, CA), and differences were considered statistically significant when $p < 0.05$.

Table 1

Sequences of primers used in this study		
Gene symbol(Accession no.)	Primer sequences(5'→3')	
Polβ(NC_000008.11)	F	5'-CCGCAGGAGACTCTCAACG-3'
	R	5'-GTACTTGTGGATAGCTTGGCTC-3'
APEX(NC_000014.9)	F	5'-CCAGCCCTGTATGAGGACC-3'
	R	5'-GGAGCTGACCAGTATTGATGAGA-3'
FEN1(NC_000011.10)	F	5'-ATGACATCAAGAGCTACTTTGGC-3'
	R	5'-GGCGAACAGCAATCAGGAACT-3'
LIG1(NC_000019.10)	F	5'-GAAGGAGGCATCCAATAGCAG-3'
	R	5'-ACTCTCGGACACCACTCCATT-3'
PCNA(NC_000020.11)	F	5'-CCTGCTGGGATATTAGCTCCA-3'
	R	5'-CAGCGGTAGGTGTCTGAAGC-3'
PARP1(NC_000001.11)	F	5'-CGGAGTCTTCGGATAAGCTCT-3'
	R	5'-TTTCCATCAAACATGGGCGAC-3'
XRCC1(NC_000019.10)	F	5'-TCAAGGCAGACACTTACCGAA-3'
	R	5'-TCCAAGTGTAGGACCACAGAG-3'
GAPDH(NC_000012.12)	F	5'-GGACCTGACCTGCCGTCTAG-3'
	R	5'-GTAGCCCAGGATGCCCTTGA-3'
Abbreviations: F, forward; R, reverse		

Abbreviations

HNSCC: head and neck squamous cell carcinoma

BER: base excision repair

mRNA-seq: mRNA sequencing

GEO: Gene Expression Omnibus

PPI: protein-protein interaction

FEN1: flap endonuclease 1

DSBs: DNA double-strand breaks

FC: Fold change

DEGs: differentially expressed genes

KEGG: Kyoto Encyclopedia of Genes and Genomes

KOBAS: KEGG Orthology Based Annotation System

GO: gene ontology

STRING: Search Tool for the Retrieval of Interacting Genes/Proteins

MCC: maximum clique centrality

GEPIA: gene expression profiling interactive analysis

Declarations

Ethics approval and consent to partic:

Not applicable

Consent for publication:

Not applicable

Availability of data and materials :

1. The datasets generated and/or analysed during the current study are available in the NCBI GEO repository, (<https://www.ncbi.nlm.nih.gov/geo/>)
2. All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests:

The authors declare that they have no competing interests

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Authors' contributions:

All authors performed the study and approved the final manuscript.

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Figures

Figure 1

Effects of metformin on the biological behaviours of HNSCC cells. A. Cal27 cells were treated with metformin at the indicated concentrations (0, 1, 5, 10, 20, 30, 40, 50, 60 mM) for 24, 48, 72 h, and cell viability was determined using the CCK-8 assay. B. FaDu cells were treated the same for 24, 48, 72 h, and cell viability was determined using the CCK-8 assay. C. IC₅₀ for metformin in Cal27 and FaDu cells. D. Cell migration experiments were performed to evaluate the altered migration ability of Cal27 and FaDu cells after treatment with different concentrations of metformin. E. Cal27, FaDu cell mobility after treatment with different concentrations of metformin. F. Apoptosis was analysed by flow cytometry, and the differences in apoptosis between Cal27 and FaDu cells treated with metformin for 48 h were detected by annexin V-FITC/and PI staining.

Figure 2

Gene expression analysis of Cal27 cells after metformin treatment. A. Gene cluster plot. The abscissa represents the sample names between the groupings, and the ordinate represents the differential transcripts. In the plot, red represents the differentially expressed transcripts with high expression values in the grouped samples, and green represents the differentially expressed transcripts with low expression values in the grouped samples. B. Volcano plotting. Those with a fold difference greater than 2 and a p-value less than 0.05 in the figure are upregulated transcripts, indicated by red dots. Those with a fold difference less than 0.5 and a p-value less than 0.05 were downregulated transcripts, indicated by green dots. Transcripts with nonsignificant differences are indicated by gray dots. C. The downregulated DEGs identified by screening were subjected to KEGG protein pathway enrichment analysis. D. GO enrichment analysis, which mainly included three categories (cellular component, molecular function, and biological process), was performed on downregulated DEGs from the screening. E. The upregulated DEGs were subjected to KEGG pathway enrichment analysis. F. GO enrichment analysis was performed for the upregulated DEGs from the screening.

Figure 3

Potential genes involved in the chemosensitizing effect of metformin on HNSCC. A. Venny analysis showing the intersection of DEGs in HNSCC patients pre- and post-chemotherapy metformin and dataset GSE102787. B. PPI relationships among DEGs; red nodes represent upregulated DEGs, and blue nodes represent downregulated DEGs. C. According to node degree size mapping, CDK2, Fen1, and Cdc6 were the key targets with the highest node degrees. D. Module 1. E. Module 2.

Figure 4

GO analysis and protein pathway enrichment analysis of the DEGs. A. Correlation analysis between the gene expression levels of CDK2, FEN1, CDC6, HNRNPA1, BUB3, RAN, BIRC5, HNRNPAB, SNRPF, FBXO5 and disease-free survival. B. GO enrichment analysis of all DEGs in three categories: cellular component, molecular function and biological process. C. Protein pathway enrichment analysis of all DEGs.

Figure 5

KEGG pathway analysis indicated that various targets in BER signalling were closely related to the pharmacological action of metformin. The green rectangular nodes represent the downregulated genes or biological pathways associated with the pharmacological action of metformin, and the red rectangles represent the genes upregulated in cells after the action of metformin ($P = 0.000616685457809$).

Figure 6

A. Validation of Cal27 RNA-seq data by RT-PCR. The x-axis represents the metformin concentration, the y-axis represents the fold change ratio (metformin group/control group), and different colours represent the key LP-BER pathway protein. B. Cal27 cells were treated with metformin 20 mM alone or in combination with cisplatin 5 mM for 48 h, and cell viability was determined using the CCK-8 assay. C. FaDu cells were treated the same for 48 h, and cell viability was determined using the CCK-8 assay (* indicates a significant difference from the control group (* $P < 0.05$; ** $P < 0.01$)).