Clinical Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
2018, Vol. 46(9) 3709–3716

DOI: 10.1177/0300060518777944
journals.sagepub.com/home/imr
SAGE

Neural precursor cell-expressed developmentally down-regulated 4-like: a new biomarker in the pathophysiology of endometrial cancer

Ercan Yilmaz[1], Mehmet Gul[2], Rauf Melekoglu[1], Ebru Inci Coskun[1], Nurhan Sahin[3], Semir Gul[2], Ayse Gulcin Bastemur[1] and Baris Ciplak[4]

Abstract

Objectives: Endometrial cancer is the most frequent tumor of the female genital tract. Ubiquitin is a small protein (8.5 kDa) found in all eukaryotic cells, binds to substrate proteins via a three-phase enzymatic pathway referred to as ubiquitination and plays an important role in cellular stability. Neural precursor cell-expressed developmentally down-regulated 4-like (NEDD4L) functions in the last phase of this enzymatic process. In this study, we investigated NEDD4L protein expression in endometrial cancer.

Methods: The study participants were divided into patients with benign endometrial pathologies (Group 1, n = 23), patients with endometrial hyperplasia (Group 2, n = 21) and patients with endometrial cancer (Group 3, n = 20). NEDD4L expression was detected by immunohistochemical staining and H scores were calculated to standardize staining intensity. Statistical analysis was performed using SPSS 16.0.

Results: NEDD4L expression levels according to H scores were significantly lower in patients diagnosed with endometrial cancer compared with those with benign endometrial pathologies.

Conclusion: NEDD4L is involved in maintaining cell stability, and reduced NEDD4L expression as a result of gene mutation may disrupt this balance in favor of tumorigenesis.

[1]Obstetrics and Gynecology Department, Inonu University Medicine Faculty, Malatya, Turkey
[2]Histology and Embryology Department, Inonu University Medicine Faculty, Malatya, Turkey
[3] Pathology Department, Bezmialem Foundation University Medicine Faculty, Istanbul, Turkey
[4]Beydağı State Hospital, Malatya, Turkey

Corresponding author:
Ercan Yilmaz, Obstetrics and Gynecology Department, Inonu University Medicine Faculty, Malatya 44000, Turkey.
Email: ercanyilmazgyn@yahoo.com

Keywords
NEDD4L, endometrial cancer, ubiquitin, tumorigenesis, endometrial hyperplasia, immunohistochemistry

Date received: 17 November 2017; accepted: 27 April 2018

Introduction

Endometrial cancer is the most frequently detected gynecologic tumor and the fourth most common cancer overall in women, representing a significant threat to women's health. However, endometrial cancer manifests symptoms at an early stage, and an early diagnosis may allow a cure to be achieved before progression to an advanced stage, thus minimizing the need for surgical intervention and adjuvant radiotherapy and chemotherapy.[1] The role of unopposed estrogen in the etiology of endometrial cancer is widely accepted, and age, parity and genetic factors are also recognized etiological factors.[2] However, although specific etiological factors in the process of tumorigenesis have been defined, a role for chronic inflammation and causative mediators has also been recognized.[1] Similarly, cellular stability and the proteins responsible for maintaining this stability are also considered to be important factors.

Ubiquitin was first discovered in 1975 and has since been found in almost all eukaryotic cells.[3] It has a molecular weight of 8.5 kDa, and acts to regulate cellular functions and maintain cellular stability.[3] The process of conjugation between a ubiquitin molecule and a substrate protein is called ubiquitination, and ubiquitinated proteins are found in many cell membranes, and among cell cycle proteins, transcription factors and tumor suppressors. The process of ubiquitination is vital for maintaining cellular stability, and impairments in the mechanism of ubiquitination have adverse effects on cellular functions, resulting in the development of cancer and other pathologies.[4]

Ubiquitination takes place through the collaborative functions of three different enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin protein ligase (E3), all of which are required for successful ubiquitination and consequent cellular stability.[5] The neural precursor cell-expressed, developmentally down-regulated 4-like (NEDD4L) protein is a member of the E3 ubiquitin enzyme family. In cancer genetics, NEDD4L protein is known to interact with transforming growth factor-β (TGF-β) and to affect the TGF-β, epidermal growth factor receptor and p53 pathways.[6]

Although numerous previous studies have demonstrated that expression of NEDD4L disrupts cellular stability leading to the development of cancer, the current study provides the first evidence for its role in the etiopathogenesis of endometrial cancer.

Materials and methods

Patient selection

Patients who attended our clinic and who underwent surgery were divided into three groups based on the histopathologic diagnosis of their endometrial tissues: Group 1, patients diagnosed with endometrial polyps; Group 2, patients with endometrial hyperplasia; and Group 3, patients with endometrial adenocarcinoma. Patient files were analyzed retrospectively to determine their demographic characteristics. This study was

approved by the ethics committee of Inonu University Medical Faculty (authentication code: 2016/13). Written informed consent was obtained from all patients for participation in the study.

Pathologic diagnosis of endometrial tissues

Tissue blocks and slides from patients diagnosed with benign endometrial lesions, endometrial hyperplasia or endometrial adenocarcinoma were selected from the pathology archives and reexamined. Lesions with a morphology indicating a low stroma/gland ratio in the endometrial sample and patchy areas of cyst formation were diagnosed as simple non-atypical endometrial hyperplasia, and lesions with morphologies showing more marked glandular crowding and more complex glandular architecture were diagnosed as complex endometrial hyperplasia.

The presence of complex glandular architecture combined with papillary and/or villous structures, cribriform architecture, stromal desmoplasia and cytologically distinct atypia was also taken into consideration in the diagnosis of endometrial carcinoma. Archival slides were reclassified based on the morphologic diagnostic criteria and tissue blocks suitable for immunohistochemical staining were selected.

Immunohistochemical analysis

Formalin-fixed and paraffin-embedded sections (5 μm) were mounted on Superfrost Plus slides (Thermo Fisher Scientific Inc., Rockford, IL, USA). Tissue sections were deparaffinized and rehydrated in graded concentrations (50%–99.9%) of ethanol. Sections were rehydrated and antigen retrieval was performed using citrate buffer (boiled at 121°C) and cooled to room temperature. Endogenous peroxidase was blocked in 3% H_2O_2 in methanol for 10 minutes, followed by protein block for 5 min in 0.5% casein in phosphate-buffered saline. The slides were then incubated with primary antibody (anti-NEDD4-2, 1:500 dilution, Abcam plc., Cambridge, UK) for 1 hour followed by streptavidin peroxidase for 10 minutes. Finally, the preparations were developed in 3-amino-9-ethylcarbazole chromogen, counterstained with hematoxylin and mounted with aqueous mounting medium.

All sections were examined systematically under an Eclipse Ni-U light microscope with a DS-Fi2 Camera and analyzed using NIS-Elements Documentation Image Analysis System (Nikon Corporation, Tokyo, Japan).

The degree of anti-NEDD4-2 immunoreactivity of the endometrial tissues was determined using the H-score method. This semi-quantitative method calculated the H score based on the percentages of positively stained cells multiplied by a weighted intensity of staining: H score $= \Sigma$ Pi (i+1), where Pi is the percentage of stained cells in each intensity category (0%–100%).[7] The intensity of specific staining was characterized as not present (0), weak but detectable above control (1), distinct (2) and very strong (3).

Statistical analysis

Statistical analysis was carried out using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Values were expressed a mean ± standard deviation where appropriate, and categorical variables were reported as number and percent. Normally distributed variables were compared by one-way ANOVA, non-normally distributed variables were compared between groups by Kruskal–Wallis tests, and comparisons among multiple groups were made by Tukey's and Conover tests.

Results

The study participants were distributed based on the histopathological architecture

of their lesion. Patients in Group 1 (n = 23; mean age 42.87 ± 8.143 years) all had benign endometrial pathologies, including endometrial polyps (n = 20), secretory endometrium (n = 2) and proliferative endometrium (n = 1). Patients in this group underwent dilatation and curettage (D&C) (n = 16) or hysteroscopy and total abdominal hysterectomy and bilateral salpingo-oophorectomy (TAH+BSO) (n = 6) to obtain endometrial tissue specimens. Group 2 (n = 21; mean age 54.90±12.3 years) consisted of patients with hyperplasia with and without atypia. Patients in this group underwent TAH+BSO (n = 14), D&C (n = 5), TAH (n = 1) and vaginal hysterectomy plus anteroposterior colporrhaphy (VAH+CAP) (n = 1). Group 3 (n = 20; mean age 60.7±9.8 years) all had malignant pathologies, including endometrial adenocarcinoma (n = 18) and endometrial serous cancer (n = 2). Thirteen patients underwent TAH+BSO+ pelvic-paraaortic lymphadenectomy-+omentectomy and seven patients underwent TAH+BSO+pelvic lymphadenectomy.

Immunohistochemical analysis of paraffin blocks revealed the weakest NEDD4L staining in Group 3 and the highest level of staining in patients in Group 1 with benign pathologies (Figures 1–3). The median H scores in Groups 1, 2 and 3 were 90 (20–240), 60 (20–280) and 20 (1–270), respectively. The H score in the endometrial cancer group was significantly lower than that in Group 1 ($P = 0.019$) and was also lower

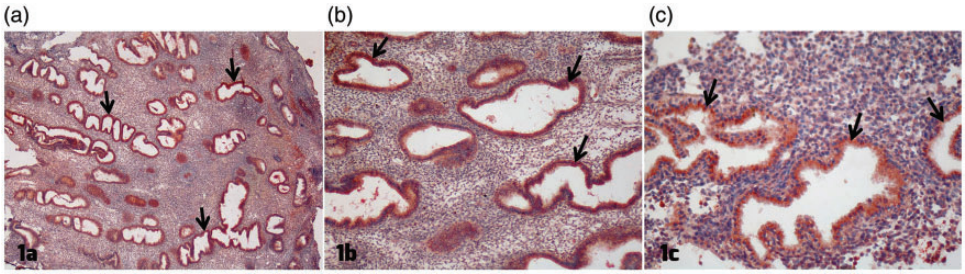

Figure 1. Immunohistochemical analysis of NEDD4L staining intensity grades in endometrial lesions. (a) Benign group: positive anti-NEDD4-2 immunoreactivity in endometrium (arrow), staining intensity grade = 3, ×4. (b) Benign group: positive anti-NEDD4-2 immunoreactivity in endometrium (arrow), staining intensity grade = 3, ×10. (c) Benign group: positive anti-NEDD4-2 immunoreactivity in endometrium (arrow), staining intensity grade = 2, ×20

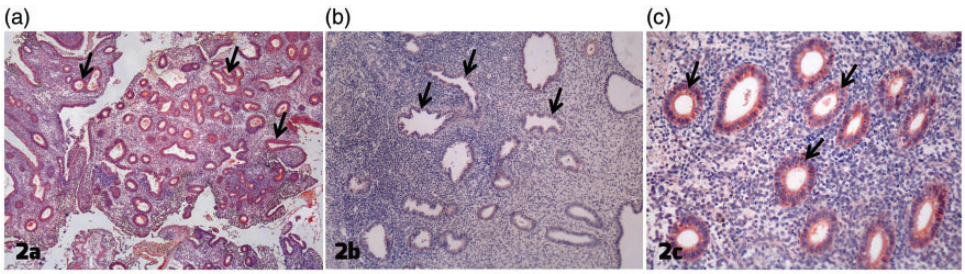

Figure 2. Immunohistochemical analysis of NEDD4L staining intensity grades in endometrial lesions. (a) Hyperplasia group: positive anti-NEDD4-2 immunoreactivity in endometrium (arrow), staining intensity grade = 3, ×4. (b) Hyperplasia group: positive anti-NEDD4-2 immunoreactivity in endometrium (arrow), staining intensity grade = 1, ×10. (c) Hyperplasia group: positive anti-NEDD4-2 immunoreactivity in endometrium (arrow), staining intensity grade = 2, ×20

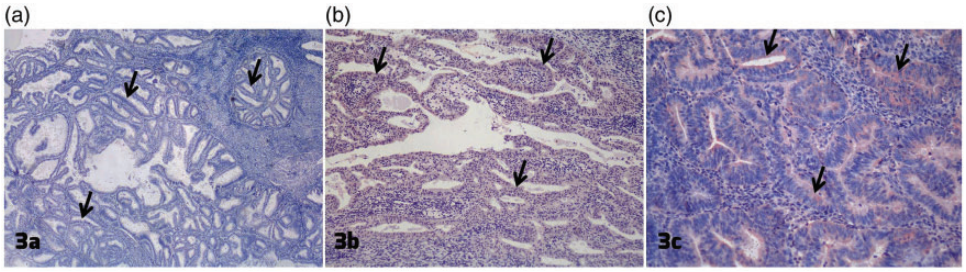

Figure 3. Immunohistochemical analysis of NEDD4L staining intensity grades in endometrial lesions. (a) Cancer group: no anti-NEDD4-2 immunoreactivity in endometrium (arrow), staining intensity grade = 0, ×4. (b) Hyperplasia group: positive anti-NEDD4-2 immunoreactivity in endometrium (arrow), staining intensity grade = 1, ×10. (c) Cancer group: positive anti-NEDD4-2 immunoreactivity in endometrium (arrow), staining intensity grade = 1, ×20

Table 1. Calculated H scores in three groups.

Group	H-score median (min–max)	*P*
Group 1 (benign endometrial lesions)	90 (20–240)*	0.019
Group 2 (endometrial hyperplasia)	60 (20–280)	
Group 3 (endometrial cancer)	20 (1–270)*	

than in the hyperplasia group, though the difference was not significant ($P = 0.054$) (Table 1 and Figure 4).

Discussion

NEDD4L is a member of the ubiquitin protein ligase (E3) family encoded by the *NEDD4L* gene on chromosome 18 (18q21), with known functions in cellular stability.[8] Its best-known task in achieving cellular stability and homeostasis is realized through regulating the functions of epithelial sodium channels (EnaCs).[9] EnaC is a proteinaceous ion channel consisting of three subunits (α, β and γ), two transmembrane regions, an extracellular loop and intracellular N and C terminals. It is a transmembrane protein mostly located in the kidney, distal colon, lungs, skin and trachea.[10] Although it exerts various organ-specific functions, many studies have demonstrated that ENaC in renal tissue is responsible for controlling sodium balance in the body and for gas exchange in the alveoli of the lungs.[4] Increased intracellular sodium concentrations prevent NEDD4L binding to the ENaC receptor and entry of sodium into the cell.[11] Recent studies demonstrated that NEDD4L protein not only regulated ion channel functions in cellular homeostasis, but also interacted with intracellular mediators to cause the development of cancer.

NEDD4L apparently exerts its effects via TGF-β, which plays key roles in tumorigenesis, invasion, angiogenesis and metastasis development. NEDD4L prevents tumor development by inhibiting the TGF-β signaling pathway.[12] However, previous studies have demonstrated different results regarding NEDD4L expression, especially in prostate cancer patients. Hu et al. found lower NEDD4L expression levels in 56 patients with prostate cancer compared with patients with a diagnosis of prostate hyperplasia,[13] while Hellwinkel et al. detected higher levels of NEDD4L expression in patients diagnosed with prostate cancer relative to the control group.[14] This apparent

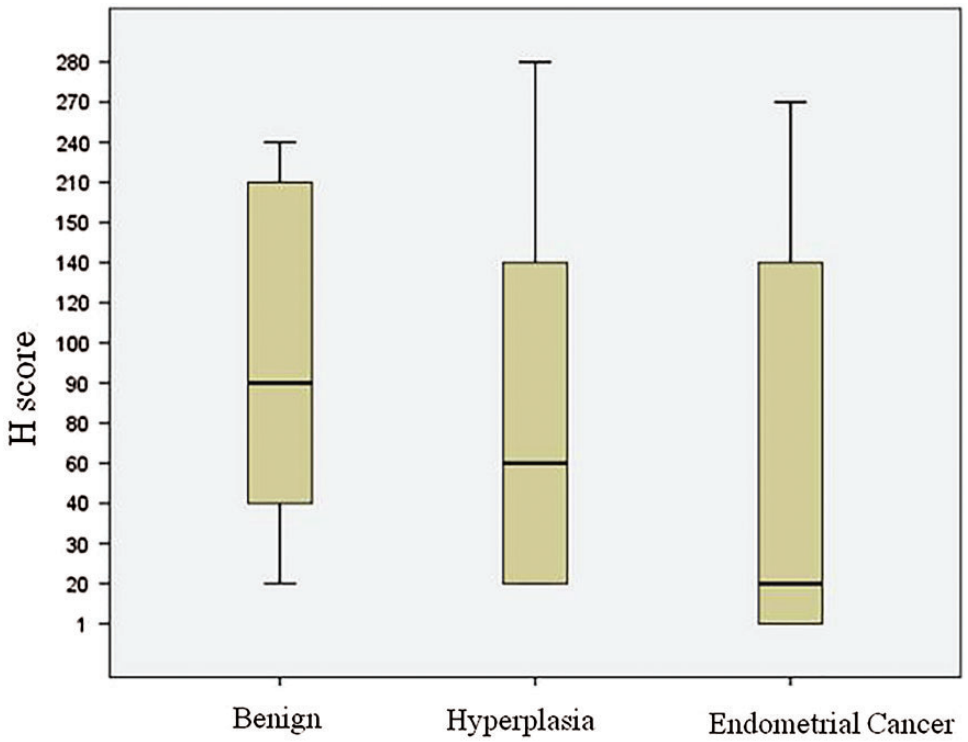

Figure 4. H scores in patients with benign endometrial pathologies, endometrial hyperplasia and endometrial cancer

discrepancy could be explained by the pleiotropic effect of the TGF-β gene in oncogenesis; TGF-β acts as a tumor suppressor gene in normal and precancerous cells, but as an oncogene during cancer progression.[15] Relevant studies revealed increased NEDD4L expression in non-small cell lung cancer, gastric cancer, glioma and colorectal cancers,[16–19] while a similar immunohistochemical study in 41 patients with cutaneous T-cell lymphoma detected increased levels of NEDD4L expression.[20]

Within this context, Yang et al. conducted the first study of NEDD4L expression in patients with gynecological malignancies. NEDD4L expression was significantly lower among 72 patients with epithelial ovarian cancer compared with those diagnosed with benign and mucinous borderline ovarian tumors ($P<0.005$). Furthermore, lower NEDD4L expression was associated with increased disease stage and grade and increased number of lymph node metastases.[15] To the best of our knowledge, the current study was only the second to investigate NEDD4L expression in gynecological cancers, and the first to compare benign endometrial pathologies and endometrial cancers. The results showed that NEDD4L expression levels were reduced in patients with endometrial cancer compared with benign endometrial pathologies.

In conclusion, this study provides the first evidence of NEDD4L protein expression in endometrial cancer tissues under *in vivo* conditions. The results showed that NEDD4L expression was reduced in cancer

tissues, in accordance with previous studies.[6,13,15] Notably, however, numerous studies have demonstrated increased NEDD4L expression in cancer tissues, and this discrepancy may be due to the pleiotropic effects of the NEDD4L-interacting factor TGF-β in oncogenesis.[17,18] The current results suggest that regulation of NEDD4L expression may play a key role in the etiology of endometrial cancer. However, the interaction of this protein with intracellular pathways remains unclear, and further comprehensive studies of NEDD4L may lead to the discovery of new proteins, gene regions and pathways as potential therapeutic targets in endometrial cancer.

Declaration of conflicting interest

The authors declare that there is no conflict of interest

Funding

This study was supported by the BAP committee of the Inonu University Scientific Research Projects (2016/74).

References

1. Elit LM, Pond G and Seow H. Time interval between endometrial biopsy and surgical staging for type I endometrial cancer: association between tumor characteristics and survival outcome. *Obstet Gynecol* 2015; 125: 1497–1498.
2. Kwon JS. Improving survival after endometrial cancer: the big picture. *J Gynecol Oncol* 2015; 26: 227–231.
3. Varshavsky A. The ubiquitin system, autophagy and regulated protein degradation. *Annu Rev Biochem* 2017; 86: 123–128.
4. Goel P, Manning JA and Kumar S. NEDD4-2 (NEDD4L): the ubiquitin ligase for multiple membrane proteins. *Gene* 2015; 557: 1–10.
5. Liang J, Wang WF, Xie S, et al. β-transducin repeat-containing E3 ubiquitin protein ligase inhibits migration, invasion and proliferation of glioma cells. *Oncol Lett* 2017; 14: 3131–3135.
6. Tanksley JP, Chen X and Coffey RJ. NEDD4L is downregulated in colorectal cancer and inhibits canonical WNT signaling. *PLoS One* 2013; 8: e81514.
7. Budwit-Novotny DA, McCarty KS, Cox EB, et al. Immunohistochemical analyses of estrogen receptor in endometrial adenocarcinoma using a monoclonal antibody. *Cancer Res* 1986; 46: 5419–5425.
8. Chen H, Ross CA, Wang N, et al. NEDD4L on human chromosome 18q21 has multiple forms of transcripts and is a homologue of the mouse Nedd4-2 gene. *Eur J Hum Genet* 2001; 9: 922–930.
9. Zhu JY, Heidersbach A, Kathiriya IS, et al. The E3 ubiquitin ligase Nedd4/Nedd4L is directly regulated by microRNA 1. *Development* 2017; 144: 866–875.
10. Kashlan OB and Kleyman TR. Epithelial Na(+) channel regulation by cytoplasmic and extracellular factors. *Exp Cell Res* 2012; 318: 1011–1019.
11. Bhalla V and Hallows KR. Mechanisms of ENaC regulation and clinical implications. *J Am Soc Nephrol* 2008; 19: 1845–1854.
12. Kuratomi G, Komuro A, Goto K, et al. NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) negatively regulates TGF-beta (transforming growth factor-beta) signalling by inducing ubiquitin-mediated degradation of Smad2 and TGF-beta type I receptor. *Biochem J* 2005; 386(Pt 3): 461–470.
13. Hu XY, Xu YM, Fu Q, et al. Nedd4L expression is downregulated in prostate cancer compared to benign prostatic hyperplasia. *Eur J Surg Oncol* 2009; 35: 527–531.
14. Hellwinkel OJ, Asong LE, Rogmann JP, et al. Transcription alterations of members of the ubiquitin-proteasome network in prostate carcinoma. *Prostate Cancer Prostatic Dis* 2011; 14: 38–45.
15. Yang Q, Zhao J, Cui M, et al. Nedd4L expression is decreased in ovarian epithelial cancer tissues compared to ovarian non-cancer tissue. *J Obstet Gynaecol Res* 2015; 41: 1959–1964.
16. Takeuchi T, Adachi Y, Nagayama T, et al. Nedd4L modulates the transcription of

metalloproteinase-1 and -13 genes to increase the invasive activity of gallbladder cancer. *Int J Exp Pathol* 2011; 92: 79–86.
17. Gao C, Pang L, Ren C, et al. Decreased expression of Nedd4L correlates with poor prognosis in gastric cancer patient. *Med Oncol* 2012; 29: 1733–1738.
18. He S, Deng J, Li G, et al. Down-regulation of Nedd4L is associated with the aggressive progression and worse prognosis of malignant glioma. *Jpn J Clin Oncol* 2012; 42: 196–201.
19. Sakashita H, Inoue H, Akamine S, et al. Identification of the NEDD4L gene as a prognostic marker by integrated microarray analysis of copy number and gene expression profiling in non-small cell lung cancer. *Ann Surg Oncol* 2013; 20 Suppl 3: S590–S598.
20. Booken N, Gratchev A, Utikal J, et al. Sézary syndrome is a unique cutaneous T-cell lymphoma as identified by an expanded gene signature including diagnostic marker molecules CDO1 and DNM3. *Leukemia* 2008; 22: 393–399.