Nardostachys jatamansi Root Extract Modulates the Growth of IMR-32 and SK-N-MC Neuroblastoma Cell Lines Through MYCN Mediated Regulation of MDM2 and p53

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Submitted: 20-04-2016 Revised: 11-07-2016 Published: 06-01-2017

ABSTRACT

Aim: The present study evaluated the effect of ethanolic extract of Nardostachys jatamansi roots (NJ) on MYCN mediated regulation of expression of MDM2 and p53 proteins in neuroblastoma cell lines, IMR-32 and SK-N-MC. Materials and Methods: The effect of NJ on cell viability was determined by MTT, and on growth kinetics was evaluated by trypan blue dye exclusion method and soft agar assay. The expression of p53, MDM2 and MYCN proteins in response to NJ treatment was evaluated by immunoblotting. Results: NJ decreased the viability of neuroblastoma cells without affecting the viability of non-cancerous, HEK-293 cells. It altered the growth kinetics of the cancer cells in a dose-dependent manner. NJ down regulated the expression of MYCN and MDM2 proteins with a simultaneous increase in the expression of tumor suppressor protein p53. Conclusions: The present data demonstrated that NJ regulated the growth of IMR-32 and SK-N-MC through reduction in MYCN expression that lead to down regulation of MDM2 protein and increase in p53 expression. These preliminary results warrant further in depth studies to explore the therapeutic potential of Nardostachys jatamansi in the management of neuroblastoma.

Key words: Neuroblastoma; Nardostachys jatamansi; MYCN; p53; MDM2

SUMMARY

- NJ reduced the viability of human neuroblastoma cell lines without affecting the viability of non-cancerous, HEK-293 cells.
- NJ regulated the growth kinetics of the cancer cells.
- NJ decreased the expression of MYCN and MDM2 proteins and simultaneously increased the expression of tumor suppressor protein p53.

INTRODUCTION

Nardostachys jatamansi (Valerianaceae), commonly known as muskroot, is indigenous to the Himalayan regions of India.[1] In Ayurveda, N. jatamansi has been used traditionally since centuries for treating various neurological disorders. It has been used as a sedative, an antispasmodic, for mind rejuvenation, promotion of sleep, alleviating mental diseases and as a brain tonic.[2] It has also been used for treating headache, insomnia, epilepsy; disorders of cardiovascular system; flatulence and intestinal colic as well as menopausal symptoms.[2] N. jatamansi has been shown to possess anti-inflammatory,[3] anti-oxidant,[4] anti-bacterial,[5] anticonvulsant[6] and hepatoprotective activities.[7] It has been shown to exhibit anti-tumour activity in lung, liver, ovary, breast and prostate cancers.[8] Recently, the higher doses of alcoholic and n-hexane extract of N. jatamansi were shown to significantly inhibit the growth of neuroblastoma cell lines, IMR 32 and SK-N-SH.[4]

In the present study, we have shown that ethanolic extract of N. jatamansi roots (NJ) significantly altered the growth kinetics of IMR-32 and SK-N-MC neuroblastoma cells at lower doses through down regulation of MYCN and MDM2 proteins and upregulation of p53.

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Cite this article as: Suryavanshi S, Raina P, Deshpande R, Kaul-Ghanekar R. Nardostachys jatamansi root extract modulates the growth of IMR-32 and SK-N-MC neuroblastoma cell lines through MYCN mediated regulation of MDM2 and p53. Phcog Mag 2017;13:21-4.
MATeRIALS AND METHODS

Drugs and chemicals
Tissue culture plasticware was purchased from BD Biosciences (CA, USA). DMEM powder, penicillin and streptomycin were obtained from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Valeric acid and primary antibody against MYCN were purchased from Sigma-Aldrich (St. Louis, MO). Primary antibody against p53 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Plant material, extract preparation and preliminary phytochemical analysis
N. jatamansi roots were purchased from Green Pharmacy (Pune, Maharashtra, India). The sample was authenticated and validated macroscopically and microscopically at the Department of Botany, Agharkar Research Institute (ARI), Pune (Maharashtra, India) (ref no.1045). Voucher specimen sample (Number R-154) has been deposited at the Department.

Cell culture
The cell lines (IMR-32, SK-N-MC and HEK-293) used in the present study were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in DMEM containing 2 mM L-glutamine supplemented with 10% fetal bovine serum and 20 U/ml of penicillin-streptomycin and incubated in a humidified 5% CO₂ incubator at 37°C.

Cell viability
IMR-32, SK-N-MC and HEK-293 were seeded at a density of 1 × 10⁴ cells/ml in a 24-well plate. The cells were treated with different concentrations of N. jatamansi root extract (0-160 µg/ml) in triplicates for 24 h. Cell viability was determined by MTT assay as described previously.供给侧

Cell growth analysis
IMR-32 and SK-N-MC were seeded at a density of 5 × 10⁴ cells/well in a 24-well plate. The cells were treated with different concentrations of N. jatamansi root extract (0-20 µg/ml) in triplicates for 24, 48 and 72 h. The effect of N. jatamansi root extract on cell growth was determined by trypan blue dye exclusion method as described previously.供给侧

Soft agar assay
IMR-32 and SK-N-MC cells (5 × 10⁴ cells/ml) treated with different concentrations of N. jatamansi root extract (0-20 µg/ml) were mixed with culture medium containing 0.35% agarose and plated over a previously gelled layer of 0.5% agarose as described previously.供给侧 The colonies were manually counted and photographed directly using an Axiosvert 200 M microscope (Carl Zeiss, Germany).

Western blotting
IMR-32 and SK-N-MC cells were plated at a seeding density of 5 × 10⁴ cells/well in a 6-well plate and incubated for 24 h at 37°C in CO₂ incubator. Next day, the cells were treated with different concentrations of N. jatamansi root extract (0-20 µg/ml) for 24 h. The cells were lysed as previously described.供给侧 The protein was estimated using Bradford reagent (Biorad Laboratories Inc, CA, USA). Equal amount of protein was loaded on a 10% SDS-polyacrylamide gel and transferred electrophoretically to Amersham Hybond-P PVDF membrane (GE Healthcare, UK) in sodium phosphate buffer (pH 6.8). The membrane was blocked in 5% BSA in TBST and incubated at room temperature for 3 h with mouse monoclonal primary antibodies against p53, MDM2 and tubulin (Santacruz, CA, USA) at 1:2000 and MYCN (Sigma-Aldrich, St. Louis, MO) at 1:500 dilutions respectively. The blots were washed in TBST and incubated with donkey anti mouse IgG HRP conjugate at 1:4000 (for p53, MDM2, tubulin) and 1:2000 (for MYCN) dilutions. Proteins were visualized using a chemiluminescence kit (Amersham ECL Advance western blotting detection kit, GE Healthcare, UK) and densitometric analysis of X-ray films was performed using the Image J gel analysis tool.

Measurement of apoptosis
The cells were plated at a seeding density of 5 × 10⁴ cells/well in a 6-well plate and treated with different concentrations of N. jatamansi root extract (0-20 µg/ml). After 24 h of treatment, the cells were harvested and washed with PBS twice. Cells were stained with Annexin V-FITC following the manufacturer’s instructions (Annexin V-FITC apoptosis kit #3, Invitrogen) and analyzed for apoptosis by FACS using Cell Quest Software.供给侧

Statistical analysis
All the results were obtained from three independent experiments, each performed in triplicates and the values have been presented as mean ± SD. Differences among means were tested for statistical significance using one-way analysis of variance (ANOVA). The analyses were carried out using Graph-pad prism 5 software (San Diego, CA, USA). *p < 0.05; **p < 0.01; ***p < 0.001 were considered to be statistically significant.

RESULTS

Chromatographic analysis of N. jatamansi root extract
HPTLC chromatographic examination of N. jatamansi root extract based on its marker compound, valeric acid [Figure 1]. The finger print profile was developed in toluene: ethylacetate: formic acid (8:2:0.5) solvent system and the peaks corresponding to valeric acid were recorded at Rf 0.75. Similar peaks were found in chromatographic profile of N. jatamansi root extract at the same Rf value of 0.75. The calibration curves were linear in the range of 200-1200 ng of valeric acid. The amount of valeric acid in N. jatamansi root extract was found to be 41.58 mg/g.

N. jatamansi altered growth kinetics of neuroblastoma cell lines
In IMR-32 and SKNMC, N. jatamansi root extract inhibited the growth of neuroblastoma cell lines exhibited IC₅₀ values of 61.43 and 63.42 µg/ml, respectively [Figure 2]. Contrarily, in non-cancerous HEK-293 cells, IC₅₀ was not obtained up to 160 µg/ml concentration of N. jatamansi root extract [Figure 2]. Thus, N. jatamansi significantly reduced the viability of neuroblastoma cells at lower doses without affecting the viability of the non-cancerous cells. Further studies were carried out at non-cytotoxic doses (0-20 µg/ml) of N. jatamansi root extract to analyze its effect at cellular and molecular level.

In IMR-32, N. jatamansi root extract reduced the cell proliferation to ~2-fold at 24 h and by ~1.5-folds at 48 and 72 h (p < 0.001) [Figure 3A]. On the other hand, in SK-N-MC, the proliferation was reduced by ~2- (p ≤ 0.001), 3—(p < 0.001) and 2- folds (p < 0.001), respectively at 24, 48 and 72 h compared to the untreated control cells [Figure 3B]. The effect of N. jatamansi root extract on cell growth was further evaluated by soft agar assay that showed a dose dependent decrease in the number of soft agar colonies. At 20µg/ml, there was ~2.5- (p ≤ 0.001) and 3.5-folds...
increased the expression of p53 through down regulation of MYCN and MDM2

**DISCUSSION**

The present study reported the mechanism of anti-cancer potential of *N. jatamansi* root extract (NJ) in the human neuroblastoma cell lines, IMR-32 and SK-N-MC. NJ reduced the viability of neuroblastoma cell lines at lower doses (40 µg/ml onwards) compared to the non-cancerous cells. At 80 µg/ml dose, NJ reduced the cell viability of IMR-32 and SK-N-MC by 70 and 62%, respectively, with IC_{50} values of 61.43 and 63.42 µg/ml, respectively. It negatively regulated the growth kinetics of neuroblastoma cells in a significant manner and effectively reduced their proliferation. Previously, Monga and Kumar in 2013 had reported the anti-proliferative potential of alcoholic extract and n-hexane fraction of roots of *N. jatamansi* against neuroblastoma cell lines, IMR-32 and SK-N-SH. They had shown that at 100 µg/ml dose, the alcoholic extract reduced the proliferation of IMR-32 and SK-N-SH by 71 and 85%, respectively, whereas at the same dose hexane fraction reduced the proliferation by 91 and 82% respectively. Interestingly, in our study NJ reduced the viability at much lower doses compared to that reported by Monga and Kumar, which is excellent for drug development point of view. The difference in these results could be due to time of collection of the roots, method of preparation of the extract and the method of cell proliferation assay. Various reports have shown anticaner activity of herbal formulations against neuroblastoma cell lines. PienTze Huang, a popular Chinese medicine for liver diseases, has been reported to decrease survival of SH-SY5Y neuroblastoma cell line. The leaf extracts of *Holarrhena antidysenterica* have been reported to be cytotoxic to SK-N-MC neuroblastoma cells. The leaf extracts of *Calotropis procera, Ocimum sanctum* and *Cannabis sativa* have been shown to be cytotoxic to IMR-32. However, neither of the studies has delineated the molecular mechanisms underlying the anticancer activity of these medicinal plants.

In the present work, we have shown the preliminary mechanism of action of NJ in neuroblastoma cell lines. We found that NJ significantly down regulated the expression of MYCN, the major negative prognostic marker in neuroblastoma with significant role in the pathogenesis and clinical onset of this aggressive malignancy. MYCN has been reported to activate proliferative cellular pathways and inhibition of p53-mediated apoptosis has been considered as the prerequisite for MYC-driven tumorigenesis. Interestingly, NJ not only decreased the expression of MYCN but simultaneously upregulated the expression of p53. Moreover, NJ significantly reduced the expression of MDM2, the negative regulator of p53. These results lead us to study the mechanism of cell death induced by NJ, which was found to induce apoptosis in IMR-32 and G2/M arrest in SK-N-MC cells (data not shown).

All these data suggested the potential of ethanolic extract of *N. jatamansi* (NJ) in regulating the growth of neuroblastoma cells.
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CONCLUSION
The present study has evaluated the mechanism of anticancer activity of N. jatamansi in IMR-32 and SK-N-MC neuroblastoma cell lines. However, further in-depth studies are warranted against other neuroblastoma cell lines as well as in vivo studies need to be done to establish the chemopreventive potential of N. jatamansi.

Acknowledgement
The authors thank Director of IRSHA for his generous support and encouragement. The authors would like to acknowledge Dr. Anand Zanwar, Department of Food and Nutrition Lab, for helping in HPTLC profiling of the extract.

Financial support and sponsorship
This work was supported by funding from the Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University.

Conflicts of interest
There are no conflicts of interest.

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Figure 3: Effect of N. jatamansi extract on growth kinetics of neuroblastoma cells. (A) IMR-32 and (B) SK-N-MC cell lines were treated with N. jatamansi (0-20 μg/ml) for 24, 48 and 72 h and the number of viable cells were counted by trypan blue dye exclusion method. Data represent means ± SD of three independent experiments (C) IMR-32 and SK-N-MC cell lines were treated with N. jatamansi (0-20 μg/ml) and grown in soft agar for two weeks. Colonies were counted from at least 10 different areas and the average of each has been plotted. The data represents mean ± SD of five independent experiments.

Figure 4: Effect of N. jatamansi on the expression of tumor regulatory markers. (A-E) IMR-32 and SK-N-MC cell lines were analyzed for the expression of p53, MDM2 and MYCN proteins by western blotting. Tubulin was used as a loading control.