

Original Research Article

MiR-25 enhances the proliferation, invasion and migration of nasopharyngeal carcinoma cells through targeted regulation of RAGE expression

Yang Jing¹, Qingxia Zhao^{2*}, Yujuan Wang¹, Pei Lin¹

¹Department of Otorhinolaryngology, ²Department of Operating Room, Shaanxi Provincial People's Hospital, Xi'an 710068, Shaanxi Province, China

*For correspondence: **Email:** qxzha02023@163.com

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Abstract

Purpose: To determine the effect of microRNA-25 (miR-25) on the proliferation, invasion, and migration of nasopharyngeal carcinoma cells, and the involvement of targeted regulation of late glycation end product receptor (RAGE) in the process.

Methods: Three groups of cells comprising; routinely cultured nasopharyngeal normal epithelial cell line NP69 without any treatment (blank control group), untreated human nasopharyngeal carcinoma cell line 5-8F, also cultured routinely (nasopharyngeal carcinoma group) and 5-8F human nasopharyngeal carcinoma cells infected with lentiviral vectors for miR-25 gene knockdown and cultured after stable transduction (miR-25 knockdown group). Cell proliferation was assessed using CCK-8 assay, while Western blot assay and quantitative polymerase chain reaction (qPCR) were used to measure protein and mRNA expression levels of relevant genes. Transwell assay was utilized to evaluate cell migration and invasion.

Results: The miR-25 expression level was significantly increased in nasopharyngeal carcinoma group ($p < 0.05$). Cell proliferation, migration, and invasion rates in miR-25 knockdown group were lower than those in nasopharyngeal carcinoma group ($p < 0.05$). Apoptosis rate of cells and levels of apoptotic proteins were significantly elevated, whereas bcl-2 level was significantly reduced in miR-25 knockdown group when compared to nasopharyngeal carcinoma group ($p < 0.05$). Protein expression levels of RAGE and S100P was significantly increased in nasopharyngeal carcinoma group, but significantly down-regulated in miR-25 knockdown group ($p < 0.05$).

Conclusion: Expression of miR-25 increases in nasopharyngeal carcinoma cells. Suppression of miR-25 results in decreased viability of nasopharyngeal carcinoma cells and inhibits cell proliferation, migration, and invasion, while enhancing apoptosis. The mechanism underlying these effects was associated with modulation of protein expressions of RAGE and S100P. Targeted regulation of late glycation end-product receptors hold promising potential as prospective biomarker for therapeutic interventions targeting specific cancers.

Keywords: Micro RNA-25, Advanced glycation end products, Nasopharyngeal cancer; Proliferation

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is one of the prevalent malignancies of the head and neck areas in China. The exact mechanisms underlying its development are not yet fully understood. However, current research suggests that NPC may be associated with various factors such as exposure to carcinogens, Epstein-Barr virus infection, genetic factors, and epigenetic variations [1]. Nasopharyngeal carcinoma (NPC) is predominantly a poorly differentiated squamous cell carcinoma which exhibits moderate sensitivity to radiation. Hence, radiation therapy is the primary modality employed in the clinical management of NPC. However, clinical studies have shown that approximately 55 % of NPC patients experience recurrence or metastasis within five years after radiotherapy, leading to treatment failure. This may be attributed to the development of radiation resistance by tumor cells, thereby rendering conventional radiation doses ineffective [2]. In recent years, targeted therapy has made significant progress, and it is increasingly being employed in the clinical treatment of various malignancies. Thus, it is of great clinical significance to further study the pathogenesis and etiology of NPC so as to identify new therapeutic targets that may reduce clinical symptoms, slow down cancer progression, and enhance the quality of life of the affected patients [3]. MicroRNAs (miRNAs) exert significant influence on the modulation of cellular processes. In particular, miR-25 is abnormally expressed in NPC patients. However, there is limited research on the impact of miR-25 on biological behaviors such as cell proliferation, migration, and invasion in NPC, as well as its underlying mechanisms [4]. Moreover, the receptor for advanced glycation end-products (RAGE) is postulated to be linked with tumor infiltration and dissemination [5]. Research has reported a strong correlation between RAGE expression and cancer progression in patients with gastric cancer, colorectal cancer, and prostate cancer [6]. This study was carried out to investigate the impact of miR-25 on NPC cell proliferation, invasion, and migration through targeted regulation of RAGE expression. The results may offer promising therapeutic avenues for the clinical management of nasopharyngeal carcinoma (NPC).

EXPERIMENTAL

Materials

Cell lines 5-8F and NP69 were procured from the American Type Culture Collection (ATCC). Fetal

bovine serum, 0.25 % trypsin with EDTA, and DMEM for growth were acquired from Thermo Fisher Scientific (USA). Primers for miR-25 mRNA and β -actin were purchased from Sigma, USA. Immunohistochemistry rabbit anti-rabbit secondary antibody, qPCR assay kit, CCK-8 kit, and Annexin V-FITC/PI apoptosis kit were products of Beyotime, Shanghai, China. Transwell chambers were bought from Corning, USA, while Matrigel was purchased from BD (USA).

The primary antibodies against Fas, bax, FasL, bcl-2, RAGE, and S100P were obtained from Abcam, UK. Lentiviral vectors for miR-25 gene knockdown in cells were obtained from JiKai Genes (Shanghai, China).

Procedures

Cell culture

Following cell retrieval, the cells were plated and cultured at 37 °C in T25 culture vessels containing DMEM with 10 % FBS. Routine passages and freezing were performed based on cell growth conditions. The experimental groups comprised blank control group, nasopharyngeal carcinoma group, and miR-25 knockdown group. The blank control group consisted of normal, conventionally cultured NP69 nasopharyngeal epithelial cells without any treatment.

Nasopharyngeal carcinoma group consisted of 5-8F human nasopharyngeal carcinoma cells without any treatment, also cultured conventionally. MiR-25 knockdown group consisted of 5-8F human nasopharyngeal carcinoma cells infected with lentiviral vectors for miR-25 gene knockdown and cultured after stable transduction. Each experiment was repeated six times.

Western blot assay for protein expressions

The cells were standardized to a concentration of 1×10^6 cells per well and plated in 6-well dishes. After stable transduction of miR-25 knockdown in the cells, total protein was extracted from the cells in each group with cell protein lysis solution at 4 °C, and the supernatant was collected after homogenization. Protein concentration was measured using the BCA method.

Gel electrophoresis was performed for 90 min, followed by gel cutting, membrane transfer for 90 minutes, membrane blocking, incubation with primary and secondary antibodies, development, and grayscale analysis of the results using Bio-Rad Image Lab software.

qPCR analysis of mRNA expression levels

The cells were seeded in 6-well plates at a density of 1×10^6 cells per well. After stable transduction of miR-25 knockdown in the cells, total RNA was extracted from each group using a kit specifically designed to isolate total RNA. The isolated RNA was reverse-transcribed into complementary DNA (cDNA) using the One Step Prime Script miRNA cDNA Synthesis Kit. Subsequently, quantitative real-time PCR was conducted using the miRNA fluorescent quantitative PCR detection kit according to the manufacturer's guidelines. The relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta CT}$ method.

CCK-8 assay for cell proliferation and apoptosis

Following the establishment of stable miR-25 knockdown in the cells, CCK-8 solution was administered to each cell group (10 μ L per well), and subsequent incubation was carried out in a constant-temperature, 5 % CO₂-incubator at 37 °C, for 4 h. An enzyme label analyzer was employed to assess the cell proliferation capacity of each group by reading absorbance at 450 nm. Apoptosis in each group of cells was determined using a flow cytometer kit after adding the corresponding reagents according to the instructions in the apoptosis kit.

Transwell assay for cell migration and invasion

Following the stable delivery of miR-25 knockdown into the cells, the cells from each group were adjusted to a density of 5×10^5 cells/well and placed in the upper compartments of Transwell plates. The low-dose and high-dose groups were exposed to propofol for a duration of 48 h, whereas the control group received an equivalent volume of DMEM in place of propofol. After fixing and staining, the purple-stained cells that migrated through the membrane were counted under a microscope, to evaluate cell migration potential. For the cell invasion assay, Matrigel was coated on the upper chambers of the Transwell plates in a clean bench, followed by the same procedures as described in the cell migration assay.

Statistical analysis

Data processing and analyses were performed utilizing the statistical software SPSS 22.0. One-way analysis of variance (ANOVA) was employed for comparing data among multiple

groups, followed by pairwise comparisons between groups using post hoc LSD-*t* test. Statistical significance of difference was assumed at $p < 0.05$.

RESULTS

Expression of miR-25 in NPC cells

The relative expression level of miR-25 in NPC cells was significantly higher than that in control cells ($p < 0.05$). These data are shown in Table 1.

Table 1: Expression of miR-25 (n=6)

Group	miR-25
Control	0.48±0.16
Nasopharyngeal cancer	1.02±0.35
<i>T</i>	-5.435
<i>P</i> -value	0.000

Effect of miR-25 knockdown on proliferation and migration of NPCs

There were significant increases in the proliferative capacity and migratory count of NPC in NPC group ($p < 0.05$), as indicated in Table 2. In contrast, in miR-25 knockdown group, the proliferative capacity and migratory count of cells were significantly decreased, relative to NPC group ($p < 0.05$).

Table 2: Effect of miR-25 knockdown on proliferation and migration of NPCs (n=6)

Group	Cell proliferation activity	Number of migrated cells
Control	0.10±0.01	73.25±15.70
Nasopharyngeal cancer	0.22±0.04 ^a	142.74±24.34 ^a
MiR-25 knockdown	0.15±0.03 ^b	106.48±17.44 ^b
<i>F</i>	25.154	19.022
<i>P</i> -value	0.000	0.000

Note: ^a $P < 0.05$, vs. control group; ^b $p < 0.05$, vs. nasopharyngeal carcinoma group

Effect of miR-25 knockdown on invasion and apoptosis of NPCs

As indicated in Table 3, the number of infiltrated cells in NPC group was significantly increased ($p < 0.05$). In contrast, there was a significant decrease in the number of infiltrated cells in miR-25 knockdown group, while the overall rate of apoptosis exhibited an increase when compared to NPC group ($p < 0.05$).

Table 3: Effect of knockdown of miR-25 on invasion and apoptosis of NPCs (n=6)

Group	Apoptosis rate (%)	Number of cell invasions
Control	9.97±1.23	51.72±10.31
Nasopharyngeal cancer	9.51±1.13	121.05±20.26 ^a
MiR-25 knockdown	31.34±4.25 ^b	93.12±16.35 ^b
<i>F</i>	134.293	26.157
<i>P</i> -value	0.000	0.000

^a*P* < 0.05, vs. control group; ^b*p* < 0.05, vs. nasopharyngeal carcinoma group

Table 4: Effect of miR-25 knockdown on the expression of apoptotic proteins in NPCs (n=6)

Group	Fas	bax	FasL	bcl-2
Control	0.35±0.14	0.37±0.09	0.41±0.14	0.54±0.03
Nasopharyngeal cancer	0.33±0.17	0.43±0.14	0.44±0.14	0.49±0.04
MiR-25 knockdown	0.84±0.18 ^b	0.86±0.16 ^b	0.78±0.12 ^b	0.31±0.03 ^b
<i>F</i>	13.446	20.294	18.641	72.177
<i>P</i> -value	0.001	0.000	0.000	0.000

^b*P* < 0.05, vs. nasopharyngeal carcinoma group

Table 5: Effect of miR-25 knockdown on the expression levels of RAGE and S100P proteins in NPCs

Group	RAGE	S100P
Control	0.28±0.04	0.30±0.0
Nasopharyngeal cancer	0.86±0.28 ^a	0.76±0.113 ^a
MiR-25 knockdown	0.57±0.08 ^b	0.52±0.09 ^b
<i>F</i>	15.521	45.156
<i>P</i> -value	0.000	0.000

Note: ^a*P* < 0.05, vs. blank control group; ^b*p* < 0.05, vs. nasopharyngeal carcinoma group

Effect of miR-25 knockdown on the expression of apoptotic proteins in NPCs

Table 4 shows that there were significantly increased expression levels of apoptotic proteins, i.e., Fas, bax, and Fas in miR-25 knockdown group, when compared to NPC group (*p* < 0.05). In contrast, there was a significant reduction in the expression of bcl-2 protein in miR-25 knockdown group, when compared to NPC group (*p* < 0.05).

Effect of miR-25 knockdown on the expression levels of RAGE and S100P proteins in NPCs

The levels of RAGE and S100P proteins were significantly elevated in the nasopharyngeal carcinoma (NPC) group (*p* < 0.05). Conversely, in miR-25 knockdown group, protein expression levels of RAGE and S100P were significantly decreased, when compared to NPC group (*p* < 0.05; Table 5).

DISCUSSION

Nasopharyngeal carcinoma (NPC) is a type of malignant neoplasm that arises from the epithelial cells lining the nasopharynx, a region

located in the head and neck. It occurs most often in the anterior wall of the nasopharynx and the fossa of Rosen muller [7]. Epidemiological studies on NPC have revealed that it is associated with distinct racial and geographic tendencies, with a higher incidence in southern China and Southeast Asia, where approximately 50 out of 100,000 individuals are affected [8]. The exact mechanisms and etiology of NPC remain largely unclear, but it is believed to be associated with three factors: Epstein-Barr virus infection, familial genetic factors, and environmental and dietary factors [9]. Currently, image-guided intensity-modulated radiotherapy is the primary treatment modality for NPC. However, treatment failure still occurs in 20 to 30 % of patients, due to distant metastasis, local recurrence, or uncontrolled local lesions, thereby leading to suboptimal quality of life [10]. Therefore, it is of clinical importance to carry out in-depth research on the pathogenesis and etiology of NPC, as well as identification of the molecular mechanisms underlying NPC cell proliferation, invasion, and migration, in addition to identification of new therapeutic targets. These efforts are aimed at reducing the clinical symptoms of NPC, slowing down the pathological progression of NPC, prolonging patient survival, and enhancing the overall quality of life of the patient.

In recent years, microRNAs (miRNAs) have emerged as an area of research focus in the field of malignant tumors. The miRNAs are a cluster of small non-coding RNA molecules which are involved in the modulation of diverse biological mechanisms including cellular proliferation, differentiation, migration, and programmed cell death by forming complexes with target genes [11]. In vertebrates, miR-25 is highly conserved, and it is a class of miRNAs involved in the regulation of cancer-associated inflammatory

responses. However, its specific regulatory role in cancer occurrence and development has not been fully elucidated [12].

Research findings have indicated that miR-25 exhibits reduced expression in NPC tissues, and its levels are strongly correlated with the stage of NPC, suggesting a significant association between miR-25 expression and the development and progression of NPC [4]. The present study revealed significantly higher relative expression levels of miR-25 in NPC cell lines when compared to the control group. The NPC cells in miR-25 knockdown group exhibited decreased proliferation and reduced migration, relative to NPC group. Furthermore, the population of infiltrating NPC cells was reduced in miR-25 knockdown group, when compared to NPC group, while the overall rate of apoptosis was increased in miR-25 knockdown group, relative to NPC group. Additionally, the aberrant expression of the Fas pathway, an extrinsic pathway of apoptosis, constitutes a vital element that impedes cell apoptosis.

The Fas and its ligand FasL are cellular surface receptors categorized as members of the tumor necrosis factor family, and their interplay results in ligand-mediated cellular demise. Suppressed levels of Fas and FasL or attenuated signal transduction are interconnected with tumorigenesis [13, 14]. One of the most prominent pro-apoptotic molecules is Bax, whereas Bcl-2 acts as an anti-apoptotic molecule. Both molecules are closely linked to tumor cell apoptosis. The Bcl-2/Bax ratio is frequently employed for assessing the extent of tumor cell apoptosis [15]. In this study, the expression levels of apoptosis-associated proteins Fas, Bax, FasL, and Bcl-2 exhibited significant elevations, reductions, reductions, and elevations, respectively, in miR-25 knockdown group, when compared to NPC group. These data suggest that the elevated expression level of miR-25 in NPC cells promoted cell viability, proliferation, migration, and invasion, while inhibiting apoptosis. Studies have shown that the expression of RAGE, a transmembrane receptor classified within the immunoglobulin superfamily, is down-regulated in most normal human tissues, apart from pulmonary tissue [16]. Perturbation of RAGE has been recognized as a pivotal stage in the emergence and dissemination of diverse malignancies associated with the head and neck. Restriction of RAGE expression or impairment of RAGE functionality impedes tumor proliferation and advancement [17]. Contemporary investigations have proposed that RAGE holds a promising potential as a prospective biomarker for therapeutic interventions targeting specific

cancers [18]. The main ligands of RAGE are S100/calgranulin proteins, bispecific antibodies, late-stage glycation end-products, and amyloid-beta peptides [19]. The S100 proteins are small (9-12kDa) calcium-binding proteins that exhibit 30 to 50% homology within the family [20]. In humans, there are more ≥ 20 members of the S100 calcium-binding proteins which are a multigene family, with each member encoded by a separate gene. These proteins include S100B, S100A2, S100A4, S100A6, S100A11, and S100P, all of which are associated with the progression and metastasis of various human cancers [21]. An S100 protein variant, i.e., S100P, was initially discovered in 1992 from placental tissue, and it is a 95-amino acid protein encoded by a gene positioned on chromosome 4p1 [22]. Investigations have revealed the involvement of S100P in interactions with multiple proteins, both in the extracellular and intracellular domains, thereby influencing the advancement and metastasis of diverse human malignancies such as breast, lung, gastric, and prostate cancers [23]. This study found higher expression levels of RAGE and S100P proteins in NPC cells, while miR-25 knockdown group resulted in significantly lower expression levels of RAGE and S100P proteins, when compared to NPC group. These results suggest that the lowering the miR-25 levels in NPC cells may decrease the expression levels of RAGE and S100P proteins.

CONCLUSION

This study has demonstrated a significant increase in the expression level of miR-25 in NPC. It has also shown that miR-25 knockdown reduces the viability, proliferation, migration, and invasion of NPC cells while enhancing cell apoptosis. These changes may be linked to the modulation of protein expressions of RAGE and S100P. Targeted regulation of late glycation end-product receptors holds promising potential as prospective biomarker for therapeutic interventions targeting specific cancers.

DECLARATIONS

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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

The authors 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 them. Qingxia Zhao designed the study, supervised the data collection, and analyzed the data. Yang Jing interpreted the data and prepared the manuscript for publication. Yujuan Wang and Pei Lin supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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