Stmn1 up-regulates *Cdx2* expression and participates in gastric intestinal metaplasia in vitro and in vivo: a randomized controlled trial

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Research article

Keywords: *Stmn1*, *Cdx2*, IM, rats, GES-1 cell line

Posted Date: August 6th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-42824/v1>

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Abstract

BACKGROUND AND PURPOSE: *Stmn1* is over-expressed in almost all pathological stages during gastric cancer development, such as chronic atrophic gastritis, dysplasia, and gastric cancer. IM is an important precancerous lesion of gastric cancer, however, whether *Stmn1* was up-regulated or down-regulated in this stage is still unknown. We aimed to evaluate the expression level of *Stmn1* in IM in vivo and its relationship with important gene of IM named *Cdx2* in vitro.

EXPERIMENTAL APPROACH: Wistar rats (n=12, sex in half) were gavaged with MNNG (167µg/ml) to induce IM model in stomach. After pathological examination with AB staining to confirm that the model was successful, relative expression level of *Stmn1* was detected between normal group and model group using RT-qPCR. Human gastric cell line GES-1 was used to investigate whether *Stmn1* influence expression level of IM essential gene *Cdx2* by over-expressing or down-expressing experiments, RT-qPCR and western blot.

KEY RESULTS: We have demonstrated that *Stmn1* was up-regulated in IM model induced by MNNG in rats in vivo, and it could significantly up-regulate *Cdx2* expression level in human gastric cell line GES-1 in vitro.

CONCLUSIONS AND IMPLICATIONS: We demonstrated that *Stmn1* was involved in IM in this model and it could up-regulating *Cdx2* in human gastric cell line GES-1 in vitro. These results suggested that *Stmn1* might be a potential biomarker or candidate treatment target of IM in stomach.

Background

There were 1,033, 701 new gastric cancer cases worldwide in 2018 (WHO). IM (IM), which refers to the transformation of gastric epithelium into intestinal phenotype, is the most common precancerous lesion of gastric cancer [1, 2]. Control and reversion of precancerous lesions is the key point to prevent deteriorating into gastric cancer. Cognition of the mechanisms of precancerous lesions is a prerequisite.

Caudal type homeobox gene 2 (*Cdx2*), a transcription factor, is believed to be essential in cell differentiation of intestinal epithelium [3]. *Cdx2* Ectopic expression in gastric tissue is an important mechanism of IM [4-8]. *Cdx2* controls the expression of multiple intestinal proteins, such as *MUC2* [9, 10], LI-cadherin [11], *ST6GalNAc-I* [12]. Besides, *Cdx2* is activated by *Oct-1* [13] and inhibited by *RUNX3* [14] and inhibit *SHH* [15] in IM in stomach.

Stathmin 1 (*Stmn1*) belongs to the stathmin gene family, and the protein it encoded prevents assembly and promotes disassembly of microtubules. In gastric cancer, *Stmn1* expression level is up-regulated [16], and it positively correlated with lymph node metastasis, TNM stages and vascular invasion, and negatively with recurrence-free survival [17]. *Stmn1* gene silencing could suppress proliferation, migration and invasion of gastric cancer cells via *AKT/sCLU* and *STAT3* signaling [17-20]. Over-expression of

Stmn1 is associated with poor prognosis of patients with gastric cancer [21]. However, its expression in IM is unknown.

The gastric cancer model [22] induced by 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) in rats can simulate the whole process from normal human gastric to chronic gastritis, chronic atrophic gastritis, IM, intraepithelial neoplasia, gastric adenocarcinoma, the most common type of gastric cancer in human [23], which provides possibility and sufficient window period for drug intervention to prevention or treatment of gastric cancer.

Previously, we found that *Stmn1* is significantly higher expressed in chronic atrophic gastritis in rats [24], here we investigate whether *Stmn1* and IM in stomach of IM model rat, and detected the effect of *Stmn1* on the expression of *Cdx2* in human gastric cells GES-1.

Methods

Rat experiments

Twenty-four 6-week-old wistar rats, weighing around 200g, were bought from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China) and randomly divided into normal control group (n=12) and model group (n=12) using stratified random sampling, sex in half. All rats were kept on a 12-hour light–dark cycle in a pathogen-free animal feeding facility at Zhejiang Academy of Traditional Chinese Medicine. The maximum caging density was six mice. The animal study protocols were approved by the Medical Ethics Committee of Tongde Hospital of Zhejiang Province [No. (2016)040] before experiments.

Rats in the model group were provided with ample fresh MNNG solution (167µg/ml) stored in light-free bottles for 20 weeks. On the last day, all rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50mg/kg, 2%), then stomachs were removed along with 0.5 cm of esophagus above the stomach and 1.5 cm of duodenum below the stomach. After these, liver and blood of each rats were collected for further experiments, as a result every rat was sacrificed on test-bed. Each stomach was vertically divided into 2 parts; one part was frozen in liquid nitrogen for total RNA isolation, and the other part was fixed in 10% formalin. AB staining was used to observe the IM of gastric mucosa (goblet cells).

For each rat, five different investigators were handled as follows: a first investigator (RZ) administered the treatment following stratified random sampling and the randomization table. A second investigator (CY) was responsible for the anaesthetic procedure, whereas a third investigator (CX, TY, CS, DG) performed the surgical and fix procedure. A fourth investigator (ZF) (also unaware of treatment) performed pathological section and evaluation of pathological results. Finally, a fifth investigator (WR) performed data analysis.

Cell experiments

Human gastric GES-1 cell line was obtained from the cell bank of Chinese Academy of Sciences (Shanghai, China) and was cultured in 1640 medium with 10% fetal bovine serum at 37°C in a 5% CO₂

atmosphere. Cells were divided into 5 groups: normal control group, *Cdx2* over-expression group, *Cdx2* over-expression + *Stmn1* over-expression group, *Cdx2* over-expression + *Stmn1* over-expression+ *Stmn1* siRNA group, *Cdx2* over-expression + *Stmn1* siRNA group. *Cdx2* plasmid, *Stmn1* plasmid and siRNA (Forward: 5'-GUAGGACUGUAUAGGUAGATT-3'; Reverse: 5'-TTCAUCCUGACAUAUCCAUCU-3') were designed and synthesized by GenePharma. Transfection was performed in 24-well (n=6; for RT-qPCR; *Cdx2* plasmid, 1 pg/well; *Stmn1* plasmid, 20 pg/well; *Stmn1* siRNA, 10 pM) or 6-well (n=3; for WB; *Cdx2* plasmid, 4 pg/well; *Stmn1* plasmid, 80 pg/well; *Stmn1* siRNA, 40 pM) culture vessels using 0.5ul/well or 2ul/well lipo2000 (invitrogen), respectively.

Histological section assay

The rat stomach samples in formalin were embedded in paraffin and sliced at 4-μm thickness. The slices were then separately stained with AB-PAS following routine procedures. Sections were examined under light microscope. The investigator was blind to group assignment and outcome assessment. IM was determined according to intestinal type cells in gastric gland cells.

RT-qPCR

The RT reaction was performed using 500 ng of total RNA according to the manufacturer's instructions (RR036A, Takara). The qPCR reactions were performed on the StepOnePlus system (ABI) with the SYBR® Premix ExTaq™ kit (RR820A, Takara). The endogenous control gene was *RPL 13A* for cell experiments and *EF-1a* for rat experiments. The relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [25]. Primers were as follows: *Stmn1* (rat, homo), Forward: 5'- TGGCAGAGGAGAACTGACC-3', Reverse: 5'- TTCTTCCGCACCTCTTCAAC-3'; *Cdx2* (homo), Forward: 5'- TTTACTACAGTCGCTACATCACCAT-3', Reverse: 5'- TTGTTGATTTTCCTCTCCTTTGCT-3' [12]; *EF-1a* (rat), Forward: 5'- CGAGCCACCATACAGTCAGA-3', Reverse: 5'- CCATTCCAACCAGAAATTGG-3'; *RPL 13K* (homo), Forward: 5'- GCCCTCCAATCAGTCTTCTG-3', Reverse: 5'- CATAGGAAGCTGGGAGCAAG-3'.

Western blot protein analysis

Protein was isolated from GES-1 cells at 0°C using Whole Cell Lysis Assay (KGP250, KeyGEN BioTECH) and quantified by BCA Protein Assay Kit (P0012, Beyotime Biotechnology). A capillary-based Wes Simple Western Analysis (ProteinSimple®) was used to detect protein abundance according to the manufacturer's protocol. In brief, 0.6 μg whole protein sample was loaded into each well, and Size Range of the separation matrix was 12-230 kDa. Target proteins were identified with following primary antibodies: *Cdx2* (12306; Cell Signaling Technology®), *Stmn1* (3352; Cell Signaling Technology®), *SOX2* (23064; Cell Signaling Technology®). *GAPDH* (60004-1-Ig; Proteintech®) was used as a reference control. According to primary antibodies, mouse or rabbit secondary antibodies provided by the manufacturer were used. Chemiluminescent signals were detected and quantified using Compass Software (ProteinSimple), the area value was used as the protein expression level.

Statistical analysis

SPSS Statistics 17.0 was used to analyze the data. Body weight and gene expression level among 5 groups were analyzed using one way *ANOVA*, and between 2 groups were analyzed using independent sample *t*-tests. Differences were considered statistically significant at $P < 0.05$.

Results

***Stmn1* was significantly higher expressed in IM in stomach tissues in rats**

To investigate whether *Stmn1* is over-expressed in IM, we detected its expression level in MNNG induced IM in stomach of rats. In typical pictures of pathological sections (Fig 1a.), there was a very small amount of blue staining in the deep part of gastric mucosa in the antrum of normal stomach; there was blue staining in the whole layer from the deep part to the shallow part of the antrum mucosa, and there were goblet cells, which are the sign of IM. In model group, there were 10 IM rats, the other 2 rats failed to be induced into IM model. Then we divided all 24 rats in the normal group and model group into two groups: the group without IM ($n=14$) and the group with IM ($n=10$). The *t*-test showed that the expression level of *Stmn1* in IM group was significantly higher than that in normal group ($P = 0.003$; Fig 1b). The relationship between *Stmn1* and important genes of intestinal meplasia, such as *Cdx2* needed to be established. However, we could not detect the expression of *Cdx2*, a key regulatory gene of IM, in rat IM gastric tissue, and no other literature has reported about *Cdx2* in IM model rats induced by MNNG.

***Stmn1* significantly up-regulates *Cdx2* expression level in GES-1 cells**

To explore the effect of *Stmn1* on *Cdx2*, we over-expressed or knock-down its expression along with over-expression of *Cdx2* in GES-1 cells (Fig 2).

Relative mRNA expression level of *Cdx2* and *Stmn1* in each group was detected by RT-qPCR. The results showed that *Cdx2* was not expressed in normal group (Fig 3). Among other 4 groups, relative expression level of *Cdx2* was tested by *Levene* variance homogeneity test and the variance of each group was not homogeneous ($P = 0.011$), then one-way *ANOVA* of grouping design was used to evaluate the difference, and the results showed that the expression level of *Cdx2* among 4 groups was significantly different ($F = 65.179$, $P = 0.000$; Fig 3). Compared with *Cdx2* over-expression group, *Cdx2* expression level in *Cdx2* over-expression + *Stmn1* over-expression group was significantly higher ($P = 0.000$; Fig 3.), indicating that *Stmn1* could significantly up-regulate *Cdx2* expression level. Compared with *Cdx2* over-expression + *Stmn1* over-expression group, *Cdx2* expression level in *Cdx2* over-expression + *Stmn1* over-expression + *Stmn1* siRNA group decreased significantly ($P = 0.000$; Fig 3.), which was lower than that in *Cdx2* group ($P = 0.046$; Fig 3.), indicating that blocking the over-expression of *Stmn1*, *Cdx2* was significantly down regulated. However, expression level of *Cdx2* was no difference between *Cdx2* over-expression group and *Cdx2* over-expression + *Stmn1* siRNA group.

Relative protein expression level of *Cdx2* and *Stmn1* in each group was detected by western blot on Wes instrument ($n=3$; Fig 4a, b). *Cdx2* was not expressed on protein level in normal group, which consistent with mRNA level results. In the other 4 groups, *Cdx2* protein abundant among groups was significantly

different ($F = 56.622$, $P = 0.000$). Compared with *Cdx2* over-expression group, the protein level of *Cdx2* in *Cdx2* over-expression + *Stmn1* over-expression group was significantly higher ($P = 0.006$), indicating that *Stmn1* could significantly up regulate *Cdx2* on protein level, which support the mRNA level results. Compared with *Cdx2* over-expression + *Stmn1* over-expression group, *Cdx2* protein abundance in *Cdx2* over-expression + *Stmn1* over-expression + *Stmn1* siRNA group was significantly lower ($P = 0.000$), indicating that blocking *Stmn1* over-expression, *Cdx2* protein expression was significantly inhibited. Compared with *Cdx2* over-expression group, *Cdx2* protein expression level in *Cdx2* over-expression + *Stmn1* siRNA group did not change ($P = 0.540$), indicating that blocking *Stmn1* expression, *Cdx2* protein level expression did not change, consistent with mRNA level change.

Discussion

Gastric cancer is one of the most common malignant tumors worldwide. The chains of process from normal gastric mucosa to intestinal type gastric carcinoma include superficial gastritis, atrophic gastritis, IM, dysplasia, carcinoma, metastasis [23]. We found it over-expressed in chronic atrophic gastritis in rats [24]. Here we found that *Stmn1* was up-regulated in IM in stomach in rats. Moreover, it could induce significant higher expression of *Cdx2*, a key gene in IM, in human gastric GES-1 cells, which suggested that *Stmn1* might involve in the development of IM by up-regulating *Cdx2*. These results initially fill the IM gap of expression level of *Stmn1* in development of gastric cancer. However, clinical evidences about whether *Stmn1* was over-expressed in IM and/or chronic atrophic gastritis in patients need to be studied further.

Over-expression of *Stmn1* alone did not induce *Cdx2* expression, indicating that *Stmn1* could not trigger *Cdx2* expression, but promoted *Cdx2* expression.

Down-regulation of *Stmn1* in *Cdx2* over-expressed group did not inhibit the expression of *Cdx2*, indicating that low expression level *Stmn1* could not regulate *Cdx2* and also knock-down low expression *Stmn1* could not effect *Cdx2*. Only over-expressed *Stmn1* could promote *Cdx2* expression, and knock-down high expression level *Stmn1* could inhibit over-expressed *Cdx2*.

Over-expression of *Cdx2* alone had no effect on *Stmn1* expression, indicating that *Cdx2* could not affect the expression level of *Stmn1*.

Conclusions

Stmn1 was up-regulated in IM in rats in vivo and it could significantly up-regulate an IM essential gene *Cdx2* in human gastric GES-1 cells in vitro. All these results suggested that *Stmn1* might involve in the development of IM by up-regulating *Cdx2*.

List Of Abbreviations

IM: IM; *Cdx2*: Caudal type homeobox gene 2; *Stmn1*: Stathmin 1; MNNG: N-methyl-N'-nitro-N-nitrosoguanidine.

Declarations

Ethics approval and consent to participate

The animal study protocols were approved by the Medical Ethics Committee of Tongde Hospital of Zhejiang Province [No. (2016)040]

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China [grant number, 81703789], Talent Project of Zhejiang Association for Science and Technology [grant number, 2018YCGC007]—major new drug discovery of important national science and technology projects [grant number, 2016ZX09101069], key social development projects of the Zhejiang province major science and technology projects [grant number, 2009C13032], Natural Science Foundation of Zhejiang Provincial [grant number, LQ18H280010]. Funding bodies did not participate in the design of the study, collection, analysis, and interpretation of data or in writing the manuscript.

Authors' contributions

XC, LC and RW generated the conception; XC and GD designed the work; XC, ZR, YT, SC completed the experiments and analyzed the data. XC was a manor contributor in writing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

Figure 1

Photographs of pathological sections of IM in stomach of rats (AB staining) (a) and expression difference of *Stmn1* between normal gastric tissue and IM (b)

Figure 2

Photographs of cells in each group (10×)

Figure 3

Stmn1 and Cdx2 relative expression level among groups using RT-qPCR Compared with CDX1 over-expression group, *P<0.05, ***P<0.001

Figure 4

Comparison of Cdx2 protein abundance (area value) in each group by western blot ** $P < 0.01$ *** $P < 0.001$ (a) and representative western blots of Cdx2, Stmn1 and GAPDH in each group (b)

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