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GPC6 Promotes Cell Proliferation, Migration, and Invasion in Nasopharyngeal Carcinoma

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Abstract

Nasopharyngeal carcinoma (NPC) is a highly metastatic tumor that occurs frequently in Southeast Asia, particularly including southern China. Epstein-Barr virus infection is well established as a primary cause of NPC; nevertheless, the mechanisms underlying NPC pathogenesis remain largely unknown. In our previous study, we conducted whole-genome sequencing to screen for genomic variations that were associated with NPC. Of the resultantly identified variations, *glypican-6* (*GPC6*), was shown, for the first time, to be frequently mutated in NPC. In the present study, we verified this finding and conducted a series of functional experiments, which demonstrated that *GPC6* promotes the migration, invasion, and proliferation of NPC cells *in vitro*. Thus, the present study identified novel biological functions for *GPC6* in NPC, and thus, showed that *GPC6* may be a promising potential therapeutic target for this disease.

Key words: NPC, *GPC6,* genomic variations, migration, invasion, proliferation

Introduction

Nasopharyngeal carcinoma (NPC) is a polygenic hereditary disease that arises in nasopharyngeal epithelial cells, and is common in southern China [1-3]. While Epstein-Barr virus infection, environmental factors, and genetic susceptibility have been shown to promote NPC progression [4-10], the molecular basis underlying NPC pathogenesis has not yet been fully elucidated.

Genetic susceptibility is strongly implicated in NPC pathogenesis [11-13]. In fact, many previous studies have revealed frequent NPC-causative chromosome rearrangements (of chromosomes 1 and 12) and deletions (of chromosomes 3, 6, 11, and 14) [14-19]. Moreover, novel NPC-susceptibility loci have recently been discovered, such as *TNFRSF19* at 13q12, *MECOM* at 3q26m, and *CDKN2A/2B* at 9p21 [11]. Similarly, various single-nucleotide variants (SNVs) have been shown to be associated with a range of

immune diseases and cancers, in which they often represent somatic mutations relative to normal tissue. With the rapid development of second-generation sequencing technologies, it is now possible to perform a comprehensive, multi-level, whole-genome analysis of the genetic factors underlying NPC pathogenesis [20-22]. For example, in our previous study, we performed a genome-wide, high-throughput resequencing of genomic DNA in paired NPC tissue and peripheral blood (control) samples to explore the molecular basis of NPC. We resultantly identified a large number of NPC tumor-specific mutations, including SNVs, insertions, deletions, and genomic-structural and copy-number variants [23]. In the present study, we selected a gene called *glypican-6* (*GPC6*) that has not been previously shown to be associated with NPC, but was found to be frequently mutated in NPC tumor tissues (i.e. 8.3% and 58.3% of

the analyzed NPC tumor samples were shown to harbor SNVs and deletions in *GPC6*, respectively) for further analysis.

Materials and Methods

PCR and Sanger-sequencing analyses

PCR primers targeted to the identified *GPC6* SNVs were designed using primer 3 software, synthesized (Tsingke Company), amplified via PCR, and subjected to Sanger sequencing (Tsingke Company). The utilized Sanger-sequencing primers comprised (forward) 5'-TCTCATTGCAGGGGAACACT-3' and (reverse) 5'-TCTCACAGGCAAAGCACAAA -3'.

Clinical tissue samples

A total of 37 NPC and 19 adjacent non-tumor nasopharyngeal epithelium tissue samples were collected from patients who were newly diagnosed with NPC at the Affiliated Tumor Hospital of Central South University and who had not undergone either radio- or chemotherapy, or surgery. Relevant clinical data were also collected. All patients provided written informed consent for their participation in the study, which was approved by the Ethics Committee of the Health Authority of Central South University, China.

Cell lines, plasmids, and transfection

The NPC cell lines used in this study, HNE2 and 5-8F, were maintained (37°C, 5% CO_2) in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Shanghai, China). They were then cultured to 50–60% confluency in 6-well plates, and transfected with an pIRESneo3 empty (Clontech, Mountain View, CA, USA) or pIRESneo3-*GPC6* *GPC6*-overexpression (containing the full-length *GPC6* open reading frame) vector using Lipofectamine 3000 (Life Technologies, Grand Island, NY) according to the manufacturer's instructions[24-27].

RNA isolation and quantitative qRT-PCR

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and reverse transcribed into complementary DNA (cDNA) using 5× All-in-One RT MasterMix (Applied Biological Materials, Richmond, BC, Canada). quantitative real-time (qRT)-PCR was performed using the MiniOpticon system (Bio-Rad, Hercules, CA, USA) with EvaGreen 2× qPCR MasterMix (Applied Biological Materials) and primers for *GPC6* (forward, 5'-ACCGAGTTTCCAAGGTCAGC-3'; reverse, 5'-CA GCCTGATTTGCCAAGCAG-3') and the selected normalizing control, *GAPDH* (forward, 5'-TCTCTGCT CCTCCTGTTC-3'; reverse, 5'-GTTGACTCCGACCTT CAC-3') (Life Technologies, Carlsbad, CA, USA).

Wound healing assay

Cells were transfected with an empty vector or pIRESneo3-*GPC6* when they reached 50–60% confluency. A 10 µL pipette tip was used to scratch the monolayers at a consistent width (and each original scratch was normalized prior to the statistical analysis). After scratching, images were captured at 0, 12, 24, and 36 h with an inverted-phase contrast microscope. The width of the healed scratch in different groups was measured and analyzed using Image Pro Plus 6.0 software[28-30].

MTT assay

A total of 800 cells in 200 µl medium were added to 96-well plates after transfection. Proliferation was determined by incubating the cells (4 h) in 20 µl of 1 mg/ml tetrazolium salt MTT reagent (Sigma Chemicals, St Louis, MO, USA) and then dissolving them in dimethyl sulfoxide (DMSO). The absorbance of the resultant suspension was measured at 490 nm simultaneously each day on days 0, 1, 2, 3, 4, and 5.

Matrigel invasion assay

The invasive capacity of the cells was determined using transwell chambers (8-µm pores; Corning, NY, USA) in 24-well plates. The chambers were evenly coated with Matrigel (BD Biosciences, San Jose, CA, USA) diluted 1:8 in serum-free medium. A total of 20,000 cells suspended in 200 µl of serum-free medium were added to the upper chamber, and the lower chamber was filled with 20% FBS to induce cell invasion. Following 24–36 h of incubation, cells that had invaded through the filter pores were fixed with methanol and stained with 5% crystal violet. The number of invasive tumor cells from five randomly selected 20× fields was counted using Image Pro Plus 6.0 software.

Colony formation assay

A total of 800–1,000 transfected cells were seeded into 6-well culture plates. After 7 days, the cultures were harvested, fixed (15 min) with 4% paraformaldehyde, and stained (15 min) with hematoxylin. The number of colonies formed was counted using Image Pro Plus 6.0 software.

Statistical analyses

A Student's t-test was used to assess differences between two groups. All data were obtained from three independent replicates of each experiment and expressed as the mean ± standard deviation. A *P* value <0.05 was considered to indicate statistical significance. All statistical analyses were performed using SPSS 13.0 and GraphPad 5 software.

Results

Validation of *GPC6* somatic mutations in NPC

Via our previously conducted study, we showed that *GPC6* SNV and deletion mutations occurred in a high proportion (8.3% and 58.3%, respectively) of the analyzed NPC cases. To verify this finding, we used a combination of PCR and Sanger-sequencing techniques to detect and analyze somatic *GPC6* mutations in the present patient cohort. We resultantly showed that *GPC6* G-C mutations occurred more frequently in the NPC tumor than in blood (control) samples, following the change of the 106th amino acid of GPC6 protein from Aspartic acid to Histidine (**Fig. 1**).

GPC6 is highly expressed in NPC tissues

To explore the potential biological function of *GPC6* in NPC, we analyzed *GPC6* expression levels using the online Gene Expression Omnibus (GEO) database. By retrieving and analyzing *GPC6*-associated datasets (GSE68799 and GSE12452), we showed that *GPC6* is more highly expressed in NPC than in adjacent control tissues (**Fig. 2A**). Since these datasets did not include patient clinical data, and to verify this finding, we collected and evaluated *GPC6* expression in 37 NPC and 19 normal control tissues from newly enrolled patients via qRT-PCR. The results of this analysis showed that *GPC6* expression was significantly elevated in NPC tumor tissues, and furthermore, that the increased *GPC6* expression was associated with higher TNM tumor stages (**Fig. 2B**).

GPC6 promotes NPC cell proliferation

After cloning the full length *GPC6* open reading frame sequence into the pIRESneo3 expression vector, HNE2 and 5-8F cells were transfected with pIRESneo3-*GPC6* and confirmed to overexpress *GPC6* mRNA (**Fig. 3A**). The subsequently conducted MTT assays demonstrated that pIRESneo3-*GPC6*-transfected 5-8F and HNE2 cells exhibited a higher absorbance value than the corresponding negative control (NC; empty vector) cells. This finding indicates that the induced *GPC6* overexpression promoted NPC cell proliferation (**Fig. 3B**).

GPC6 increases NPC cell colony-formation and invasion capacities

We next conducted colony formation and Matrigel invasion assays to examine whether changes to *GPC6* expression affected the ability of NPC cells to form colonies and/or become invasive. The results of these assays showed that overexpressing *GPC6* increased colony formation in both 5-8F and HNE2 cells compared to that in the controls (**Fig. 4A**), and

similarly, that NPC cells transfected with pIRESneo3-*GPC6* exhibited an increased capacity for invasion (**Fig. 4B**).

GPC6 promotes NPC cell migration

We also examined whether *GPC6* overexpression impacted NPC cell migration. Cells were seeded in 6-well plates and transfected with pIRESneo3-*GPC6* or an empty vector, before their migration capacity was evaluated via a wound-healing assay. The results of this analysis showed that the migratory potential of the *GPC6*-overexpressing cells was dramatically increased compared to that of the NC NPC cells (**Fig. 5**).
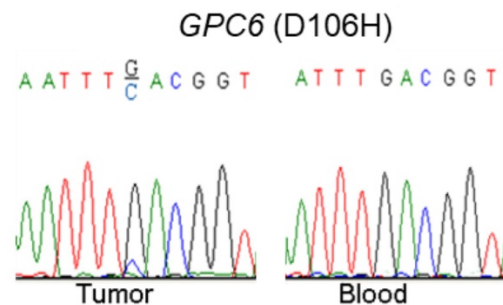

Figure 1. Validation of somatic mutations in nasopharyngeal carcinoma (NPC). A combination of polymerase chain reaction (PCR) and Sanger-sequencing techniques was used to validate previously identified somatic mutations in tumor and blood (negative control) samples collected from patients with NPC. The depicted *GPC6* G-C mutation was found to occur more frequently in the analyzed tumor than in blood samples.
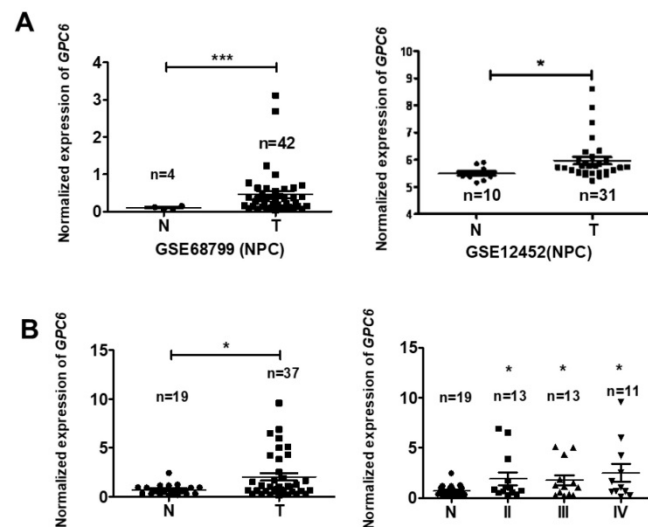

Figure 2. *GPC6* is highly expressed in nasopharyngeal carcinoma (NPC). (A) *GPC6* expression was shown to be significantly increased in NPC biopsies via the analysis of publicly available datasets GSE68799 (Normal, n = 4; Tumor, n = 42; $p = 0.0006$) and GSE12452 (Normal, n = 10; Tumor, n = 31; $p = 0.041$) that were sourced from the Gene Expression Omnibus database. **(B)** *GPC6* expression was measured by RT-qPCR and was shown to be significantly increased in 37 NPC compared to 19 non-tumor tissues collected from newly enrolled patients (left), and this increase in *GPC6* expression was shown to be positively associated with higher TNM stages (right). N, normal tissue sample; T, tumor tissue sample.
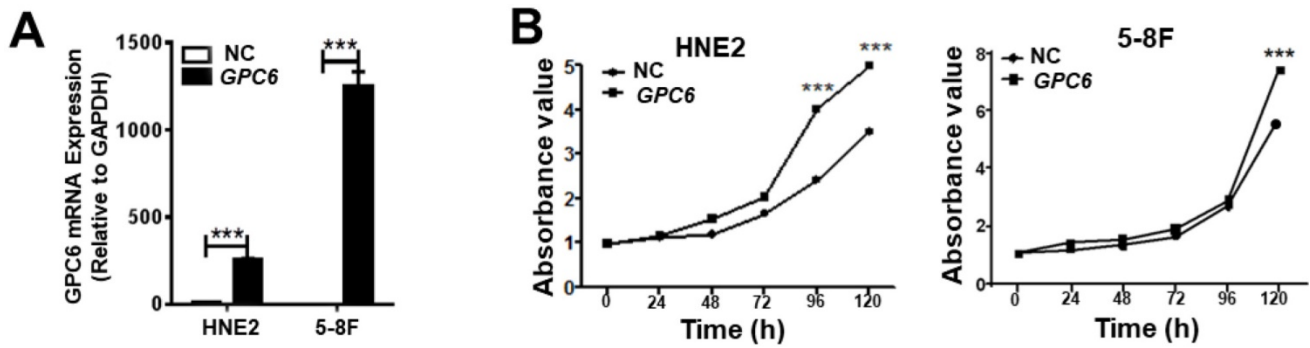
Figure 3. *GPC6* overexpression promotes nasopharyngeal carcinoma (NPC) cell proliferation. (A) Transfection of an *GPC6* overexpression construct, pIRESneo3-*GPC6*, dramatically increased *GPC6* expression in 5-8F and HNE2 cells, as shown via qRT-PCR. **(B)** Cells were seeded in 6-well plates, transfected with pIRESneo3-*GPC6* or an empty vector (negative control, NC), and allowed to proliferate for a further 24 h. They were then digested, counted, suspended (800 cells/200 µl), added to a 96-well plate, and subjected to an MTT assay. The results of this analysis showed that *GPC6* overexpression significantly promoted the proliferation of NPC cells.
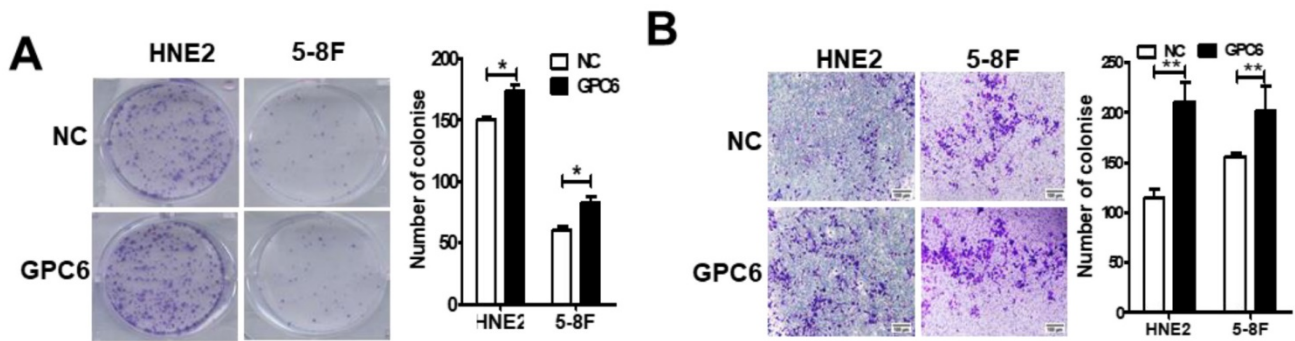
Figure 4. *GPC6* increased the capacity of nasopharyngeal carcinoma (NPC) cells to form colonies and become invasive *in vitro*. 5-8F and HNE2 cells were transfected with pIRESneo3-*GPC6* or an empty vector (negative control, NC), and allowed to proliferate for 24 h before being subjected to **(A)** a colony formation and **(B)** a Matrigel invasion assay. The results of these analyses showed that *GPC6* overexpression increased both the number of colonies that were formed by the cells, and the invasive capacity of the cells, respectively.
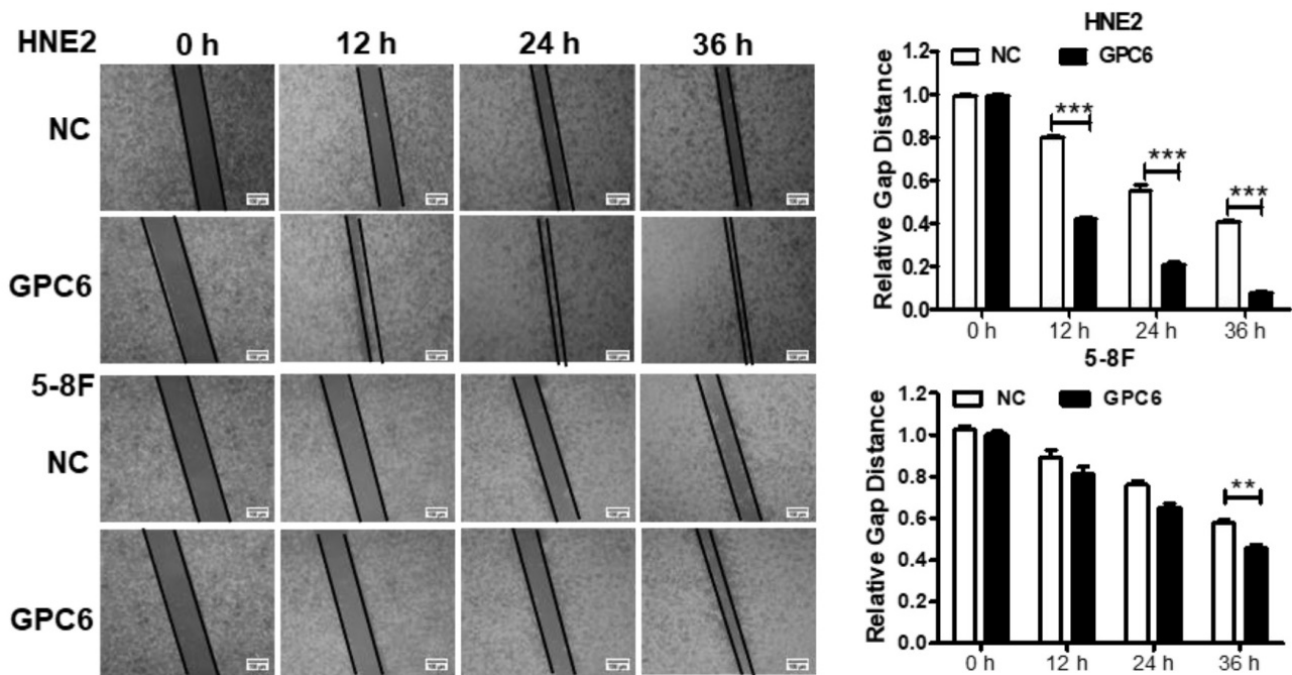
Figure 5. Overexpression of *GPC6* promoted nasopharyngeal carcinoma (NPC) cell migration. 5-8F and HNE2 cells were transfected with pIRESneo3-*GPC6* or an empty vector (negative control, NC), and cultured to 100% confluency before being subjected to a wound healing assay. After scratching, images were captured at 0, 12, 24, and 36 h with an inverted phase contrast microscope. Data are presented as the width ratio of migratory promotion.

Discussion

NPC is a highly metastatic tumor that occurs more or less frequently with remarkable racial and regional specificity and that is most common in South China, Southeast Asia, and North Africa [31-33]. Because of its concealed location in the body, lymph-node and/or distant metastases are often already present at the time of NPC diagnosis. The current clinical treatment for NPC mainly comprises a combination of radiotherapy and chemotherapy [34, 35]; however, these therapies incur severe side-effects, and treatment is frequently hindered by tumor heterogeneity [36-38]. Therefore, recent studies have focused on screening for novel NPC pathogenic mutations with the aim of identifying potential drug targets and/or molecular prognostic markers that can be used to improve current detection and treatment methods, and thus improve the outcome of patients with this disease [8, 39-46].

In recent years, whole-genome sequencing has been used to identify pathogenic mutations in various tumor types [47-49]; however, to date, NPC-mutation screening has only been conducted at the exon level. For example, Lin *et al.*[50] performed exon sequencing for NPC cases from Singapore and identified a large number of novel "driver" genes, many of which were found to be mutated and dysfunctional in NPC cells, and shown to thus mediate NPC pathogenesis. Following this, our previous study employed whole-genome sequencing to screen for recurring pathogenic NPC mutations. We resultantly showed that *GPC6* is frequently mutated in NPC, and thus, it was selected for further research in the present study. The conducted *in vitro* functional experiments herein showed, for the first time, that *GPC6* likely performs a regulatory role in NPC.

The conducted analysis of publicly reported NPC gene expression profile data (in the GEO datasets GSE12452 and GSE68799) and the q-PCR examination of the present patient cohort indicated that *GPC6* expression was upregulated in NPC compared to adjacent non-tumor samples, suggesting that it may promote oncogenesis. *GPC6* is a member of the GPC family of thiohepatic glycoproteins and is a cell surface phosphatidylinositol glycan [51]. To date, six members (comprising *GPC1–6*) of this family have been identified in mammals and *Drosophila*. Glypicans are primarily expressed during development and play a pivotal role in morphogenesis [52]. While previous studies have shown that *GPC6* mutations and copy-number variations frequently occur in various tumor types, the function of *GPC6* in human cancers remains controversial. For example, *GPC6* mutations have been identified in terminal cases of prostate cancer (via exon sequencing) [53], and *GPC6* copy-number variations have been suggested to promote the progression of non-small cell lung cancer (via a whole-genome microarray analysis) [54]. Moreover, *GPC6* was found to be frequently mutated and aberrantly methylated in colorectal cancer (in 12 paired colorectal tumors and adjacent healthy mucosal tissues using the Illumina Infinium Human Methylation 450 BeadChip) [55]. Similarly, a previous study demonstrated that increased *GPC6* expression was significantly correlated with a reduced progression-free survival time in patients with melanoma, and furthermore, that this effect occurred in an HIF1α-dependent/hypoxia-responsive manner (as shown via a ROC curve) [56]. Likewise, high *GPC6* expression has been shown to be closely associated with gastric cancer progression [57]. In contrast, *GPC6* has been found to function as a tumor suppressor, and high *GPC6* expression has been shown to be positively correlated with T-lymphocyte infiltration and increased overall survival in patients with early-stage ovarian cancer [58]. Likewise, reduced *GPC6* mRNA expression in retinoblastoma (RB) is associated with non-random allelic loss at 13q31, which is thought to promote RB progression [59]. Nevertheless, to date, the function of *GPC6* is unknown in NPC. The *in vitro* functional assays conducted by the present study suggest that *GPC6* likely promotes NPC cell proliferation, migration, and invasion, supporting the previously reported and present genome sequencing results. Taken together, our findings implicate *GPC6* as a likely oncogenic factor in NPC, and thus, as a promising prognostic biomarker. Further studies are needed to elucidate the mechanisms by which GPC6 mediates NPC pathogenesis. Notably, *GPC6* has been shown to promote breast cancer progression by inhibiting β-catenin and Wnt signaling pathways. This effectively upregulates Wnt5A signaling, which, in turn, inhibits JNK and p38 MAPK signaling, thereby promoting cancer-cell metastasis and invasion [60]. Moreover, mounting evidence suggests that GPCs may regulate several Hedgehog signaling pathways [61]. Thus, further research is required to examine the functional roles of *GPC6* in NPC.

Aspartic acid is an important amino acid. In the synthesis of amino acids, aspartic acid can function as a precursor of various amino acids; in the degradation of amino acids, it participates in the urea cycle and in the malic acid shuttle system. It also facilitates the transfer of NADHs from the mitochondrial membrane to the mitochondrial matrix. Our results indicated the presence of a G/C mutation in NPC samples that led to the change of the 106th amino acid of GPC6 protein from aspartic acid to histidine. Histamine, a well-known mediator of inflammation, is released

from mast cells and basophils. The histamine-producing enzyme, histidine decarboxylase (HDC), is commonly induced at inflammatory sites, enabling the regulation of various functions through the production of cytokines/chemokines/growth factors [62]. Baly et al. introduced a His19 to Ala mutant of melanoma growth-stimulating activity (MGSA) to antagonize the CXCR2 receptor, which offers the opportunity to design antagonists of MGSA that could have clinical utility [63]. Interestingly, accumulating evidence suggests that histamine synthesis induced in several types of tumor tissues modulates tumor immunity via inducing IFN production [64, 65]. His15 residue is necessary for tumor necrosis factor-alpha (TNF) to exert its cytotoxic activity [66]. Moreover, histidine can promote vasodilation. Formation of histamine by histidine decarboxylase contributes to histamine-mediated vasodilation following aerobic exercise [67]. Histidine is involved in structural rearrangement in the heme pocket and signal transduction [68]. Other studies have shown that histidine catabolism is a major determinant of methotrexate sensitivity [64]. The proliferation of different cancer cells requires the consumption of a large number of specific amino acids, and we speculated whether this mutation (aspartic acid to histidine) promotes the malignancy of tumor cells. The search for an analog of this amino acid, a metabolic antagonist, will provide effective means to treat tumors.

In summary, we herein employed a series of functional assays to validate the role of *GPC6* in NPC, and resultantly showed that increased *GPC6* expression promotes the migration, invasion, and proliferation of NPC cells *in vitro*. These findings provide an invaluable foundation for further research into the association of genomic variations with NPC development, and into the molecular mechanisms and signaling pathways underlying NPC pathogenesis.

Abbreviations

NPC: nasopharyngeal carcinoma; EBV: Epstein-Barr virus; *GPC6*: *glypican-6*; NC: negative control; MTT: 3-(4,5-dimethylthiazole-2-yl)-2.5-diphenyl-tetrazolium bromide; DMSO: dimethyl sulfoxide; GEO: Gene Expression Omnibus; SNV: single-nucleotide variant.

Acknowledgements

Ethics approval

All patients provided written informed consent for their participation in the present study, the design of which (including the use of human tissues) was approved by the Ethics Committee of the Health Authority of Central South University, Changsha, Hunan, China.

Competing Interests

The authors have declared that no competing interest exists.

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