

Original Article

Insulin-like growth factor II mRNA binding protein 3 regulates proliferation, invasion and migration of neuroendocrine cancer cells

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Abstract: This study aimed to investigate the role of insulin-like growth factor II mRNA binding protein 3 (IMP3) in neuroendocrine tumor (NET). Mouse NET STC-1 cell line was chosen as the experimental model and three IMP3-targeting siRNAs and a non-specific scramble siRNA were transfected into STC-1 cells. The efficiency of IMP3 siRNA to knockdown IMP3 was evaluated by immunocytochemical staining. Cell proliferation was detected by MTT assay. Cell migration and invasion was analyzed with Transwell chamber assay. Protein expression was detected by Western blot analysis. We found that IMP3 silencing inhibited the proliferation of STC-1 cells potentially by down-regulating the expression of cell proliferation associated proteins EGFR and Ki67. Furthermore, IMP3 silencing inhibited the migration and invasion of STC-1 cells potentially by downregulating the expression of metastasis associated proteins IGF1R, MMP2 and MMP9. In conclusion, this study provides the first evidence that IMP3 plays an oncogenic role in Net and is a promising therapeutic target for NET.

Keywords: IMP3, neuroendocrine tumor, STC-1

Introduction

Neuroendocrine tumor (NET) is a relatively rare tumor arising from diffuse neuroendocrine system. NET has a low incidence, accounting for only 2% of all gastroenteropancreatic malignancies [1, 2]. Due to technological advances in the diagnosis, such as pathological diagnosis and classification, tumor imaging techniques with endoscopic ultrasound and somatostatin receptor fusing imaging, the incidence of NEN has gradually increased in the last three decades [3-5]. The incidence of gastroenteropancreatic neuroendocrine neoplasm (GEP-NEN) was increased by 380%, rising to 5.25/10 million from 1.09/100,000 between 1973 and 2004 in USA [6]. The clinical treatment option is still surgical resection, and targeted chemotherapy, radiotherapy, interventional therapy and biological target therapy are under developing.

Currently, the pathogenesis of GEP-NEN is poorly understood. Further study on the factors related to the occurrence, development, proliferation and invasion of GEP-NEN is important

to identify molecular targets for prevention and therapy of GEP-NEN. Insulin-like growth factor II mRNA binding protein3 (IMP3), a newly identified mRNA binding protein, is a kind of carcinoembryonic protein. Recent studies have shown that IMP3 is expressed in many malignancies, and its expression is closely associated with the expression of tumor-associated molecules (ki67 and P53) and clinic pathological features and postoperative tumor recurrence and metastasis [7, 8]. However, the role of IMP3 in GEP-NEN remains elusive. In this study we aimed to investigate the role of IMP3 genes in neuroendocrine tumor using loss-of-function approach. NET cell line STC-1 was transfected with IMP3-targeting small interfering RNA (siRNA) to examine the effects of IMP3 knockdown on the proliferation and invasion of NET cells.

Materials and methods

Cell culture and transfection

Murine small intestinal NET cell line STC-1 was purchased from American Typical Cell

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Collection (USA), and cultured in RPMI 1640 medium containing 1% non-essential amino acids and 10% fetal bovine serum (FBS) at 37°C with 5% CO₂. IMP3-siRNA and negative control NS-siRNA were synthesized in RiboBio Co. Ltd (Guangzhou, China). Cells were transfected with siRNAs using RiboFECT™ CP Reagent (RiboBio Co. Ltd). After transfection, cells were divided into three groups: transfected with IMP3-siRNA (IMP3-siRNA group), transfected with NS-siRNA (NS-siRNA group) and blank control group without any siRNA transfection (Con group).

RT-PCR analysis

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol, and subjected to reverse transcription for synthesis of cDNA. The primers were as follows: IMP3 upstream: 5'-GATGTCCTATTTCTTGCTTCTCTA-3', and downstream: 5'-GTTTCTCCTTCTACTCTCCTGTTTC-3'; GAPDH upstream: 5'-CGCTGAGTACGTCGTGGAGTC-3' and downstream: 5'-GCTGATGATCTTGAGGCTGTTGTC-3'. PCR reaction was initiated with a cycle of 95°C for 30 s, followed by 40 cycles consisting of 95°C for 5 s and 72°C for 20 s. The relative mRNA expression of target genes was calculated using 2^{-ΔΔCt} method. Each experiment was replicated for three times.

Western blot analysis

Total protein was extracted from the cells for quantification, and polyacrylamide gel electrophoresis was used to separate proteins in the lysates. The proteins were transferred to polyvinylidene fluoride membranes, blocked in skim milk solution for 1 h, and incubated with antibody for IMP3, IGF1R, MMP2, MMP9, ki67, p27, CyclinD1, EGFR or GAPDH antibody (ImmunoWay Biotech, USA) overnight. The blots were washed, incubated with horseradish peroxidase-coupled secondary antibody, and developed using enhanced chemiluminescence kit. GAPDH served as an internal reference. The automated electrophoresis gel image analyzer was used to calculate relative protein expression. Each experiment was performed three times.

Immunocytochemical analysis

After being trypsinized, cells from IMP3-siRNA group, NS-siRNA group and CON group were

seeded in 6-well plates on 5 mm × 5 mm coverslips. When cells adhering to coverslips grew to 70% confluency at 37°C in 5% CO₂, they were washed with phosphate-buffered saline (PBS; 20 mM NaH₂PO₄, 80 mM Na₂HPO₄, 65 mM NaCl, pH 7.4), and fixed in 4% paraformaldehyde for 30 min. Cells were washed with PBS and incubated with IMP3 antibody overnight at 4°C. Cells were then washed with PBS and incubated with the appropriate secondary antibodies for 1 h at room temperature. Finally, cells were incubated with diaminobenzidine and observed under microscope.

Cell proliferation assay

STC-1 cells in each group were cultured in 96-well plates for 0 h, 12 h, 24 h, 36 h and 48 h, then the medium was discarded and the cells were incubated with 20 μl MTT (5 mg/mL) at 37°C for 4 h, then 150 μl dimethyl sulfoxide was added to each well and the plates were incubated at room temperature for 10 min. Absorbance value (OD) at 490 nm wavelength was measured with a microplate reader. Six replicate wells were made in each group to obtain the average OD value.

Migration and invasion assays

Migration and invasion assays were performed as described previously [9]. For migration assay, STC-1 cells were cultured in serum-free medium for 6 h, and then 0.5 ml cell suspension (1 × 10⁵ cells/ml) were seeded in the upper Transwell chamber. The lower chamber was added with 1640 medium supplemented with 10% FBS. The cells were incubated at 37°C for 24 h. Cells that did not penetrate to the lower chamber were carefully wiped with cotton swabs, and the number of cells in each visual field in the lower chamber was counted and averaged. For invasion assay, Transwell chambers were added with 100 μl pre-diluted Matrigel gel and incubated at 37°C for 30 min. The prepared chambers were sterilized with ultraviolet radiation for 2 h for full polymerization of Matrigel. 200 μl cell suspension in each group were seeded in the upper chamber. The lower chamber was added with RPMI 1640 supplemented with 10% FBS. The cells were incubated at 37°C for 24 h. A cotton swab was used to wipe Matrigel gel and cells on the surface of the chamber. The membranes were fixed with methanol for 10 min. After crystal

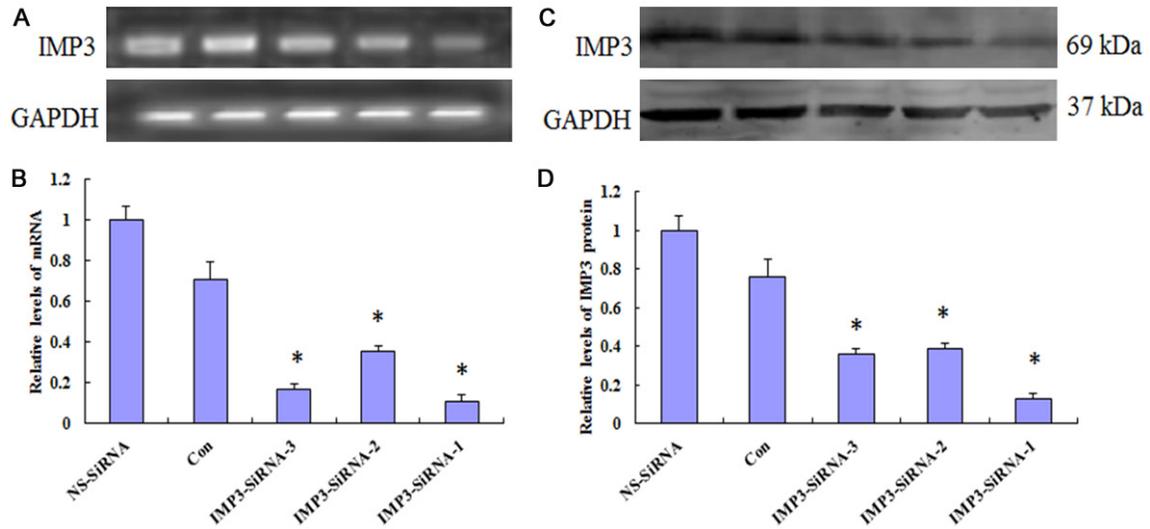


Figure 1. IMP3-siRNA inhibited the expression of IMP3 in STC-1 cells. A. STC-1 cells were transfected with the indicated siRNAs, 24 h later IMP3 mRNA expression levels were detected by PCR. B. Quantitative analysis of IMP3 mRNA levels relative to GAPDH (n=3). C. STC-1 cells were transfected with the indicated siRNAs, 24 h later IMP3 protein expression levels were detected by Western blot analysis. D. Quantitative analysis of IMP3 protein levels relative to GAPDH (n=3). *P<0.05 versus control (untransfected).

violet staining; the number of cells in five visual fields of the membranes were counted and averaged.

Statistical analysis

SPSS16.0 software was used for statistical analysis. The data were tested with normality and homogeneity of variance test. One-Way-ANOVA method was used for comparison, and Mann-Whitney U test was performed to analyze non-normal variables. Unpaired t-test was applied to normally distributed variables. P<0.05 was considered statistically significant.

Results

Knockdown of IMP3 in STC-1 cells

STC-1 cells were transfected with three specific IMP3-siRNAs. qRT-PCR and Western blot analysis showed that compared with the Con group and NS-siRNA group, IMP3-siRNA-1, IMP3-siRNA-2 and IMP3-siRNA-3 reduced IMP3 mRNA and protein levels significantly (**Figure 1**). Among the three IMP3-siRNAs, IMP3-siRNA-1 showed the highest silencing efficiency (**Figure 1**). Therefore, we used IMP3-siRNA-1 for the following experiments.

Immunocytochemistry of IMP3 expression in three cell lines from IMP3-siRNA-1 group, Con group and NS-siRNA group revealed that IMP3-siRNA-1 significantly inhibited IMP3 expression (**Figure 2**).

IMP3 knockdown inhibited the proliferation of STC-1 cells

MTT assay demonstrated that cell proliferation was decreased significantly in IMP3-siRNA-1 group compared to CON group and NS-siRNA group (P<0.05) (**Figure 3A**). Furthermore, we detected cell proliferation related proteins and found that EGFR and Ki67 protein expression levels were significantly reduced while CyclinD1 and p27 protein expression levels showed no obvious change in IMP3-siRNA-1 group compared to CON group and NS-siRNA group (**Figure 3B, 3C**).

IMP3 knockdown inhibited the migration and invasion of STC-1 cells

Transwell cell migration and invasion assay showed that cell migration and invasion ability was decreased in IMP3-siRNA-1 group, compared with NS-siRNA group and Con group (P<0.05) (**Figure 4**). Compared with Con group, cell migration and invasion in NS-siRNA group

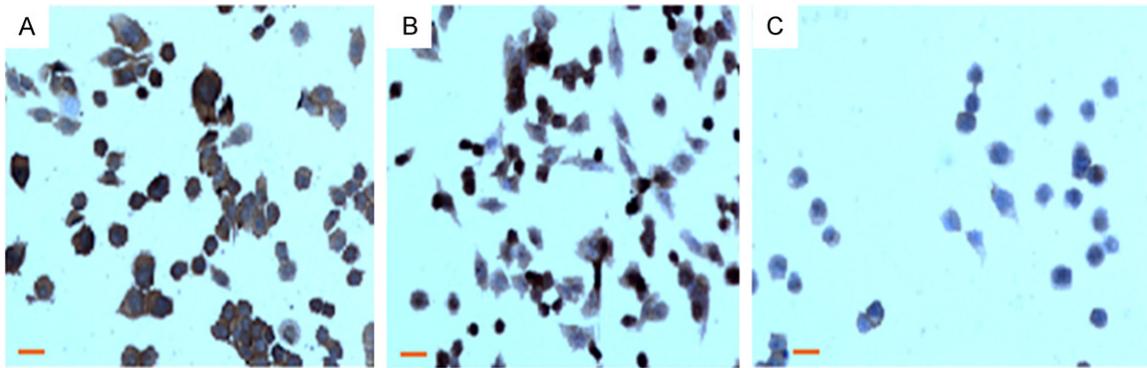


Figure 2. Immunocytochemical staining of IMP3 in STC-1 cells. A: NS-siRNA; B: Con; C: IMP3-siRNA-1. Scale bar: 20 μ m.

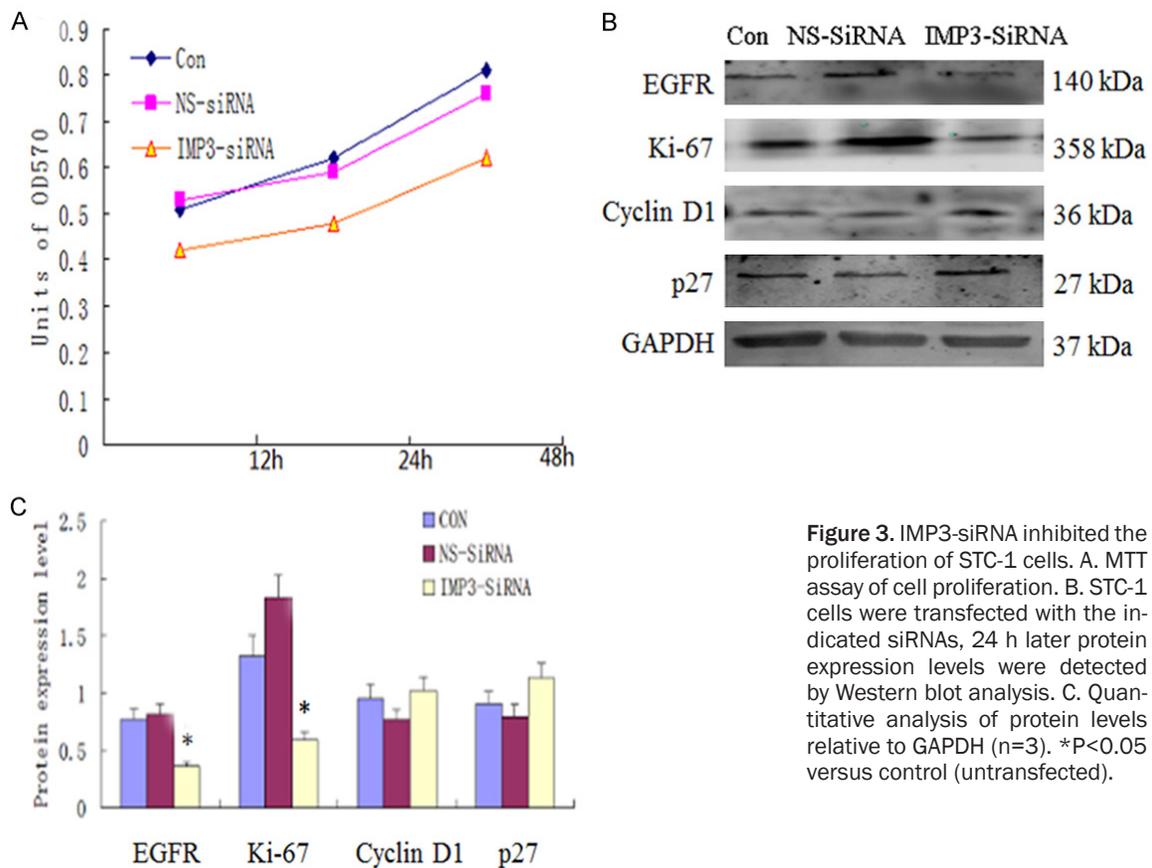


Figure 3. IMP3-siRNA inhibited the proliferation of STC-1 cells. A. MTT assay of cell proliferation. B. STC-1 cells were transfected with the indicated siRNAs, 24 h later protein expression levels were detected by Western blot analysis. C. Quantitative analysis of protein levels relative to GAPDH (n=3). *P<0.05 versus control (untransfected).

showed no significant difference (Figure 4). Furthermore, we detected cell invasion related proteins and found that IGF1R, MMP2, and MMP9 protein expression levels were significantly reduced compared to CON group and NS-siRNA group (Figure 5A, 5B).

Discussion

IMP3 is one member of IMPs (IMP1, IMP2 and IMP3) in mRNA binding protein family, its gene

is located in the 2nd sub-band of the first band in zone 1 on short arm of human chromosome 7, which belongs to KOC (Khomology domain containing protein overexpressed in cancer) gene [10]. IMP3 is currently recognized as a carcinoembryonic protein with high expression in various malignancies, including uterine cancer and pancreatic cancer. In addition, IMP3 expression is associated with tumor progression, indicating the important role of IMP3 in

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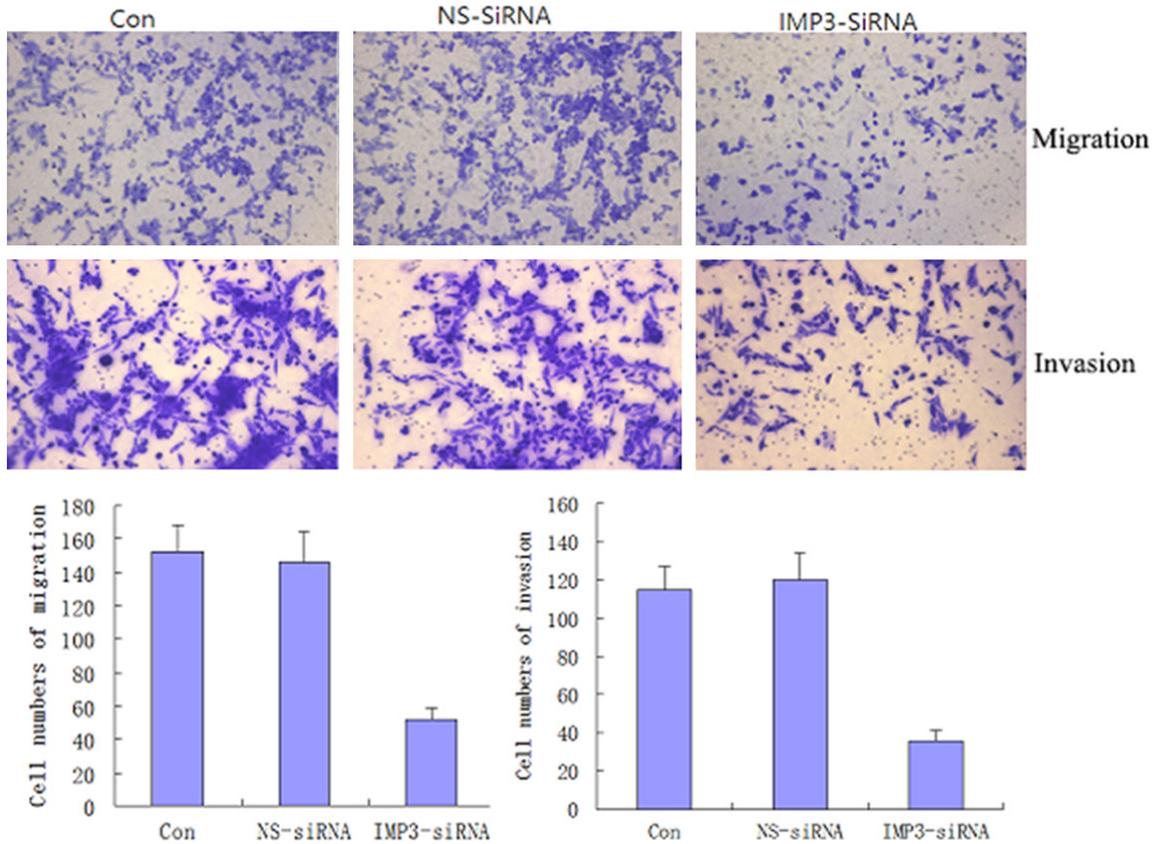


Figure 4. IMP3-siRNA inhibited the migration and invasion of STC-1 cells. Magnification: 200 ×.

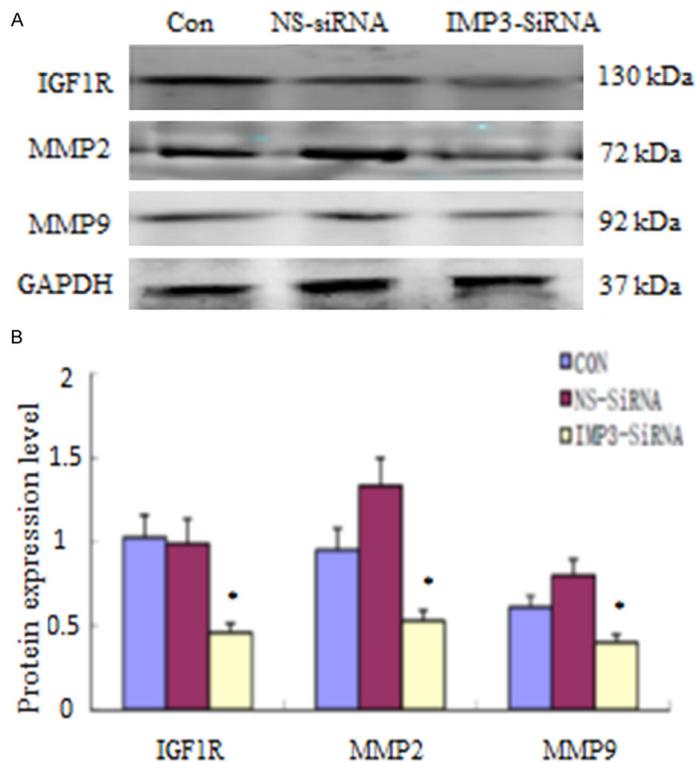


Figure 5. IMP3-siRNA regulated the expression of migration and invasion related proteins in STC-1 cells. A. STC-1 cells were transfected with the indicated siRNAs, 24 h later protein expression levels were detected by Western blot analysis. B. Quantitative analysis of protein levels relative to GAPDH (n=3). *P<0.05 versus control (untransfected).

tumor invasion and metastasis [11-14]. In present study, we confirmed that silencing STC-1 effectively inhibited the proliferation, invasion and migration of NET cells.

siRNA technology is an effective approach to investigate the function of gene [15]. Using specific siRNA targeting IMP3, we depleted IMP3 expression at mRNA and protein levels in STC-1 cells. To explore the role of IMP3 in NET, MTT assay was performed to examine the effect of IMP3 knockdown on the prolifera-

tion of STC-1 cells. The results revealed that the inhibition of IMP3 expression suppressed the proliferation of STC-1 cells. Next we explored molecular mechanisms and confirmed that IMP3 silencing downregulated the expression of Ki67 and EGFR. Ki67, an indicator of cell proliferation, is an antigen of non-histone nuclear protein present in the nuclear matrix of the proliferating cells. Ki67 level is highly correlated with the progression, metastasis and prognosis of malignancies [16, 17]. EGFR can regulate cell mitosis and proliferation. Previous study reported that IMP3 indirectly regulated EGFR signaling pathway, thereby promoting the occurrence and development of breast cancer [18].

Moreover, we conducted Transwell assay to examine migration and invasion ability of NET cells after IMP3 knockdown. The results demonstrated that the numbers of cells migrated and invaded across the membrane were significantly lower in IMP3-siRNA group than in control group. These data suggest that migration and invasion ability of NET cells was markedly reduced after IMP3 knockdown. We further investigated molecular mechanism underlying the changes in invasion and metastasis of NET cells. Insulin-like growth factor 1 receptor (IGF1R), a transmembrane receptor, is a member of insulin-like growth factor system (IGFs) family [19]. IGF1R may promote metastasis of tumor cells mainly by upregulating the expression levels of metalloproteinases (MMP) and urokinase-type plasminogen activator (uPA) and accelerating the degradation of extracellular matrix [20]. As expected, IMP3 knockdown led to significantly reduced protein expression levels of IGF1R, MMP2, and MMP9, which may contribute to the inhibition of NET cell migration and invasion.

In summary, our data demonstrated that the depletion of IMP3 expression in NET cells inhibited cell proliferation, migration and invasion, and this is related to the downregulation of EGFR, Ki67, IGF1R, MMP9 and MMP2 expression. Therefore, IMP3 is a promising therapeutic target for NET.

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Disclosure of conflict of interest

None.

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