EJGO European Journal of Gynaecological Oncology

# Tissue expression of HE4 and its correlation with CA125 and p53 in high grade serous ovarian carcinoma

**T. Bulut[1], B. Celik[1], A. D. Yalcin[2], S. Keser[3]**

*[1]Pathology Unit, Antalya Training Hospital, Antalya; [2]Allergy and Immunology Unit, Antalya Training Hospital, Antalya*
*[3]Pathology Unit, Dr Lutfi Kirdar Training Hospital, Istanbul (Turkey)*

**Summary**

*Background:* The aim of this study was to evaluate the predictive efficacy of the human epididymis secretory protein 4 (HE4) and to compare the results with the other tumor markers CA125 and p53 in ovarian serous cancer patients. The authors also aimed to find a potantial molecular target for serous carcinoma treatment. *Materials and Methods:* A total of 31 patients with a diagnosis of high grade serous ovarian carcinoma were enrolled. All patients underwent surgical resection. Final original diagnosis was reached by histopathological features of the tumor and by combined immunohistochemistry using CA125 and p53 immunostains. The results were compared to HE4 immunostaining. *Results:* Of the tumor tissues studied, HE4 immunostaining was seen in a majority of the cases (28 out of 31 cases) (90.32%). Neither CA125 nor p53 results were available in six cases based on pathology reports, in which HE4 expression was observed in five cases. *Conclusions:* Immunohistochemical staining pattern of HE4 serves as a surrogate marker for p53 in high grade serous ovarian carcinoma and it is superior to p53 and CA125. It may be a potential candidate for therapeutic targets.

*Key words:* HE4; Ovary; Carcinoma; CA125; p53.

## Introduction

High grade ovarian serous cancer differs in its genetic profiles, biological behavior and outcome, and should be treated as a distinct disease. Thus, a specific pathological diagnosis is crucial for selection of optimal treatment strategy. Currently the pathological diagnosis of serous ovarian carcinoma is based on distinctive histopathological features along with the immunohistochemical expression of p53 and cancer antigen 125 (CA125). To be considered significant, p53 staining should be strong and diffuse [1]. However the percentage of p53 staining is 60-90% and it is only78% for CA125 [2, 3]. Moreover, p53 is altered in 30–80% of other types of ovarian carcinoma in general. CA125 is widely used in clinical practice as a serum biomarker for ovarian carcinoma as well as immunohistological subtyping of the tumor; however it lacks specificity and sensitivity [4].

Human epididymis 4 (HE4) protein belongs to whey acidic 4-disulfide center protein family [5]. The protein shows characteristics of a secretory protein, with an acidic and cysteine-rich polypeptide [6, 7]. It is a protease inhibitor and is involved in the innate immunity defence of the respiratory tract and nasal cavity [8].

Formerly found in epididymis, it is now shown that serum level is the most predictive marker detecting endometrial and adnexal malignancy [9-13]. This finding is promising since there is not a good marker available for endometrial cancer (EC) diagnosis, prognosis, and monitoring in routine practice. A meta-analysis conducted on patients with EC also confirmed that serum HE4 level is found to be superior to CA125 in terms of screening accuracy of EC [14].

There are more important aspects of serum HE4 level. Preoperative assesment of serum HE4 level has been found a good predictor for outer-half myometrial invasion [15, 16]. It identifies Stage IA versus Stage IB tumor with a sensitivity of 94% [17]. A prospective multicenter study confirmed its correlation with histological grade, lymph node metastases, myometrial invasion, cervical involvement [18-20], and prognosis [21].

There are several in vitro studies using ovarian cell lines: one of them revealed that HE4 plays an important role in cultured ovarian cell adhesion and motility [22]. Additionally, a specific region of the HE4 promoter (-530 bp from the ATG start site) is shown to be highly transcriptionally active in various ovarian carcinoma (OC) cell lines compared to minimal activity in normal ovarian tissue [23]. There is also an interaction between HE4 and steroid hormones: Treatment of ovarian cancer cells with steroid hormones promoted nuclear translocation of HE4 and cells became less responsive to hormonal therapy, which was re-

Revised manuscript accepted for publication November 15, 2016

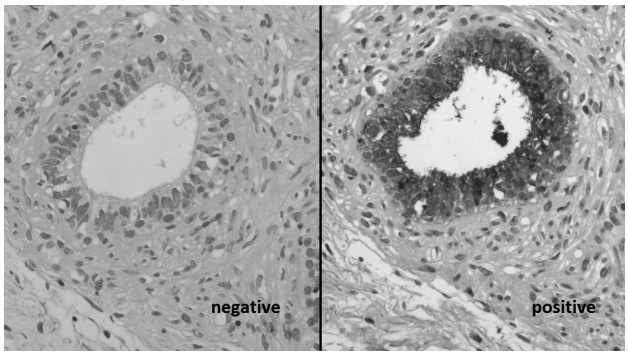


Figure 1. — Human epididymis. The cells found in the ductus epididymidis are immunoreactive with HE4 antibody. Negative control is obtained by omitting the primary antibody. Stroma may be used as internal negative control (anti-HE4, ×20).

stored by blocking HE4 from entering the nucleus [24].

Together with these in vitro studies, the present authors aimed to examine tissue expression of HE4 in human subjects and compared their results to well known tumor markers, to see whether it may be a substitute for p53 or CA125 in pathological practice, and to open a gate for future gene therapies.

## Materials and Methods

A total of 31 patients who were diagnosed with high grade serous carcinoma and who underwent surgical excision at Antalya Training Hospital between 2012-2016 were retrospectively enrolled in the study. The authors excluded patients with a diagnosis of malignant mixed Müllerian tumor. In each case, the patient's characteristics and presence or absence of CA125 and/or p53 immunostaining were extracted from the patient's pathology reports.The ethics committee at Antalya Training Hospital approved the study. Then, from the pathology department archives, the authors obtained 31 consecutive ovarian carcinoma tissue specimens from the study population. All specimens were used to measure the HE4 expression through immunohistochemistry.

Formalin-fixed, paraffin-embedded sections were de-waxed with xylene and rehydrated through gradient ethanol into a phosphate buffered solution (PBS). Endogenous peroxidase activity was quenched with 0.3% H2O2 in methanol for ten minutes at room temperature. At the same time 2 ml Tris-EDTA Buffer (ab93684) was added to 198 ml of distilled water, and swirled. Prepared retrieval solution was added to the microwaveable vessel. When the time elapsed, slides were washed in PBS three times and placed into the microwaveable vessel. The vessel was placed inside the domestic microwave, set to full power for ten minutes, at a second highest power for five minutes, and at medium power for five minutes. The procedure was monitored for evaporation and watched for boiling over during the procedure and did not allow the slides to dry out. When the retrieval solution evaporated during the boil, hot retrieval solution was added. When 20 minutes elapsed, the vessel was removed. When it cooled, the slides were washed in PBS three times before application of the rabbit polyclonal antibody to HE4 (anti-HE4 antibody [EPR16658] [ab200828], 1:2000 dilution). After two hours incubation with the primary antibody, the slides were washed in PBS and biotinylated goat anti-rabbit IgG secondary antibody was applied and incubated for ten minutes at room temperature. Slides were washed three times in PBS and streptavidin peroxidase was applied for ten minutes at room temperature. At the same time 20 µl DAB chromogen was added to 1 ml of DAB substrate and swirled. When the time elapsed, the slides were washed in PBS three times and prepared chromogen was applied to the tissues for ten minutes at room temperature. Slides were then washed in PBS three times and lightly counterstained with hematoxylin, followed by dehydration and coverslip mounting. The tissue sections of the human epididymis were processed in a comparable manner and provided a positive control. Negative control was obtained by omitting the primary antibody (Figure 1). Cytoplasmic staining was graded for intensity (0-negative, 1-weak, 2-moderate, and 3-strong) and percentage of positive cells [0, 1 (1–24%), 2 (25–49%), and 3 (50–100%)]. The grades were multiplied to determine an H-score. The H-scores for tumors with multiple cores were averaged. Protein expression was then defined as negative (H-score=0), weak (H-score=1–3), or strong (H-score ≥ 4).

Table 1. — *HE4, CA125, and p53 immunostaining among patients*

| Patient number | HE4 | CA125 | p53 |
|---|---|---|---|
| 1 | 3+ | 1 | neg |
| 2 | neg | 1 | 1 |
| 3 | 2 | 1 | NA |
| 4 | 3+ | 1 | NA |
| 5 | 1+ | 1 | NA |
| 6 | 3+ | NA | NA |
| 7 | 3+ | 1 | 1 |
| 8 | neg | NA | NA |
| 9 | 3+ | 1 | neg |
| 10 | 3+ | 1 | NA |
| 11 | 3+ | foc | NA |
| 12 | 3+ | NA | NA |
| 13 | 1+ | 1 | NA |
| 14 | 3+ | 1 | neg |
| 15 | 3+ | 1 | 1 |
| 16 | 3+ | 1 | 1 |
| 17 | 3+ | NA | 1 |
| 18 | 2+ | 1 | NA |
| 19 | 1+ | foc | NA |
| 20 | 3+ | foc | neg |
| 21 | 3+ | NA | NA |
| 22 | neg | 1 | NA |
| 23 | 3+ | 1 | NA |
| 24 | 3+ | 1 | neg |
| 25 | 3+ | 1 | NA |
| 26 | 3+ | 1 | 1 |
| 27 | 3+ | 1 | 1 |
| 28 | 2+ | 1 | 1 |
| 29 | 3+ | 1 | 1 |
| 30 | 3+ | NA | NA |
| 31 | 3+ | NA | NA |

*Abbreviation: foc: focal; NA: not available. 1: positive.*

## Results

Among 31 patients, CA125 or p53 were not applied in seven tumor tissues immunohistochemically. Among them,

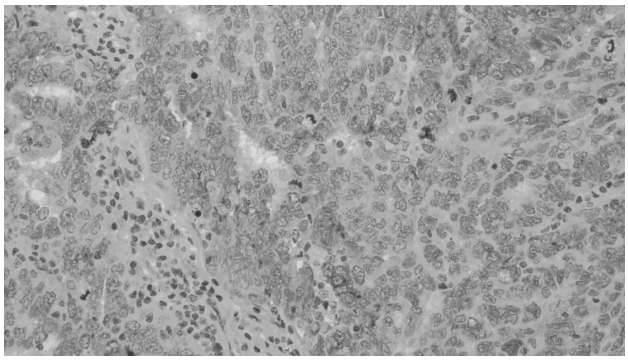

Figure 2. — Immunostaining is quantified as described in Materials and Methods. Weak immunostaining is seen throughout the tissue in this case (anti-HE4, ×20),

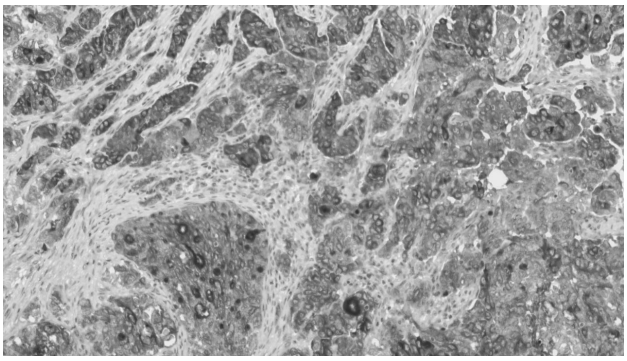

Figure 3. — Immunostaining is strong and occupies more than 50% of the tissue (anti-HE4, ×10).

all but one case had 3+ HE4 immunostaining. In addition to these seven cases, CA125 immunostaining was not present in one case, which was HE4 and p53 positive. The rest of the 24 cases were positive with CA125 immunostaining. Despite the fact that CA125 staining was ordered in the majority of the cases, p53 immunostaining was ordered in only 14 cases in which nine of the 14 cases were positive, while five of the 14 cases were negative immunohistochemically. However, all p53 negative cases were positive with either CA125 or HE4 (Table 1).

Of the 31 cases successfully stained with HE4, there were three 1+ (Figure 2), three 2+, and 22 3+ (Figure 3) immunostaining, whereas three cases (9.68%) were negative with HE4. Overall, HE4 tissue expression among ovarian serous carcinoma was 90.32% (Table 1).

## Discussion

Among gynecological malignancies, ovarian cancer is the most common cause of cancer-related mortality. A reliable marker that allows early detection of ovarian carcinoma is absent and there are no specific symptoms that allow diagnosis of ovarian cancer at an earlier stage in the majority of the patients [25]. Even with an earlier stage, primary treatment with surgery and chemotherapy, usually paclitaxel and carboplatin, have very little improvement for serous type ovarian carcinoma. As a recent ovarian carcinoma patient was the cousin of one of the present authors, they are aware of correct subtyping of ovarian carcinoma histopathologically.

Ovarian serous carcinoma responds to platinium-based chemotherapy very well. Despite this response, the majority of women ultimately relapse and develop resistance to chemotherapy. Thus, there is a need with this cancer to consider the use of gene therapies and better understanding of the cancer pathways.

HE4, also known as whey acidic protein four-disulfide core2 (WFDC2) was originally identified as a transcript exclusively expressed in the epididymis [7]. It is a 124-amino acid long polypeptide that has two WFDC domains. Whey acidic proteins (WAP) belong to a large gene family of antibacterial peptides that perform critical immune system functions [26]. The recombinant HE4 protein is shown to exhibit proteinase inhibitory activity towards trypsin, elastase, and matrix metallopeptidase 9 [27]. Although not expressed on normal epithelium [28], HE4 or WFDC2, a secretory glycoprotein is found to be elevated in the serum of adnexal malignancies and could discriminate between malignant and benign tumours with high specificity [29-31]. This finding has raised a question in the role of HE4 protein in cancer development. In vitro studies confirmed its functional role in cellular processes: When an exogenous HE4 gene is transfected into ovarian cancer cell lines, promoted cell apoptosis and inhibition of cell proliferation are seen [32], while, when HE4 is knockdown, the invasion and adhesion ability of the transfected cells are reduced [33]. More interestingly, HE4-overexpressed cell clones display resistance to platinium-based chemotherapy [34-36]. Silencing HE4 in the ovarian carcinoma cell lines also confirmed that proliferation is inhibited and G(0)/G(1) phase is arrested [37]. Consistent with these results, in vitro study with endometrial cancer cell lines has found that purified, extracellular HE4 protein increased cell viability and proliferation, DNA synthesis, and modulated the mRNA and the protein level of cell cycle inhibitor p21 [38]. As it is known, p21 plays an essential roles in the cellular response to DNA damage, and functions as a regulator of cell cycle progression, that its absence results in cell cycle acceleration [39]. One study examined ovarian cancer cell lines and found a significantly negative correlation between cytoplasmic p21 immunostaining and response to cisplatin based treatment. Knockdown of cytoplasmic p21 increased cisplatin-induced apoptosis, while induction of p21 enhanced the resistance to cisplatin [40]. With the result of 90.32% HE4 tissue expression seen in the present ovarian carcinoma cases, the authors may conclude that HE4 may

influence or may be influenced by cisplatin resistance in ovarian carcinoma.

The present authors observed 90.32% HE4 tissue expression in their ovarian carcinoma cases, which is higher than p53 and CA125. One of the present cases was devoid of CA125 and p53 immunostaining which is also negative with HE4 (case number 8). Despite this negative staining, the hobnail appearance of the tumor cells was easily appreciated on the H&E stain, which is very characteristic of the serous type ovarian carcinoma.

This is the first report showing HE4 tissue expression in human serous high grade ovarian carcinoma and comparing results to p53 and CA125. In routine practice when CA125 and p53 immunostainings are focal and/or negative, HE4 will be a surrogate marker with an easy application protocol. Besides its additive discriminating role in the differential diagnosis among epithelial ovarian carcinoma, as the present authors demonstrated, it is widely expressed and may be a selective molecular target against high grade serous ovarian cancer tumor growth.

In conclusion, the present authors identified HE4 as a potential biomarker for subtyping specific diagnosis of high grade serous ovarian cancer. This study found that it is superior to p53 and CA125 and the authors suggest that combining HE4 with p53 and CA125 could provide added value for histopathological diagnosis of ovarian cancer subtyping. Because of the known interaction between HE4 and cell proliferation, high expression makes it a potential candidate for the therapeutic target.

## Acknowledgements

This work was funded by The Educational Commission at Antalya Training Hospital.

The authors thank Mert Cesur and Onur Ergun for their prompt help obtaining archival formalin-fixed, paraffin-embedded tissues and David M. Silverman for English grammar editing.

## References

[1] Seidman J.D., Cho K.R., Ronnet B.M., Kurman R.J.: "Surface epithelial tumors of the ovary". *In:* Kurman R.J., Ellenson L.H., Ronnet B.M. (eds). *Blaustein's pathology of the female genital tract.* 6th ed. New York: Springer, 2011, 679.

[2] Vang R., Shih I.M., Kurman R.J.: "Ovarian low-grade and high grade serous carcinoma: pathogenesis, clinicopathologic and molecular biologic features, and diagnostic problems". *Adv. Anat. Pathol.*, 2009, *16*, 267.

[3] Yemelyanova A., Vang R., Kshirsagar M., Lu D., Marks M.A., Shih IeM., *et al.*: "Immunohistochemical staining patterns of p53 can serve as surrogate marker for TP53 mutations in ovariancarcinoma: an immunohistochemical and nucleotide sequencing analysis". *Mod. Pathol.*, 2011, *24*, 1248.

[4] Tamir A., Gangadharan A., Balwani S., Tanaka T., Patel U., Hassan A., *et al.*: "The serine protease prostasin (PRSS8) is a potential biomarker for early detection of ovarian cancer". *J. Ovarian Res.*, 2016, *9*, 20.

[5] Ma Q., Wang Q., Zhong D.: "Advances of human epididymis protein 4 in lung cancer". *Zhongguo Fei Ai Za Zhi*, 2015, *18*, 184.

[6] Kirchhoff C.: "Molecular characterization of epididymal proteins". *Rev. Reprod.*, 1998, *3*, 86.

[7] Kirchhoff C., Habben I., Ivell R., Krull N.: "A major human epididymis-specific cDNA encodes a protein with sequence homology to extracellular proteinase inhibitors". *Biol. Reprod.*, 1991, *45*, 350.

[8] Bingle L., Cross S.S., High A.S., Wallace W.A., Rassl D., Yuan G., *et al.*: "WFDC2 (HE4): a potential role in the innate immunity of the oral cavity and respiratory tract and the development of adenocarcinomas of the lung". *Respir. Res.*, 2006, *7*, 61.

[9] Zapardiel I., Gorostidi M., Ravaggi A., Allende M.T., Silveira M., Macuks R.: "Utility of human epididymis protein 4 serum marker for the detection of adnexal malignancy: a multicentric prospective study". Eur. J. Cancer Prev., 2016, Apr 25. [Epub ahead of print]

[10] Angioli R., Miranda A., Aloisi A., Montera R., Capriglione S., De Cicco Nardone C., *et al.*: "A critical review on HE4 performance in endometrial cancer: where are we now?" *Tumour Biol.*, 2014, *35*, 881.

[11] Presl J., Novotny Z., Topolcan O., Vlasak P., Kucera R., Fuchsova R., et al.: "CA125 and HE4 levels in a Czech female population diagnosed with endometrial cancer in preoperative management". *Anticancer Res.*, 2014, *34*, 327.

[12] Yanaranop M, Anakrat V, Siricharoenthai S, Nakrangsee S, Thinkhamrop B.: "Is the Risk of Ovarian Malignancy Algorithm Better Than Other Tests for Predicting Ovarian Malignancy in Women with Pelvic Masses?" *Gynecol. Obstet. Invest.*, 2016, May 21. [Epub ahead of print]

[13] Huhtinen K., Suvitie P., Hiissa J., Junnila J., Huvila J., Kujari H., *et al.*: "Serum HE4 concentration differentiates malignant ovarian tumours from ovarian endometriotic cysts". *Br. J. Cancer*, 2009, *100*, 1315.

[14] Hu L., Du S., Guo W., Chen D., Li Y.: "Comparison of Serum Human Epididymis Protein 4 and Carbohydrate Antigen 125 as Markers in Endometrial Cancer: A Meta-Analysis". *Int. J. Gynecol. Cancer*, 2016, *26*, 331.

[15] Brennan D.J., Hackethal A., Metcalf A.M., .Coward J., Ferguson K., Oehler M.K., *et al.*: "Serum HE4 as a prognostic marker in endometrial cancer--a population based study". *Gynecol. Oncol.*, 2014, *132*, 159.

[16] Minar L., Klabenesova I., Jandakova E., Zlamal F., Bienertova-Vasku J.: "Prognostic value of human epididymis protein 4 in endometrial cancer and its utility for surgical staging". *J. Obstet. Gynaecol. Res.*, 2015, *41*, 1644.

[17] Moore R.G., Miller C.M., Brown A.K., Robison K., Steinhoff M., Lambert-Messerlian G.: "Utility of tumor marker HE4 to predict depth of myometrial invasion in endometrioid adenocarcinoma of the uterus". *Int. J. Gynecol. Cancer*, 2011, *21*, 1185.

[18] Antonsen S.L., Høgdall E., Christensen I.J., Lydolph M., Tabor A., Loft Jakobsen A., *et al.*: "HE4 and CA125 levels in the preoperative assessment of endometrial cancer patients: a prospective multicenter study (ENDOMET)". *Acta Obstet. Gynecol. Scand.*, 2013, *92*, 1313.

[19] Kemik P., Saatli B., Yıldırım N., Kemik V.D., Deveci B., Terek M.C., *et al.*: "Diagnostic and prognostic values of preoperative serum levels of YKL-40, HE-4 and DKK-3 in endometrial cancer". *Gynecol. Oncol.*, 2016, *140*, 64.

[20] Li J., Chen H., Mariani A., Chen D., Klatt E., Podratz K., *et al.*: "HE4 (WFDC2) promotes tumor growth in endometrial cancer cell lines". *Int. J. Mol. Sci.*, 2013, *14*, 6026.

[21] Li X., Gao Y., Tan M., Zhuang H., Gao J., Hu Z., *et al.*: "Expression of HE4 in endometrial cancer and its clinical significance". *Biomed. Res. Int.*, 2015, *2015*, 437468.

[22] Lu R., Sun X., Xiao R., Zhou L., Gao X., Guo L.: "Human epididymis protein 4 (HE4) plays a key role in ovarian cancer cell adhesion and motility". Biochem. Biophys. Res. Commun., 2012, *419*, 274.

[23] Berry N.B., Cho Y.M., Harrington M.A., Williams S.D., Foley J.,

Nephew K.P.: "Transcriptional targeting in ovarian cancer cells using the human epididymis protein 4 promoter". *Gynecol Oncol.*, 2004, *92*, 896.

[24] Lokich E., Singh R.K., Han A., Romano N., Yano N., Kim K., *et al.*: "HE4 expression is associated with hormonal elements and mediated by importin-dependent nuclear translocation". *Sci. Rep.*, 2014, *4*, 5500.

[25] Tamir A., Jag U., Sarojini S., Schindewolf C., Tanaka T., Gharbaran R., *et al.*: "Kallikrein family proteases KLK6 and KLK7 are potential early detection and diagnostic biomarkers for serous and papillary serous ovarian cancer subtypes". *J. Ovarian Res.*, 2014, *7*,109.

[26] Bouchard D., Morisset D., Bourbonnais Y., Tremblay G.M.: "Proteins with whey-acidic-protein motifs and cancer". *Lancet Oncol.*, 2006, *7*, 167.

[27] Hua L., Liu Y., Zhen S., Wan D., Cao J., Gao X.: "Expression and biochemical characterization of recombinant human epididymis protein 4". *Protein Expr. Purif.*, 2014, *102*, 52.

[28] Drapkin R., von Horsten H.H., Lin Y., Mok S.C., Crum C.P., Welch W.R., *et al.*: "Human epididymis protein 4 (HE4) is a secreted glycoprotein that is overexpressed by serous and endometrioid ovarian carcinomas". *Cancer Res.*, 2005, *65*, 2162.

[29] Kristjansdottir B., Levan K., Partheen K., Sundfeldt K.: "Diagnostic performance of the biomarkers HE4 and CA125 in type I and type II epithelial ovarian cancer". *Gynecol. Oncol.*, 2013, *131*, 52.

[30] Gucer F., Kiran G., Canaz E., Kilinc M., Ekerbicer H.C., Avci F., *et al.*: "Serum human epididymis protein 4 can be a useful tumor marker in the differential diagnosis of adnexal masses during pregnancy: A pilot study". *Eur. J. Gynaecol. Oncol.*, 2015, *36*, 406.

[31] Gao L., Cheng H.Y., Dong L., Ye X., Liu Y.N., Chang X.H., *et al.*: "The role of HE4 in ovarian cancer: inhibiting tumour cell proliferation and metastasis". J. *Int. Med. Res.*, 2011, *39*, 1645.

[32] Zhou L., Xiao R., Chen Y., Zhang J., Lu R.Q., Guo L.: "Effect of down-regulation of HE4 gene expression on biologic behavior of ovarian cancer cells". *Zhonghua Bing Li Xue Za Zhi*, 2013, *42*, 687.

[33] Ribeiro J.R., Schorl C., Yano N., Romano N., Kim K.K., Singh R.K., *et al.*: "HE4 promotes collateral resistance to cisplatin and paclitaxel in ovarian cancer cells". *J. Ovarian Res.*, 2016, *9*, 28.

[34] Wang H., Zhu L., Gao J., Hu Z., Lin B.: "Promotive role of recombinant HE4 protein in proliferation and carboplatin resistance in ovarian cancer cells". *Oncol. Rep.*, 2015, *33*, 403.

[35] Moore R.G., Hill E.K., Horan T., Yano N., Kim K., MacLaughlan S., et al.: "HE4 (WFDC2) gene overexpression promotes ovarian tumor growth". *Sci. Rep.*, 2014, *4*, 3574.

[36] Zhu Y.F., Gao G.L., Tang S.B., Zhang Z.D., Huang Q.S.: "Effect of WFDC 2 silencing on the proliferation, motility and invasion of human serous ovarian cancer cells in vitro". *Asian Pac. J. Trop. Med.*, 2013, *6*, 265.

[37] Lu Q., Chen H., Senkowski C., Wang J., Wang X., Brower S., et al.: "Recombinant HE4 protein promotes proliferation of pancreatic and endometrial cancer cell lines". *Oncol. Rep.*, 2016, *35*, 163.

[38] Gartel A.L., Radhakrishnan S.K.: "Lost in transcription: p21 repression, mechanisms, and consequences". *Cancer Res.*, 2005, *65*, 3980.

[39] Xia X., Ma Q., Li X., Ji T., Chen P., Xu H., et al.: "Cytoplasmic p21 is a potential predictor for cisplatin sensitivity in ovarian cancer". *BMC Cancer*, 2011, *11*, 399.

Corresponding Author:
B. CELIK, M.D.,
Department of Pathology, Training Hospital
Varlik Mah, Antalya
07100 (Turkey)
e-mail: bet_celik@yahoo.com