Ovarian carcinomas express HE4 epitopes independently of each other

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ABSTRACT

Background: The gene encoding HE4 undergoes alternative splicing to yield multiple protein isoforms. We investigated anti-HE4 mAbs which recognize epitopes on the C (2H5 and 3D8) or N (12A2 and 14E2) terminals.
Methods: A Luminex assay was applied to determine mAb affinity. Binding of mAbs to sections from formaline fixed ovarian carcinomas was determined by immunohistology, binding to cultured ovarian carcinoma cells was tested by flow cytometry and immunocytochemistry, and HE4 secretion to patient serum or supernatants of cultured ovarian carcinoma was assayed by ELISA.
Results: mAb 12A2 bound to formalin-fixed sections from 18 of 19 ovarian carcinomas, while 14E2, 2H5 and 3D8 bound to sections from 14, 7 and 4 patients, respectively. The mAbs bound independently of each other, i.e. a sample bound one mAb most strongly while another sample with similar affinity strongest bound another mAb. The intensity of binding to sections did not significantly correlate with the serum level of HE4 except for mAb 14E2, but there was a significant correlation between HE4 expression and its detection in supernatants of cultured ovarian carcinomas.
Conclusions: HE4 epitopes are expressed independently of each other and their expression of HE4 by cultured ovarian carcinoma cells correlates with the release of HE4 into culture supernatants. he epitope recognized by mAb 14E2 was significantly more expressed by platinum resistant tumors.
Impact: Expression of HE4 by most ovarian carcinomas makes it an excellent biomarker.. Further studies are needed to investigate the clinical relevance of overexpression of a particular epitope.

Introduction

There is a clinical need for non-invasive assays to aid the detection of ovarian cancer and monitoring of treated patients. For many years, testing serum for CA125 was the 'gold standard' for this need [1]. However, CA125 is frequently elevated in women with benign pelvic disease or inflammation [2] and much effort has been given to find more tumor selective markers.

The WFDC2 gene encodes a family of secreted 4-disulfide core proteins including HE4 which was first observed in epithelial cells from the epididymis [3,4]. Other family members are Wp, a secretory leukocyte protease inhibitor and elafin [5–7]. Overexpression of HE4 in serous and clear cell ovarian carcinoma [8] suggests that it can be a useful biomarker for these tumors. Hybridomas making mAbs to HE4 were therefore raised by immunizing [9], and two mAbs, 2H5 and 3D8, which recognize different HE4 epitopes, were used to construct a double determinant ("Sandwich") ELISA. The ELISA is less frequently positive than CA125 in women who have benign disease [9] or tumors other than ovarian carcinoma [10] and it complements CA125 for

detecting ovarian carcinoma in women who have a pelvic mass [11–13]. High preoperative HE4 level is associated with poor prognosis [14], and a combination of serum assays for CA125 with HE4 improves detection of ovarian carcinoma beyond measuring only one of these two biomarkers [15–19]. Testing urine for HE4 rather than serum has a slightly better sensitivity [20,21] and may aid the identification of patients who after cytoreductive therapy are at higher risk for platinum resistance [21].

The WFDC2 genes undergoes splicing to yield various isoforms [22]. The two mAbs applied to construct the clinically approved ELISA kit are both specific for epitopes expressed on the C-terminal of the HE4 molecule. Monoclonal antibodies (mAbs) have been generated also to epitopes at its N-terminal end. We have compared two such mAbs, 12A2 and 14E2, with mAbs 2H5 and 3D8 for binding to sections from 16 serous, 2 clear cell and 1 endometrial ovarian carcinoma as well as to cultured cell lines from 6 ovarian cancers. Ovarian carcinoma cell lines which strongly expressed several HE4 epitopes secreted HE4 antigen into culture supernatants while cell lines with little such expression did not. Expression of the epitope recognized by mAb 14E2 was

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more frequent in tumors that were resistant to platinum based chemotherapy.

Materials and methods

Construction and initial testing of mAbs to HE4

Fusion proteins with an Ig tail of either mouse (HE4-mIgG) or human (HE4-hIgG) origin were constructed applying a published technique [23] and were designed to incorporate the full length HE4 gene product, HE4-V4 and HE4-V2 variants. Mice were immunized against fusion proteins with a mouse Ig tail, and hybridomas were screened against fusion proteins with a human Ig tail as previously described [9]. The mRNA sequence for HE4 as originally published [4,24] provided the basis for oligonucleotide primer design to clone cDNA that encodes HE4.

Studies performed at Fujirebio Diagnostics showed that mAbs 12A2 and 14E2 react with full length HE4 and HE-V4 indicating that they are binding to the HE4 N-WFDC domain, while mAbs 2H5 and 3D8 bind to the HE4 C-WFDC domain [9].

Preparation of antibody conjugated Luminex™ beads and of biotinylated HE4 peptide

Coupling of 2H5, 3D8, 12A2 and 14E2 mAbs to fluorescent magnetic beads (Bio-Rad; Hercules, CA) was performed as previously described [25]. All couplings to beads were confirmed using serial dilutions of phycoerythrin (PE) labeled anti-mouse IgG detection antibodies (Jackson ImmunoResearch; West Grove, PA), and the beads were read using a LiquiChip Luminex instrument (QIAGEN; Germantown, MD). Recombinant HE4 peptide was biotinylated using a One-step Antibody Biotinylation kit (Miltenyi; Auburn, CA).

Luminex assay to determine HE4 binding affinity to 2H5, 3D8, 12A2 and 14E2 mAbs

Bead coupled mAbs 2H5, 3D8 and 12A2 were incubated with a dilution series of biotinylated HE4 peptide ranging from 0.49 ng to 500 ng. For 3D8 we also included 1000 ng and 2000 ng of biotinylated HE4 peptide. Bead coupled mAb 14E2 was incubated with a dilution series of biotinylated HE4 peptide ranging from 3.91 ng to 500 ng. For each bead-coupled mAb we also included a reagent blank with no biotinylated HE4 peptide using 2500 beads per reaction and a 3-h incubation period. After this first incubation, beads were washed twice with phosphate-buffered saline (PBS) and then incubated for 1 h in PBS containing 10 µg/ml PE. Then, beads were washed twice with PBS, resuspended in PBS and their fluorescent intensities (FIs) read to using the Bio-Plex® 200 Luminex instrument. Six replicates per condition were used for each bead-coupled mAB. FIs were then used to calculate the equilibrium constant (Kd) for each antibody using Prism software (GraphPad: San Diego, CA) and the following formula: $y = \frac{Bmax \times X}{Kd + X}$, with Bmax equal to the maximum number of binding sites, X the concentration of biotinylated HE4 and Y the specific binding (blank-subtracted FI) of each mAb.

Sandwich Luminex assays for detection of different HE4 epitopes

We incubated the bead-coupled mAbs (2H5, 3D8, 12A2) described above with recombinant HE4 peptide and with biotinylated mAbs 14E2 or 2H5 (same biotinylation procedure as described above for HE4). We used the following combinations of bead-coupled and biotinylated mAbs: 2H5-bead/14E2-biotin, 3D8-bead/2H5-biotin and 12A2-bead/

14E2-biotin. Based on our Kd calculations for the HE4 mAbs, we incubated bead-coupled mAbs 2H5 and 12A2 (2500 beads per reaction) for 2 h with a dilution series ranging from 0.03 ng –to 500 ng of HE4 peptide, and bead-coupled mAb 3D8 (5000 beads per reaction) over night using the same HE4 dilution series. Beads were washed with PBS and then incubated with biotonylated mAbs (1000 ng/ml) for 3 h, after which beads were washed, incubated with PE for 1 h and their FIs read to using the Bio-Plex® 200 Luminex instrument as described above.

Detection of HE4 by immunohistochemical evaluation of tumor sections

Paraffin embedded slides were prepared from ovarian carcinoma tissues obtained at primary surgery and deparaffinized in xylene followed by incubation with 100%, 95%, 70% ethanol and water. They were then microwaved in a target retrieval solution containing 10 mM citrate buffer pH 6.0 (Agilent Technologies, Santa Clara, CA) for 10 min and incubated in warmed target retrieval solution for 30 min. The slides were washed in PBS, and endogenous peroxidase was quenched by incubation in 3% H_2O_2 for 5 min, after which they were incubated for 1 h at room temperature with 5 µg/mL of anti-HE4 mAbs 2H5, 3D8, 12A2 and 14E2 (provided by Fujirebio Diagnostics, Inc.) and mouse IgG1 kappa isotype control (Thermo Fisher Scientific). After washing in 0.05% Tween/PBS, the slides were incubated with anti-mouse EnVision + System-HRP labelled polymer (Agilent Technologies) for 30 min. The Liquid DAB+ substrate-chromogen system (Agilent Technologies) was used according to the manufacturer's instructions for visualization of mAb bound to tumor cells. After counterstaining with hematoxylin (Fisher Scientific, Waltham, MA) the slides were mounted and analyzed by microscopy. The binding of mAbs to cells was scored 0–4 from undetectable to strong staining of the majority of tumor cells in the section.

Measuring HE4 antigen in serum or culture supernatants

HE4 antigen in sera from ovarian carcinoma patients was measured with the clinically approved quantitative enzyme-linked immunosorbent assay Fujirebio Diagnostics ELISA using mAbs 2H5 and 3D8 according to manufacturer's instructions. The plates were read at 405 nm wavelength with a microplate reader (Fusion universal microplate analyzer, Fusion instrument company, Meriden, CT). To relate the HE4 expression in culture supernatants to cell number, we divided the HE4 (in pM) measured in ELISA by the MTS optical density value.

Secretion of the HE4 biomarker was also measured in supernatants of cultured ovarian carcinoma cells, which were seeded at 5000 cells/well 100uL/well in 96-well plates (EMD Millipore, Billerica, MA) in DMEM/F-12 media with GlutaMAX supplement (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine sera (Atlanta Biologicals, Flowery Branch, GA) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, MA) at 5000 cells/well on day 1. Supernatants were collected on day 4 to measure HE4 secreted into the cultured media, and three wells from each cell line was used to determine the number of living tumor cells by the MTS assay (Promega, Madison, WI) following the manufacturer's protocol.

Establishment of cell lines from serous ovarian carcinoma

Samples obtained at surgery from 5 patients with primary ovarian carcinoma (code numbered 225 (serous neoplasm with borderline features and desmoplastic implants), 239 (poorly differentiated carcinoma), 249 (clear cell), 272 (serous) and 289 (endometriod and serous) were suspended mechanically and via liberase (Roche, Indianapolis, IN) digestion and cultivated in vitro. In addition, we purchased the Ovcar3 cell line from ATCC.
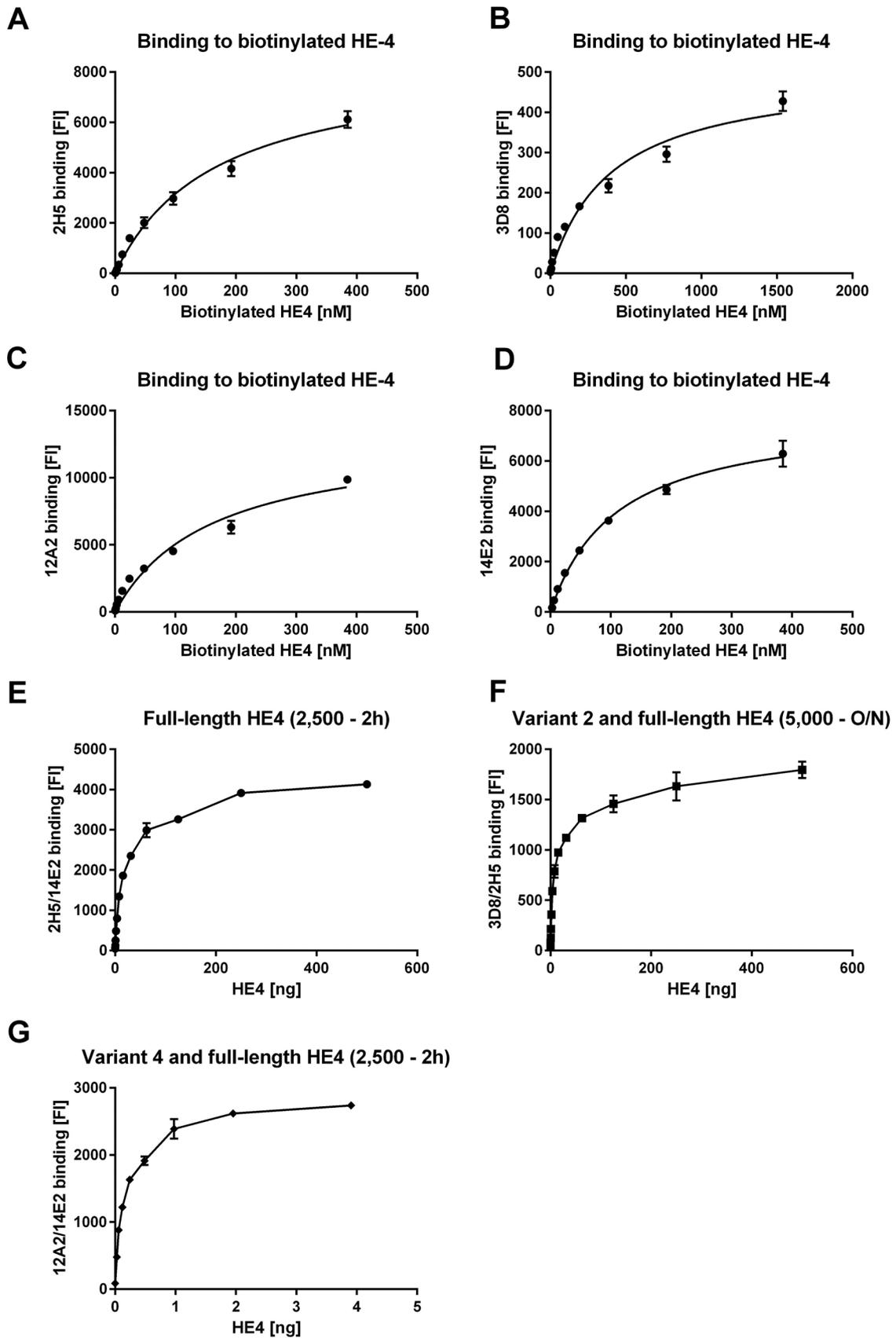
Fig. 1. Affinities of mAB binding to HE4 peptide and detection of different HE4 epitopes with specific mAB combinations. Binding of (A) 2H5, (B) 3D8, (C) 12A2 and (D) 14E2 mABs to increasing concentrations of biotinylated HE4 peptide. (E) Detection of an epitope present on full-length HE4 using a combination of bead-coupled mAB 2H5 and biotinylated mAB 14E2. (F) Detection of epitopes present on variant 2 and full-length HE4 using a combination of bead-coupled mAB 3D8 and biotinylated mAB 2H5. (G) Detection of epitopes present on variant 4 and full-length HE4 using a combination of bead-coupled mAB 12A2 and biotinylated mAB 14E2.

Table 1
Binding affinities of HE4 mABs.

HE4 mAB	Number of replicates	Mean Kd (nM)	SD of Kd (nm)
2H5	6	166.9	12.4
3D8	6	405.9	39.3
12A2	6	161.4	16.2
14E2	6	109.4	6.2

Determination of intracellular expression of HE4 by flow cytometry of cultured ovarian carcinoma cells

The cells were incubated in DMEM/F-12 media with GlutaMAX supplement (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine sera (Atlanta Biologicals, Flowery Branch, GA) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, MA). Cultured cells were detached by incubation with TrypLE™ Express Enzyme (Thermo Fisher Scientific, Waltham, MA), and single cell suspensions were washed with phosphate buffered saline (PBS). The cells were fixed and permeabilized applying the Cytofix/Cytoperm kit (BD Bioscience, San Jose, CA) according to manufacturer's instructions. The anti-HE4 mAbs 2H5, 3D8, 12A2 and 14E2 were diluted to 1 μg/mL with permeabilization wash buffer from the Cytofix/Cytoperm kit and incubated with the tumor cells for 30 min after which the cells were washed with permeabilization wash. A PE- labelled goat anti-mouse IgG Fc cross-adsorbed secondary antibody (Thermo Fisher Scientific, Waltham, MA) was used at 1:200 dilution for detecting bound anti-HE4 mAbs. Flow cytometry was performed using FACSCalibur (BD Bioscience) and the tumor cell populations were selected. Negative cells

were determined based on the control staining (mouse IgG1 kappa isotype, Thermo Fisher Scientific, Waltham, MA). The data were analyzed using FlowJo 10 (FlowJo, Ashland, OR), and statistical significance was calculated by one-way ANOVA. All flow cytometry experiments were performed 6 times.

Detection of HE4 in cultured ovarian carcinoma cells

The cells were cultured in 8-well chamber slides (EMD Millipore, Billerica, MA) in DMEM/F-12 media with GlutaMAX supplement (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine sera (Atlanta Biologicals, Flowery Branch, GA) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, MA) until full confluency. Cells were fixed with 10% formalin in phosphate buffered saline for 10 min and permeabilized by 0.5% Triton-X 100 in PBS for 30 min at room temperature. Endogenous peroxidase was quenched by incubation in 3% H_2O_2 for 5 min after which the slides were blocked with 5% BSA/PBS for 1 h at room temperature followed by incubation with 5 μg/mL of HE4 and isotype control mAbs overnight at 4 °C. The following day, the slides were washed with 0.05% Tween/PBS after which they were incubated with anti-mouse EnVision+ System-HRP labelled polymer (Agilent Technologies) for 30 min. For visualization of mAb bound to tumor cells the Liquid DAB+ substrate-chromogen system (Agilent Technologies) was used according to the manufacturer's instructions. The slides were mounted with mounting medium (Thermo Fisher Scientific, Waltham, MA) and analyzed by microscopy. A score ranging from 0 to 4 was assigned based on the intensity of the stain and the frequency of the stained cells.
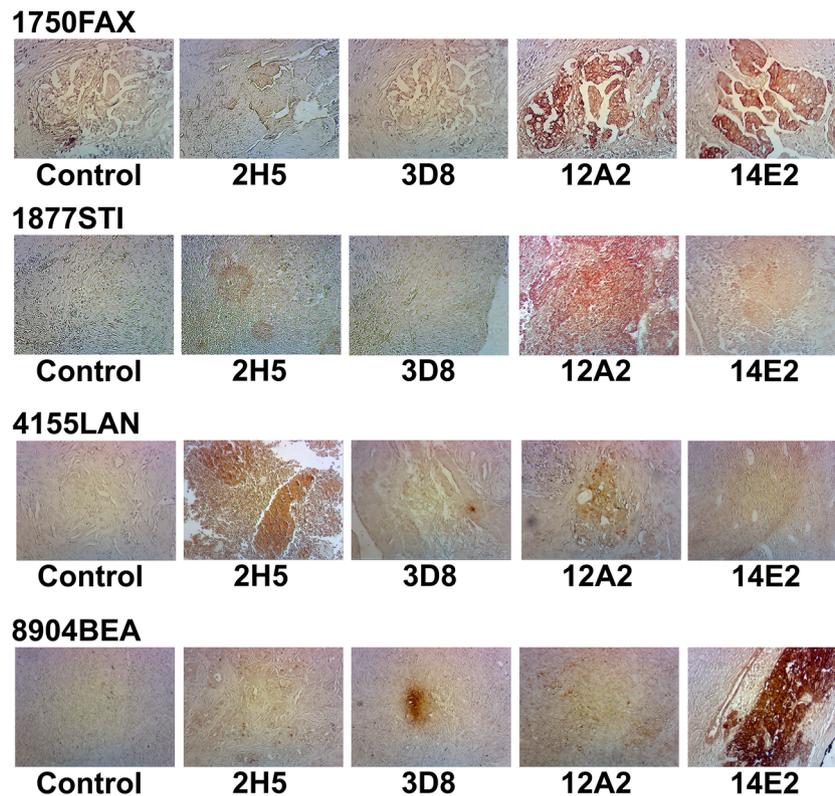

Fig. 2. (a). Photographs of formalin fixed sections of human ovarian carcinomas stained with mouse anti-HE4 mAbs 2H5, 3D8,12A2 or 14E2 using mouse kappa IgG1 as isotype control. (b). Expression of HE4 in sections from ovarian carcinoma as detected by IHC using anti-HE4 mAbs 2H5, 3D8, 12A2 and 14E2, as compared to mouse IgG1 kappa isotype control.
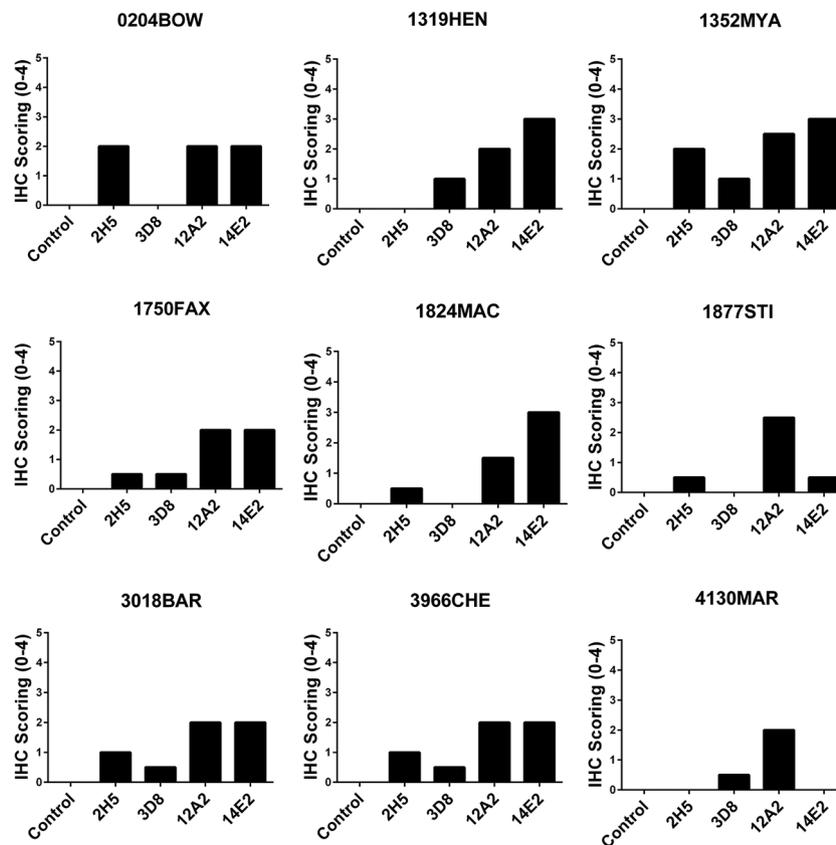
Fig. 2. (*continued*)

Results

Characterisation of the epitopes recognized by mAbs 12A2 and 14E2

The type of epitopes recognized by the 12A2 and 14E2 mAbs was determined by testing the reactivity of the mAbs to denatured and reduced HE4 antigens. Spent medium from a cell line producing full-length HE4-hIgG1, was concentrated five times, denatured at 70 °C and separated with SDS-PAGE under reducing conditions and the proteins were blotted onto a PVDF membrane. After incubating the membrane with the 12A2 or 14E2 mAbs, their binding to the PVDF membrane was traced by a HRP swine anti mouse antibody (Dako/ Agilent) which was detected by chemiluminescence using the Pierce™ ECL™ detection system (Thermo Fisher Scientific, Waltham, MA). MAbs 12A2 and 14E2 had no reactivity against denatured and reduced HE4 antigen indicating that they recognize a conformational dependent epitope.

HE4 binding affinity to mAbs 2H5, 3D8, 12A2 and 14E2

The binding affinity of mAbs to HE4 was determined by testing the binding of each mAb to biotinylated HE4. Binding was measured as fluorescent intensity using a Luminex assay with fluorescent-bead conjugated 2H5, 3D8, 12A2 and 14E2 mAbs (Fig 1 Panels A-D). We calculated an average Kd of 166.9 nM for mAb 2H5, an average Kd of 405.9 nM for mAb 3D8, an average Kd of 161.4 nM for mAb 12A2 and an average Kd of 109.4 nM for mAb 14E2 (Table 1), indicating that the binding to HE4 was stronger for mAbs 2H5, 12A2 and 14E2 than for mAb 3D8.

Sandwich Luminex assays for detection of different HE4 epitopes

We used 3 different combinations of bead-coupled and biotinylated HE4 mAbs to detect epitopes present on full-length HE4, variant 2 HE4 and variant 4 HE4. All three Luminex sandwich conditions detected HE4 with increasing signal intensities for increasing amounts of HE4 (Fig 1A-D). We observed that the 2H5/14E2 and 3D8/2H5 combinations resulted in similar HE4 input curves (Fig 1E and F) which started to level out when the amount HE4 exceeded 250 ng, and that produced reliable FI signals for as little as 1.95 ng of HE4 (HE4 concentration per reaction 19.5 ng/ml). The 12A2/14E2 combination (Fig 1G) resulted in a leveling of the HE4 input curve when the amount HE4 exceeded 1.95 ng, and that produced reliable FI signals for as little as 0.06 ng of HE4 (HE4 concentration per reaction 0.6 ng/ml).

Binding of anti-HE4 mAbs to tissue sections of ovarian carcinoma

We evaluated the binding of the 4 mAbs to sections from formalin-fixed blocks of 19 ovarian carcinomas, which are listed in Supplementary Table 1. Photographs of sections from 4 patients are shown in Fig. 2a, and Supplementary Fig. 1 provides photos of sections from all patients. As illustrated in Fig. 2a and b, epitopes defined by the 4 mAbs were found to be expressed independently of each other with different epitopes being most prominent in different patients. mAb 12A2 bound at a level of > 0.5 to sections from 18 patients and mAbs 14E2, 2H5 and 3D8 had a corresponding binding for 14, 7 and 4 patients, respectively.
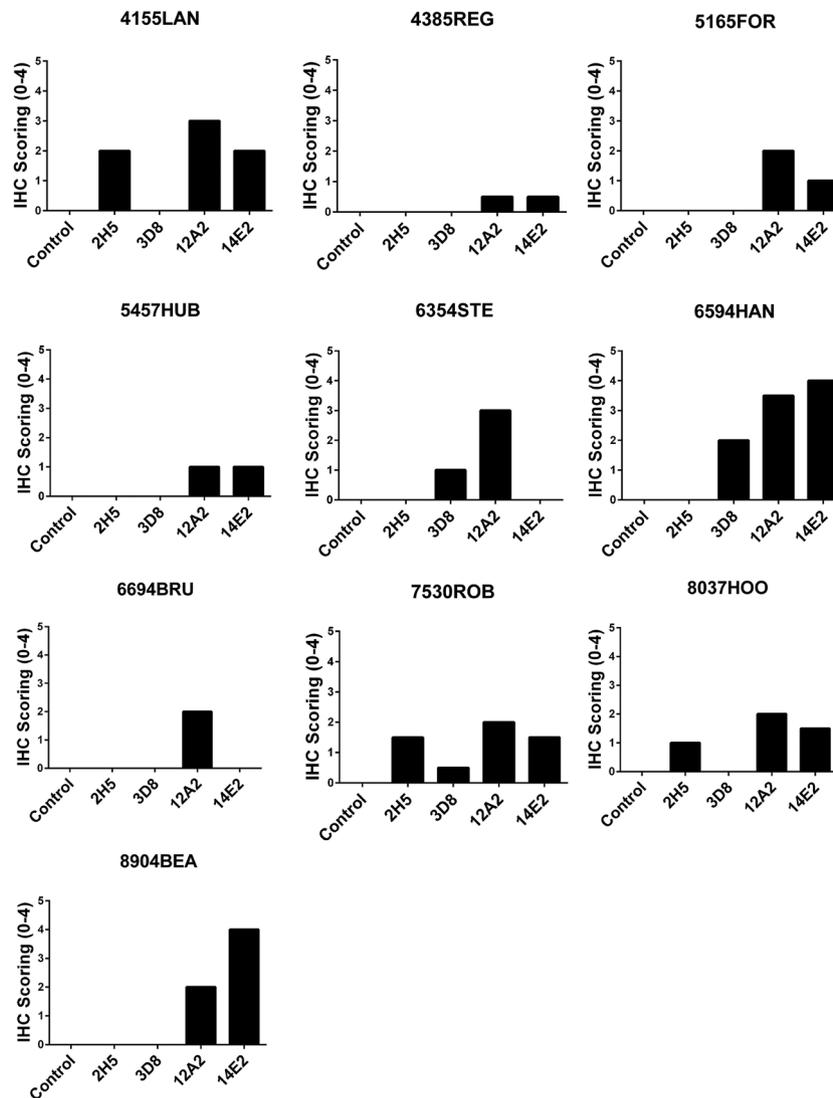
4155LAN

4385REG

5165FOR

5457HUB

6354STE

6594HAN

6694BRU

7530ROB

8037HOO

8904BEA

Fig. 2. (*continued*)

Detection of HE4 in serum from patients whose tumor sections were analyzed by immunohistology

Sera from 12 of 19 patients were positive for HE4 according to the commercially available ELISA and the standard 150pM cutoff (Supplementary Table 1). While binding of anti-HE4 mAbs to ovarian carcinoma cells was slightly higher for the group of patients whose serum was positive for HE4, this difference was not statistically significant except for the epitope recognized by mAb 14E2 (Fig. 3).

Binding of anti-HE4 mAbs to cultured ovarian carcinoma cells

We first applied flow cytometry to study the intracellular binding of mAbs 2H5, 3D8, 12A2 and 14E2 to cell lines established from 6 different serous ovarian carcinomas. As shown in Fig. 4, mAb 14E2 bound to all 6 cell lines and was the only mAb which gave a detectable binding to lines 225, 249 and 272.

As shown in Fig. 5a and b, according to immunohistochemistry, all 4

mAbs bound to cultured cells from 3 ovarian carcinomas (239, 289 and Ovcar 3). In contrast, only 2H5 bound to 225 cells, 2H5 plus 3D8 bound to 249 cells and none of the mAbs bound to 272 cells, i.e. 12A2 and 14E2 which bound well to cells from three ovarian carcinomas were negative on 225, 249 and 272 cells. Similar to the findings by flow cytometry, mAb 12A2 bound slightly better than 14E2 to 239, 289 and Ovcar 3.

Secretion of HE4 into supernatants of cultured ovarian carcinoma cells

Cultured cells from 3 ovarian carcinoma lines, 239, 289 and Ovcar 3, secreted the HE4 molecule as detected by testing culture supernatants with the HE4 kit (350, 120, 430 pM, respectively), while cells from lines 225, 249 and 272 did not secrete any detectable HE4. While the 3 secreting cell lines significantly bound all 4 mAbs, the non-secretors only bound 14E2 according to flow cytometry, while lines 225 and 249 bound 2H5 and l249 also bound 3D8 according to immunohistochemistry. We conclude that ovarian carcinoma cells that
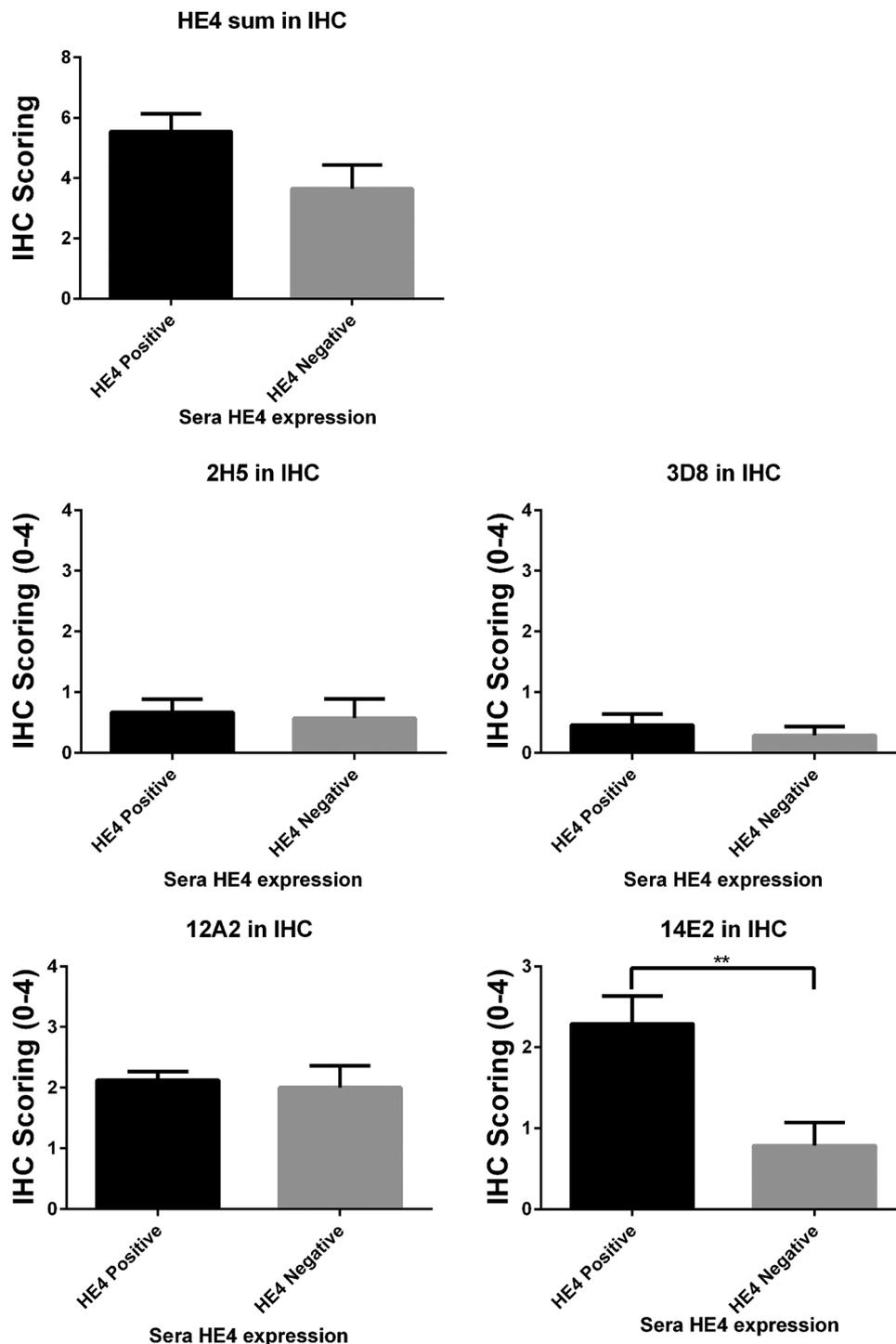
Fig. 3. HE4 secretion and IHC scoring.

express high levels of HE4 are more prone to secrete this molecule than cells which express low levels of HE4.

Platinum resistance and expression of HE4 epitopes

There was no difference in the presence of the HE4 antigen in sera from patients who were sensitive or resistant to platinum-based chemotherapy. However, the epitope recognized by mAb 14E2 was significantly more expressed by platinum resistant tumors than on

sensitive ones ($p < 0.01$), while no such difference was observed for the other 3 epitopes. These data need to be confirmed before we want to draw any conclusion about HE4 expression and platinum resistance.

Discussion

We first measured the affinity for the binding of the 4 mAbs to the HE4 protein, applying a Luminex assay and found that 14E2 had the highest affinity followed by 2H5 and 12A2 which were similar while
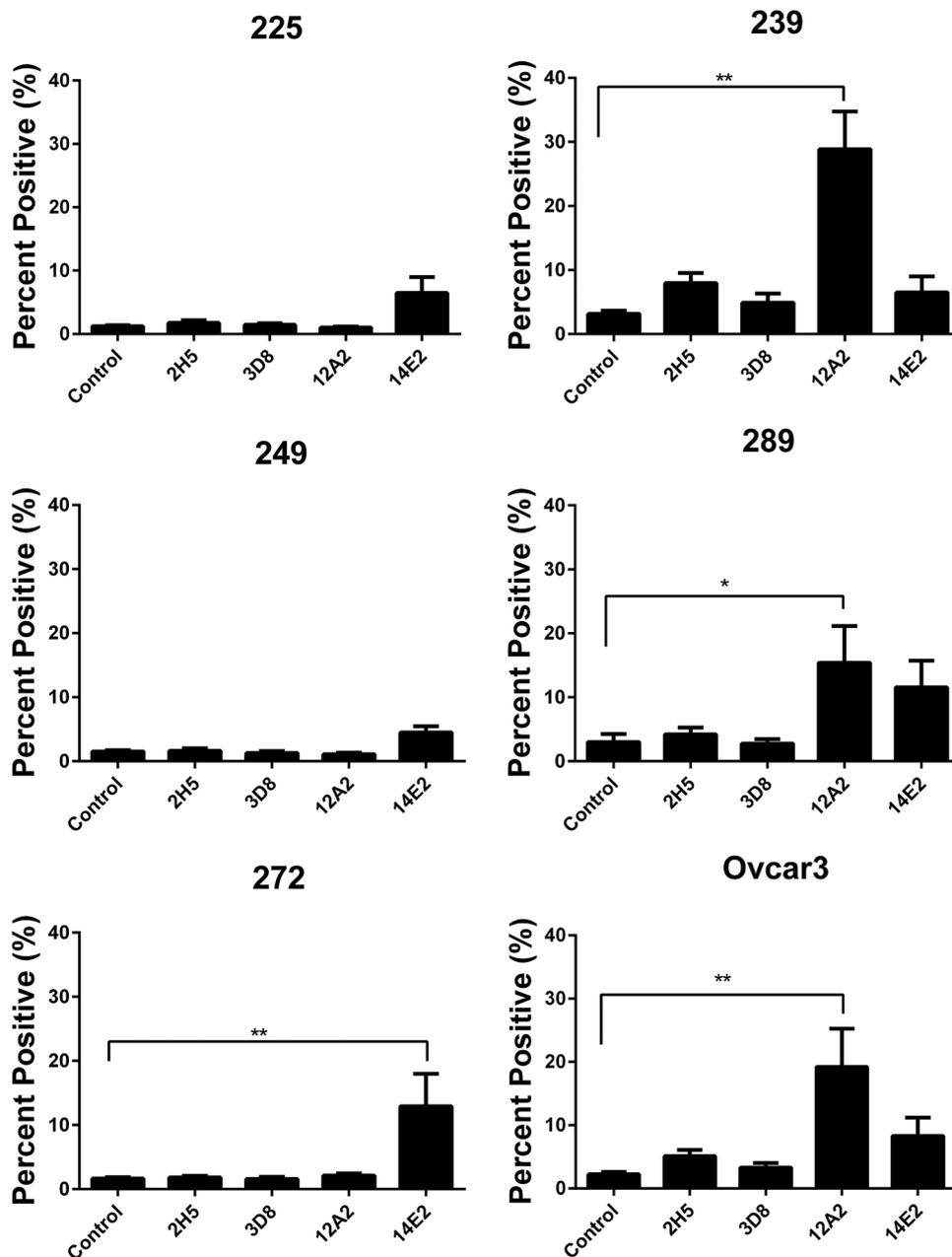
Fig. 4. Expression of HE4 in 6 ovarian carcinoma lines according to flow cytometry with mAbs 2H5, 3D8, 12A2 and 14E2 as compared to mouse IgG1 kappa isotype control. Statisitical analysis was performed using one-way ANOVA. The data were summaried from at least 5 tests.

3D8 had the lowest affinity.

We then investigated the binding of 4 anti-HE4 mAbs to formalin fixed sections from 19 patients with ovarian carcinoma and tried to relate the findings to the presence of the HE4 biomarker in serum. As illustrated in Fig. 2a and b, our data indicated that the epitopes to which the 4 mAbs bind were expressed independently of each other so that one mAb was the strongest binder to section from one patient while another mAb best bound to sections from another patient. Anti-12A2 mAbs bound to sections from 18 patients while 2H5 which has approximately the same affinity as 12A2 bound to sections from 7 patients

and sections from 4 patients bound to 3D8 which has the lowest affinity. Patients whose tumors expressed much HE4 had slightly higher HE4 levels in serum but the difference was not statistically significant. It is noteworthy, however, that the epitope recognized by mAb 14E2 was significantly more often expressed by tumors from patients whose sera were positive for HE4 according to the standard 150 pM cutoff.

To further investigate the relation between cellular expression of HE4 epitopes and secretion of the biomarker, we established cell lines from 5 ovarian carcinomas and also studied a commercially available line, Ovcar3. By applying both flow cytometry and immunohistology
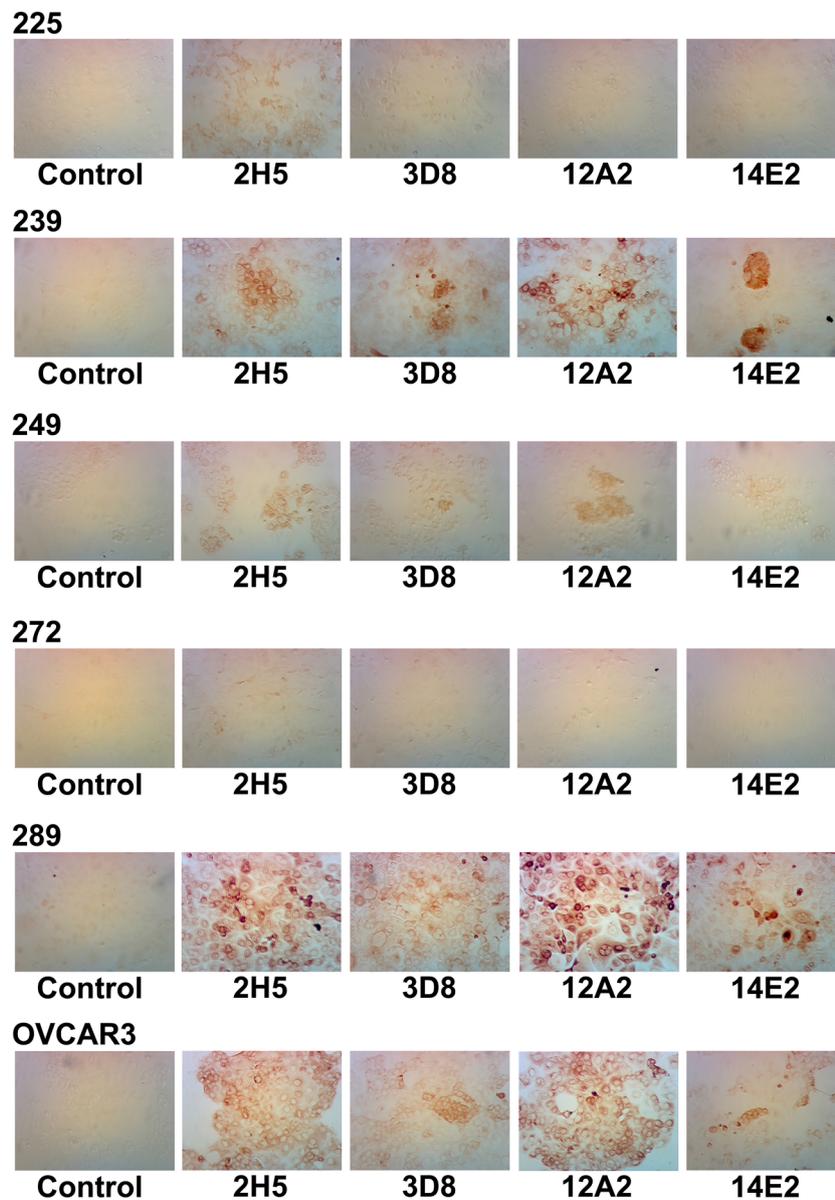
Fig. 5. (a) Cells from human ovarian carcinoma lines were stained with mouse anti-HE4 mAbs 2H5, 3D8,12A2 and14E2 using mouse IgG1 kappa as isotype control. (b) Expression of HE4 in human ovarian carcinoma lines as detected by immunocytochemistry using anti-HE4 mAbs 2H5, 3D8, 12A2 and 14E2, as compared to mouse IgG1 kappa isotype control.

we found that 3 of the lines expressed high levels of the HE4 epitopes while 3 other lines did not and that only the strongly positive lines secreted HE4 into culture supernatants. Based on these findings we conclude that there is a relationship between HE4 expression and secretion also in ovarian carcinoma patients. Yet it is hard to get reliable quantitative data analyzing tumor sections with immunohistology.

Interestingly, tumors that were resistant to platinum bound more anti-14E2 mAb, but a difference, albeit statistically significant needs to be established on a larger study cohort.

The strong expression of epitopes identified by mAbs 12A2 and 14E2 indicates that these epitopes may be potential targets for immunotherapy and further studies are needed to show whether an ELISA combining mAbs 12A2 and 14E2 with each other or with 2H5 has better sensitivity than the commercially available HE4 kit without compromised specificity.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.
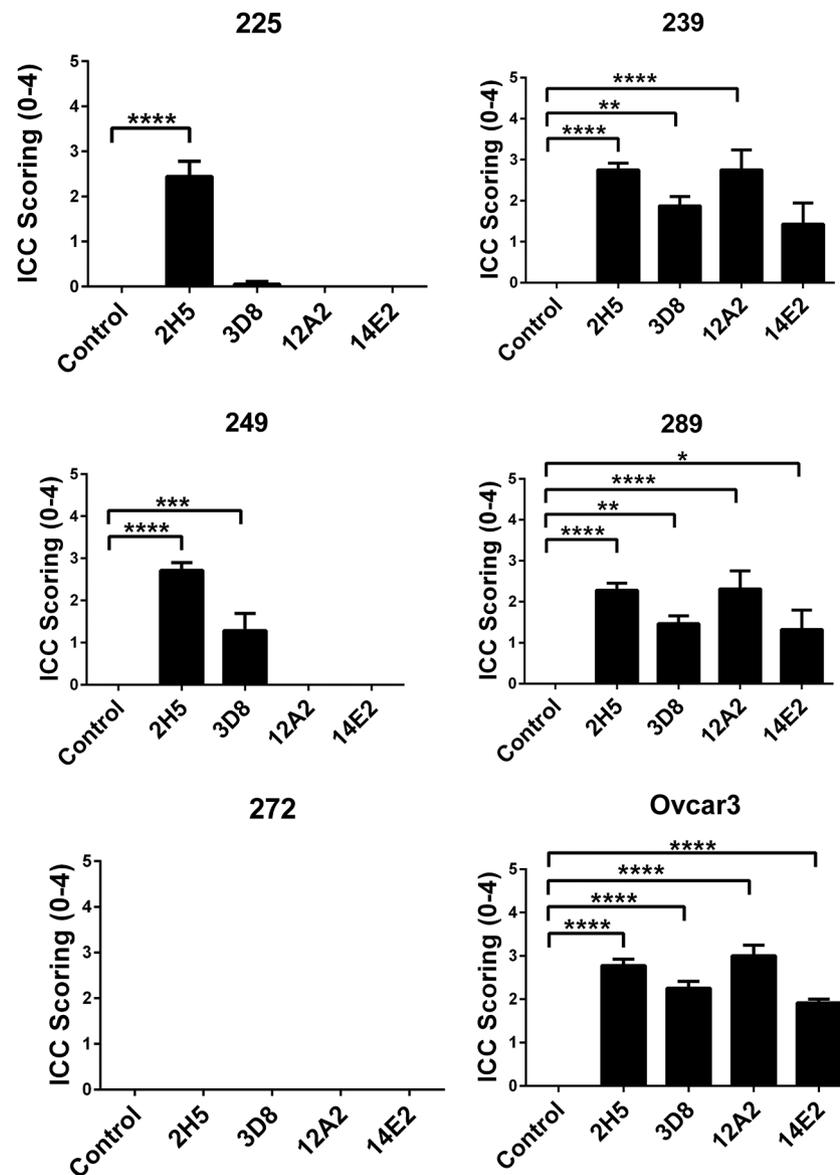
Fig. 5. (*continued*)

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ctarc.2019.100152.

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