

Ovarian cancer and normal fallopian tube high WFDC2 expression does not correlate with HE4 serum level

Wysoka ekspresja WFDC2 w raku jajnika oraz prawidłowym jajowodzie nie koreluje z poziomem HE4 w surowicy krwi

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Abstract

Objectives: Recent evidence suggests that epithelial ovarian cancer (EOC) does not derive from ovarian surface epithelium but from the tissues of Mullerian origin, particularly from the fallopian tube. HE4, a protein of Mullerian origin, seems to be promising marker for EOC detection and treatment monitoring. This study was designed to compare the expression of WFDC2 gene, encoding HE4 protein, in normal tissue of the ovary, fallopian tube and EOC. The correlation between WFDC2 expression in cancer tissue and serum level of HE4 was additionally measured.

Material and methods: Tumor specimens were obtained from EOC patients during primary surgery before chemotherapy. Samples of normal ovaries and fallopian tubes were collected from healthy patients operated for other reasons. Total RNA was isolated from the tissues and relative WFDC2 expression was evaluated by Real Time RT-qPCR. HE4 serum level in cancer patients was measured using COBAS System.

Results: EOC samples were distinguished by much higher WFDC2 expression in comparison to normal ovaries ($p=0.000016$). Transcriptional activity of WFDC2 in EOC specimens and in normal fallopian tubes was comparable ($p=1.00$). Additionally, lack of correlation between WFDC2 expression in cancer tissue and serum level of HE4 protein in ovarian cancer patients was observed ($p=0.3$).

Conclusions: High expression of WFDC2 was demonstrated for both, EOC and fallopian tube, as opposed to its low expression observed in normal ovaries suggesting that EOC is derived from fallopian tube rather than ovary. Elevated HE4 serum concentration in EOC patients is not correlated with higher gene expression in cancer tissue.

Key words: **ovarian cancer / HE4 / WFDC2 / tumor marker /**

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Streszczenie

Cel pracy: Ostatnie badania sugerują, że rak jajnika (EOC) nie pochodzi z nabłonka jajnika lecz z tkanek pochodzenia Mullerowskiego, a najprawdopodobniej z jajowodu. HE4, białko pochodzenia Mullerowskiego, wydaje się być obiecującym markerem raka jajnika. To badanie zostało przeprowadzone, aby porównać ekspresję genu *WFDC2* kodującego białko HE4 w tkance prawidłowego jajnika, jajowodu oraz raka jajnika. W raku jajnika dodatkowo została oceniona korelacja między ekspresją *WFDC2* a stężeniem jego produktu białkowego w surowicy krwi.

Materiał i metody: Materiał tkankowy został pobrany od pacjentek podczas pierwotnej operacji cytoredukcyjnej raka jajnika. Fragmenty prawidłowych jajników i jajowodów zostały pobrane od pacjentek operowanych z powodu mięśniaków macicy. Z tkanek zostało wyizolowane całkowite RNA. Za pomocą Real-Time RT-qPCR została określona ekspresja *WFDC2*. Stężenie białka HE4 w surowicy krwi pacjentek zostało zmierzone za pomocą systemu COBAS.

Wyniki: Stwierdziliśmy istotnie wyższą ekspresję genu *WFDC2* w tkankach EOC niż w tkankach prawidłowego jajnika ($p=0.000016$). Nie stwierdziliśmy natomiast różnic między ekspresją *WFDC2* w jajowodzie oraz w raku jajnika ($p=1.00$). Dodatkowo, wykazaliśmy brak korelacji między ekspresją tkankową *WFDC2* oraz stężeniem HE4 w surowicy krwi u pacjentek z rakiem jajnika. ($p=0.3$).

Wnioski: Wysoka ekspresja *WFDC2* stwierdzona zarówno w raku jajnika jak i prawidłowym jajowodzie w przeciwieństwie do niskiej ekspresji stwierdzonej w jajniku sugeruje, że rak jajnika może wywodzić się raczej z jajowodu niż z jajnika. Wysokie stężenie HE4 w raku jajnika nie koreluje z nadekspresją *WFDC2* w tkance nowotworowej.

Słowa kluczowe: **rak jajnika / HE4 / WFDC2 / markery nowotworowe /**

Introduction

Epithelial ovarian cancer (EOC) encompasses various histological types that differ in terms of clinical course of the disease, presence of genetic mutations, response to treatment and survival outcome [1]. Recently, there has been growing evidence that most of ovarian cancer types are morphologically similar to tissues other than ovarian epithelium [2]: poorly differentiated serous ovarian cancer, the most common EOC type, was found to resemble the epithelium of fallopian tubes, endometrioid ovarian cancer – the endometrium and mucinous ovarian cancer – the epithelium of the endocervical canal [2, 3]. Moreover, it was postulated that poorly differentiated serous and endometrioid ovarian cancers develop from fallopian tubes, whereas well differentiated and clear cell ovarian cancers arise from nonmalignant conditions such as endometriosis [2].

Due to histological variety of EOC, there is ongoing research that aims at identifying specific biomarkers for each histological subtype. One of the biomarkers that was recently introduced into clinical practice is HE4 (human epididymis protein 4), a protein helpful in differentiating between early EOC and nonmalignant ovarian tumors [4, 5]. Nonetheless, HE4 cannot be considered a universal EOC marker, as its applicability is limited to certain types of EOC – its concentration remains low in most of the mucinous, clear cell, G1 serous and G1 endometrioid ovarian cancers [6, 7]. This fact can be explained by taking into account the histological origin of those tumors, as HE4 was shown to be overexpressed in tissues that derive from Müller ducts, such as fallopian tubes, endometrium and endocervical epithelium [3, 6, 8]. HE4 is also referred to as WAP-4-disulfide core domain 2 (*WFDC2*) and it is classified as a WAP-protein family member [9, 10].

Apart from ovarian cancer, increased expression of WAP-proteins is also observed in gastric, oral cavity, large intestine

and breast cancer. It was suggested that the involvement of WAP-proteins in cell cycle acceleration and cell differentiation indicated their role in carcinogenesis, tumor progression and metastasis [9-11].

In our study we analyzed the expression of *WFDC2* gene encoding HE4 protein in the tissues of EOC, normal fallopian tubes and normal ovaries in the context of ovarian cancer origin. Additionally, the correlation between tissue gene expression and protein level in serum of EOC patients was evaluated.

Materials and methods

After receiving the approval of the Local Bioethics, tissue and serum samples were collected from 30 patients diagnosed with ovarian cancer undergoing primary cytoreductive surgery in Gynecologic Oncology Department of the Poznan University of Medical Sciences. Histopathological examination was performed by a qualified pathologist following the FIGO classification.

The control group consisted of tissue samples of normal fimbriae and ovaries (that included epithelium and stroma) obtained from 10 patients operated due to reasons other than malignant tumors.

Age of the patients ranged from 30 to 74 (with an average of 55) in the experimental group and from 37 to 73 (with an average of 54) in the control group. 27 patients were diagnosed with FIGO stage III EOC, while 3 had FIGO stage I or II EOC. (Table 1).

Patients from the control group were operated due to uterine myomas. HE4 serum concentration was measured before surgical treatment.

All tissue samples were placed in RNAlater (Sigma-Aldrich) stabilization reagent immediately after surgery and stored at -20°C until RNA isolation. Total RNA was isolated from the tissues and relative *WFDC2* expression was measured by RT-PCR (real-time polymerase chain reaction).

RT-qPCR

RNA extraction and analysis

Total RNA from the analyzed tissues was extracted using TriPure Isolation Reagent (Roche Diagnostics) according to manufacturer's protocol. Purity and concentration of RNA was analyzed spectrophotometrically ($A=260$ nm, $A=280$ nm) and electrophoretically on agarose denaturing gel. RNA samples were stored at -80°C until reverse transcription.

Reverse transcription

1 μg of RNA was employed individually for one reverse transcription reaction with oligo(dT) universal primer (Roche Molecular Diagnostic) and Transcriptor Reverse Transcriptase (Roche Molecular Diagnostic) according to manufacturer's protocol.

Real-Time PCR

To quantify *WFDC2* expression, a quantitative PCR (qPCR) was performed using TaqMan-based Real Time Ready (Roche Diagnostics) kit (Assay ID 108184). Expression of a reference gene, *HPRT*, was assessed applying Universal Probe Library Human *HPRT* Gene Assay (Roche Diagnostics). Amplification of both genes was carried out in conditions specified by the manufacturer of the assay in Light Cycler 2.0® instrument. In all cases relative expression of a target gene was normalized against *HPRT* and relative level was calculated using Light Cycler 2.0 software. All experiments were performed in triplicates using newly synthesized cDNA.

PCR efficiencies were calculated from standard curves (generated using serial dilutions of *in vitro* generated transcripts). The PCR results were assembled using the LightCycler® Data Analysis (LCDA) Software version 4.0.5.415 dedicated for the LightCycler® 2.0 instrument.

HE4 serum level measurement

The analysis of HE4 serum concentration was performed in the Central Hospital Laboratory Unit following standard procedure. Analytical procedures were performed using the Cobas unit and reagent kits delivered by Roche Diagnostics.

Statistical analysis

RT-qPCR data were log transformed and analyzed using Statistica ver. 10.0 software package (StatSoft, Krakow, Poland). Kruskal-Wallis ANOVA, Spearman Pearson tests were performed and the results were considered to be statistically significant if the p-value was lower than 0.05. Figures were created in Excel 2010 (Microsoft, USA).

Ethics Statement

The study design, protocol and written information for the patients were accepted by the Local Bioethics Commission of Poznan University of Medical Sciences (No. 695/12). Written informed consent was obtained from all patients enrolled in the study.

Results

Relative expression of *WFDC2* gene in EOC, ovary and fallopian tube

Statistical analysis of qPCR results illustrating *WFDC2* expression confirmed the presence of gene transcripts in all studied tissue samples. Median level of *WFDC2* tissue expression equaled 0.0035 (range 0.000061-0.1) in EOC, it 0.0042 (range

Table I. Clinical characteristics of the study group.

Median age (range)	55 (30-74)	
FIGO stage	I-II	3
	III	27
Histology	Serous	18
	Mucinous	2
	Endometrioid	2
	Undifferentiated	6
	Carcinosarcoma	2
Grade	G1	2
	G2	6
	G3	22

Table II. Relative *WFDC2* gene expression in ovarian cancer, fallopian tube and ovary (RT-qPCR results).

	n	min	max	median
ovarian cancer	30	0.000061	0.1	0.0035
fallopian tube	10	0.000033	0.01	0.0042
normal ovary	10	0.000006	0.0005	0.00004

Table III. Statistical differences in *WFDC2* expression between study groups (Kruskal-Wallis test).

Group	p
ovarian cancer vs ovary	0.000016
ovarian cancer vs fallopian tube	1.000000
ovary vs fallopian tube	0.001775

Table IV. *WFDC2* tissue gene expression and HE4 serum concentration in EOC.

EOC group	median	range
HE4 serum concentration	528 pmol/dl	54-6930 pmol/dl
<i>WFDC2</i> expression	0.0035	0.000061-0.1

0.000033-0.01) in normal fallopian tube and 0.00004 (range 0.000006-0.0005) in normal ovary (Table II, Figure 1). Thus, *WFDC2* expression in EOC tissue and normal fallopian tube was comparable ($p=1.000000$). In contrast, the comparison of *WFDC2* expression in normal ovary and EOC revealed a statistically significant difference ($p=0.00016$). Similarly, *WFDC2* expression in fallopian tube and normal ovary differed significantly ($p=0.001775$) (Table III).

HE4 serum level in EOC

In EOC patients, median HE4 serum level equaled 528 pmol/dl (range 54-6930 pmol/dl) and median *WFDC2* expression was 0.0035 (Table 4). Thus, no statistically significant correlation

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between the relative *WFDC2* gene expression in EOC tissue and preoperative serum concentration of its protein product HE4 was demonstrated. The result was confirmed by two statistical tests: Spearman ($p > 0.05$) and Pearson ($p = 0.3$).

Discussion

Despite extensive research some aspects of ovarian cancer pathogenesis remain unclear. For many years EOC was considered a uniform pathology that originated from the epithelium of the ovary (mesothelium). Only recently it has been suggested that some types of EOC develop from tissues derived from Mullerian ducts, such as fallopian tubes [3]. A detailed analysis performed on tissue samples obtained from women with BRCA1/2 mutations surprisingly revealed no pathology in the ovaries. However, foci of microinvasive serous tubal intraepithelial carcinoma (STIC) were found in the fallopian tubes identifying the organ as a potential starting point of carcinogenesis. [12]

In a meta-analysis of gene expression profiles in ovarian cancer, *WFDC2* was identified as the most commonly overexpressed gene [13]. Its product, HE4, is a protein of Mullerian origin and a relatively new ovarian cancer biomarker whose usefulness in differential diagnosis of EOC was confirmed in various studies [5, 14, 15].

In immunohistochemical studies, the highest HE4 expression was observed in ovarian serous carcinoma, whereas moderate to high levels of HE4 expression were registered for endometrioid EOC, endometrial cancer and other: lung, occasional breast and pancreas adenocarcinomas [16]. Furthermore, moderate HE4 expression was demonstrated in normal glandular epithelium of the female genital tract (fallopian tubes, endometrium and endocervix), respiratory tract and kidneys [16].

These results are consistent with our present findings that show elevated expression of *WFDC2* in ovarian cancer and normal fallopian tubes as opposed to its low expression in healthy ovaries. The amount of *WFDC2* transcripts identified in the ovaries differed significantly from the amount detected in EOC and the fallopian tubes. These results seem to confirm the molecular similarity between ovarian cancer and normal fallopian tube tissue.

In contrast to our study, Galgano *et al.* reported lack of HE4 in normal ovary [16]. Moreover Clauss *et al.* using semi-quantitative PCR did not observe *WFDC2* expression in immortalized OSE [24]. These discrepancies may be a consequence of adopting different methods for measuring HE4 expression: Galgano's group used immunohistochemistry, Clauss and coworkers utilized semi-quantitative PCR while we applied RT-qPCR, which is the most sensitive method enabling detection of even single copies of *WFDC2* transcripts present in the tissue.

Very recently, it was demonstrated that the overexpression of *WFDC2* in EOC stimulates tumor growth and disease progression [17]. Another study observed a higher HE4 expression in high-grade serous ovarian carcinoma than in low-grade EOC [18]. Thus, the relationship between *WFDC2* elevated expression and a more aggressive clinical course of the disease was established [17, 18].

Similarly, in our experiments we observed lower *WFDC2* gene expression in low-stage and low-grade ovarian cancer than in other EOCs, although the difference was statistically insignificant, possibly due to insufficient number of samples.

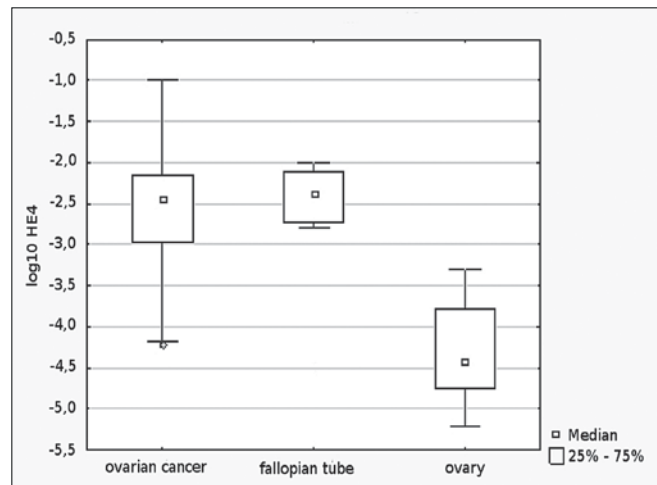


Figure 1. Relative *WFDC2* expression in ovarian cancer, normal fallopian tube and normal ovary.

One of the most interesting findings of the present study is the lack of association between HE4 serum level and *WFDC2* expression in EOC tissue. Whereas both, the protein level and mRNA level, are high in EOC patients and they independently correlate with poorer prognosis and shorter overall survival [6, 18, 19], the lack of correlation between them is surprising and demands further analysis and explanation.

Studies on the relationship between mRNA and protein expression of selected genes showed that in many cases the level of mRNA does not correlate with the amount of protein produced in cells. It is known that protein expression may be post-transcriptionally regulated [20]. Another explanation for our findings is provided by Bingle *et al.* who report the existence of at least five splicing variants of HE4 in lung cancer [21].

It is known that HE4 is composed of two WAP domains. Each WAP domain has a dual function related to apoptosis and proliferation processes [22]. Based on the already discovered function of other WAP-domain family members – Elafin and SLPI – it was hypothesized that HE4 has a similar antiproteinase function and might be a part of host defense of the airways [21]. However, HE4 splicing forms contain only one WAP domain and therefore their function may be impaired or changed. Thus, due to alternative splicing, the functions of regular HE4 form can be altered and lead to EOC development. This theory seems especially plausible in light of the paper by Tokushi *et al.* who studied the association of HE4 with clinicopathological factors in lung cancer and proved that the presence of all splicing forms of HE4 closely correlate with the prognosis. Surprisingly, the level of full-length HE4 did not show such correlation [23].

The existence of splice variants could also elucidate the resemblance of ovarian cancer and healthy fallopian tubes in relation to *WFDC2* gene expression, even though the groups differed in HE4 serum levels (in ovarian cancer patients HE4 serum concentration was significantly higher than in healthy patients) [24, 25]. Therefore, further analysis of *WFDC2* expression and alternative mRNA splicing in EOC is needed to identify splice variants correlated with poorer prognosis and/or disease progression.

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The results of our study verify the genetic similarity between ovarian cancer and healthy fallopian tube in terms of *WFDC2* expression and support the theory of fallopian tube involvement in ovarian cancer carcinogenesis. These findings can be crucial for EOC prevention.

The lack of correlation between HE4 serum level and tissue *WFDC2* gene expression in EOC patients indicates the necessity of further investigation on regulation of the gene expression, especially in terms of HE4 splicing variants and their role in EOC carcinogenesis.

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Disclosure

The authors report no conflicts of interest in this work.

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