Original Article
Solasonine inhibits gastric cancer proliferation and enhances chemosensitivity through microRNA-486-5p

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Received December 29, 2019; Accepted May 29, 2020; Epub July 15, 2020; Published July 30, 2020

Abstract: Previous studies have proved the anticancer effects of solasonine against a number of human cancers. Considering this, the present study was designed to explore the anticancer effects of solasonine against the human gastric cancer cells with an emphasis on elucidation of the underlying molecular mechanism. The results showed that solasonine significantly \( P < 0.05 \) inhibited the cancer cell proliferation and also reduced the colony forming potential of gastric cancer cells. The antiproliferative effects of solasonine were due to the induction of apoptosis in the gastric cancer cells as evident from the DAPI, AO/EB and PI staining assays. Further, the chemosensitivity of gastric cancer cells was seen to be enhanced markedly under solasonine administration. Solasonine was shown to exert its anticancer effects through miR-486-5p and its treatment increased the expression of miR-486-5p significantly. The up-regulation of miR-486-5p imitated the growth inhibitory effects of solasonine treatment on gastric cancer cells. The miR-486-5p in turn exerted its molecular role by targeting PIK3R1. The results of this study are suggestive of anticancer role of solasonine against the gastric cancer via modulation miR-486-5p/PI3KR1 axis.

Keywords: Gastric cancer, solasonine, anticancer, glyalkoloid, micro RNA

Introduction
Cancer is one of the lethal human disorders involves the loss of normal control over cell division and growth [1]. This malignancy is associated with very high mortality rates. The current anticancer strategies employ the usage of low-molecular weight drug molecules and radioactive radiation therapies [2]. These strategies are focused at the selective targeting of cancerous tissues with little or no ill-effects on the normal body tissues. However, such ideal effects are very difficult to achieve and the researchers are continuously looking for the improvement of the results of anticancer approaches. A vast number of studies of recent times were conducted to elucidate the anticancer effects of plant based natural products. The plant derived natural compounds possess health beneficial properties and have antioxidant, anti-inflammatory, antimicrobial and antitumor activities which make them ideal to serve as lead molecules in drug discovery [3]. Keeping these facts in mind, the current research study was aimed to investigate the anticancer role of a plant based compound, solasonine, against the human gastric cancer. Gastric cancer is among the most prevalent human disorders in Eastern Asia [4]. It ranks 4th in terms of the prevalence rates. Additionally, it is ranked as 3rd principal cause of cancer related mortality worldwide [5]. The conditions in China are particularly worse and in 2015 near about 0.5 million deaths occurred because of this malignancy. Although the recent anticancer efforts have attained a fair bit of success in combating the human gastric cancer but the overall 5-year survival rates are still very low suggesting an urgent need for exploring the more effective anticancer measures against the gastric cancer [6]. In this regard, the current study analyzed the anticancer role of solasonine against the human gastric cancer cells. Solasonine is a steroidal glycoalkaloid, extracted from *Solanum nigrum* plant [7]. It has been found to possess anti-proliferative effects against the human cancer cell lines and acts as a vital antitumor agent [8]. Taking a lead from these findings, observed that solasonine repressed the proliferation of cancer cells in a dose dependent manner. Interestingly, the proliferation of normal gastric cell line was not affected much. The effects
Solasonine inhibits gastric cancer proliferation

were also manifested as reduction in the colony forming potential of gastric cancer cells when administered with solasonine. The results also indicated that the gastric cancer cells when treated with solasonine became comparatively more susceptible to paclitaxel drug molecules. At molecular level, the anticancer effects of solasonine were seen to be exerted through targeting of microRNA-486-5p (miR-486-5p). Solasonine effectively enhanced the expression of miR-486-5p. Additionally, miR-486-5p targeted phosphotidylinositide-3-kinase, regulatory subunit 1 gene (PIK3R1), post-transcriptionally to exert its regulatory control in gastric cancer. The enrichment of microRNA-486-5p expression was found to mimic the anticancer effects of solasonine on the gastric cancer cells. Taken together, the results of this study highlighted the importance of plant based natural compounds as lead molecules in anticancer drug discovery.

Materials and methods

Procurement of cell lines and their culture, maintenance and transfection

The normal human gastric cell line (GES-1) along with the cancer cell lines (SNU1 and SNU5) were purchased from the American Type Collection Center (ATCC), USA. The Dulbecco’s modified Eagle’s medium (DMEM, Thermo Scientific) was used for culturing of cell lines at 37°C. The CO$_{2}$ humidified incubator was used for maintaining the cell lines with 5% CO$_{2}$ concentration. At the 80% growth saturation, the AGS and SNU1 cell lines transfected with miR-486 mimics or its negative control construct (miR-NC) alone or in combination with luciferase reporter constructs of ST5. The transfection was done using the reagent Lipofectamine 2000 (Thermo Scientific) using the manufacturer protocol.

Determination of cell proliferation rate

The analysis of cell proliferation was made by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide reagent (MTT, Thermo Scientific), as per the manufacturer guidelines. In this assay, the cells were cultured for 24 h at 37°C in 96-well plates with initial cell count of $1 \times 10^5$ cells/well. The wells were incubated with MTT reagent and 37°C incubation was prolonged for 4 h. Afterwards DMSO was added to each well to dissolve the product formed, i.e., formazan. The samples were processed for estimations of formazan concentrations using spectrophotometric absorbance-based quantification method by recording absorbance of each sample at 570 nm. The absorbance readings were taken as the measure for estimating the cell proliferation rate.

Clonogenic assay

The cell lines were cultured in the 6-well plates for 2 weeks period with 5% CO$_{2}$ concentration and 37°C temperature. The cultures were collected by centrifugation and the pellets were fixed with methanol after being washed several times with phosphate buffered saline (PBS) buffer. The wells were then stained with solution of 0.1% crystal violet (Thermo Scientific). The samples were then assessed for the respective colony numbers and the photographs were also taken.

Apoptosis assays

The SNU1 and SNU5 cancer cells treated with 0, 10, 20 and 40 µM solasonine for 24 h were centrifuged and collected cell pellets were washed with PBS, fixed using 70% ethanol. The cells were then stained with 4',6-diamidino-2-phenylindole (DAPI), acridine orange and ethidium bromide (AO/EB) dual staining mix or propidium iodide (PI) and analyzed for the morphological examinations under the fluorescent microscope.

Annexin V-FITC/PI fluorescent staining

The annexin V-FITC/PI staining assay was performed to assess the level of cell apoptosis under 0, 10, 20 and 40 µM solasonine administrations for 24 h. The treated SNU1 and SNU5 cancer cells were then fixed using methanol and stained with dual annexin V-FITC/PI staining solution. Then, the cells were examined by flowcytometry to determine the percentage of apoptotic cells.

RNA isolation, cDNA synthesis and qRT-PCR

The Trizol reagent (Thermo Scientific) was used to isolate the total RNA from the cell lines, following the manufacturer guidelines and the DNA was purified from the RNA contamination by DNase treatment. The RNA was then reverse transcribed to complementary DNA (cDNA) using First Strand cDNA synthesis kit (Thermo Scientific) as per kit protocol. The gene expression studies for analyzing the expression leve-
Solasonine inhibits gastric cancer proliferation

Is of genes of interest employed the usage of SYBR Green mix (Thermo Scientific) to perform the quantitative Real Time PCR (qRT-PCR) using StrepOnePlus™ Real-Time PCR system (Thermo Scientific). The relative expression levels of respective genes was inferred using 2^−ΔΔCt method The human GADPH gene was used as an internal control in the qRT-PCR experiments.

**In silico microRNA target inference and dual luciferase reporter assay**

To identify the intracellular transcripts targeted specifically by the miR-486-5p, in silico analysis was performed by using online bioinformatics software tools like TargetScanHuman7.2 (http://www.targetscan.org/vert_72/), microRNA.org (http://34.236.212.39/microrna/home.do) and miRDB (http://www.mirdb.org/). Dual-Luciferase Reporter Assay System (Promega) was used for the interactional assessment of miR-486-5p and its intracellular identified target PI3KR1. The 3’ upstream fragment of PI3KR1, as native (WT) or mutated (MUT) was cloned into psiCHECK-2 luciferase vector. The luciferase construct bearing either 3’ UTR-WT or 3’ UTR-MUT was co-transfected with miR-NC or miR-486 mimics into gastric cancer cells. After 48 h and using renilla luciferase activity as control, the luciferase activities were estimated for different co-transfection combinations to assess the level of interaction of miR-486-5p with reporter constructs of PI3KR1.

**Western blotting analysis**

Prior to performing the western blotting technique, the total proteins of cell lines were extracted using RIPA lysis buffer (Thermo Scientific) and their concentrations were estimated through Bradford method. Equal loading was done for each sample on the PAGE gel. The PVDF membranes were using for blotting the gel contents. The protein concentrations of proteins of interest were assessed through chemiluminescence method after their exposure to respective primary and secondary antibodies specifically designed for the purpose.

**Statistical analysis**

For the statistical validity of experimental results, the mean was calculated as final results were given as mean ± standard deviation (SD). Student’s t test was performed using the Minitab 18 software. The calculated p values less than 0.05 were representative of statistically significant difference between two values.

**Results**

**Inhibition of gastric cancer cell proliferation and colony formation by solasonine**

The administration of gastric cancer cell lines (SNU1 and SNU5) and normal cell line GES-1

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**Figure 1.** Solasonine selectively inhibits proliferation of gastric cancer cells. (A) Molecular structure of solasonine (B) MTT assay for proliferation rate assessment of the normal GES1 and Gastric cancer SNU1 and SNU5 cells. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).
Solasonine inhibits gastric cancer proliferation

![Graph showing solasonine concentration vs. colony formation](image)

**Figure 2.** Colony forming assay of gastric cancer cells. Solasonine inhibited the proliferation of the human SNU1 and SNU5 gastric cancer cells dose dependently. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).

with 0, 2.5, 5.0, 10, 20, 50, 80 or 110 µM concentration of solasonine, a glyalkoloid (Figure 1A), was made for time duration of 24 h. The cultures were then processed for proliferation rate estimations through MTT assay and proliferation rates were compared in terms of percent values. The results showed that the solasonine inhibited the proliferation of cancer cell lines (SNU1 and SNU5) in a dose dependent manner. The IC\textsubscript{50} value of solasonine against the SNU1 was 10 µM and it was 12.5 µM against the SNU5 cell line (Figure 1B). Although, the inhibition of proliferation rate was also observed against the normal cell line, GES-1 but the effects were not so significant where very high IC\textsubscript{50} value of about 110 µM was observed. Hence, it is evident that solasonine inhibits the proliferation of gastric cancer cells, selectively without much harm on the normal gastric cells. The growth inhibitory role of solasonine against gastric cancer was also noticed in terms of its effect on the colony forming potential of gastric cancer cell lines where the colony number was seen to be reducing in a solasonine concentration dependent fashion (Figure 2). The DAPI, AO/EB as well as the PI staining showed that Solasonine exerts its anti-cancer effects by inducing apoptotic cell death in SNU1 and SNU5 cells in a dose dependent manner (Figure 3A and 3B). Additionally, the annexin V/PI staining revealed that the percentage of the SNU1 and SNU5 gastric cancer cells increased remarkably upon solasonine treatment (Figure 3C).

**Solasonine enhances chemosensitivity of gastric cancer cells**

To infer about the potential of solasonine in enhancing the chemotherapeutic effect of paclitaxel anticancer drug molecules, the gastric cancer cells (SNU1 and SNU5) were treated with 4 µM paclitaxel and administered with 0, 10, 20 or 40 µM solasonine for 24 h. The results indicated that the solasonine treatment increased the chemosensitivity of both SNU1 and SNU5 cancer cells to paclitaxel molecules (Figure 4A and 4B). The increase in the chemosensitivity of gastric cancer cells was more prominent at higher doses of solasonine treatment.

**The miR-486-5p modulated the anticancer effects of solasonine against gastric cancer**

To infer about the molecular mechanics governing the anti-cancer effects of solasonine against the gastric cancer cells, the normal and gastric cancer cell lines were processed for RNA isolation, cDNA synthesis and then qRT-PCR analysis was performed. It was observed that one of the important regulatory microRNAs, miR-486-5p was significantly down-regulated in solasonine (20 µM) treated gastric cell...
Solasonine inhibits gastric cancer proliferation

A

SNU1
Salsonine (µM)

0 10 20 40

AO/EB DAPI

PI

B

Salsonine (µM)

0 10 20 40

AO/EB DAPI

PI

C

Salsonine (µM)

0 10 20 40

SNU1

SNU5
Solasonine inhibits gastric cancer proliferation

Figure 3. Solasonine induces apoptosis in the gastric cancer cells. A. The DAPI, AO/EB and PI staining assays showed that Solasonine activated apoptosis in the SNU1 cells. B. The DAPI, AO/EB and PI staining assays showed that Solasonine activated apoptosis in the SNU1 and SNU5 cells. C. Annexin V/PI staining assay showing the percentage of apoptotic cells at indicated concentrations of solasonine. The experiments were performed in triplicate.

Figure 4. Effect of solasonine on chemosensitivity of gastric cancer cells. Proliferation rates of SNU-1 and SNU5 cancer cells at indicated dosage of Solasonine and 4 µM paclitaxel. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).

Figure 5. miR-486-5p modulates the effects of solasonine. (A) qRT-PCR for miR-486-5p expression in cancer cell lines and normal cell line treated with solasonine (20 µM) (B) Over-expression of miR-486-5p in cancer cell lines (C) miR-486-5p mimics the role of solasonine (20 µM). The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).

miR-486-5p was obtained by transfecting the gastric cancer cell lines with miR-486-5p mimics and confirmed by qRT-PCR (Figure 5B). The miR-486-5p expression was up-regulated by 8.1 and 7.3 folds, respectively in SNU1 and SNU5 cancer cell lines. This up-regulation of miR-486-5p not only declined the proliferation of cancer cells (Figure 5C) but also increased their chemosensitivity towards the paclitaxel drug molecules. These findings advocate that miR-486-5p is the molecular regulator for exerting the anticancer effects of solasonine against the gastric cancer.

Pi3KR1 is targeted by miR-486-5p to exert its regulatory role in gastric cancer

The in silico target analysis through bioinformatics tools indicated that miR-486-5p specifically targets Pi3KR1 (Figure 6A). miR-486-5p targets Pi3KR1 was further supported by the gene expression pattern and western blotting of Pi3KR1 in normal and cancerous gastric cell lines (Figure 6B).
Solasonine inhibits gastric cancer proliferation

Figure 6. miR-486-5p targets PI3KR1 in gastric cancer. A. In silico target analysis of miR-486-5p target identification. B. Gene expression analysis of PI3KR1 in cell lines. C. Protein expression analysis of PI3KR1 in cell lines. D. Dual luciferase assay. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).

Figure 7. PI3KR1 modulates the anticancer effects of solasonine. Effects of miR-PI3KR1 on the viability of the SNU1 and SNU5 gastric cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*P < 0.05).

interaction was finally confirmed by the dual luciferase assay. The co-transfection of 3’ UTR-WT or 3’ UTR-MUT fragment of PI3KR1 with miR-NC or miR-486 mimics into SNU1 and SNU5 cancer cell lines indicated that the luciferase activity was significantly lower in both cancer cell lines where PI3KR1 3’ UTR-WT was co-transfected with the miR-486 mimics (Figure 6C). The attenuation of growth inhibitory effects of solasonine treatment or miR-486-5p up-regulation by the transfection of cancer cell lines with pCDNA-PI3KR1 indicated that the anticancer effects of either solasonine or miR-486-5p are exerted through PI3KR1 intracellularly (Figure 7). Taken together, the results support that solasonine experiences its anticancer role against human gastric cancer through miR-486-5p/PI3KR1 axis.

Discussion

Over the years, studies have attempted to explore the role of natural compounds in restricting the growth and tumorigenesis of human which has lead to the several anticancer drugs [9, 10]. These studies have not only enhanced our understanding about the potential measures of cancer treatment but also the mechanics of the deadly disease has become more evident. Human gastric cancer is one of the major health issues which is seen with high mortality and low 5-year survival rates because of the lack of early diagnosis and relatively inefficient treatment strategies [11]. There is a growing need for evaluating the battery of natural compounds against the human gastric cancer to search and design better chemothera-
Solasonine inhibits gastric cancer proliferation

Conclusion

To conclude, the current study revealed the anticancer role of solasonine against the growth and progression of gastric cancer and the molecular mechanism of its action. The study indicated that the anticancer effects of solasonine are exerted via miR-486/Pi3KR1 axis. These findings point towards the therapeutic potential of solasonine in the treatment of gastric cancer.

Disclosure of conflict of interest

None.

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Solasonine inhibits gastric cancer proliferation


