Human Epididymis Protein 4 (HE4) Is a Secreted Glycoprotein that Is Overexpressed by Serous and Endometrioid Ovarian Carcinomas

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Abstract

Among the genes most commonly identified in gene expression profiles of epithelial ovarian carcinomas (EOC) is the gene for human epididymis protein 4 (*HE4*). To ascertain its clinical utility, we did a comprehensive assessment of HE4 protein expression in benign and malignant ovarian and nonovarian tissues by immunohistochemistry. In comparison with normal surface epithelium, which does not express HE4, we found that cortical inclusion cysts lined by metaplastic Mullerian epithelium abundantly express the protein. Its expression in tumors was restricted to certain histologic subtype: 93% of serous and 100% of endometrioid EOCs expressed HE4, whereas only 50% and 0% of clear cell carcinomas and mucinous tumors, respectively, were positive. Tissue microarrays revealed that the majority of nonovarian carcinomas do not express HE4, consistent with our observation that HE4 protein expression is highly restricted in normal tissue to the reproductive tracts and respiratory epithelium. HE4 is predicted to encode a secreted protein. Using reverse transcription-PCR, we identified ovarian cancer cell lines that endogenously overexpress HE4. Cultured medium from these cells revealed a secreted form of HE4 that is N-glycosylated. This observation is consistent with the recent report that HE4 circulates in the bloodstream of patients with EOC. Therefore, HE4 is a secreted glycoprotein that is overexpressed by serous and endometrioid EOCs. Its expression in cortical inclusion cysts suggests that formation of Mullerian epithelium is a prerequisite step in the development of some types of EOCs. (Cancer Res 2005; 65(6): 2162-9)

Introduction

Ovarian cancer is a leading cause of cancer-related death of women in the United States. It is estimated that in 2004, ~24,000 American women will be diagnosed with ovarian cancer and that 14,000 women will die of the disease (1). Worldwide, the incidence is estimated to include 190,000 new cases and 114,00 deaths annually (2). At the time of diagnosis, three quarters of patients have locally advanced or disseminated disease that is characterized by diffuse intraperitoneal spread and in many cases, malignant ascites (reviewed in refs. 3, 4). Cures are rare at advanced clinical stages, placing emphasis on early detection to reduce ovarian cancer mortality.

A sensitive and specific screening test that could detect ovarian cancer at a curative stage has yet to be developed. The application of cDNA and oligonucleotide microarray analyses to ovarian cancer has resulted in the identification of many genes that are overexpressed in primary tumors and ovarian cancer cell lines (5–19). We previously validated the expression of several of the most frequently identified and most highly expressed genes (*Mucin1*, *EpCAM*, *Mesothelin*, and *CD9*) in human ovarian cancer by immunohistochemistry on tumor samples (20).

Among the genes most commonly overexpressed in ovarian cancers relative to normal tissues is the gene for human epididymis protein 4 (*HE4*; refs. 5, 6, 8, 9, 11, 13, 19). *HE4* was first described as an epididymis-specific gene using Northern blot analysis and *in situ* transcript hybridization (21, 22). Subsequent studies using RNA dot blots, reverse transcription-PCR (RT-PCR), and Northern blot analysis suggested that HE4 RNA expression might be more widespread (23, 24). HE4 is also designated WFDC2 because it contains two whey acidic protein (WAP) domains and a "four disulfide core" made up of eight cysteine residues. The *HE4* gene resides on human chromosome 20q12-13.1, a region that harbors a locus of 14 genes encoding protein domains that have homology with WAP (Fig. 1*A*; ref. 24). Among these WAP genes is secretory leukocyte protease inhibitor (*SLPI*), which is also overexpressed in ovarian carcinomas (11, 12). Significantly, comparative genomic hybridization studies have shown that 20q13 is among the most frequently amplified chromosomal regions in ovarian carcinomas (25–27). However, the scope of HE4 protein expression in epithelial ovarian carcinomas versus nonovarian carcinomas and normal tissues is not known.

In this report, we focused on the characterization of the *HE4* gene product in benign and malignant tissues with the aim of defining features that would aid in its further clinical development as a biomarker for ovarian cancer.

Materials and Methods

Cell Lines. Fourteen established ovarian carcinoma cell lines were used to evaluate the mRNA and protein expression levels of HE4. They included OVCAR-5, OVCAR-8, IGROV-1 (kindly provided by Dr. Michael Seiden, Massachusetts General Hospital, Boston, MA), OVCA420, OVCA429 (Laboratory of Gynecologic Oncology, Brigham and Women's Hospital, Boston, MA), MCAS, TOV-112D (Japanese Collection of Research Bioresources, Tokyo, Japan), CaoV3, OVCAR-3, SKOV-3, OV-90, ES-2, and TOV-21G (American Type Culture Collection, Rockville, MD). The SNU-251 endometrioid ovarian cancer cell line, which harbors a *BRCA1* mutation,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

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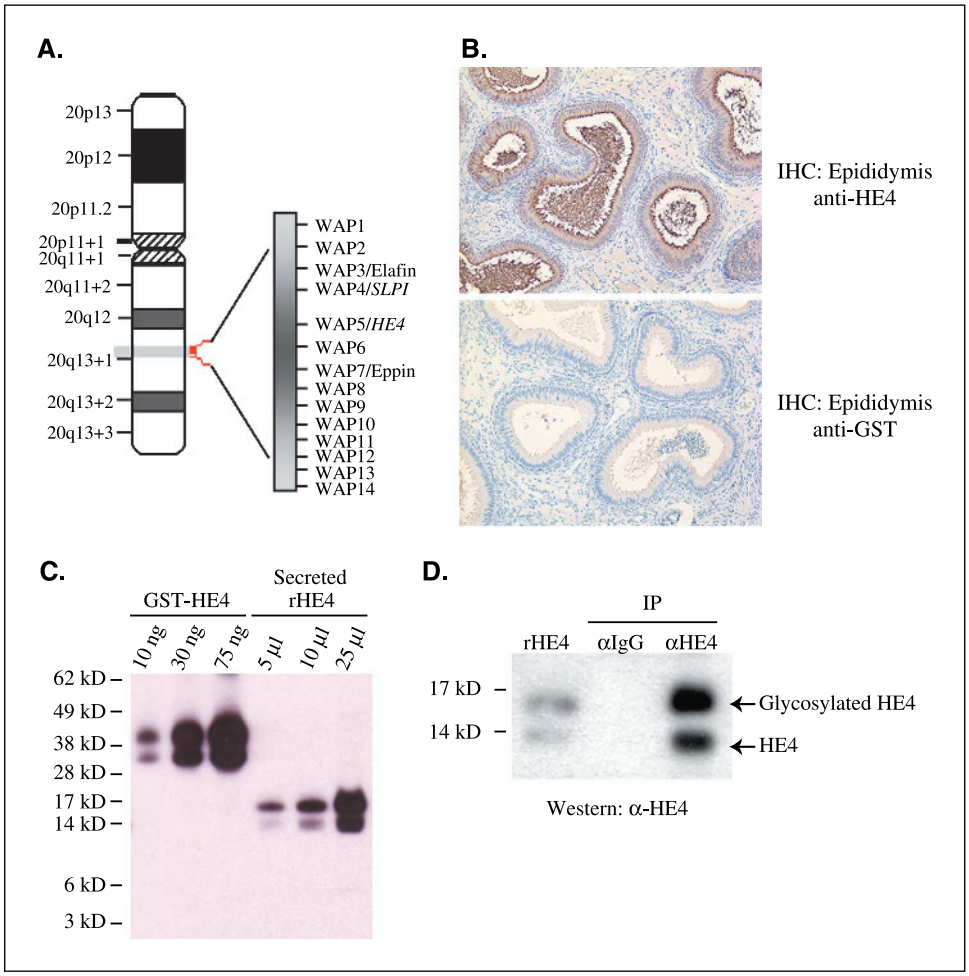

Figure 1. HE4 is expressed in the male epididymis. *A,* illustration of the region on chromosome 20q13 that harbors the WAP gene cluster, including *HE4*, *SLPI*, and *Eppin-1*. *B,* immunohistochemical (*IHC*) staining of human epididymis with HE4 antibodies (20× objective) shows an apical and luminal distribution. GST antibodies at higher concentrations than those used for HE4 did not elicit a specific signal in the epididymis. *C,* HE4 antibodies can detect GST-HE4 and recombinant HE4 synthesized in High Five insect cells by Western blot. *D,* HE4 antibodies can specifically immunoprecipitate (*IP*) recombinant HE4, including the glycosylated form. Preimmune normal rabbit IgG served as a negative control.

was purchased from the Korean Cell Line Bank, Seoul National University, Seoul, Korea (28, 29). All ovarian cancer cell lines, except SNU-251 and ES-2, were propagated in 1:1 MCDB105 and Media 199 (Sigma-Aldrich, St. Louis, MO) supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Inc., Gaithersburg, MD) at 37°C in a 10% CO_2-containing atmosphere. The ES-2 clear cell carcinoma line was propagated in McCoy's 5A modified medium with 10% fetal bovine serum. The SNU-251 line was propagated in RPMI 1640 supplemented with 10% fetal bovine serum. In addition, HeLa and IMR90 cells were obtained from the American Type Culture Collection and propagated in DMEM with 10% fetal bovine serum. HE4 expression in these cancer cell lines was compared with that of primary human ovarian surface epithelial cells (HOSE) and to human telomerase-immortalized OSE (IOSE).

RNA Extraction and RT-PCR. Cells were grown to 80% to 90% confluence. Medium was carefully aspirated from cell cultures and cells were lysed in 1 mL/dish of Trizol reagent (Life Technologies). Total RNA was extracted according to the manufacturer's recommendation. RNA was quantitated using a spectrophotometer. cDNA was synthesized from each cell line using 2 μg of total RNA. Oligo(dT) primers were used for the first-strand synthesis as described in the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). PCR primers for HE4 were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primer pairs for HE4 were (forward) 5′-CGGCTTCACCC-TAGTCTCAG-3′, (reverse) 5′-CCTCCTTATCATTGGGCAGA-3′ and those for β-actin were (forward) 5′-ACAGAGCCTCGCCTTTGC-3′), (reverse) 5′-AGGATGCCTCTCTTGCTCTG-3′. PCR primers for Eppin-1 were previously described (30). Testes cDNA was obtained from BD Biosciences, Clontech (Palo Alto, CA). PCR products were resolved and visualized on a 2% TAE agarose/ethidium bromide gel. The identity of the HE4 PCR product was confirmed by DNA sequencing.

Recombinant HE4 Proteins. To generate a baculovirus gene construct, the HE4 complementary DNA without its intrinsic signal peptide-coding sequence was modified for insertion into the pMel-BacB transfer vector (Invitrogen) by PCR with oligonucleotides that incorporated *Bam*HI and *Hin*dIII enzyme restriction sites into the ends. Inserts were ligated into the pMelBacB-vector after restriction digest, transformed, and amplified in *Escherichia coli* Top10. Insert-containing clones were selected and confirmed by dideoxynucleotide sequencing on both strands. After cotransfection of the transfer vector with a linearized defective baculovirus DNA (Bac-N-Blue linear DNA, Invitrogen) into Sf9 insect cells, viable recombinant baculoviral clones were selected from plaque assay. Pure recombinant baculovirions were propagated to obtain high titer viral stock and used to infect High Five insect cells at a multiplicity of three plaque-forming units per cell.

HE4 Antibodies. The NH_2 terminus of HE4 contains a signal peptide with a predicted cleavage site for a signal peptidase between codons 30 and 31 (21). Therefore, we raised HE4-specific antibodies by immunizing rabbits with a glutathione *S*-transferase (GST) fusion protein composed of the mature form of HE4 (amino acids 31-125) and GST. New Zealand White rabbits were immunized eight times against the glutathione *S*-transferase fusion protein. Serum was harvested after the rabbits showed significant anti-GST-HE4 and anti-rHE4 titer. Affinity purified antibodies were generated by adsorption of the crude antisera to a GST affinity column (Pierce Biotechnology, Inc., Rockford, IL) to remove all the GST antibodies. The GST antibody-depleted serum was then affinity-purified by passing it over a GST-HE4 column generated using an AminoLink Coupling Gel column

(Pierce Biotechnology). The antibodies were eluted with 100 mmol/L glycine (pH 2.8), neutralized, and finally dialyzed against PBS (pH 7.4) with 50% glycerol. The final concentration of the affinity-purified HE4 antibody was 205 ng/μL. The GST antibodies were eluted and dialyzed in a similar fashion and served as a negative control.

Tissue Specimens. Following institutional review board approval, the records of the Division of Women's and Perinatal Pathology in the Department of Pathology at Brigham and Women's Hospital (Boston, MA) were reviewed for ovarian carcinomas from 2001 to 2004. Inclusion criteria included (a) primary ovarian carcinoma, including the four major histopathologic subtypes (serous, endometrioid, clear cell, and mucinous) and (b) ovarian carcinoma with residual histologically identified normal ovary. Exclusion criteria were (a) metastatic carcinoma to the ovary and (b) tumors of low malignant potential (borderline tumors). All in all, 92 cases were identified. All the tumors were stages III and IV; all the serous tumors and the majority of endometrioid tumors were of high grade, thus correlation of HE4 staining with grade and stage could not be evaluated. Each case was reviewed and appropriate blocks designated for immunohistochemistry. In addition, we identified 11 cases in which normal ovaries were removed incidentally for another procedure unrelated to malignancy. These were used to evaluate the expression pattern of HE4 in normal ovarian tissues. To further study the expression of HE4 protein in these and other normal human tissues, we embedded four to six samples of formalin-fixed tissue from different patients for several tissues, including brain, esophagus, stomach, duodenum, gallbladder, pancreas, colon, liver, kidney, spleen, lymph node, thyroid, lung, trachea, heart, prostate, testes, breast, fallopian tubes, endometrium, cervix, testis, and epididymis. To examine HE4 expression in other nonovarian carcinomas, we used paraffin-embedded in-house whole sections and a multiple tumor tissue microarray provided by the Dana-Farber Harvard Cancer Center Core Facilities.

Immunohistochemistry. Immunohistochemical localization of HE4 protein was done on 4-μm sections from formalin-fixed, paraffin-embedded tissue. The HE4 affinity-purified rabbit polyclonal antibody was used at a dilution of 1:10,000 with heat-induced epitope retrieval. This antibody was detected using the Envision + system (K4011, DakoCytomation, Carpinteria, CA) that employs horseradish peroxidase–labeled polymer conjugated to goat anti-rabbit immunoglobulin antibodies. The immune complexes were identified using a peroxidase reaction with 3,3′-diaminobenzidine-plus as chromogen. The positive control for HE4 staining was human epididymis. Polyclonal nonimmune rabbit IgG antibodies and anti-GST antibodies, generated as a byproduct of the HE4 antibody purification, were used as negative controls to show the specificity of the HE4 affinity-purified antibodies. Slides were counterstained with Mayer's hematoxylin. Antibodies against SLPI were purchased from R&D Systems (Minneapolis, MN).

Immunofluorescence. SKOV-3, CaoV3, and OVCAR-5 cells were grown on coverslips, fixed with 70% methanol, and permeabilized with 0.5% Triton X-100 as previously described (31). Cells were double labeled by incubation with the rabbit polyclonal antibodies to HE4 (1:5,000), and monoclonal antibodies against the 58K Golgi protein formiminotransferase cyclodeaminase (1:150; Abcam, Inc., Cambridge, MA), or KDEL monoclonal antibodies against the endoplasmic reticulum protein Grp78 (1:300; EMD Biosciences, Inc., San Diego, CA) in PBS with 5% goat serum. After washing, appropriate species-specific, fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were applied as recommended by the manufacturer, and fluorescence was visualized with a Zeiss Axioskop 2 microscope using AxioVision Software for Digital Microscopy.

Glycosylation Analysis. CaoV3 and OVCAR-5 cells were grown in 1:1 MCDB105 and Media 199 (Sigma-Aldrich) supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies) to 80% confluence. The medium was then changed to 1:1 MCDB105 and Media 199 without any fetal bovine serum and the cells were cultured for an additional 48 hours. The cultured medium was then cleared by centrifugation and concentrated using a Millipore Amicon Ultra-15 centrifugal filter with a 5,000 molecular weight cutoff. Twenty micrograms of the concentrated cultured medium were then denatured in denaturing buffer (5% SDS, 10% β-mercaptoethanol) at 100°C for 10 minutes. One-tenth volume of both G7 buffer [0.5 mol/L sodium phosphate (pH 7.5) at 25°C] and 10% NP40 surfactant were then added, followed by 2 μL of *N*-Glycosidase F (PNGase F; New England BioLabs, Beverly, MA). The reaction was then incubated at 37°C for 1 hour. Reaction products were denatured and resolved on a 4% to 12% SDS-polyacrylamide gel and analyzed by Western blot using the affinity-purified HE4 antibody (1:4,000).

Statistical Analysis. Comparison of papillary serous immunostaining with endometrioid, mucinous, and clear cell subtypes was accomplished by using exact version of Kruskal-Wallis nonparametric test for singly ordered RxC contingency tables (Software: StatXact, version 6.1 2003, from Cytel Software, Cambridge, MA).

Results

Expression of HE4 Protein in Normal Human Tissues. *HE4* was first described as an epididymis-specific gene using Northern blot analysis and *in situ* transcript hybridization (21, 22). To characterize the distribution of HE4 protein in benign and malignant tissues we raised and affinity purified HE4 antibodies and assessed their immunohistochemical profile in human epididymis. The HE4 antibodies localized to the duct of the epididymis in a granular apical pattern, consistent with prior studies using *in situ* transcript hybridization (Fig. 1*B*; ref. 21). No HE4 protein was detected in the surrounding stroma or vasculature. Importantly, affinity-purified antibodies against GST, a byproduct of the HE4 antibody purification, and purified nonimmune IgG failed to produce a signal in this tissue under similar conditions (Fig. 1*B* and data not shown). In addition, the HE4 antibodies could detect the immunizing antigen (GST-HE4) and a recombinant form of HE4 generated by baculovirus infection of High Five insect cells by Western blot analysis (Fig. 1*C*). Finally, these antibodies could specifically immunoprecipitate the insect cell-derived recombinant HE4, including the glycosylated 16-kDa form (see Fig. 4*D*), whereas the preimmune immunoglobulins could not (Fig. 1*D*). We then assembled a collection of formalin-fixed, paraffin-embedded normal human tissues to study the presence and distribution of HE4 in these tissues. Under conditions where the HE4 antibody shows specific immunostaining for HE4 in the epididymis, we found that the expression of the HE4 protein is highly restricted in normal human tissues (Table 1). Specifically, HE4 was expressed most highly in the epididymis and in the female reproductive tract (fallopian tubes, endometrium, and endocervix). HE4 expression was also present in the respiratory epithelium, especially in the trachea. Occasional staining was also observed in the epithelium of the renal convoluted tubules and salivary gland ducts. HE4 protein was notably absent from the GI tract, liver, pancreas, spleen, lymph nodes, mesenchymal tissues (heart, skeletal muscle), breast, and brain. There was nonspecific staining of colloid in the thyroid but the epithelium was negative. Under similar conditions, antibodies against another WAP domain containing protein, SLPI (Fig. 1*A*), show widespread expression in multiple epithelia in the body (Supplementary Table 1). Therefore, expression of HE4 protein is highly restricted in normal tissues and tends to consistently identify epithelia in the reproductive tracts and central respiratory airways.

HE4 Protein Is Expressed in the Mullerian Epithelium of Cortical Inclusion Cysts in Normal Ovaries. We had previously reported that Mullerian metaplasia of the OSE, frequently seen in cortical inclusion cysts (CIC), heralds the expression of a number of ovarian cancer biomarkers, including EpCAM, Mucin 1, Mesothelin, and CD9 (20). To address whether HE4 is expressed by surface epithelial cells and/or by the Mullerian epithelia in CICs, we stained

Table 1. HE4 protein expression in normal human tissues

Normal tissues	Positive/tested	Histological description
Esophagus	0/2	
Stomach	0/4	
Gallbladder	0/5	
Duodenum	0/6	
Colon	0/6	
Pancreas	0/4	
Liver	0/5	
Spleen	0/5	
Lymph node	0/7	
Skeletal muscle	0/4	
Cardiac muscle	0/4	
Lung	1/4	Proximal respiratory epithelium
Trachea	5/5	Respiratory epithelium and minor salivary glands
Thyroid	0/6	
Kidney	5/6	Focal in a minority of tubules
Brain	0/4	
Breast	4/5	Predominantly in secretions with focal reactivity in a minority of ducts
Ovary	0/7	Negative in OSE; positive in metaplastic, ciliated CICs
Fallopian tubes	10/10	
Endometrium	4/4	
Cervix	4/4	Endocervical glands only
Epididymis	5/5	Strongest staining in duct of the epididymis, weaker staining in efferent ducts
Testes	0/4	
Prostate	4/7	Weak, focal glandular epithelial staining

11 ovaries that were histologically proven benign. We used the calcium binding protein calretinin as a positive control for the OSE, because we and others previously showed that calretinin stains normal OSE but not metaplastic or neoplastic ovarian epithelium (20, 32). Neither the flat nor the more cuboidal and stratified OSE expressed any HE4 protein under conditions where calretinin could easily be detected (Fig. 2*A*). Conversely, in cortical inclusion cysts with Mullerian epithelium, HE4 was readily detected whereas calretinin was negative in such CICs (Fig. 2*A*). However, in CICs that still maintained the flat morphology of the OSE the opposite was true; HE4 was negative and calretinin was positive (Fig. 2*B*). Therefore, expression of HE4 protein, like certain other ovarian biomarkers identified by gene expression profiling, is acquired during the normal, age-related process of CIC formation and Mullerian metaplasia.

HE4 Is Overexpressed in Serous and Endometrioid Ovarian Carcinomas. To address whether HE4 protein is overexpressed by ovarian carcinomas, 92 unique ovarian carcinoma tissue blocks were obtained with institutional review board approval from the Women's and Perinatal Division of Pathology in the Department of Pathology at Brigham and Women's Hospital. These samples were from women treated at the Dana-Farber/Brigham and Women's Cancer Center between 2001 and 2004. The cases included 60 serous carcinomas, 16 endometrioid carcinomas, 10 mucinous carcinomas, and 6 clear cell carcinomas. Human epididymis served as a positive control and all cases were selected to include some residual nontumor stromal, adipose, and/or vascular tissue as a negative control for HE4 staining. HE4 immunoreactivity was readily detected in the serous and endometrioid tumors as perinuclear, cytoplasmic, and membranous staining (Fig. 3). Of the serous carcinomas, 93% exhibited moderate-to-strong staining and the vast majority (90-100%) of these cases showed diffuse immunoreactivity throughout the tumor epithelia. Every endometrioid carcinoma was diffusely immunoreactive with HE4 antibodies. Of the 10 mucinous carcinomas, nine were completely negative for HE4 (Fig. 3; Table 2). These differences were statistically significant. The one case of mucinous carcinoma that was positive was a high-grade carcinoma in which both epithelial and stromal components showed immunoreactivity. We have not detected stromal HE4 immunostaining in any other case, nor do we see HE4 RNA expressed by normal human diploid fibroblasts (IMR90 cells; see below). HE4 immunoreactivity in clear cell carcinomas was intermediate, with three of six cases exhibiting moderate-to-strong staining (Fig. 3, Table 2). The distribution of staining in these tumors was heterogenous (50-100% of cells).

We then asked whether the overexpression of HE4 was unique to ovarian carcinomas or is distributed across a spectrum of otherwise disparate epithelial tumors. A combination of multitumor tissue microarrays and in-house surgical specimens were used. Surprisingly, the majority of common nonovarian carcinomas did not exhibit HE4 immunostaining, including colon, breast, non–small cell lung, bladder, kidney, thyroid, and prostate cancers (Supplementary Table 2). The exception was endometrial carcinoma, a finding that is not surprising given the Mullerian origins of this carcinoma.

HE4 Is Expressed and Secreted as a Glycoprotein by Ovarian Carcinoma Cells. HE4 expression in the epididymis was reported to be apical/membranous and within the duct lumen where it is in contact with spermatozoa (22). This pattern of expression is consistent with the fact that the cDNA for HE4 predicts a small, secretory protein with hydrophobic amino acids at the NH_2 terminus consistent with a signal peptide (21). Cleavage of the signal peptide is predicted to yield a mature secretory polypeptide with a consensus site for N-glycosylation at amino acid position 15 (N-C-T). Our observation that HE4 protein is overexpressed in human ovarian carcinomas prompted us to ask whether HE4 is also secreted by ovarian cancer cells, as is seen in the epididymis. To address this possibility, we assembled a collection of 14 established ovarian carcinoma cell lines and used semiquantitative RT-PCR to identify those lines that endogenously overexpress HE4 relative to normal primary HOSE and IOSE. Consistent with our immunohistochemical data (Fig. 2), we did not detect any HE4 RNA in HOSE or IOSE (Fig. 4*A*). We also did not detect HE4 RNA in HeLa or IMR90 cells. Conversely, the majority of the ovarian carcinoma lines expressed varying degrees of HE4 RNA. Equal loading was confirmed by RT-PCR for β-actin. Moreover, the specificity of HE4 expression was challenged by asking whether Eppin-1, another WAP domain containing protein encoded on chromosome 20 next to HE4 (Fig. 1*A*), is also expressed in ovarian carcinomas. We could not detect any Eppin-1 RNA in the ovarian cancer cells lines, although Eppin-1 was clearly expressed in the testes, as previously reported (Fig. 4*A*; ref. 30).

To determine the intracellular localization of HE4 in ovarian cancer cells, SKOV-3, OVCAR-5, and CaoV3 cells were subjected to indirect immunofluorescence using the HE4 polyclonal antibodies. HE4 antibodies localized into perinuclear structures (Fig. 4*B*,

bottom) and in some cells into dome-shaped perinuclear structures with a polarity that resembled the Golgi apparatus (Fig. 4*B*, *top*). Indeed, double staining with antibodies against 58K formiminotransferase cyclodeaminase, a known resident enzyme of the Golgi complex, showed partial colocalization with HE4 (Fig. 4*B*). Antibodies against Grp78, a component of the endoplasmic reticulum also showed partial colocalization with HE4, a finding consistent with proteins that are processed through the endoplasmic reticulum and Golgi for extracellular transport. To determine whether ovarian cancer cells can secrete HE4 we cultivated cells that express HE4 RNA (OVCAR-5 and CaoV3) and those that only express minute amounts or none at all (IOSE, TOV-21G, and ES-2). The cells were grown to 80% confluence, the medium was replaced with medium lacking serum and the cells were incubated for an additional 48 hours. The conditioned medium was then harvested, concentrated, and analyzed by Western blot for the presence of secreted HE4. Both CaoV3 and OVCAR-5 secreted a modified form of HE4 that migrated larger than the recombinant HE4 produced in insect cells (Fig. 4*C*). We could not detect any secreted HE4 from HOSE, IOSE, or the two cancer lines that lacked significant HE4 expression by RT-PCR. Because HE4 is predicted to undergo glycosylation, we asked whether the altered migration of HE4 in the cultured medium could be explained by such a post-translational modification. Indeed, treatment of the cultured medium with the deglycosylating enzyme *N*-glycosidase F (PNGase F) dramatically altered the migration of the secreted HE4, resulting in a form that comigrates with recombinant HE4 (Fig. 4*D*). Therefore, our results show that ovarian carcinoma cells express HE4 and that the resulting gene product is N-glycosylated and secreted into the extracellular environment. Interestingly, the pattern of HE4 glycosylation in human cancer cells was different that the one seen in the High Five embryonic ovarian insect cells. Glycosylated HE4 in OVCAR-5 and CaoV3 migrated with an approximate molecular weight of 25 kDa whereas the glycosylated form of HE4 in the insect cells migrated as a 16-kDa species (Fig. 4*D*).

Discussion

Ovarian carcinoma remains a major worldwide health care issue in large part due to the lack of satisfactory screening methods for early detection of the disease. The application of cDNA microarrays has resulted in the identification of numerous candidate ovarian cancer biomarkers (reviewed in refs. 3, 33). Determining which, if any, of the candidate tumor markers have clinical utility requires rigorous validation in human tissues and basic characterization of the individual proteins. In this report, we present a comprehensive

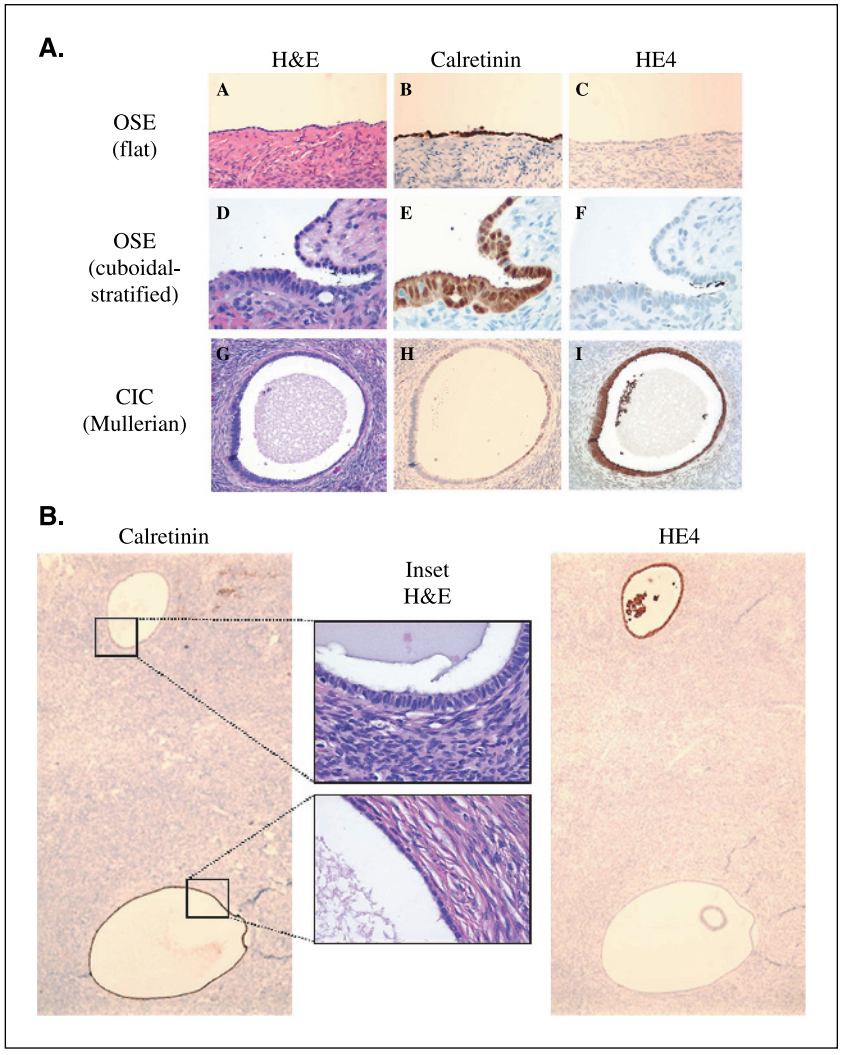

Figure 2. Analysis of HE4 expression in normal ovarian tissue. *A,* examples of OSE and CICs stained with H&E, the normal OSE marker calretinin, and HE4. HE4 expression is absent in flat (*C*) or cuboidal (*F*) OSE but is abundantly present in the epithelium lining metaplastic CICs (*I*). Calretinin expression (*middle*) is limited to the OSE. (*A-C*, 20× objective; *D-F*, 40× objective; *G-I*, 20× objective). *B,* HE4 and calretinin exhibit differential expression in cortical inclusion cysts. A section of normal ovary containing two different CICs was identified and stained with antibodies against calretinin and HE4 (10× objective). Histologic examination (*H&E insets,* 40× objective) showed the top CIC lined by Mullerian type epithelium and the bottom CIC lined by flat mesothelium. HE4 expression is limited entirely to the CIC with Mullerian epithelium.

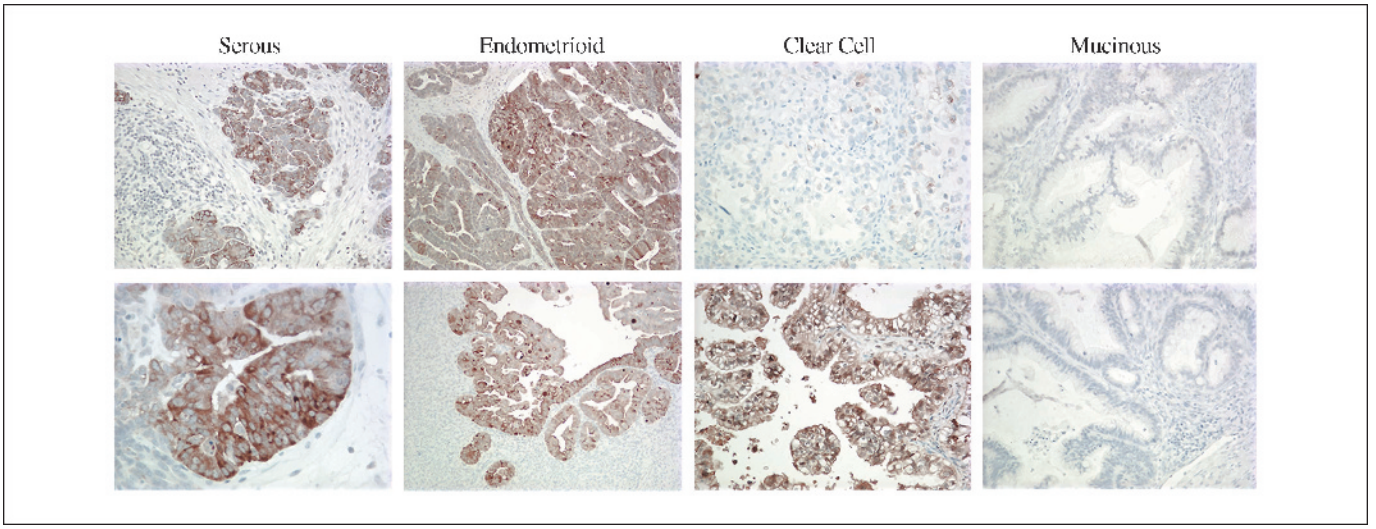

Figure 3. HE4 is expressed by subtypes of ovarian carcinomas. Two examples each of HE4 expression in serous, endometrioid, clear cell, and mucinous carcinomas. Immunoreactivity is common in serous and endometrioid subtypes and tends to be diffusely distributed in these tumors. Expression in clear cell carcinomas is less common and completely absent in mucinous carcinomas. Surrounding stroma and inflammatory cells are negative.

analysis of HE4 protein expression in normal and malignant human tissues and show that expression of HE4 protein is highly restricted in normal human tissues and is largely limited to the epithelium of the reproductive tracts and to the respiratory epithelium of the proximal airways. In malignant tumor tissues, HE4 is a biomarker for certain subtypes of ovarian carcinomas (i.e., serous and endometrioid types). This is a significant result since serous and endometrioid carcinomas account for the vast majority of malignant ovarian epithelial tumors. Moreover, we did not observe significant overexpression of HE4 in a limited collection of other nonovarian epithelial tumors, including colon, breast, nonsmall cell carcinomas of the lung, kidney, or thyroid. Notably, three of four carcinomas of the endometrium were positive for HE4, a finding that is perhaps not surprising given that HE4 is normally expressed in the endometrium. Therefore, HE4 is a robust tissue biomarker for serous and endometrioid ovarian carcinomas and may be a significant marker for other Mullerian-derived tumors.

The observation that HE4 is expressed in Mullerian-type epithelium of ovarian cortical inclusions cysts suggests that this marker is actually a marker of Mullerian differentiation, not a bona fide marker of neoplastic transformation. We recently reported a similar observation with other markers commonly identified as ovarian carcinoma markers by cDNA microarrays, including Mucin 1, EpCAM, Mesothelin, and CD9 (20). These observations lend further support to the hypothesis that the OSE is not the direct precursor of ovarian epithelial neoplasms. Rather, this process requires the emergence of Mullerian epithelium, in which a series of genetic events develop, leading to neoplasia. A number of observations support the emergence of Mullerian type epithelium in CICs as part of the developmental pathway to ovarian carcinomas: (a) there is an increased frequency of CICs in apparently normal ovaries contralateral to ovarian cancer compared with ovaries from age-matched women without ovarian cancer, (b) ovaries removed prophylactically from women with a family history of ovarian cancer show more CICs than controls, and the epithelium lining these cysts shows abnormalities detectable by image analysis, (c) nuclear accumulation of mutant p53 protein has been detected in Mullerian-type epithelial cells lining CICs, and (d) rare cases of ovarian intraepithelial neoplasia, manifested by epithelial atypia in an inclusion cyst, have been reported (reviewed in refs. 34, 35). How this epithelium develops is not established. One proposed model suggests that loss of the basement membrane in cells lining CICs may contribute to early tumorigenesis (4, 36). In this scenario, exposure of epithelial cells in CICs to direct stromal contact may

Table 2. Summary of immunohistochemical staining of ovarian carcinomas with HE4 antibodies

	Intensity of immunostaining*				
Histologic subtytpes	Strong (% diffuse†)	Moderate (% diffuse)	Weak (% diffuse)	Negative	P‡
Papillary serous (n = 60)	24 (100)	32 (90)	4 (75)	0	
Endometrioid (n = 16)	12 (100)	4 (100)	0	0	0.014
Mucinous (n = 10)	0	1§	0	9	0.001
Clear cell (n = 6)	2 (100)	1 (50)	0	3	0.10

*All cases were independently reviewed and scored by four pathologists (R.D., C.P.C., W.R.W., J.L.H.).
†Diffuse staining is defined as immunoreactivity over ≥75% of the tumor area.
‡Compares distribution of papillary serous immunostaining with endometriod, mucinous, and clear cell subtypes, using exact version of Kruskal-Wallis nonparametric test for singly ordered RxC contingency (Software: StatXact, version 6.1 2003, Cytel Software).
§Represents a high-grade mucinous tumor. HE4 immunoreactivity was present throughout, including stromal components.

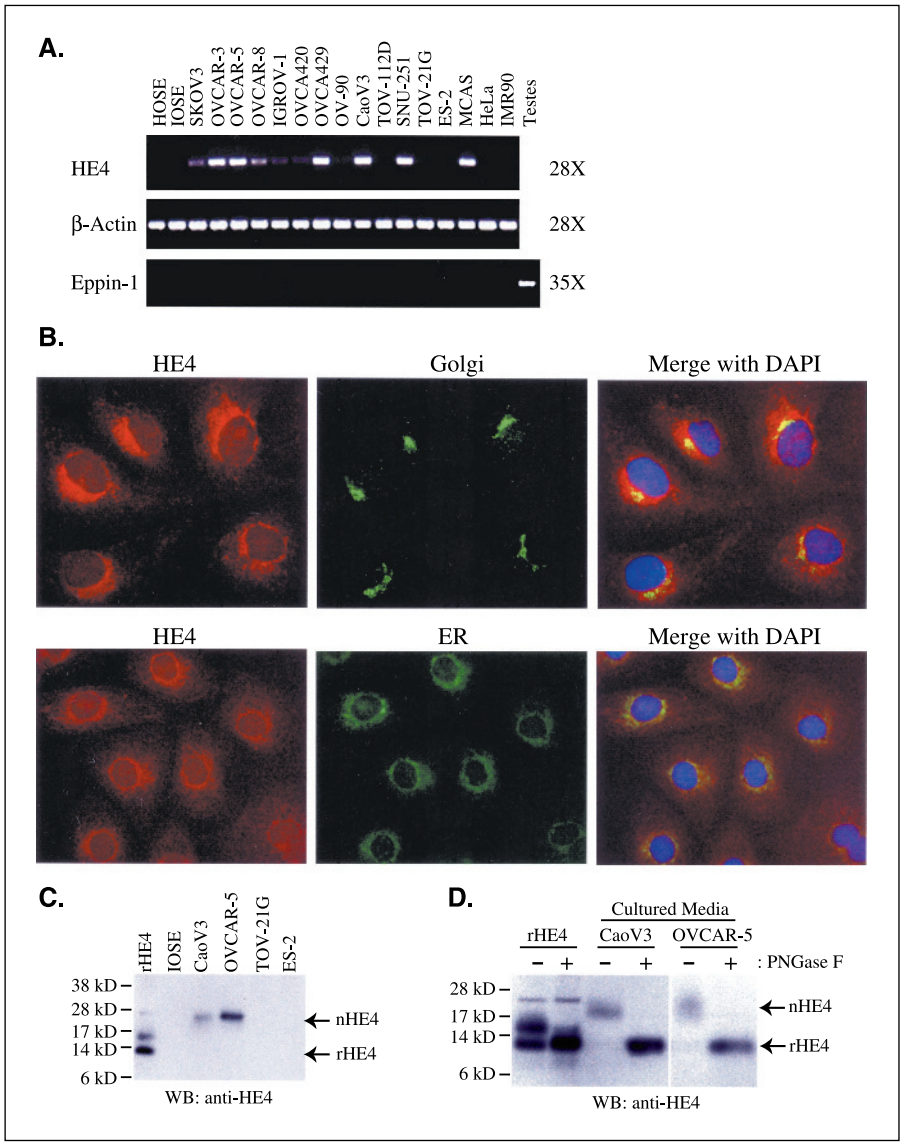

Figure 4. HE4 is overexpressed and secreted as a glycoprotein by ovarian carcinoma cells. *A,* expression of HE4 was determined by semiquantitative RT-PCR. Normal OSE (HOSE) and IOSE RNA served as negative controls for HE4 expression. Expression of β-actin served as a loading control. *Eppin-1* is another WAP gene located on chromosome 20q13 (Fig. 1*A*). *Right,* number of PCR cycles. HE4 is overexpressed by most ovarian carcinoma cells lines and is absent in HeLa and IMR90 cells. *B,* HE4 is localized to the perinuclear Golgi apparatus and endoplasmic reticulum (*ER*). Immunofluorescence localization of HE4 in SKOV-3 cells revealed a perinuclear pattern that partially colocalized with the 58K Golgi protein and Grp78, an ER marker. Images were merged with 4′,6-diamidino-2-phenylindole to show the nucleus. Similar results were obtained in CaoV3 and OVCAR-5 cells. *C,* HE4 is secreted by ovarian cancer cells. Conditioned medium from IOSE, CaoV3, OVCAR-5, TOV-21G, and ES-2 cells was concentrated and analyzed by Western blot for HE4. Secreted HE4 migrates as a higher molecular weight species compared with recombinant HE4. *D,* secreted HE4 is N-glycosylated. Cultured medim was incubated with PNGase F, a deglycosylation enzyme. Western blot analysis of the migration of the recombinant glycosylated HE4 and the HE4 secreted by CaoV3 and OVCAR5 cells comigrate with unmodified recombinant HE4 after enzymatic deglycosylation. Experiments were repeated at least three independent times.

trigger the metaplasia of these cells into a Mullerian-type epithelium. If Mullerian metaplasia of OSE plays a role in tumorigenesis, one would expect that a comparison of the expression profile of the Mullerian-like epithelium lining CICs and ovarian carcinomas may result in the identification of markers of neoplastic transformation, rather than surrogates of Mullerian differentiation. This possibility notwithstanding, the expression of HE4 in CICs with Mullerian type epithelium and in ovarian carcinomas suggests that HE4 expression would be present in early stage ovarian carcinomas.

The fact that established ovarian cancer cell lines express endogenous HE4 by RT-PCR presented an opportunity to begin characterizing the cellular biology of the protein. Intracellular immunofluorescence studies revealed that HE4 is distributed in a region of the cytoplasm with a perinuclear pattern reminiscent of the endoplasmic reticulum and the Golgi apparatus. Double immunostaining with antibodies directed against known constituents of the endoplasmic reticulum and Golgi apparatus confirmed that HE4 is present in these organelles. This observation is consistent with the prediction that HE4 is a secreted protein, at least in the normal milieu of the reproductive tract (epididymis). Our results predicted that ovarian carcinoma cells also secrete HE4. Indeed, HE4-expressing carcinoma cells secreted HE4 protein into the extracellular medium. Secreted HE4 was only seen in cell lines that express endogenous HE4 RNA; HOSE cells were negative. Interestingly, the secreted form of HE4 migrated with an apparent molecular weight of 25 kDa on SDS-polyacrylamide gels, almost double the size of the predicted recombinant protein, and larger than the form secreted by High Five insect cells. Because the mature HE4 polypeptide contains one consensus N-glycosylation site at position 14 (N-C-T), we reasoned that the altered migration of the secreted product might be a consequence of glycosylation. Enzymatic deglycosylation confirmed this suspicion. Therefore, our results show that ovarian carcinomas secrete HE4 as an N-glycosylated protein. Because there is only one predicted glycosylation site in HE4, the difference between the insect cell secreted HE4 and the form secreted by ovarian carcinoma cells may simply reflect species-specific differences in glycosylation patterns. However, because alterations in cellular glycosylation are frequently observed in tumor cells (37–39), the difference in migration of secreted HE4 also raises the possibility that the glycosylation

pattern of HE4 may be different in cancer cells compared with benign human cells, such as those in the epididymis or ovarian CICs.

A logical prediction from our studies is that HE4 may be secreted into the bloodstream of patients with ovarian carcinoma. In fact, during the course of this work, Hellstrom et al. reported that HE4 circulate in the bloodstream of patients with ovarian cancer and not in age-matched controls (40). Their study indicates that the specificity and sensitivity of HE4 serology is comparable to that of CA125 and that HE4 is less frequently positive in patients with nonmalignant disease, a factor that has greatly limited the utility of CA125 as a screening tool (41).

Our study complements the Hellstrom report and supports the further development of HE4 in the clinical setting. The development of more quantitative serum assays will enable us to assess the sensitivity of HE4 in the presurgical, postsurgical, and chemotherapeutic settings and eventually enable us to determine whether this marker will be useful in the detection of early stage ovarian carcinoma. A very real possibility is that the combination of HE4 and CA125 serology may result in a test with sufficient sensitivity and specificity to be used for the detection of early ovarian cancer. These studies are in progress and will enable us and others to define the clinical utility of HE4. In addition, our results show that HE4 is a small secreted glycoprotein. Therefore, it is formally possible that HE4 is also filtered by the kidneys into the urine. If true, HE4 may also represent an interesting target for the development of a urine test for ovarian cancer.

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