Print ISSN: 2319-2003 | Online ISSN: 2279-0780

IJBCP International Journal of Basic & Clinical Pharmacology

DOI: http://dx.doi.org/10.18203/2319-2003.ijbcp20180653

Original Research Article

Differential genotypical expression of a NEDD9 in normal and tumor tissues: a possible pharmacological target

Pavan Kumar G. S.[1], Krishna V.[2], Prabahkar B. T.[1]*

[1]Department of Molecular Biomedicine Laboratory, Sahyadri Science College (A), Kuvempu University, Shivamogga, Karnataka, India
[2]Department of Studies and Research in Biotechnology and Bioinformatics, Jnanasahyadri, Kuvempu University, Shankaraghatta, Shivamogga, Karnataka, India

Received: 11 January 2018
Accepted: 02 February 2018

***Correspondence to:**
Dr. Prabahkar B. T.,
Email: prabhakarbt@gmail.com

ABSTRACT

Background: Neural precursor cell expressed developmentally down regulated-9 (NEDD9) is a scaffolding metastatic marker protein in multiple cancer types. Generally, the expression occurs during the embryonic development and depletes in adults. Expression of NEDD9 in adults leads to the progression of tumor which is sufficient for the cellular invasion. Elevated behavior of the gene mediates metastatic movement which includes protease dependent neovessel formation, invasion and migration of tumor cells from the site of origin to the distant tissues.
Methods: The current study involves the screening and elucidation of differential expression of NEDD9 in normal and tumor subtypes with various tissues of mice by immunohistochemistry.
Results: The validating approaches in the study, low expression of NEDD9 was observed in the normal tissues and predominance in the tumor subsets.
Conclusions: The experimental analysis proven that NEDD9 expression is merely associated with tumor progression and the molecular mechanism of NEDD9 is restricted in the establishment of metastatic cascade. NEDD9 association in tumor prognosis which helps in the emergence of diagnostic and therapeutic approaches.

Keywords: Angiogenesis, Diverse tissues, In-vivo tumor, Immunohistochemistry metastasis, NEDD9

INTRODUCTION

Tumorigenesis is a serious process with a multiple pathogenesis, which threats the life seriously. Progression of tumor is associated with several hallmarks comprised of cell signaling, angiogenesis, sustained growth signals and other characteristics.[1] Several investigations shown, many biological agents involved in the tumor differentiation are termed as marker genes, there expression plays a vital part in the tumor microenvironment fallout with tumor aggressiveness. Several markers such as, HIF-1α, VEGF, TGF-β, Vimentin, NF-κB etc., are obeying the rules during tumor metastatic cascade.[2-6] To investigate, the role of tumor markers in-vivo, animal models help to understand the molecular mechanism in the establishment of tumor. Because, various types of tumor shows numerous characteristic behavior in the expression of genes that altered in normal condition. The tumor environment includes a set of marker genes, it describes a group of

genes that are expressed in various tumors. In tumor progression necessitated with major cellular signals involves unlimited cell proliferation, angiogenesis, survival in circulation and establishing metastatic colonization.[7]

Neural Precursor Cell Expressed Developmentally Down regulated-9 (NEDD9), it is a CRK-associated substrates family member gene, commonly termed as Cas-L and HEF-1. It has a set of genes with corresponding sequence tags predominantly expresses during embryonic development in brain, but deficient in matured mice. It is a scaffolding non-catalytic multi-domain protein which plays a crucial role in tumorigenesis and metastasis.[8,9] Several researches understanding of NEDD9, it has own pleiotropic mechanism in carcinogenesis by comprising of complex structures that regulates the oncogenic signals with various kinases. The NEDD9 signaling cascade involves multiple processes of cell growth, anoikis resistance, tumor neoplasia, invasion, migration and metastases.[10,11] The function NEDD9 linked with potentiality in tumor prognosis, metastatic latency and chemotherapeutic resistance. Because of these functions, it is well marked as to contribute in the cause of malignancy. Certainly, animal cancer models will show the predominance of these protein functions in multiple stages of tumor progression.[12] Important mechanism of NEDD9 in cancer metastases and neoplastic transformation involves the epithelial plasticity. A downstream biological change includes the elevated expression of tumor associated biomarkers, neovessel sprouting factors, resistance to cell death and release of extracellular matrix mechanism. Elevated expression of NEDD9 leads to the extracellular activity in the degradation of endothelial basement membrane of normal capillary tissues results in the formation of tumor angiogenesis. Consequently, tumor cells infiltration into the lymphatic vessels and finally ends up with metastases.[13]

Overarching goal of our current investigation is to evaluate the predominance of NEDD9 association in tumor prognosis which helps in the emergence of diagnostic and therapeutic approaches. In the present study, we screened the NEDD9 expression from solid tumor and ascites tumor tissue types and assessed against the normal tissues to confirm the role of gene in tumor development.

METHODS

Chemicals and reagents

Ehrlich ascites tumor cell line (EAT) for the tumor development was obtained from the Amala Cancer Research Centre, Kerala, India. Immunohistochemistry (IHC) reagents (Leica Biosystems, Germany), NEDD9 antibody (Santacruz Biotechnoloy, Europe) were employed in the study. General chemical for experimental analysis were procured from Merck Millipore, Himedia. For photographic documentation inverted Fluorescent microscope from Thermoscientific, USA was used.

Animal use and ethics

In the current study Swiss albino strain mice with 20-25 gm (n=6/group) were used. The animals were maintained at standard laboratory condition. For animal experimentation the ethical clearance was approved by IAEC (Institutional animal ethics committee) Ref. SJMCP/IAEC/06/2015-16 and NCP/IAEC/CL/101/05/2012-13.

Tumor induction

EAT cells were drawn from donor tumor bearing mouse and the cells were quantified to $5x10^6$ cells/animal. The cells were taken in 26 gauze needle syringes and infused into the intraperitoneal (i.p.) hollow space of albino mouse and permissible for tumor growth with massive abdominal swelling. The cells in the ascites model the life span of mice is restricted to 10-12 days.[14,15]

For the induction of solid tumor, Cells were taken from the tumor mouse and without dilution along with the ascetic fluid $1x10^6$ cells/animal were injected to thigh region of experimental mouse and observed for the solid tumor to final stage. The animal with solid tumor type will survive for 40-60 days.[2,16]

Quantification of tumor growth

On the 12^{th} day on ascites injection the ascites carcinoma mice were sacrificed and dissected to examine the tumor growth like packed cell volume (PCV) and peritoneum angiogenesis.[14] On 45^{th} day of solid tumor induction, the animal was sacrificed and the tumor was dissected, tumor weight was noticed and tumor mass was documented using vernier calipers.[16,17]

Tissue isolation and histological assessment

For histological examination to understand the tissue modulation against normal tissue level was validated. Lung, Skin portion (Solid tumor in solid tumor model and peritoneum in ascites model), stomach and colon were surgically removed and fixed in formalin solution histology. The formalin fixed tissues were processed for histological analysis by as described previously.[18,19] In brief, the tissues were embedded in paraffin and sectioned of 5µm thickness. The sections were fixed on glass slide and H and E staining was performed. The images were taken using phase contrast inverted fluorescent microscope.

Immunohistochemistry (IHC) examination for NEDD9

For the analysis of protein expression level in normal and tumor tissue types immunohistochemistry was performed to assert the correlation. The paraffin embedded tissues

were subject for IHC analysis as mentioned earlier.[20] In short, the slides were de-paraffinized at 60^0c subsequently dipped in xylene, alcohol for 5-10 min each and rehydrated with water. Antigen retrieval was done by keeping the slides in 0.01M sodium-citrate buffer at (P^H 6.0) for 20 min under steaming pressure. Using IHC reagents (Novolink Polymer Detection System from Leica Biosystems) protease and proteins were blocked and washed in Tris Buffered Saline and incubated with NEDD9 primary antibody at 1:50 dilution, washed and blocked with post primary block, and secondary antibody for 30 min each at room temperature. Reaction intensity was developed with DAB reagent, counterstained by haematoxylin. The stained slides were processed again with xylene, gradient alcohol (80%, 90%, 100%) followed by DPX mount. The images were documented using fluorescent microscope.

Statistical analysis

The investigated values in the experimentation were expressed in standard error mean (SEM) and the statistical significance of the outcome was assessed by ANOVA and Student's t-test. The significance values were expressed in ≤ 0.05.

RESULTS

Tissue level modulation in normal and tumor types

The experiment was performed in ascites carcinoma and solid tumor model system using Ehrlich Ascites Carcinoma (EAC) cells in Swiss albino mice. The results showed a massive change in the morphology and physiology of the mice in both ascites and solid tumor mice compared to normal (Figure 1A, Figure 1B).

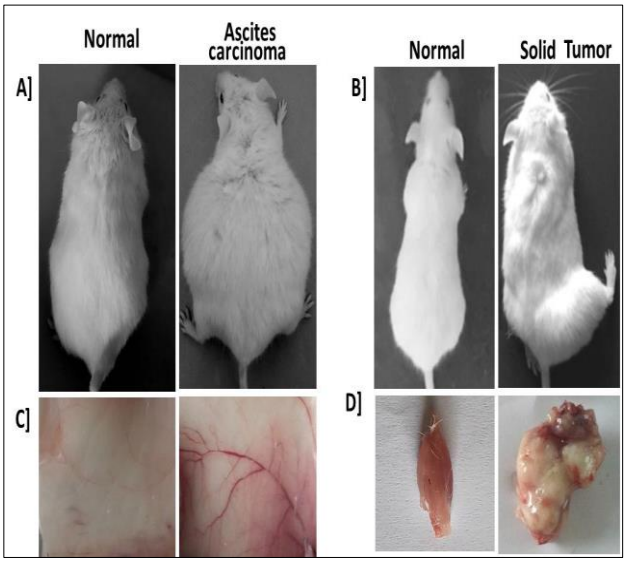

Figure 1: Tumor Morphology: (A, B) Mice bearing ascites and solid tumor compared with normal mice. (C) Peritoneum image showing the pattern of neovascularization (angiogenesis) normal and tumor bearing mice. (D) Solid tumor morphology.

In ascites an abnormal swelling at the peritoneum cavity and the enormous neovessel formation was observed (Figure 1C). Localization of tumor at the thigh region and establishment of cellular mass was in solid tumor observed and the outcome was compared against normal mice (Figure 1D). The resultant tumor was analyzed for tumor mass and angiogenesis quantification.

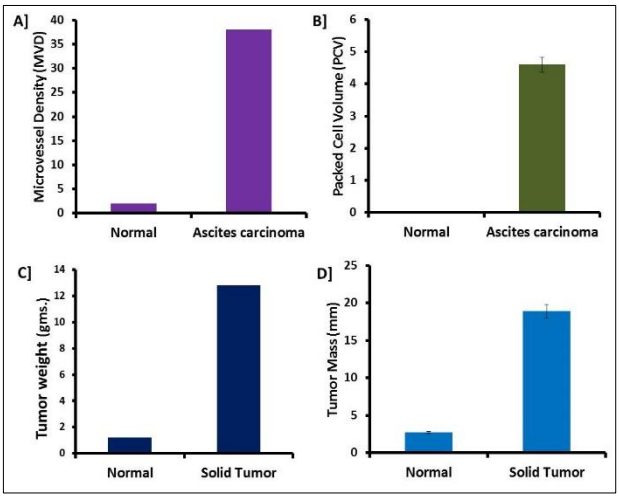

Figure 2: Tumor Parameters: (A) Microvessel density of tumor bearing mice with normal mice. (B) Packed cell volume of ascites carcinoma cells compared to normal mice. (C, D) Solid tumor weight and tumor mass compared to the normal counterpart.

MVD of peritoneal angiogenesis and tumor growth

The development of the tumor is depends on the process of neovascularization. In ascites carcinoma, enormous sprouting of neovessel (microvesssel density) was observed and quantified at the peritoneum, compared to normal mice and the packed cell volume was showed a better tumor progression in the short duration of 12 days. (Figure 2A, Figure 2B).

In solid tumor, tumor growth was evaluated by measuring the tumor weight and tumor mass. The results inferred that the tumor was localized to a particular region and showed a huge compact mass with tumor aggressiveness compared to normal mice (Figure 2C, Figure 2D).

Tumor modulates the normalized tissue into tumor type

EAC cells were used for generating two carcinoma models (Ascites and Solid tumor) in Swiss albino mice. Upon induction of cells on 10^{th} day of tumor growth different tissue section such as peritoneum, Lung, Stomach and Colon from ascites, and solid tumor region (on 45^{th} day) and their respective from normal mice was isolated. Histological analysis interpreted that skin, lung, thigh region from solid tumor showed tumor modulation, whereas stomach and colon showed moderate effect compared with normal mice respectively. The result

showed a significant progress in the tumor volume and compared to normal animals (Figure 3).

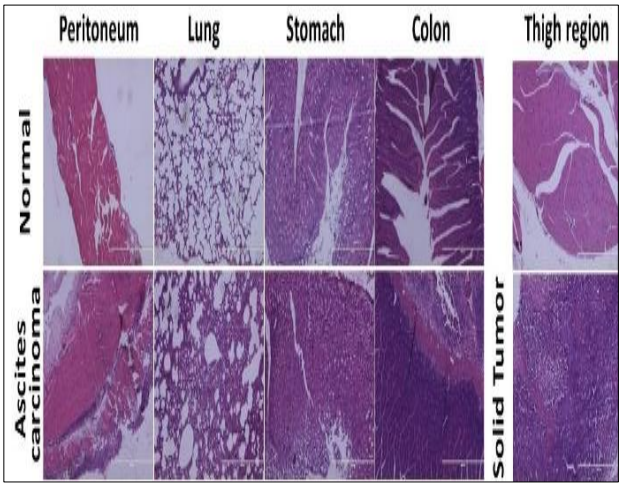

Figure 3: Histological analysis: the images representing the tumor induced tissue modulation in various murine tumor bearing tissues compared against normal tissues. (Magnification at 10x).

NEDD9 expression is associated tumor prognosis

Strong expression of NEDD9 was observed in the lung, colon from ascites carcinoma and solid tumor tissue of solid tumor model and tumor adjacent tissue of ascites carcinoma skin, stomach tissues showed a moderately positive for NEDD9 expression compared with normal tissue (Figure 4).

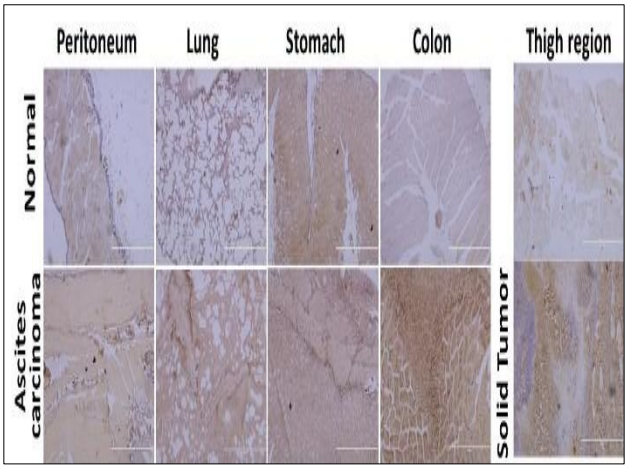

Figure 4: Immuno profile of NEDD9 expression: Tissue level expression of NEDD9 in murine ascites and solid tumor versus normal tissue in various tissue sections respectively. (Magnification at 10x).

The results Summarizes that, the prognosis of tumor is associated with tumor modulation and tumor modulation correlated with NEDD9 gene expression (Figure 5).

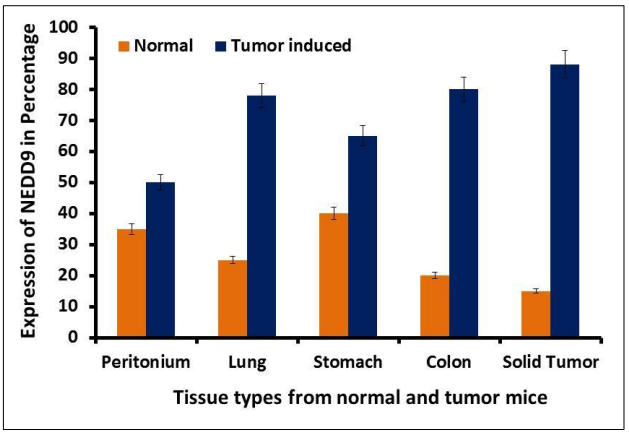

Figure 5: Association of NEDD9 expression correlates with the tissue modulation in diverse tissue in normal and tumor counterparts. Significance value is ≤0.05.

DISCUSSION

Gene identification and there differential expression compared between normal and tumor associated counterpart is one of the challenging concerns in the field of research. NEDD9 protein is multifunctional signaling non-catalytic scaffolding domain which is involved in normal and pathological mechanism including tumor development.[21] NEDD9, CRK-associated substrates family member gene has an integral role in cellular signalling. In broader view point, actively involved in the process of cell growth, mortality and escaping apoptosis in tumor development.[22] In tumor metastatic pathway, cellular coordination during invasion, migration signalling network mediated by NEDD9 to invade from primary site by formation of neovessels for circulation and surviavlity, secondary tumor establishment to form metastatic colonies at secondary tissue.[23] Several in-vitro, in-vivo validation revealed that, NEDD9 is the key component in the pathway of tumorigenesis and metastasis.[24,25] Elevated expression of NEDD9 in the tumor environment led to the activation and is linked to the functions of downstream factors such as VEGF, MMPs, etc., results in the neovascularization through disease progression in multiple caner types. The NEDD9 dependent molecular mechanism involves the stimulation of VEGF expression with elevated matrix metalloproteinase (MMPs) activity and interaction with numerous oncogenic molecules helps in the metastatic behavior of tumor cells lead to the formation of invadopodia leading to the cancer poor prognosis.[26-28] This kind of biomarker which helps in promoting tumor stage and useful in assessing the tumor metastatic role are clinically significant, and identification of such promoter is essential for targeted therapeutics. Hence, to find the potential target therapies can be achieved by identifying the tumor promoting biomarkers and this modulating activity in tumor development and metastasis.

In this study, the ascites tumor showed a massive growth and swelling was observed. At the peritoneal region we observed formation of numerous blood vessels whereas in

normal peritoneal part it was absent. Consequently, in solid tumor bearing mice showed a gigantic establishment of tumor and their localization of tumor at thigh region were noticed. And also we demonstrated that NEDD9 expression is associated with the progression of tumor that results in the establishment of tumor at the primary site and always linked within the tumor microenvironment helps in the metastasis. In fact, we investigated that NEDD9 overexpression affects the tumor progression but not in normal tissues. On the other hand, the NEDD9 expression positively correlates with the tumor aggressiveness of cell proliferation, evading cellular apoptosis and poor prognosis of tumor within the microenvironment of mice. Considering the results outcome, we showed the differential expression of NEDD9 in normal and tumor bearing mice and their significance in tumor growth by immunohistochemistry. This will bring out the significance of NEDD9 and helps in the further investigation in targeted therapy.

ACKNOWLEDGEMENTS

The Author Mr. Pavankumar G.S., INSPIRE-JRF appreciatively acknowledges to DST-INSPIRE programme (No.DST/INSPIRE Fellowship/2015/IF150975 Dated:17/11/2016.) for their financial support. Corresponding Author Dr. Prabhakar B.T., gratefully covey their thanks to DBT for financial support through DBT pilot research project (6242-P37/RGCB/PMD/DBT/PBKR/2015, Dated:11/9/2015). All the authors in the publication are thankful to The Principal, Sahyadri Science College (Autonomous), A constituent college of Kuvempu University, Shivamogga for their support and providing laboratory facilities for research.

Funding: Funding sources from DBT Pilot Research Project
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Animal Ethics Committee (SJMCP/IAEC/06/2015-16 and NCP/IAEC/CL/101/05/2012-13)

REFERENCES

1. Man S, Bocci G, Francia G, Green SK, Jothy S, Hanahan D, et al. Antitumor effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. Cancer Research. 2002 May 15;62(10):2731-5.
2. Thirusangu P, Vigneshwaran V, Ranganatha VL, Vijay Avin BR, Khanum SA, Mahmood R, et al. A tumouralangiogenic gateway blocker, Benzophenone-1B represses the HIF-1α nuclear translocation and its target gene activation against neoplastic progression. Biochem Pharmacol. 2017 Feb 1;125:26-40.
3. Chekhonin VP, Shein SA, Korchagina AA, Gurina OI. VEGF in tumor progression and targeted therapy. Curr Cancer Drug Targets. 2013 May;13(4):423-43.
4. Katsuno Y, Lamouille S, Derynck R. TGF-β signaling and epithelial-mesenchymal transition in cancer progression. Curr Opin Oncol. 2013 Jan;25(1):76-84.
5. Desouki MM, Kallas SJ, Khabele D, Crispens MA, Hameed O, Fadare O. Differential vimentin expression in ovarian and uterine corpus endometrioid adenocarcinomas: diagnostic utility in distinguishing double primaries from metastatic tumors. Int J Gynecol Pathol. 2014 May;33(3):274-81.
6. Yu L, Mu Y, Sa N, Wang H, Xu W. Tumor necrosis factor α induces epithelial-mesenchymal transition and promotes metastasis via NF-κB signaling pathway-mediated TWIST expression in hypopharyngeal cancer. Oncol Rep. 2014 Jan;31(1):321-7.
7. Chapman KB, Prendes MJ, Sternberg H, Kidd JL, Funk WD, Wagner J, West MD. COL10A1 expression is elevated in diverse solid tumor types and is associated with tumor vasculature. Future Oncol. 2012 Aug;8(8):1031-40.
8. Kumar S, Tomooka Y, Noda M. Identification of a set of genes with developmentally down-regulated expression in the mouse brain. Biochem. Biophys. Res. Commun. 1992;185(3):1155-61.
9. Karabulut M, Alis H, Afsar CU, Karabulut S, Kocatas A, Oguz H, et al. Serum neural precursor cell-expressed, developmentally down regulated 9 (NEDD9) level may have a prognostic role in patients with gastric cancer. Biomed Pharmacother. 2015 Jul;73:140-6.
10. Shagisultanova E, Gaponova AV, Gabbasov R, Nicolas E, Golemis EA. Preclinical and clinical studies of the NEDD9 scaffold protein in cancer and other diseases. Gene. 2015 Aug 1;567(1):1-11.
11. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. Biochim Biophys Acta. 2013 Dec;1833(12):3481-98.
12. Guerrero MS, Parsons JT, Bouton AH. Cas and NEDD9 Contribute to Tumor Progression through Dynamic Regulation of the Cytoskeleton. Genes Cancer. 2012 May;3(5-6):371-81.
13. Morimoto K, Tanaka T. Association of NEDD9 with TGF-β-triggered epithelialmesenchymal transition and cell invasion in prostate cancer cells: implications for cancer aggressiveness. Cancer Cell and Microenvironment. 2015;2:e342.
14. Al-Ghorbani M, Pavankumar GS, Naveen P, Thirusangu P, Prabhakar BT, Khanum SA. Synthesis and an angiolytic role of novel piperazine-benzothiazole analogues on neovascularization, a chief tumoral parameter in neoplastic development. Bioorg Chem. 2016 Apr;65:110-7.
15. Prabhakar BT, Khanum SA, Shashikanth S, Salimath BP. Antiangiogenic effect of 2-benzoyl-phenoxy acetamide in EAT cell is mediated by HIF-1alpha and down regulation of VEGF of in-vivo. Invest New Drugs. 2006 Nov;24(6):471-8.
16. Vijay Avin BR, Thirusangu P, Lakshmi Ranganatha V, Firdouse A, Prabhakar BT, Khanum SA. Synthesis and tumor inhibitory activity of novel coumarin

analogs targeting angiogenesis and apoptosis. Eur J Med Chem. 2014 Mar 21;75:211-21.
17. Mukherjee A, Dutta S, Sanyal U. Evaluation of antitumor efficacy and toxicity of novel 6-nitro-2-(3-chloropropyl)-1H-benz[de]isoquinoline-1,3-dione in vivo in mouse. J Cancer Res Ther. 2013 Jul-Sep;9(3):442-6.
18. Batista AP, da Silva TG, Teixeira AA, de Medeiros PL, Teixeira VW, Alves LC, Dos Santos FA. Melatonin effect on the ultrastructure of Ehrlich ascites tumor cells, lifetime and histopathology in swiss mice. Life Sci. 2013;S0024-3205(13)00614-0.
19. Zabiulla, Vigneshwaran V, Bushra AB, Pavankumar GS, Prabhakar BT, Khanum SA. Design and synthesis of conjugated azo-hydrazone analogues using nanoBF(3)·SiO(2) targeting ROS homeostasis in oncogenic and vascular progression. Biomed Pharmacother. 2017 Nov;95:419-428.
20. Thirusangu P, Vigneshwaran V, Vijay Avin BR, Rakesh H, Vikas HM, Prabhakar BT. Scutellarein antagonizes the tumorigenesis by modulating cytokine VEGF mediated neoangiogenesis and DFF-40 actuated nucleosomal degradation. Biochem Biophys Res Commun. 2017 Feb 26;484(1):85-92.
21. Ismail RS, Baldwin RL, Fang J, Browning D, Karlan BY, Gasson JC, Chang DD. Differential gene expression between normal and tumor-derived ovarian epithelial cells. Cancer Res. 2000 Dec 1;60(23):6744-9.
22. Wang Z, Shen M, Lu P, Li X, Zhu S, Yue S. NEDD9 may regulate hepatocellular carcinoma cell metastasis by promoting epithelial-mesenchymal-transition and stemness via repressing Smad7. Oncotarget. 2017;8(1):1714-24.
23. Guerrero MS, Parsons JT, Bouton AH. Cas and NEDD9 Contribute to Tumor Progression through Dynamic Regulation of the Cytoskeleton. Genes and Cancer. 2012;3(5-6):371-81.
24. Lucas JT Jr, Salimath BP, Slomiany MG, Rosenzweig SA. Regulation of invasive behavior by vascular endothelial growth factor is HEF1-dependent. Oncogene. 2010 Aug 5;29(31):4449-59.
25. Elena S, Gaponova AV, Gabbasov R, Nicolas E, Golemis EA. Preclinical and clinical studies of the NEDD9 scaffold protein in cancer and other diseases. Gene. 2015;567:1-11.
26. Singh MK, Cowell L, Seo S, O'Neill GM, Golemis EA. Molecular basis for HEF1/NEDD9/Cas-L action as a multifunctional co-ordinator of invasion, apoptosis and cell cycle. Cell biochemistry and biophysics. 2007 May 1;48(1):54-72.
27. O'Neill GM, Seo S, Serebriiskii IG, Lessin SR, Golemis EA. A new central scaffold for metastasis: parsing HEF1/Cas-L/NEDD9. Cancer research. 2007 Oct 1;67(19):8975-9.
28. Jin Y, Li F, Zheng C, Wang Y, Fang Z, Guo C, et al. NEDD9 promotes lung cancer metastasis through epithelial–mesenchymal transition. International Journal of Cancer. 2014 May 15;134(10):2294-304.

Cite this article as: Kumar PGS, Krishna V, Prabahkar BT. Differential genotypical expression of a NEDD9 in Normal and Tumor tissues: A possible pharmacological target. Int J Basic Clin Pharmacol 2018;7:423-8.