

## Original Research Article

# Potential effect of MSR1 and C6-ceramide small molecules on nasopharyngeal carcinoma based on GSEA analysis

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Sent for review: 16 June 2023

Revised accepted: 2 November 2023

### Abstract

**Purpose:** To investigate some potential therapeutic agents and targets for nasopharyngeal carcinoma (NPC).

**Methods:** Some potential therapeutic agents and target genes for NPC were identified by integrating bioinformatic analysis and in vitro experimental validation. Three datasets of NPC patients were gathered to reveal 26 upregulated and 344 downregulated differentially expressed genes (DEGs). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway analysis (KEGG) of the DEGs were implemented. Separately, 316 drug and small molecule target genes were acquired from the SEA database, allowing for detection of C6-ceramide as a small molecule of high relevance to NPC. Then, the differentially upregulated genes were intersected with potential target genes of C6-ceramide small molecules to obtain macrophage scavenger receptor 1 (MSR1). Finally, the study validated the potential roles of MSR1 and C6-ceramide in NPC cell lines.

**Results:** Knockdown of MSR1 expressions in NPC cells significantly decreased cell viability. Treatment with 10  $\mu\text{mol/L}$  C6-ceramide also significantly reduced NPC cell viability ( $p < 0.0001$ ). Furthermore, C6-ceramide attenuated the increase in MSR1 levels induced by MSR1 overexpression in NPC cells ( $p < 0.0001$ ). Concurrently, MSR1 knockdown decreased expression of PI3K and AKT, while MSR1 overexpression upregulated AKT and PI3K levels.

**Conclusion:** MSR1 modulates viability of NPC cells by regulating PI3K and AKT expression. Additionally, C6-ceramide exerts therapeutic effect on NPC by regulating MSR1 expression. These findings reveal new therapeutic targets and strategies for the clinical management of NPC. These results establish a rationale for further exploration of MSR1 and ceramides as novel targets in NPC.

**Keywords:** Nasopharyngeal carcinoma, Macrophage scavenger receptor 1, C6-ceramide, PI3K/AKT pathway, Cell viability

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## INTRODUCTION

The nasopharyngeal mucosa can develop a malignant epithelial tumor called a

nasopharyngeal carcinoma (NPC), which is common in southern China and Southeast Asia. Unlike other cancers, NPC cells easily spread to lymph nodes and other regions even in the early

stages of tumorigenesis [1]. The curative value of current treatments, such as radiation, chemotherapy and molecular targeted therapy, is limited, as evidenced by the high rates of recurrence, metastasis and mortality in NPC patients [2]. Thus, developing appropriate molecular targets for NPC therapy is essential.

Macrophage scavenger receptor 1 (MSR1), also known as class A scavenger receptor (SR-A) and cluster of differentiation 204 (CD204) [3], is primarily produced in macrophages. Its functions include scavenging and modifying lipoproteins. The adhesion and phagocytosis of macrophages are related to MSR1 [4]. The MSR1 is a marker of M2 tumor-associated macrophages, which promotes the development and metastasis of tumor.

The MSR1 is also a prognostic biomarker for glioma (LGG), uveal melanoma (UVM), lung squamous cell carcinoma (LUSC) and other tumor types [5], but the function of MSR1 in NPC is not clear. In addition, MSR1, as a membrane receptor, binds to ligands to activate signaling pathways including mitogen-activated protein kinase, nuclear factor kappa B (NF- $\kappa$ B) and PI3K/AKT [6], therefore affecting the progression of cancer.

Ceramides are a class of bioactive sphingolipids in cell membranes that promotes apoptosis in response to cell stressors and have demonstrated antitumor activities in preclinical studies [7-9]. C6-ceramide is a cell-permeable ceramide analogue that promotes apoptosis and suppresses the growth of various types of tumors [10,11]. However, the therapeutic potential and mechanisms of action of ceramide analogs remain to be elucidated in NPC. Ceramides respond to various changes caused by biological factors and chemotherapy by inhibiting AKT-dependent mitosis to suppress tumor cell proliferation [7]. Endogenous ceramides as effector molecules enhance the cytotoxic effects of drugs on tumor cells, while exogenous ceramides demonstrate greater cytotoxicity towards tumor cells compared to normal cells [8]. Additionally, ceramides inhibit tumor growth in breast cancer xenograft models [9]. Ceramides and their analogs exhibit antitumor potential, but their specific mechanisms of action in NPC warrant further investigation.

This study is aimed to identify MSR1 as a putative target of C6-ceramide in NPC, using a bioinformatics method. The study is further aimed to verify the roles of MSR1 and C6-ceramide in inhibiting NPC cell viability and modulating PI3K/AKT signaling.

## EXPERIMENTAL

### Data acquisition

The gene expression matrix of NPC was retrieved from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>). The desired genes of pharmaceuticals and small compounds were gathered from the SEA database (<https://sea.bkslab.org/>).

### Differential gene expression analysis

The "Limma" pack in R was utilized to perform Bayesian differential expression analysis on the downloaded NPC dataset. DGEs were identified based on  $|\log_2FC| > 0.2$  and adjusted  $p$ -values  $< 0.05$ .

### DEG pathway analysis

Gene Ontology (GO) functional and KEGG pathway enrichment analyses were implemented by applying the "ClusterProfiler" package in R. Terms with adjusted  $p < 0.05$  were considered significantly enriched.

### Potential drug analysis

The  $t$ -values for all genes in the dataset were obtained through Bayesian testing and ranked. The genes in targets for medications and small compounds were acquired as SET files from the SEA database. GSEA was then performed to identify potential drugs and target genes associated with NPC [12].

### Cell culture

The human normal nasopharyngeal epithelial cell line NP69 and NPC cell lines HNE-1, HONE-1, CNE2 and CNE1 were obtained from the ATCC. HNE-1, HON-1 and CNE2 cells were raised in DMEM, while CNE1 and NP69 were raised in RPMI-1640 medium (both from GIBCO, USA). The media in which the cells were maintained was enriched with 10 % FBS (Hyclone, USA), 100  $\mu$ g/mL streptomycin, and 100 U/mL penicillin and maintained at 37 °C in 5 % CO<sub>2</sub>.

### RNA isolation and qPCR

Total RNA was extracted from cell lines using Trizol reagent (Invitrogen), in line with the directions provided by the manufacturer. Reverse transcription was performed with the Reverse Transcriptase Kit (Madison, Wisconsin, USA) and qPCR was done by applying SYBR Green Supermix (Invitrogen) on an ABI Prism 7500 system (Applied Biosystems, Foster City, CA,

USA). Relative mRNA transcription was quantified by the  $2^{-\Delta\Delta CT}$  technique with three technical replicates. Based on the results obtained, two NPC cell lines were selected for subsequent experiments.

### Western blotting

Cells were dissolved in RIPA buffer (Thermo Fisher Scientific) and protein concentrations measured using the Pierce Protein Assay Kit (Thermo Fisher Scientific). Proteins were separated by SDS-PAGE, put on PVDF membranes and detected by incubation with primary antibodies overnight at 4°C. Proteins bands were visualized using WesternBright™ enhanced chemiluminescence (Bio-Rad, USA).

### Cell transfection and drug treatment

The si-MSR1 and si-NC were separately transfected into cells and incubated for 24, 48, 72, and 96 h. The absorbance at 450 nm was read on a microplate reader following which cells were incubated with 10 mL of CCK-8 reagent for 2 h at 37 °C with 5 % CO<sub>2</sub>. HONE1 and HNE1 cells were exposed to varied doses of C6-ceramide for 24, 48, 72 and 96 h before assessing cell survival by CCK-8 assay. Cells were transfected with pcDNA-NC, pcDNA-MSR1, C6-ceramide, or pcDNA-MSR1 + C6-ceramide

according to experimental groups. The MSR1 mRNA levels were quantified using qPCR post-transfection. Cells were also transfected with MSR1 knockdown or overexpression vectors while PI3K and AKT mRNA levels were examined using qPCR.

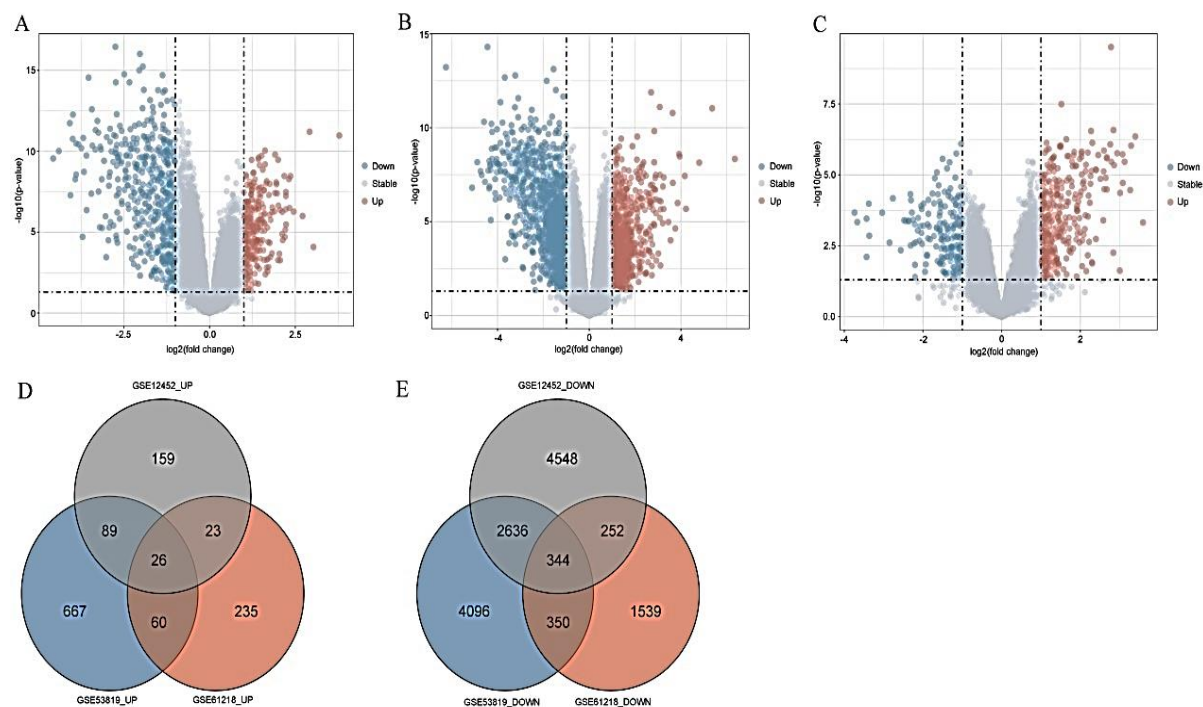
### Statistical analysis

Statistical analyses were done using SPSS 20.0 software. Variations between groups were evaluated using Student's *t*-test or one-way ANOVA. Statistical significance was evaluated by *p*-values < 0.05 (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001).

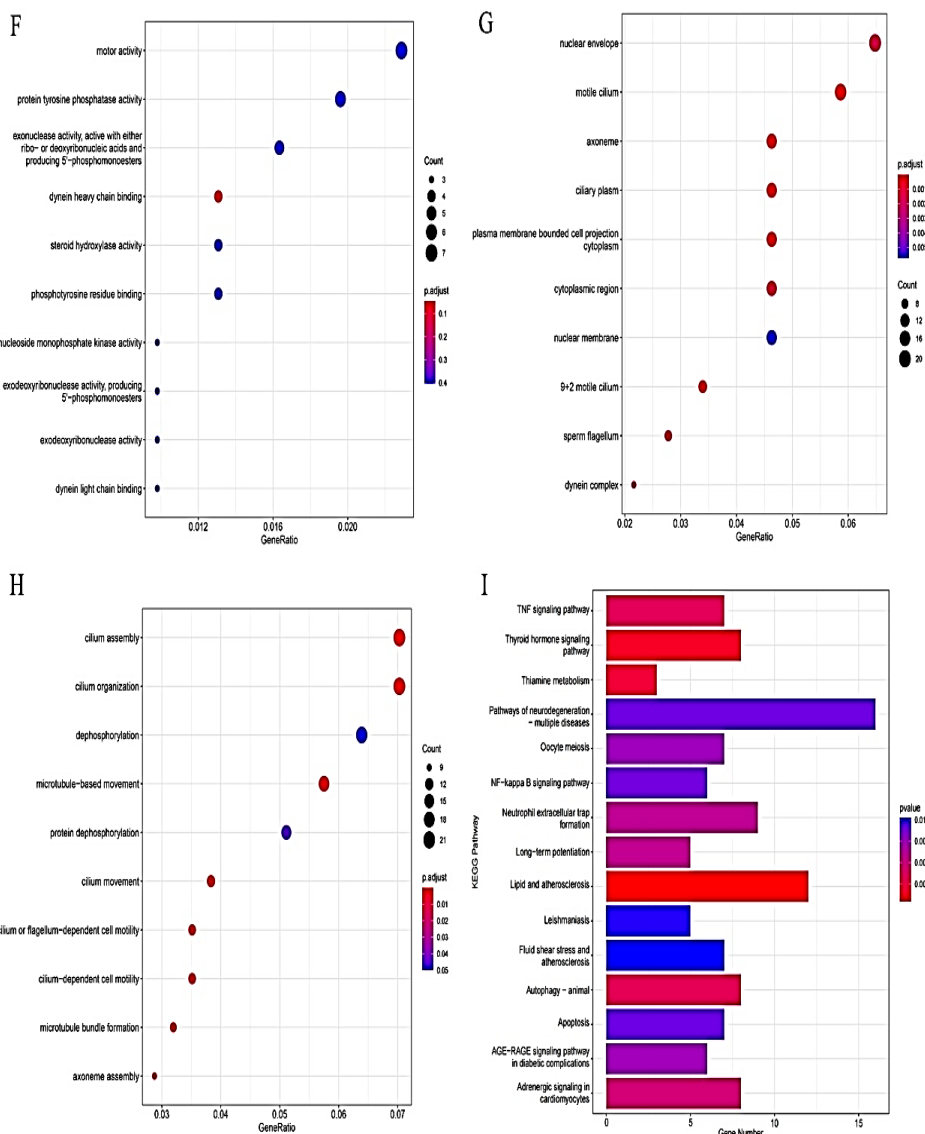
## RESULTS

### Differential gene expression and pathway analysis

Gene expression data from three NPC datasets (GSE12452, GSE53819 and GSE61218) were obtained from the GEO database. Bayesian differential analysis was performed to identify DGEs between NPC and control samples in each dataset. In GSE12452 (31 NPC and 10 control), 7780 downregulated and 297 upregulated DEGs were identified (Figure 1 A).



**Figure 1:** Differential gene expression and pathway analysis. DEGs between NPC and control samples were identified by Bayesian analysis in three datasets: GSE12452 (A), GSE53819 (B), and GSE61218 (C); Venn diagrams show the overlap of upregulated (D) and downregulated (E)



**Figure 1 continued:** Differential gene expression and pathway analysis. DEGs between NPC and control samples were identified by Bayesian analysis in three datasets: DEGs across the three datasets; Ontology enrichment analysis revealed altered BP (F), CC (G) and MF (H); The top 15 enriched KEGG pathways are shown in (I)

In GSE53819 (18 NPC and 18 control), 7426 downregulated and 842 upregulated DEGs were found (Figure 1 B) while 2485 downregulated and 344 upregulated DEGs were identified in GSE61218 (10 NPC and 6 control; Figure 1 C). The overlap of DEGs across the three datasets indicated 26 commonly upregulated and 344 commonly downregulated DEGs (Figure 1 D and E).

GO and KEGG enrichment analyses were performed on the 380 DEGs to investigate altered biological pathways and processes. The top 10 enriched GO terms included cilium assembly, cilium organization and microtubule-based movement for Biological Process (BP) (Figure 1 F); nuclear envelope and acrosome for

Cellular Component (CC) (Figure 1 G); and dynein heavy chain binding for Molecular Function (MF) (Figure 1 H). The top 15 enriched KEGG pathways included thyroid hormone signaling, lipid metabolism, atherosclerosis, TNF signaling and autophagy (Figure 1 I).

#### Potential drug candidates for NPC

To identify potential drug candidates for NPC, GSEA was performed using the 316 drug and small molecule target gene sets from the SEA database. The top 10 significantly enriched drugs ( $p < 0.05$ ,  $ES > 0.3$ ) were identified in NPC samples for each dataset (Figure 2). The top three enriched drugs in GSE12452 were C6-ceramide, bexarotene and chlorambucil (Figure 2

A). For GSE53819, the top hits were C6-ceramide, cabozantinib and etoposide (Figure 2 B). In GSE61218, C6-ceramide, AZD6482 and ML162 were most enriched (Figure 2 C). Venn diagram shows C6-ceramide as the only drug enriched across all three datasets (Figure 2 D). The enrichment scores of C6-ceramide in the three datasets are presented in Figure 2 E and G.

### Identification of MSR1 as a potential target of C6-ceramide in NPC

To identify the potential therapeutic targets of C6-ceramide in NPC, its chemical properties were first analyzed. C6-ceramide has a CAS registry number of 124753-97-5, a molecular formula of  $C_{24}H_{47}NO_3$  and a molecular weight of 397.63 Da. Its chemical structure is shown in Figure 3 A. Differentially upregulated genes from the three NPC datasets were intersected with the potential target genes of C6-ceramide, identifying MSR1 as a common target (Figure 3 B).

Molecular docking using AutoDockTools-1.5.6 revealed the potential binding interaction between MSR1 and C6-ceramide (Figure 3 C).

### Effect of MSR1 on NPC cells

The levels of MSR1 was analyzed in four NPC cell types and healthy nasopharyngeal epithelial cells by qPCR. When compared to typical cells, MSR1 mRNA amounts were significantly higher in NPC cells (Figure 4 A). Western blotting (WB) confirmed increased MSR1 protein expression in NPC cells versus normal cells (Figure 4 B) indicating that MSR1 is greatly upregulated in NPC. In order to determine the function of MSR1 in NPC, two NPC cell lines were transfected with si-NC or si-MSR1. The results of qPCR confirmed transfection with si-MSR1 significantly reduced MSR1 mRNA compared to si-NC control cells, confirming the efficient knockdown of MSR1 (Figure 4 C). Cell viability results show that MSR1 knockdown inhibited cell viability in both NPC lines (Figure 4 D).

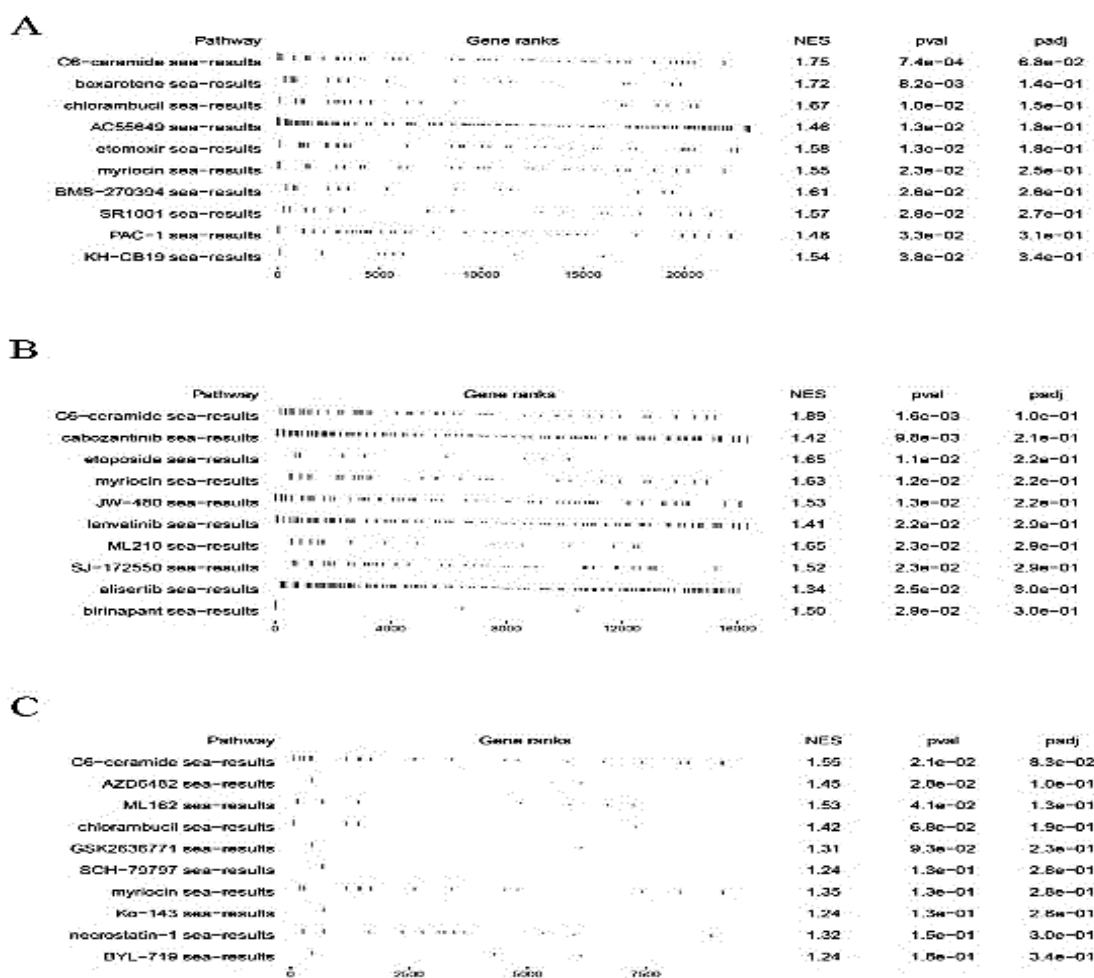
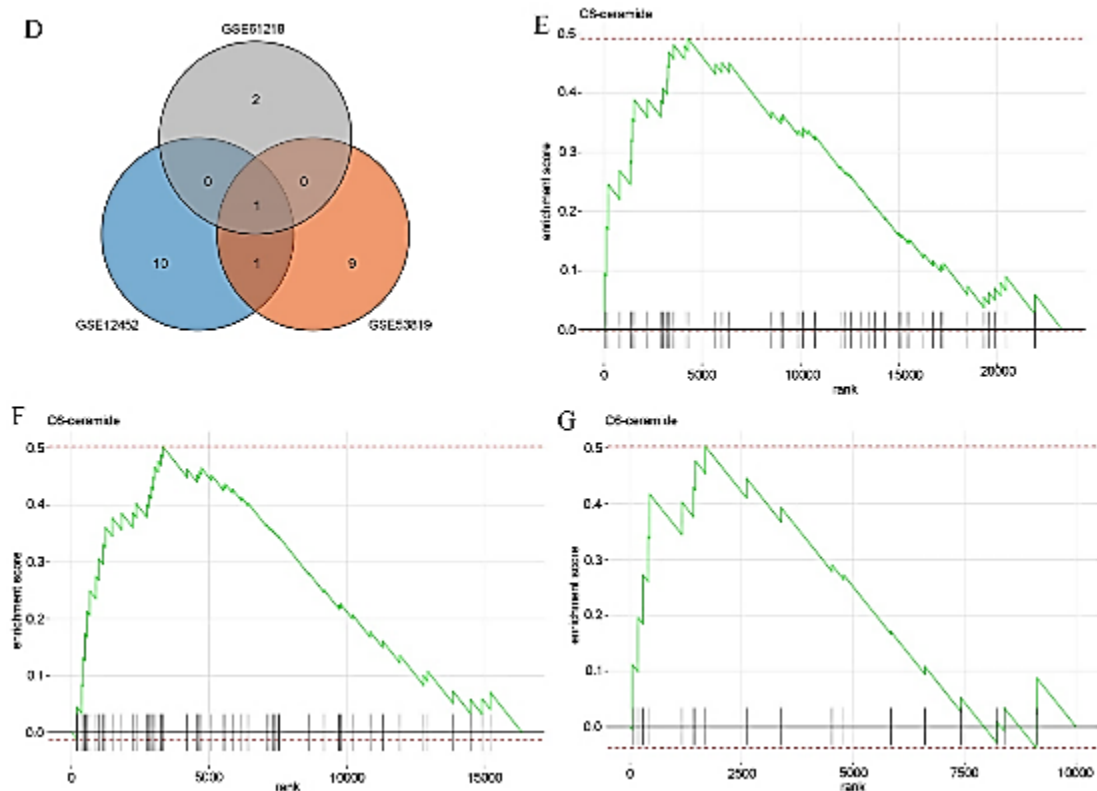
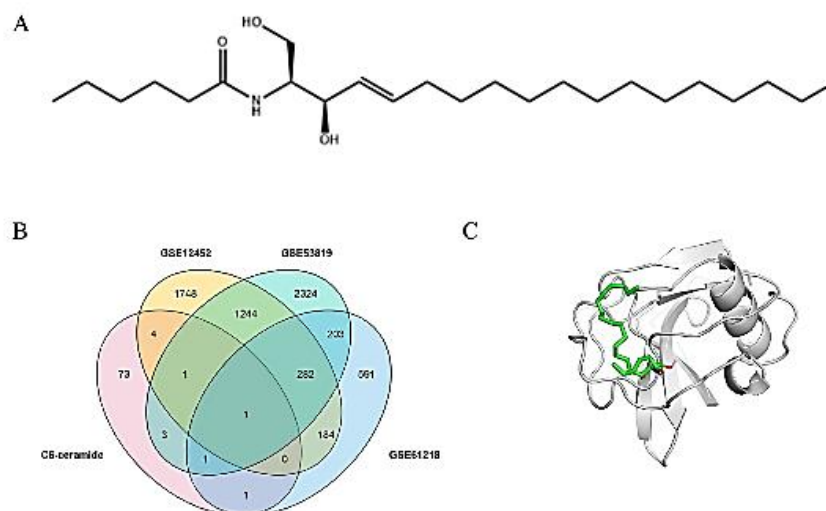


Figure 2: GSEA identifies C6-ceramide as a potential therapeutic for NPC (A-C) The top 10 enriched drugs in nasopharyngeal carcinoma samples from the three datasets



**Figure 2 continued:** GSEA identifies C6-ceramide as a potential therapeutic for NPC (D) Venn diagram showing C6-ceramide as the only drug enriched across all three datasets; (E-G) Enrichment rank scores of C6-ceramides in the three datasets.



**Figure 3:** Identification of MSR1 as a potential therapeutic target of C6-ceramide in nasopharyngeal carcinoma; (A) Chemical structure of the C6-ceramide small molecule; (B) Venn diagram showing the intersection of upregulated genes in three NPC datasets with potential target genes of C6-ceramide; (C) Molecular docking diagram depicting the potential binding interaction between C6-ceramide and MSR1

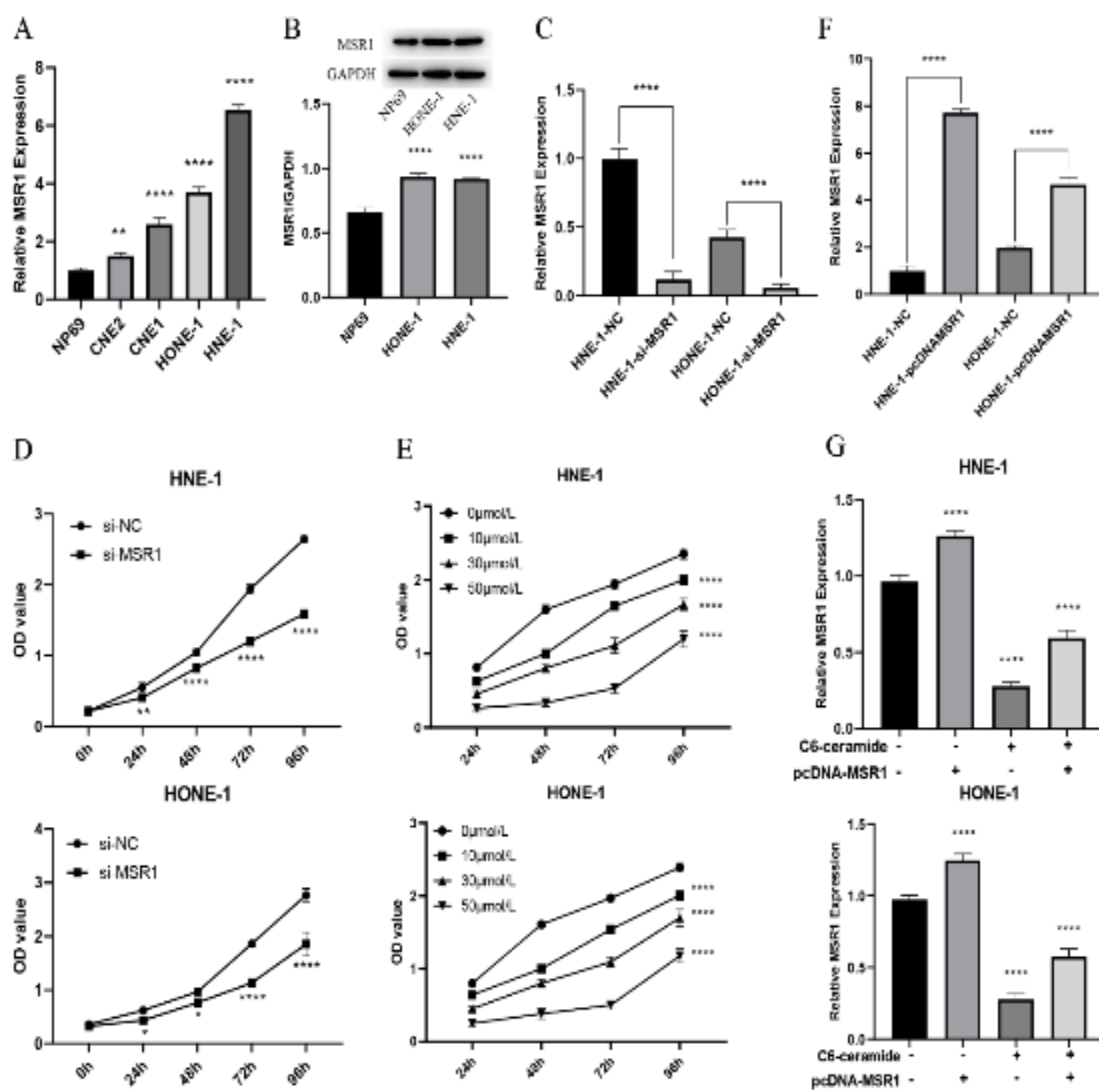
To investigate the effect of C6-ceramide on NPC cells, two cell lines were exposed to increasing amounts of the drug. The results of CCK8 assay showed decreased cell viability with higher C6-ceramide doses (Figure 4 E), indicating that C6-ceramide has anti-proliferative effect. To further investigate if this effect involved MSR1,

nasopharyngeal carcinoma cells were transfected with pcDNA-MSR1 (for MSR1 overexpression) or control pcDNA-NC. The results of qPCR confirmed increased MSR1 mRNA in pcDNA-MSR1 versus pcDNA-NC cells (Figure 4 F). Cells were then divided into four groups: pcDNA-NC, pcDNA-MSR1, C6-ceramide

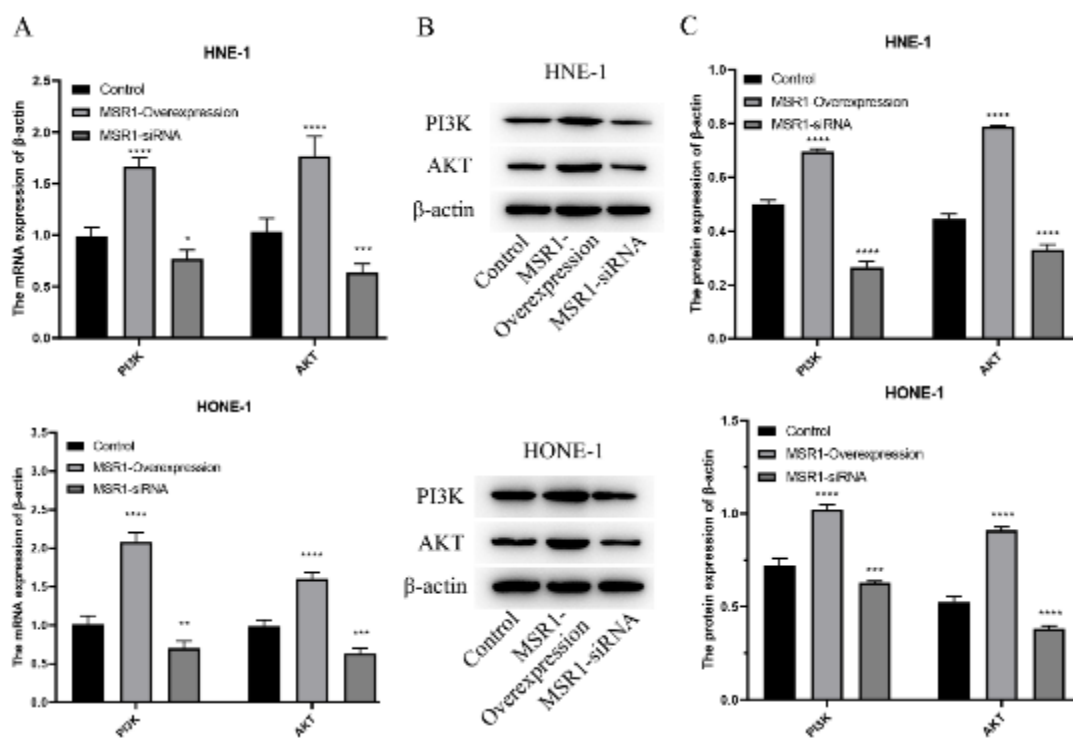
treatment and pcDNA-MSR1 + C6-ceramide. Results of qPCR showed MSR1 mRNA was significantly increased in the pcDNA-MSR1 group compared to the pcDNA-NC group. However, MSR1 amounts were significantly lower in the C6-ceramide treatment category versus the pcDNA-MSR1 group, while the pcDNA-MSR1 + C6-ceramide group exhibited an intermediate level of MSR1 expression (Figure 4 G). These results indicated that C6-ceramide inhibited the upregulation of MSR1 gene expression in NPC cells caused by MSR1 overexpression. In other words, MSR1 may be a potential target gene of C6-ceramide on NPC cells.

### MSR1 modulates PI3K/AKT signaling in NPC cells

The study previously demonstrated that MSR1 knockdown reduces NPC cell viability. Here, the study investigated the interaction between MSR1 and the PI3K/AKT signaling pathway. Compared to control cells, PI3K and AKT expression reduced with MSR1 knockdown but increased with MSR1 overexpression (Figure 5). These data indicate that modulating MSR1 reciprocally regulates PI3K and AKT levels in NPC cells. Thus, MSR1 appears to exert downstream effects on NPC cells via the activation of PI3K/AKT pathway.



**Figure 4:** Effect of MSR1 knockdown and C6-ceramide treatment on NPC cells. MSR1 mRNA (A) and protein (B) expression in NPC cell lines compared to NP69 cells; (C) MSR1 knockdown efficiency in NPC cells by shRNA; (D) Cell viability of NPC cells following MSR1 knockdown; (E) Viability of NPC cells treated with C6-ceramide; (F) MSR1 overexpression in NPC cells by plasmid transfection; (G) MSR1 expression in NPC cells with MSR1 overexpression and C6-ceramide treatment



**Figure 5:** MSR1 regulates PI3K and AKT expression in NPC cells (A) PI3K and AKT mRNA levels following MSR1 knockdown or overexpression in NPC cells; (B) PI3K and (C) AKT protein levels in NPC cells after MSR1 knockdown and overexpression

## DISCUSSION

Nasopharyngeal carcinoma is rarely detected early and current therapies have limited efficacy in advanced disease, contributing to high mortality. Approximately 95.5 % of NPC cases present at late stages, with 5-year overall and metastatic survival rates of only 61 and 50 %, respectively [13]. Elucidating NPC pathogenesis and identifying novel therapeutic targets are critical to improve patient's outcomes. Here, by integrating three NPC datasets, 26 upregulated and 344 downregulated genes were identified. GSEA nominated the small molecule C6-ceramide as a top potential NPC therapeutic drug across datasets. By overlapping differentially upregulated genes with predicted C6-ceramide targets, the study identified MSR1 as a candidate downstream effector. The study therefore investigated the functional effects of C6-ceramide and MSR1 in NPC.

Macrophage scavenger receptor 1 is expressed primarily in phagocytic and antigen-presenting cells, including dendritic cells, macrophages and lung endothelium [14]. It recognizes molecules and participate in disease pathogenesis [15]. Cluster of differentiation 204 (CD204) is a protein encoded by the MSR1 gene. Compared to normal tissues, its expression in various breast cancer subtypes is markedly higher.

Furthermore, CD204 is overexpressed in tumor-associated macrophages (TAM) and is considered a potential therapeutic target within the tumor microenvironment [16]. Other studies suggest that MSR1-mediated activation of PI3K/AKT, NF- $\kappa$ B and PPAR $\alpha$  signaling pathways helps macrophages adapt to diverse microenvironments [3,17]. Collectively, these studies indicate that MSR1 plays a crucial role in cancer treatment.

Building on this prior work, this study examined MSR1 gene transcription in one regular nasal epithelial cell line and four NPC cell types. MSR1 mRNA amounts were much higher in the NPC lines in comparison to the typical cells. Western blot analysis further showed MSR1 overexpression in NPC cells. These data indicate high MSR1 expression in NPC cells. Thereafter, the connection between MSR1 and NPC carcinogenesis was investigated. MSR1 knockdown significantly reduced MSR1 levels and inhibited NPC cell viability, indicating MSR1 is indeed involved in NPC carcinogenesis.

Previous studies have demonstrated that MSR1 may boost nuclear translocation of  $\beta$ -catenin, hence promoting stimulation of PI3K/AKT pathway [16]. The PI3K/AKT pathway activation are crucial for tumorigenesis. Therefore, the investigation further examined the effect of MSR1 on the PI3K/AKT pathway during NPC development by overexpressing or knockdown of



MSR1 in NPC cells. MSR1 overexpression resulted in increased expression of MSR1, PI3K and AKT compared to control cells. In contrast, MSR1 knockdown significantly reduced PI3K and AKT protein levels. These findings reveal that MSR1 also promotes NPC pathogenesis by stimulating PI3K/AKT signaling pathway in NPC cells.

C6-ceramide is a cell-permeable ceramide analogue that mimics endogenous ceramides [18]. The antitumor effects of C6-ceramide have been demonstrated in various tumor models [19], but its effects on NPC are unknown. In this study, GSEA analysis of three GEO datasets revealed C6-ceramide as a feasible NPC treatment. This investigation then investigated the implications of C6-ceramide upon NPC cells *in vitro*. Treatment with a specific C6-ceramide concentration decreased NPC cell viability. These data demonstrate C6-ceramide exerts cytotoxicity and significantly reduces NPC cell viability.

Previous studies have also demonstrated C6-ceramide liposomes induce cell death in chronic lymphocytic leukemia cells [20]. Next, this study investigated links between C6-ceramide cytotoxicity and MSR1 expression. MSR1 overexpression alone increased MSR1 expression. In contrast, treatment with C6-ceramide alone markedly reduced MSR1 expression. Co-treatment with overexpression and C6-ceramide resulted in MSR1 levels between individual treatment groups. This indicates C6-ceramide could mitigate increased MSR1 expression induced by overexpression in NPC cells. These studies indicate C6-ceramide may have therapeutic efficacy by targeting MSR1 in NPC.

## CONCLUSION

Using GSEA, this investigation has identified C6-ceramide and its target gene, MSR1, as therapeutic candidates for NPC. The findings verified their functional effect *in vitro*, employing NPC cell line experiments. MSR1 knockdown inhibits NPC cell proliferation, while C6-ceramide exhibits anti-NPC effect by regulating MSR1 expression. Although this study lacks patient sample data and detailed mechanistic analysis, it provides foundational data and reveals new research avenues for targeting MSR1 and ceramide signaling in NPC treatment. Going forward, validation in patient tumor tissues and elucidation of the precise molecular mechanisms mediating MSR1 and C6-ceramide effect will provide critical insight into their therapeutic potential. This work establishes a rationale for

further exploration of MSR1 and ceramides as novel targets in NPC.

## DECLARATIONS

### Acknowledgements

This study was approved by the Education Department of Heilongjiang Province (no. 2021-KYYWF-0359).

### Funding

None provided.

### Ethical approval

None provided.

### Availability of data and materials

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

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Haitao Wen, Guangrun Yang and Jiafu Zhou conceived and designed the study, and drafted the manuscript. Zhong Guo, Lixia Fan, Bowen Chen and Dapeng Zhang collected, analyzed and interpreted the experimental data. Dapeng Zhang and Jiafu Zhou revised the manuscript for important intellectual content. All authors read and approved the final manuscript draft for publication.

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