

# Comprehensive analysis of HE4 expression in normal and malignant human tissues

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The *HE4* (*WFDC2*) gene encodes a WAP-type four disulphide core domain-containing protein with a presumptive role in natural immunity. Multiple studies have consistently identified upregulation of *HE4* gene expression in carcinomas of the ovary; however, the expression in normal and malignant adult tissues has not been examined in detail. Here, we examined the expression of the *HE4* gene and protein in a large series of normal and malignant adult tissues by oligonucleotide microarray and tissue microarray, respectively. *HE4* gene expression was highest in normal human trachea and salivary gland, and to a lesser extent, lung, prostate, pituitary gland, thyroid, and kidney. In a series of 175 human adult tumors, gene expression was highest in ovarian serous carcinomas. However, adenocarcinomas of the lung, and occasional breast, transitional cell and pancreatic carcinomas had moderate or high levels of *HE4* expression. Using tissue microarrays and full tissue sections of normal and 448 neoplastic tissues, HE4 immunoreactivity was found in normal glandular epithelium of the female genital tract and breast, the epididymis and vas deferens, respiratory epithelium, distal renal tubules, colonic mucosa, and salivary glands, consistent with *HE4* gene expression. In addition to consistent positivity in ovarian carcinoma, some pulmonary, endometrial, and breast adenocarcinomas, mesotheliomas, and less often, gastrointestinal, renal and transitional cell carcinomas were also positive. Knowledge of the expression patterns of HE4 in our survey is useful for application in histopathologic diagnosis, and should be taken into consideration in future studies that examine the role of HE4 as a serological tumor biomarker or as a target for gene-based therapy.

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Human epididymis protein 4 (HE4) was first identified in the epithelium of the distal epididymis and originally predicted to be a protease inhibitor involved in sperm maturation.<sup>1,2</sup> The gene, also known as *WFDC2*, encodes a protein with a WAP-type four disulphide core (WFDC) domain.<sup>3</sup> Given its homology and comparable transcription profile with known leukocyte protease inhibitors in the WFDC family of proteins, HE4 also presumably has a role in natural immunity.<sup>3–5</sup> In malignant neoplasms, gene expression profiling studies have consistently identified upregulation of *HE4* in carcinomas of the ovary,<sup>6–14</sup> and several studies have analyzed HE4 protein expression in ovarian neoplasms, providing the opportunity for its appli-

cation in histopathologic diagnosis.<sup>12,13,15,16</sup> Moreover, recent studies have shown elevated HE4 protein levels in serum from patients with ovarian tumors, demonstrating a similar sensitivity to CA125, but increased specificity for malignant tumors as compared to benign disease.<sup>17</sup> However, the examination of HE4 in other common malignancies, benign lesions, and normal tissues has not occurred to a large scale. Here, we used oligonucleotide and tissue microarrays to survey *HE4* gene and protein expression in a large group of malignant and normal tissues from adults. We discuss the implications of our results in terms of the potential role for HE4 in histopathologic diagnosis, as a serum tumor biomarker, and as a therapeutic target.

## Materials and methods

### Oligonucleotide Microarray

*HE4* is one of more than 8900 different human genes represented on U95a GeneChips (Affymetrix, Santa

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Clara, CA, USA), which we used previously to develop a molecular classification of 175 human carcinomas based on patterns of gene expression.<sup>18</sup> With the exception of the ovarian neoplasms, the same carcinomas analyzed for gene expression were also examined for HE4 by immunohistochemistry on tissue microarrays (see below). In brief, frozen sections of profiled tumors were examined by hematoxylin-and-eosin, and areas rich in neoplastic cells were cut from the frozen blocks. RNA extraction and hybridization onto oligonucleotide microarrays (U95a GeneChip; Affymetrix) were performed as reported previously.<sup>19</sup> The processing and scaling of the hybridization data were also performed as described previously.<sup>20,21</sup> Gene expression was quantitated in relative intensity units, and divided into three groups: low (<300), moderate (300–999) and high ( $\geq 1000$ ).

### Tissue Microarray

The University of Virginia Human Investigation Committee approved the use of human tissues in this study. Tissue microarrays containing 1–3 0.6-mm cores from a comprehensive selection of formalin-fixed, paraffin-embedded normal tissues (Table 1) and neoplasms from adult patients (Table 2) were constructed using a tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA). Selected whole tissue sections were stained for comparison with the tissue microarrays. In addition, whole tissue sections were used for most of the renal papillary and chromophobe carcinomas, oncocytomas, ovarian clear cell carcinomas, and basal cell carcinomas of the skin.

### Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were placed in citrate buffer (pH 6.0) and heated in a microwave oven for 20 min before application of the rabbit polyclonal antibody to HE4 (1:20 dilution; Signet Laboratories Inc., Dedham, MA, USA). After incubation with the primary antibody, and addition of the biotinylated secondary antibody, avidin-biotin immunoperoxidase was applied. Diaminobenzidine was used as the chromogen. Sections were then counterstained with hematoxylin. Tissue sections of human epididymis processed in a comparable manner provided a positive control with surrounding soft tissue providing an internal negative control. Immunoreactivity was evaluated independently by two observers without specific knowledge of the oligonucleotide microarray results. 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%), 3 (50–74%), and 4 (75–100%)) with discrepancies resolved by consensus. The grades were multiplied to determine an H-score. The H-scores

**Table 1** Normal tissues examined for HE4 by immunohistochemistry

<i>Aerodigestive</i>	<i>Placenta</i>
Alveoli	Amnion
Bronchial epithelium	Chorionic villi
Salivary gland, parotid and minor	Umbilical cord
Squamous mucosa, oropharyngeal	Vulva
<i>Endocrine</i>	<i>Hematolymphoid</i>
Adrenal cortex	Bone marrow
Adrenal medulla	Lymph node
Parathyroid	Spleen
Pituitary	Thymus
Thyroid	Tonsil
<i>Gastrointestinal</i>	<i>Hepatic and Pancreaticobiliary</i>
Anal canal	Bile ducts
Appendix	Liver
Colon	Gallbladder
Duodenum	Pancreas
Esophagus	<i>Musculoskeletal</i>
Ileum	Cartilage
	Fat
<i>Neural</i>	Skeletal muscle
Cerebral cortex	Synovium
Choroid plexus	
Ependyma	<i>Skin and Lacrimal gland</i>
Ganglia	Dermis and epidermis
Hippocampus	Lacrimal gland
Meninges	<i>Urogenital</i>
Nerve, peripheral	Bladder
Motor neurons	Epididymis
Purkinje cells	Kidney
White matter	Prostate
	Seminal vesicles
<i>Gynecological and Breast</i>	Seminiferous tubules
Breast	Vas deferens
Ectocervix	
Endocervix	<i>Vascular</i>
Endometrium	Aorta
Decidualized	Heart
Proliferative	Lymphatics
Secretory	Pulmonary artery
Fallopian tube	Small vein, intestinal
Myometrium	
<i>Ovary</i>	
Corpus luteum	
Primary oocytes	
Surface epithelium	
Stroma	

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  $\geq 4$ ).

## Results

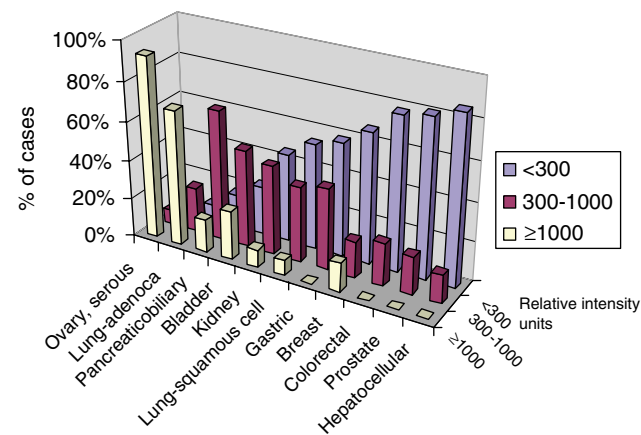
### Oligonucleotide Microarray Analysis

Among a series of 43 normal human tissues previously reported,<sup>22</sup> expression of *HE4* was restricted, with highest levels in trachea and salivary gland and to a lesser extent in lung, prostate, thyroid, pituitary gland and kidney (data not shown;

**Table 2** Neoplasms stained for HE4

	Negative	Weak	Strong
Bladder and ureter, transitional cell carcinoma	23	7	2
Breast, invasive ductal carcinoma	14	9	3
Endometrial carcinoma, endometrioid type	2	3	11
<i>Gastrointestinal, hepatic, pancreaticobiliary</i>			
Colorectal carcinoma	21	6	0
Hepatocellular carcinoma	5	0	0
Pancreaticobiliary carcinoma	1	3	4
Upper gastrointestinal carcinoma	15	2	0
<i>Kidney</i>			
Renal cell carcinoma, clear cell type	25	1	0
Renal cell carcinoma, papillary type	7	1	5
Chromophobe carcinoma	4	5	4
Oncocytoma	4	3	5
<i>Lung</i>			
Adenocarcinoma	9	5	7
Squamous cell carcinoma	6	3	0
<i>Mesothelioma</i>			
Epithelioid type	12	8	8
Spindle cell type	7	3	9
<i>Ovary</i>			
Serous papillary carcinoma	0	6	26
Endometrioid carcinoma	2	3	5
Clear cell carcinoma	2	2	9
Poorly differentiated carcinoma	0	3	1
Mucinous tumor	6	1	0
Germ cell tumor	5	0	0
Sex cord stromal tumor	9	0	0
Gastrointestinal metastasis	1	3	1
Prostatic adenocarcinoma	20	0	0
Salivary gland, various	0	12	12
Skin, basal cell carcinoma	10	1	0
Thyroid, various	13	12	10

see <http://symatlas.gnf.org>). In malignant tissues, the highest expression levels were observed in serous ovarian carcinomas ( $N=26$ ) (Figure 1). High expression was also noted in nine of 13 adenocarcinomas of the lung and a single squamous cell carcinoma; the remainder of squamous lung tumors (12 of 13) had moderate or low levels of expression. High expression was detected in two of eight transitional cell carcinomas of the urinary tract, while only a single renal cell carcinoma (clear cell type) demonstrated high expression ( $N=11$ ). Twenty of 27 invasive ductal carcinomas of the breast had low levels of HE4 expression, with occasional tumors demonstrating high levels of expression. Eighteen of 23 colon carcinomas had low expression, while five of 12 gastric adenocarcinomas had moderate expression levels. Four of six pancreatic adenocarcinomas had moderate expression, and one had a high level of HE4 expression. Hepatocellular carcinomas ( $N=7$ ) had low expression. Prostate adenocarcinomas occasionally had moderate HE4 expression (five of 26).



**Figure 1** HE4 gene expression in 175 human carcinomas. Levels of mRNA expression were determined by hybridization to Affymetrix U95a GeneChips. Relative fluorescence units were divided into three groups: low (<300), moderate (300–1000), and high ( $\geq 1000$ ).

### Immunohistochemistry of Normal Adult Tissues

Immunohistochemical staining for HE4, when present, was limited to epithelium. It was detected in the normal epithelium of the female genital tract, including endocervical glands, endometrial glands, fallopian tube, and Bartholin's glands. Breast epithelium was variably positive, but typically stronger in the ducts than in the lobules. We observed very rare focal staining within the epithelium of the prostate gland. The epithelium of the vas deferens was also positive. There was strong staining within the distal convoluted tubules of the kidney, with equivocal staining of the proximal convoluted tubules, likely non-specific uptake. Bronchial epithelium was immunoreactive. Both minor and major salivary glands demonstrated strong staining of the ductal epithelium, consistent with the high levels of gene expression, with weaker and focal staining of the acinar cells. Scattered cells in the anterior pituitary gland were strongly positive. Oxyphilic thyrocytes were weakly positive. We observed focal staining within the lacrimal gland, and weak staining within eccrine glands. Colonic epithelium was variably positive, while rare pancreatic acinar cells were weakly immunoreactive. The remaining tissues examined (Table 1) lacked immunoreactivity for HE4.

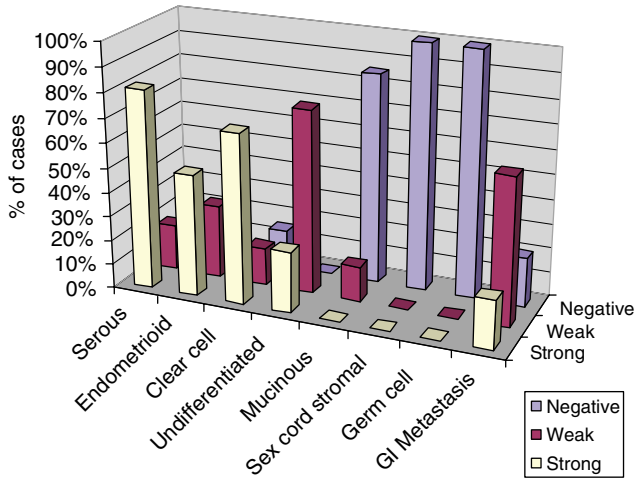
### Immunohistochemistry of Neoplasms

Staining for HE4 in 448 neoplasms is summarized in Table 2. Of ovarian tumors (Figure 2), all serous ( $N=32$ ), including two borderline tumors, and all poorly differentiated ( $N=4$ ) ovarian carcinomas were positive (Figure 3a), but with intratumoral variability, confirmed by selected immunostaining of standard tissue sections. Endometrioid carcinomas ( $N=10$ ) and clear cell carcinomas ( $N=13$ ) were

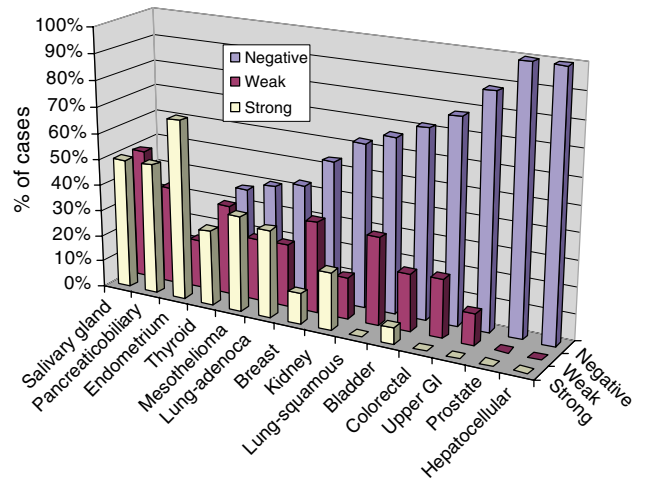
mostly positive, while mucinous carcinomas were negative (except for one that stained weakly). Granulosa cell tumors ( $N=5$ ), fibromas ( $N=4$ ), dysgerminomas ( $N=4$ ), and a single yolk sac tumor were negative for HE4. All four metastatic colorectal tumors showed some positivity, while a single metastatic gastric carcinoma was negative.

Staining for HE4 in non-ovarian neoplasms is summarized in Figure 4. Nearly 90% of endometrial

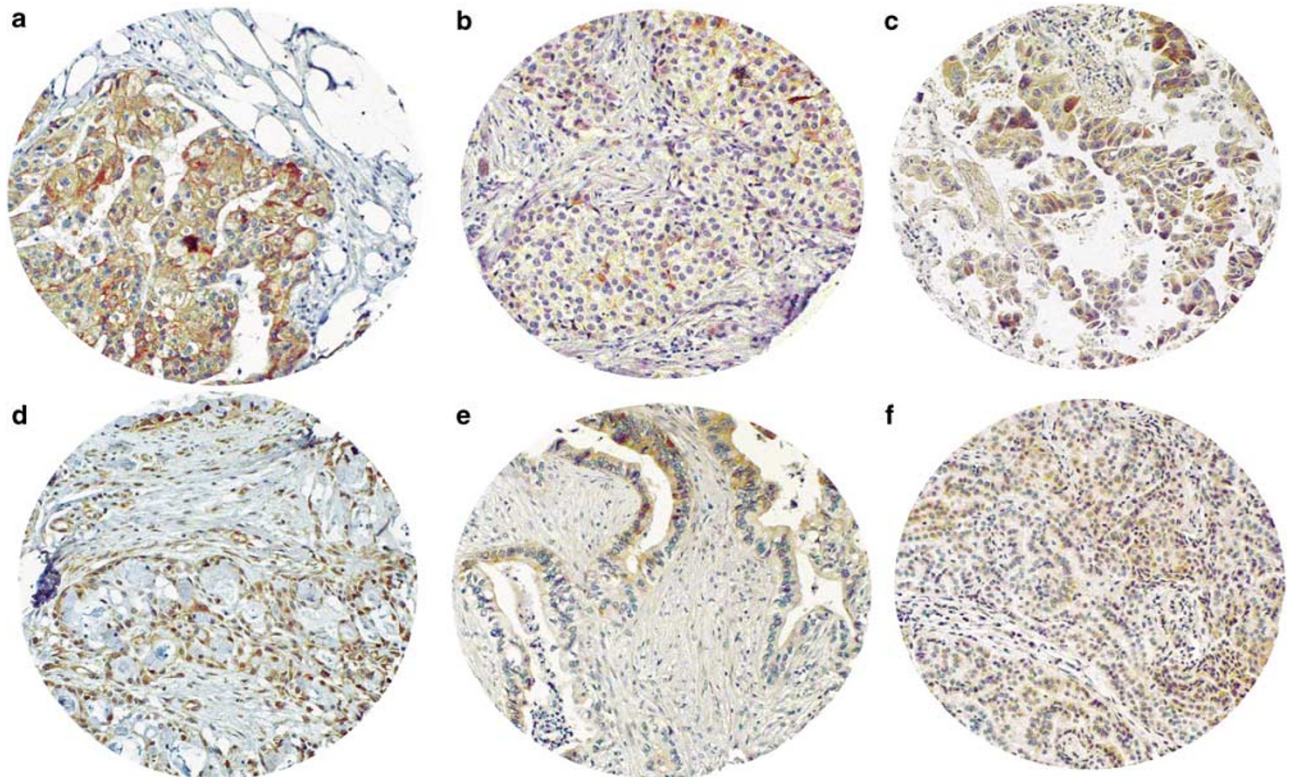
carcinomas, endometrioid type, were positive for HE4. Breast carcinomas showed variable expression, as 14 were negative, nine were weakly positive, and three were strongly positive (Figure 3b). All prostate carcinomas were negative, while nine of 32 transitional cell carcinomas showed some immunoreactivity. Only one of 26 renal cell carcinomas, clear cell type, was positive, while five of 13 papillary renal cell carcinomas demonstrated strong



**Figure 2** HE4 immunoreactivity in tissue microarrays of ovarian tumors. Degree of staining was scored as negative (H-score = 0), weak (H-score = 1–3), or strong (H-score  $\geq 4$ ).



**Figure 4** HE4 immunoreactivity in tissue microarrays of non-ovarian tumors. Degree of staining was scored as negative (H-score = 0), weak (H-score = 1–3), or strong (H-score  $\geq 4$ ).



**Figure 3** Examples of HE4 immunoreactivity in tissue microarrays of (a) ovarian serous carcinoma, (b) invasive ductal carcinoma of the breast, (c) adenocarcinoma of the lung, (d) mucoepidermoid carcinoma, (e) pancreatic carcinoma, and (f) epithelioid malignant mesothelioma.

immunoreactivity. Of 13 chromophobe carcinomas, four were strongly positive, with weak staining in an additional five cases. Oncocytomas showed similar staining, with strong immunoreactivity in five cases, weak in three, and no staining in four tumors.

Twelve of 21 adenocarcinomas of the lung (Figure 3c) were positive while three of nine squamous cell carcinomas were weakly positive for HE4. All salivary gland neoplasms demonstrated some reactivity. Prominent staining was identified in the epithelium of Warthin's tumors ( $N=7$ ) and in two mucoepidermoid carcinomas (Figure 3d), while variable staining was seen in mixed tumors ( $N=5$ ) and salivary duct carcinomas ( $N=2$ ). Focal, weak staining occurred in adenoid cystic carcinomas ( $N=7$ ) and a single polymorphous low-grade adenocarcinoma.

Colorectal (six of 27) and upper gastrointestinal tract (two of 17) adenocarcinomas occasionally demonstrated weak positivity. All hepatocellular carcinomas were negative, while five of six pancreatic carcinomas (Figure 3e), a single gallbladder carcinoma, and a single cholangiocarcinoma were immunoreactive. Of thyroid carcinomas, immunopositivity was observed in 10 of 13 papillary and nine of 13 follicular carcinomas. No reactivity was found in an anaplastic, and two medullary carcinomas. Three of the six follicular adenomas were also positive. Positive staining was present in 12 of 19 spindle cell and 16 of 28 epithelioid malignant mesotheliomas (Figure 3f). Ten of 11 basal cell carcinomas lacked immunoreactivity. A single pituitary adenoma (GH secreting) was also negative.

## Discussion

*HE4* (*WFDC2*) is one of 14 homologous genes on chromosome 20q12–13.1 which encode proteins with a WAP-type four disulphide core (WFDC) domain.<sup>3</sup> Two of these genes, *SLPI* and *elafin*, encode known leukocyte protease inhibitors<sup>23,24</sup> which are co-expressed with *HE4* in the upper aerodigestive, reproductive, and urological tracts.<sup>4</sup> The genes at the WFDC locus are variably conserved across species, and presumably share a role in natural immunity with both antimicrobial and anti-inflammatory activity.<sup>5</sup> Expression of *SLPI* and *elafin* has been identified in various carcinomas, and these genes may play a role in cancer development or progression.<sup>25–29</sup> In addition, amplification of 20q12–13 has been demonstrated specifically in breast and ovarian carcinomas.<sup>30,31</sup>

Recent interest in *HE4* has been generated by the consistent demonstration of overexpression in ovarian carcinomas in comparison to normal ovarian tissue. Although a meta-analysis of gene expression profiles of ovarian carcinomas found *HE4* to be the most frequently upregulated gene,<sup>32</sup> a large scale evaluation of *HE4* gene and protein expression in

both normal tissues and common nonovarian carcinomas has not been performed thus far.

We found that ovarian serous carcinomas had, on average, considerably higher levels of *HE4* gene expression than other carcinomas. Adenocarcinomas of the lung demonstrated the next highest average level of intensity, while squamous cell carcinomas had low expression levels. Transitional cell, breast, renal, and pancreatic carcinomas had moderate levels of *HE4* expression, but several of them had comparable levels to that for serous ovarian carcinomas. Colonic, gastric, hepatic and prostatic carcinomas had mostly low levels of *HE4* expression.

In our study, we found evidence of HE4 protein in the normal glandular epithelium of the female genital tract, while the normal ovarian surface epithelium was negative. In the male genital tract, only the epididymis and vas deferens were strongly immunopositive. Elsewhere, respiratory epithelium, distal renal tubules, and salivary glands were also consistently immunoreactive. Colonic mucosa was positive with variable intensity.

In agreement with other studies,<sup>12,13,15,16</sup> most of the ovarian surface epithelial tumors, with the exception of mucinous type, were immunoreactive for HE4. Significant staining was also identified in endometrial, lung, breast, and pancreaticobiliary carcinomas, as well as various renal, thyroid and salivary gland neoplasms, and malignant mesotheliomas.

Our immunohistochemical and oligonucleotide microarray results show that while upregulation of HE4 in ovarian serous tumors is considerably higher than that of other carcinomas, occasional non-ovarian tumors achieve similar levels of protein and mRNA expression. As HE4 can be detected by immunohistochemistry in a variety of common carcinomas that may be in the differential diagnosis of ovarian cancer, an anti-HE4 antibody obviously should not be used in isolation, but may complement the selection of other antibodies for diagnosis. Although HE4 was consistently expressed in serous, poorly differentiated, and endometrioid-type carcinomas, the staining was sometimes variable, and therefore a lack of staining in a limited biopsy sample should be interpreted with caution. While most of the cancers positive for HE4 showed diffuse cytoplasmic staining, rare adenocarcinomas contained cells with prominent apical accentuation.

In addition to its potential utility in histopathologic diagnosis, HE4 has been analyzed as a serological biomarker for ovarian carcinoma. In a report using a HE4-based ELISA assay tested in women with benign or malignant ovarian disease as well as in age-matched controls, HE4 showed comparable sensitivity to CA125, but had better specificity for malignant tumors.<sup>17</sup> Based on our results, further serological evaluation should include patients with a variety of benign and malignant diseases of other major organs, including lung,

thyroid, salivary gland, pancreas, endometrium, breast, urothelium, and mesothelium.

Given the identification of an *HE4* promoter which is differentially active in ovarian cancer cell lines as compared to normal controls, transcriptional targeting for gene-based therapy remains an area of active investigation.<sup>33</sup> Similar studies have evaluated the use of the promoter for the WFDC family member *SLPI* for gene therapy in ovarian and cervical carcinomas.<sup>34–36</sup>

In summary, our results characterize the distribution of HE4 in normal adult tissues and various malignancies. We found significant gene expression and strong immunoreactivity in some pulmonary, endometrial, and breast adenocarcinomas, and less often, in gastrointestinal and urological carcinomas. Additionally, we noted the intratumoral variability of staining within ovarian serous carcinomas. Knowledge of the protein expression patterns of HE4 in our survey of carcinomas is useful for its application in histopathologic diagnosis, and should be taken into consideration in future studies that examine the role of HE4 as a serological tumor biomarker or as a target for gene-based therapy.

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## Duality of interest

None declared.

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