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ORIGINAL PAPERS

# An HSP90-mimic peptide revealed by fingerprinting the pool of antibodies from ovarian cancer patients

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**To gain insight into the mechanisms of molecular recognition and humoral immune response in ovarian cancer, we used fingerprinting, a phage display-based combinatorial selection to isolate peptide ligands to tumor-related antibodies present in ascites from patients with advanced disease. First, we have isolated a consensus motif (sequence CVPELGHEC) in 86% of the peptides screened; this enriched motif was selected from a total of $10^8$–$10^9$ unique random sequences present in the library. Next, we identified the heat-shock protein 90 kDa (HSP90) as the native antigen mimicked by the motif. Finally, we evaluated the expression of HSP90 and the presence of antibodies against the HSP90-mimic peptide in a large panel of ovarian cancer patients and controls. In tissue microarrays, we show that the expression of HSP90 is ubiquitous. However, the corresponding humoral immune response against HSP90 is restricted to a subset of patients with stage IV disease. Together, these results show that screening humoral response can identify tumor antigens that may serve as molecular targets in ovarian cancer. Recognition of such relevant proteins in the immunobiology of malignant tumors may lead to the development of therapies.**
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## Introduction

Ovarian cancer is among the most lethal of all gynecologic malignancies (Jemal *et al*., 2003). The presence of advanced disease (stages III and IV) at diagnosis and high-grade pathology adversely affects clinical outcome (Holschneider and Berek, 2000). Currently, half of the women treated for ovarian cancer develop recurrent disease after primary treatment, less than 10% of these women respond to second-line therapy, and the remainder have a poor median survival (Bookman and Young, 2000; Marsden *et al*., 2000). Of the known serum markers for this type of cancer, only CA125 has been validated as an indicator of response or progression (Maggino and Gadducci, 2000; Meyer and Rustin, 2000). Thus, the lack of molecular profiles to distinguish an aggressive clinical phenotype makes treatment outcome in this disease relatively difficult to predict.

Phage display is an established technology that allows detection and characterization of protein–protein interactions (Barbas *et al*., 2001). We have described methodology to select peptide motifs recognized by tumor-associated antibodies; this phage display-based approach (termed 'fingerprinting') enables isolation of mimic peptides of antigens eliciting an humoral response (Mintz *et al*., 2003). In our working definition, antibody fingerprinting is a combinatorial screening in which phage display random peptide libraries are selected on *in vitro* pools of immobilized patient-derived immunoglobulins; when successful, such procedure can lead to epitope mapping and enable identification of the corresponding native tumor antigens.

Since cancers express many such tumor antigens, fingerprinting might yield markers of disease aggressiveness or targets for therapy. Thus, here we fingerprinted the pool of antibodies purified from a patient with ovarian cancer-derived ascites. First, we identified a consensus peptide motif that is selectively recognized by antibodies derived from ascites from ovarian cancer

patients. Next, by using affinity chromatography, we purified, isolated, and identified the corresponding native antigen mimicked by the motif: the heat-shock protein 90 kDa (HSP90). Finally, we show that, while HSP90 is widely expressed in ovarian cancer, the humoral response against HSP90 is tumor-associated and stage-specific. These results demonstrate that screening the humoral immune response can identify tumor antigens that may serve as molecular targets in ovarian cancer.

## Results

### *Selection, isolation, and validation of peptide motifs recognized by the pool of antibodies present in ascites from ovarian cancer patients*

To enhance the chance of selecting peptides that bind to immunoglobulins associated with ovarian cancer, we used a two-step procedure (Figure 1A). Following bulk amplification in successive rounds of selection, we observed the relative enrichment of a phage population capable of binding immunoglobulins in the patient analysed over control immunoglobulins or bovine serum albumin (BSA; Figure 1b). After selection, we randomly picked individual clones ($n=85$) for DNA amplification and sequence analysis. Essentially, only a single dominant peptide motif (sequence CVPELGHEC) was recovered (Table 1). This result attests to the power of selection and/or reflects the ease of propagation of that clone relative to others in the general population, particularly if one considers the original library size of $10^8$–$10^9$ unique sequences. To validate the enriched consensus peptide as an epitope recognized by the pool of antibodies purified from ascites of the index patient, phage binding assays and competitive inhibition ELISAs were performed. Antibodies isolated from the index ovarian cancer patient recognized the CVPELGHEC peptide (Figure 1c). To test the protein interaction out of phage context, a recombinant glutathione S-transferase (GST)-fusion protein was produced and immobilized onto microtiter wells, along with GST (negative control). The GST–CVPELGHEC-fusion protein specifically bound to antibodies purified from ascites and serum of the index patient; this reactivity was not observed with antibodies obtained from several age- and gender-matched controls. Finally, binding of purified immunoglobulins to the GST–CVPELGHEC-fusion protein was inhibited in a dose-dependent manner by the synthetic peptide CVPELGHEC (Figure 1d).

### *Reactivity against the selected peptide motif CVPELGHEC is tumor-associated and stage-specific*

The reactivity against the peptide CVPELGHEC was analysed in a large population of samples derived from ovarian cancer patients and control patients (Figure 2). In total, ascites from patients with advanced ovarian cancer (stages III and IV), metastatic gastrointestinal cancer, and nonmalignant cirrhosis were evaluated for the presence of antibodies capable of recognizing the motif CVPELGHEC. In addition, serum from age-matched blood donor women and women with benign gynecological diseases was evaluated for the presence of anti-CVPELGHEC antibodies. Among control ascites and serum samples tested, small percentages had positive reactivity: only two out of 15 (13.3%) of the gastrointestinal cancer ascites and none out of four (0%) of the cirrhotic ascites exhibited positive reactivity. Likewise, only eight out of 57 (14.0%) of the age-matched blood donor serum samples and one out of 30 (3.3%) of the serum samples from patients with nonmalignant gynecological diseases had anti-CVPELGHEC antibodies present. In ascites from patients with ovarian cancer, only three out of 42 (7.1%) patients with stage III disease had antibodies present in their ascites that recognized the CVPELGHEC peptide; in contrast, 10 out of 17 (58.8%) patients with stage IV disease exhibited positive reactivity (*t*-test; $P<0.0001$) when compared to all the other types of ascites tested (Figure 2). Kaplan–Meier curve estimates were applied to compare survival within stage IV patients whose ascites were of papillary serous origin reacting against the CVPELGHEC peptide; survival of the group of stage IV ovarian cancer patients reacting against the peptide CVPELGHEC ($n=10$, median = 174 months) was not significantly different from survival of stage IV ovarian cancer patients not reacting against the peptide CVPELGHEC ($n=7$, median = 106 months) in this small subset analysis (data not shown).

Next, the purified specific immunoglobulins reacting with the peptide CVPELGHEC were used to perform immunostaining on tissue sections from the corresponding index patient with ovarian cancer. Strong immunostaining was observed in the tumor (Figure 3a), but no staining was observed with purified blood-donor antibodies (negative control) at the same concentration (Figure 3b) or with secondary antibody alone (data not shown). Moreover, the recombinant GST–CVPELGHEC-fusion protein inhibited staining with the affinity-purified antibody (Figure 3c), but GST alone did not (Figure 3d). Taken together, these results suggest that the antigen mimicked by the peptide motif CVPELGHEC is tumor-associated.

### *Identification and validation of HSP90 as an antigen mimicked by CVPELGHEC*

The protein mimicked by the CVPELGHEC consensus peptide was identified by purification of cytoplasmic/membrane fraction preparations obtained from ovarian cancer HEY cells (Buick *et al*., 1985). Multiple protein bands were found when the cytoplasmic/membrane fraction was separated on an SDS–PAGE and Coomassie-stained (Figure 4a, left panel). A dominant band of $\sim$98 kDa was detected on a Western blot on this fraction by probing with antibodies purified from the ascites of the index patient on the peptide CVPELGHEC (Figure 4a, middle left panel), but no band was observed with control donor antibodies (Figure 4a, middle right panel). Next, we partially purified the

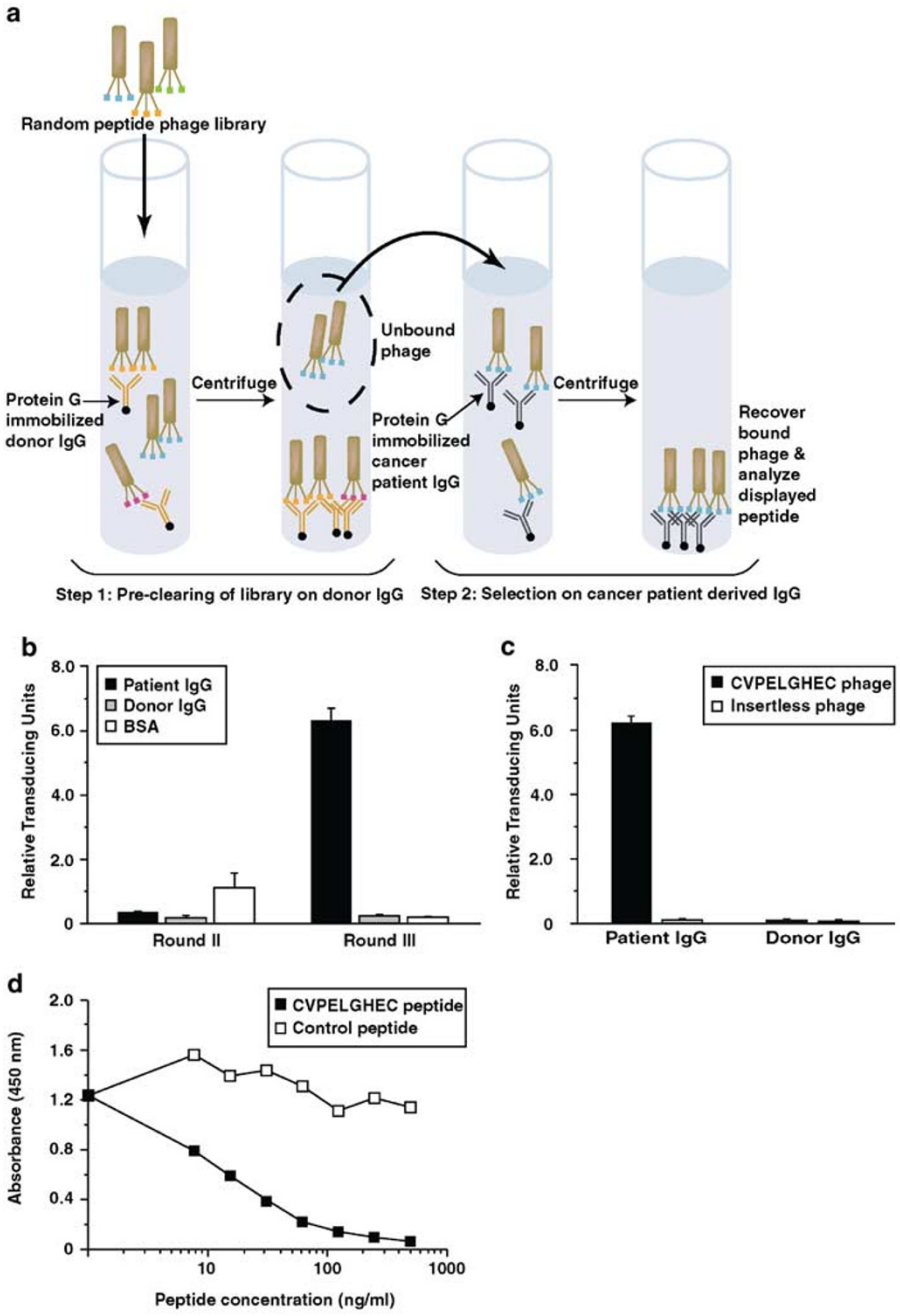



**Figure 1** Phage display can be used to enrich for a phage population capable of binding ovarian cancer IgGs. Selection strategy scheme. Selection of a phage display random peptide library on antibodies purified from an ovarian cancer index patient (**a**). Successive rounds of selection show an increase in selectivity relative to the serum control (**b**). Phage clones displaying the enriched peptide CVPELGHEC, but not insertless phage, bind IgGs from the patient analysed (**c**). Binding inhibition of the antibodies from the patient analysed to immobilized GST-peptide-fusion proteins is specific (**d**). The interaction occurs in a dose-dependent manner and can be inhibited with the corresponding synthetic peptide. Error bars represent the standard deviation of triplicate plating

**Table 1** Peptide sequences displayed by phage binding to purified IgGs from ascites

| *Motif* | n | *%* |
|---|---|---|
| VPELGHE | 73 | 86 |
| ELGFELG | 1 | 1 |
| FFLRDWF | 2 | 2 |
| Other | 9 | 11 |
| Total | 85 | 100 |

98 kDa gel band from the Coomassie-stained gel (Figure 4a, left panel, boxed region) and probed it by Western blot with the immunopurified antibodies from the index patient (Figure 4a, far right panel). The partially purified protein was processed for mass spectrometry and results of the analyses were compared to protein databases: HSP90 was found to be the corresponding antigen (MOWSE score $1.031 \times 10^8$) by

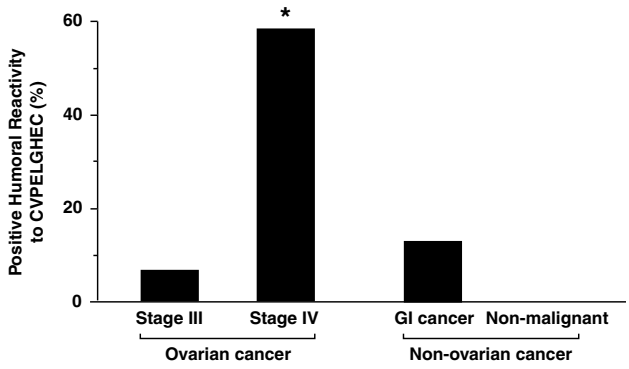



**Figure 2** Antibodies from ovarian cancer patients react with the selected peptide. Ascites from late stage ovarian cancer patients, non-ovarian (gastrointestinal cancer) patients, and cirrhotic patients were tested for their reactivity against the enriched peptide motif. For each sample, serial dilutions determined the optimal reactivity by ELISA. Positive reactivity was determined by a ratio of GST-peptide to GST alone $\geqslant 1.5$

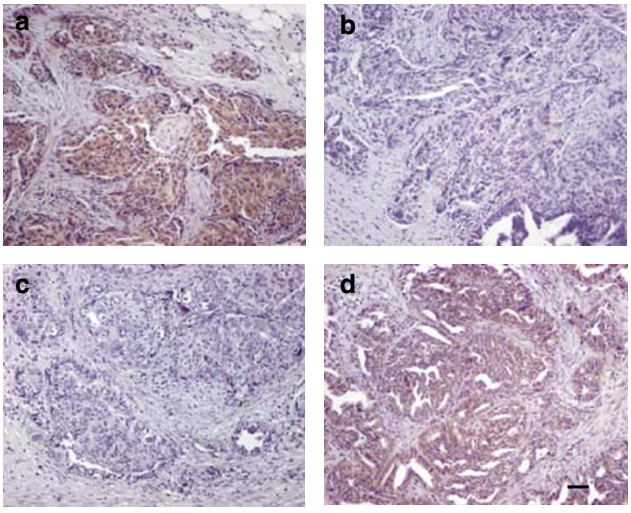



**Figure 3** The antigen mimicked by the enriched peptide epitope is tumor associated. Immunohistochemistry using the affinity-purified patient IgG was used to stain the autologous tumor (**a**). No staining was observed with control donor IgG (**b**). The staining was specific and could be inhibited with the corresponding GST-fusion peptide (**c**), but not with GST alone (**d**). Scale bar (**a–d**) 100 $\mu$m

mass mapping (Figure 4b). An alignment of the HSP90 sequence with the identified peptide epitope, CVPELGHEC (Figure 4c), suggested that the site recognized by the affinity-purified anti-CVPELGHEC antibody is likely to be conformational since a clear linear sequence of residues with homology to the motif was not initially found in the HSP90 protein. To refine this hypothesis, we used the crystal structure of HSP90 to determine the proximity of the amino acids from the peptide sharing the homology identity with HSP90 sequence. We found that amino acids from the peptide clustered with the highest homology towards the N-terminus of HSP90 (residues 66–75), suggesting the possibility of a conformational epitope (Figure 4c, left panel, boxed region). This structural analysis is consistent with a conformational epitope within an exposed area of HSP90 (Figure 4c, right panel, yellow highlight). Finally, to confirm the identity of HSP90, we identified the partially purified 98 kDa protein with an anti-HSP90 antibody on a Western blot (Figure 4d), whereas no band was detected with control donor antibodies.

Next, we sought to validate our findings with reagents derived from the index patient: First, we showed that affinity-purified antibodies from the patient bind to human HSP90 in a dose-dependent manner (Figure 5a). Moreover, the interaction between the affinity-purified antibodies from the patient and HSP90 is inhibited by CVPELGHEC and also dose-dependent (Figure 5b). These results suggest biochemical specificity. Finally, we assessed the protein expression of HSP90 in ovarian cancer. Consistently, immunoreactivity observed with anti-HSP90 antibodies (described below) had a pattern similar to that seen with CVPELGHEC-affinity-purified immunoglobulins from the index patient (Figure 5c, left panel), while pre-immune antibodies (Figure 5c, right panel) or secondary antibody alone (data not shown) did not show staining. These data indicate that HSP90 is a native antigen reacting with an affinity-purified pool of antibodies.

### *Protein expression of HSP90 in ovarian cancer is widespread and independent of tumor stage or grade*

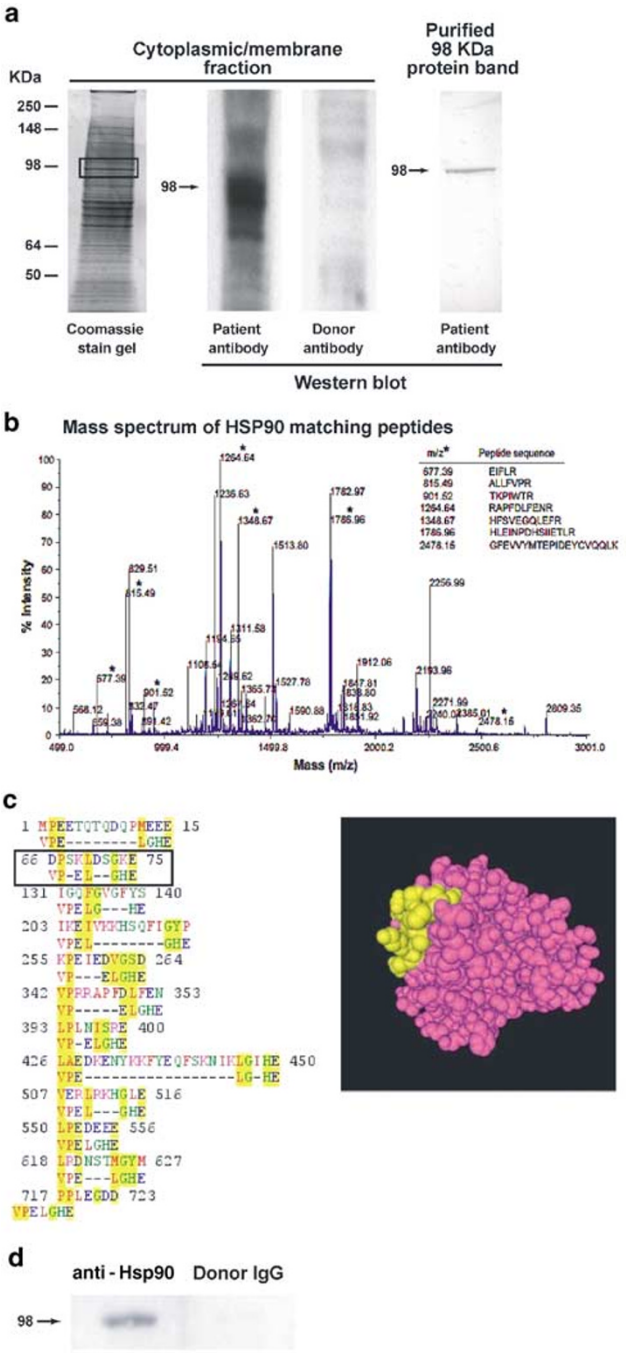
Having shown that HSP90 was an antigen eliciting a humoral immune response in the index patient analysed, we set out to survey the expression of HSP90 in a population of unrelated patients with ovarian cancer. Thus, we analysed a panel of tissue samples of normal ovarian surface epithelium and ovarian tumors (Table 2). Normal ovarian surface epithelium, tumors of low malignant potential, and tumors of various stages and grades – including serous, endometroid, clear cell, mixed malignant müllerian tumor, undifferentiated, mucinous, and transitional cell carcinomas – were represented in the tissue arrays. Results of this analysis showed that HSP90 protein expression was present in all ovarian cancer tissue sections examined (Figure 6); the broad HSP90 expression in human ovarian cancer did not appear to correlate with tumor stage, histology type, or degree of anaplasia.

## Discussion

Recognition of molecular targets that are relevant in the biology of malignant tumors is a key step for the development of therapies. Here we used a fingerprinting procedure (Mintz *et al*., 2003) to identify tumor or tumor-associated proteins – and potential targets – in ovarian cancer. First, we selected a consensus cyclic peptide motif (sequence CVPELGHEC) that is recognized by both the pool of the ascites-purified and the repertoire of circulating antibodies in an index ovarian cancer patient. Moreover, we show that the binding of the selected ligand peptide to the antibodies purified

from the patient (i) is specific, (ii) dose-dependent, (iii) also occurs out of the context of the phage, and (iv) can be inhibited by a cognate recombinant fusion or synthetic peptide. Finally, we show that HSP90 is an antigen mimicked by CVPELGHEC and that HSP90 is expressed in ovarian tumors independent of the disease stage.

The biological basis for a humoral immune response against HSP90 in disease is still not entirely clear. However, consistent with our results, an ovarian cancer cDNA expression library screening has also yielded HSP90, among other tumor antigens (Luo *et al*., 2002).




Given that cell surface expression is considered a prerequisite for direct immune recognition of cells by the humoral arm of the immune system, one might speculate on the reason for the observation that only Stage IV patients mount humoral responses since all tumors express HSP90. The simplest explanation is that lysis of tumor cells can lead to the exposure and presentation of cryptic intracellular HSP90-derived immunogenic determinants, hence a detection of humoral response in the most advanced stage (and with more tumor cell death) disease. However, proteomic approaches have recently brought forward the intriguing finding that stress-response proteins from the heat-shock family – including HSP90 – are present on tumor cell surfaces (Shin *et al*., 2003). In addition, it has also been reported that, although HSP90 is an intracytoplasmic protein, surface expression of HSP90 can exist, rendering the protein available for antibody recognition (Kenderov *et al*., 2002); another plausible (and nonmutually exclusive) alternative explanation might conceivably involve the membrane expression and/or trafficking of HSP90 for cell surface presentation. Thus, the relative immunogenic contributions of the intracellular fraction versus cell surface fraction of HSP90 expression remains an open question.

The fact that HSP90 is a complex tumor target can also be illustrated by other independent lines research that might be related to the differential humoral immune response observed here. Srivastava *et al*. (2001) have long proposed that HSP90 plays an 'MHC-like' role in presenting immunogenic peptides derived from other proteins. More recently, other investigators have also shown that a high-affinity conformation of HSP90 may exist in tumors, enhancing the selectivity of certain HSP90 inhibitors (Kamal *et al*., 2003; Neckers and Lee, 2003). Therefore, activation states and other as yet unidentified antigens may play a role in the immunogenicity of HSP90. Of note, the association of HSPs with foreign peptides may also lead to the development of immunity to both the foreign antigens and associated

**Figure 4** Identification of the corresponding antigen mimicking CVPELGHEC. The HEY human ovarian cancer cell line was used fractionated into membrane and cytosolic contents and separated on 4–20% gradient SDS–PAGE followed by Coomassie blue staining (**a**, left panel). Western blot analysis with the affinity-purified patient IgGs were used to detect the presence of the antigen. A dominant band of ~98 kDa was immunodetected, but not with the control donor antibodies (**a**, middle, left, and right panels, respectively). The corresponding band was excised and partially purified from the Coomassie blue staining gel (**a**, left panel, boxed region). The partially purified protein was used for a Western blot performed with the affinity-purified patient IgGs (**a**, far right panel). This band was excised and sent for mass spectrometry analysis by MALDI-TOF. HSP90 was identified as the antigen as shown by the mass spectrum of HSP90 (**b**). Scattered homology between the peptide CVPELGHEC and HSP90 was searched with the alignment software program ClustalW (**c**, left panel) and a high homology region was found in residues 66–75 of HSP90 (**c**, left panel, boxed region). A structural analysis of this region indicated it to be in an exposed protein region (**c**, right panel). The identity of the purified 98 kDa protein was confirmed by the use of an anti-HSP90 antibody (**d**)

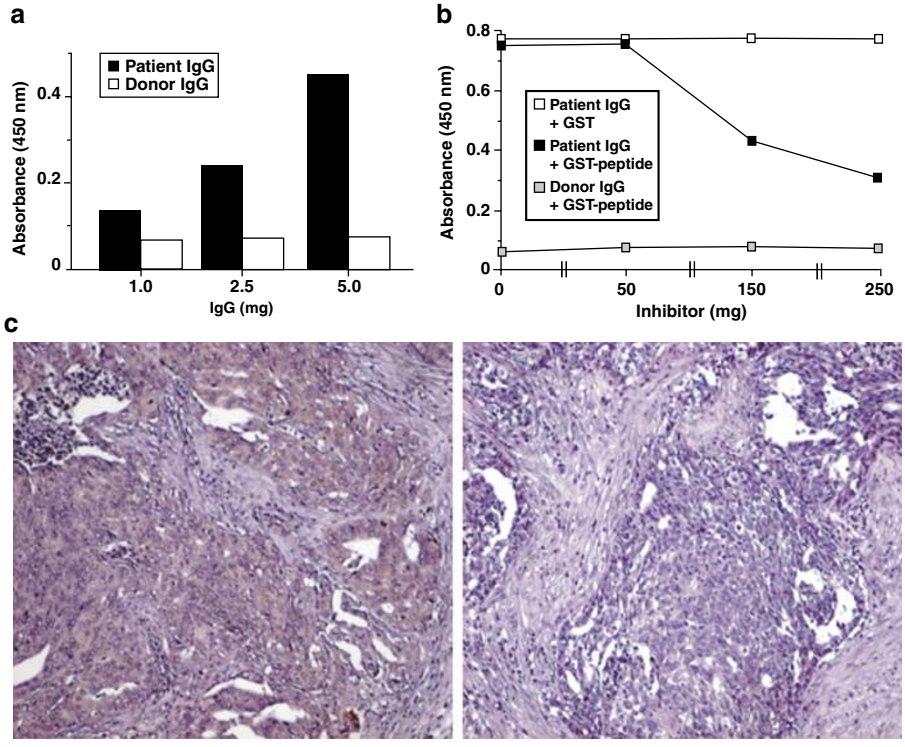



**Figure 5** HSP90 is the antigen mimicked by CVPELGHEC. Patient-derived antibodies, but not control donor antibodies, bind to recombinant HSP90 in a dose-dependent manner (**a**). The binding activity of the patient-derived IgG to recombinant HSP90 is inhibited with a recombinant GST–CVPELGHEC-fusion protein (**b**). Strong immunostaining was observed with affinity-purified patient IgG on tumor sections from the index ovarian cancer patient in whom the screening was performed (**c**, left panel), but no staining was seen with rabbit reagent grade IgG (**c**, right panel)

**Table 2** Tissue array characteristics

| | *High-grade ovarian carcinoma array* | *Ovarian progression array* |
|---|---|---|
| Total cases | $n = 309$ | $n = 65$ |
| Histotype | Serous carcinoma 162 (52.4%) | Benign |
| | Serous carcinoma mixed with another histotype 69 (22.3%) | Normal surface epithelium 5 (7.7%) |
| | Endometroid carcinoma 32 (10.4%) | Cystadenomas 10 (15.4%) |
| | Clear cell carcinoma 16 (5.2%) | Low malignant potential |
| | Mixed malignant müllerian tumor 14 (4.5%) | Mucinous carcinoma10 (15.4%) |
| | Undifferentiated carcinoma 8 (2.6%) | Serous carcinoma 5 (7.7%) |
| | Mucinous carcinoma 6 (1.9%) | Low grade |
| | Transitional cell carcinoma 2 (0.6%) | Serous carcinoma 10 (15.4%) |
| | | Endometroid carcinoma 5 (7.7%) |
| | | High grade |
| | | Serous carcinoma 10 (15.4%) |
| | | Endometroid carcinoma 5 (7.7%) |
| | | Clear cell carcinoma 5 (7.7%) |
| Tumor stage | Stage I 30 (9.7%) | |
| | Stage II 25 (8.1%) | |
| | Stage III 195 (63.1) | |
| | Stage IV 59 (19.1) | |

HSPs (Witkin, 2001). In fact, humoral immune responses to HSPs may arise from molecular mimicry, as HSP epitopes have been found in other proteins (Cohen, 1990). Aberrant expression and post-translational modification of HSPs have been invoked to explain the presence of autoantibodies. Abnormal regulation of the immune system in ovarian cancer may also lead to generation of autoantibodies (Taylor and Gercel-Taylor, 1998), and other reports suggest that anti-HSP90 antibodies have prognostic value in malignant tumors (Conroy *et al*., 1998; Trieb *et al*., 2000). Clearly, more work will be necessary

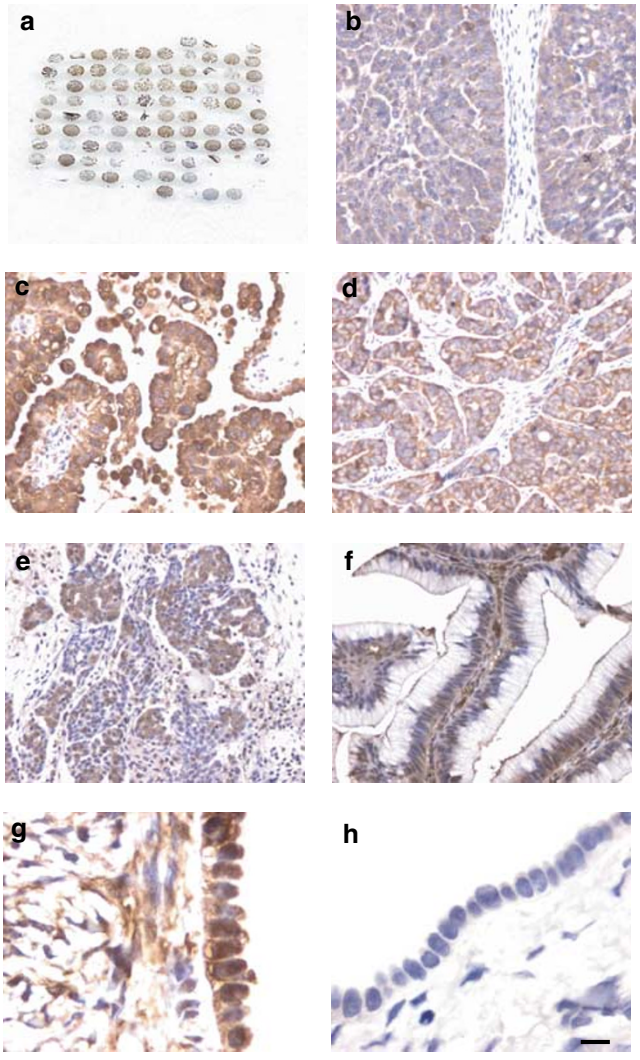



**Figure 6** HSP90 is expressed in normal ovarian surface epithelium and ovarian tumors. View of high-grade ovarian cancer array (**a**); undifferentiated ovarian carcinoma (**b**); papillary serous carcinoma (**c**); clear cell carcinoma (**d**); endometroid carcinoma (**e**); mucinous carcinoma (**f**); normal ovarian surface epithelium (**g**). No staining was observed with control rabbit IgG (**h**). Scale bar (**b–f**) 50 $\mu$m; (**g–h**) 10 $\mu$m

to clarify a functional role for HSP90-related immunity in cancer.

The mechanism(s) causing the marked differential immune reactivity between stages III and IV of the disease also remains unknown. As stage IV ovarian cancer is infrequent relative to other stages (Brinton and Hoover, 2000), there are little data to assess whether the biology of this stage is indeed different than that of earlier ones, particularly stage III. Currently, ovarian cancer patients with advanced disease (stages III and IV) are generally treated in the same manner. In our study, despite the small number of patients, there was a trend towards a better survival in the stage IV patients that reacted against CVPELGHEC (unpublished observations). Further studies are required to evaluate whether the reactivity against HSP90 or its mimic peptide has any prognostic value as a biomarker in ovarian cancer. If the work presented here is validated, possibilities include exploiting the humoral response against CVPELGHEC as a serological marker for earlier identification of stage IV patients or clinical correlation of the outcome with reactivity to this or other antibody-binding motifs.

In summary, antibody fingerprinting can be used to identify the peptide(s) associated with ovarian cancer. We show that ovarian cancer patients can develop a specific humoral immune response elicited against a selected HSP90-mimic peptide motif or against native HSP90. On a broader context, this work may lend support to HSP90 as a candidate therapeutic target or antigen for development of vaccines against ovarian cancer.

## Materials and methods

### *Patient samples*

The Institutional Review Board (IRB) at The University of Texas MD Anderson Cancer Center (UTMDACC) approved the use of all samples in this study. Ascites and tumor samples from ovarian cancer patients were obtained from the Specialized Program of Research Excellence (SPORE) Gynecologic Tumor Bank. The index patient screened in this study had stage IV, high-grade, papillary serous carcinoma of the ovary. Other patient ascites were obtained from cases of advanced ovarian cancer ($n=59$), including stage III ($n=42$) and stage IV ($n=17$); control ascites were obtained from nonovarian malignant tumors such as metastatic gastrointestinal cancer ($n=15$) and nonmalignant diseases such as cirrhosis ($n=4$). All fresh ascites were collected into sterile containers, centrifuged to separate the cell-free fraction, and stored in aliquots at $-20^{\circ}$C. Serum samples from healthy age-matched blood-donor women ($n=57$, Gulf Coast Regional Blood Center, Houston, TX, USA) and women with benign gynecological diseases ($n=30$, UTMDACC) also served as controls. All blood samples were allowed to clot at room temperature (RT), centrifuged, separated into aliquots, and frozen.

### *Screening of the phage display peptide library*

We adopted a previously reported fingerprinting procedure (Mintz *et al*., 2003). In brief, we pre-cleared the peptide library on a pool of immunoglobulins from age-matched blood-donor women to remove nonspecific peptides. Next, we incubated the pre-cleared library with antibodies purified from the ascites of the index ovarian cancer patient (described above). For the pre-clearing step, $10^9$ transducing units (TU) of the $CX_7C$ (C, cysteine; X, any residue) random peptide library were incubated with antibodies immobilized on protein G from 50 $\mu$l of serum for 1 h at 4°C. The pre-cleared phage library was then selected for 2 h at 4°C on affinity-purified antibodies from ascites of the index patient. Protein G beads (Gibco-BRL, Rockville, MA, USA) were used for immunoaffinity purification of antibodies (Mintz *et al*., 2003). Briefly, the immobilized protein G gel, binding buffer (acetate pH 5.0; Gibco-BRL), and elution buffer (glycine buffer, pH 2.8; Gibco-BRL) were allowed to warm to RT. The gel was equilibrated with binding buffer and ascites or serum, diluted 1 : 1 with binding buffer, was added. Samples were incubated for 30 min at RT and then

centrifuged (1000 *g* for 1 min). Samples were washed twice with 500 $\mu$l binding buffer per wash. Bound antibodies were eluted with elution buffer and immediately neutralized with 1 M Tris–HCl (pH 9.0). The resulting antibody-bound phage complexes obtained from binding to antibodies from ascites were recovered by elution of the phage with 0.1 M glycine buffer (pH 2.2), neutralized with 0.1 v 1 M Tris–HCl (pH 9.0), and infected with *Escherichia coli* strain K91. Serial dilutions of phage-infected K91 were plated onto Luria-Bertani (LB) agar plates containing tetracycline (40 $\mu$g/ml) and grown overnight at 37°C. Recovered phage were bulk amplified and precipitated for subsequent rounds of panning as described (Smith, 1985; Smith and Scott, 1993; Barbas *et al*., 2001). Three rounds of pre-clearing and selection were performed. Selected phage clones from the third round of panning were isolated and amplified. DNA sequence analyses were used to determine the sequences corresponding to displayed sequences.

*Individual phage clone-binding assays*

Binding of individual phage clones to affinity-purified antibodies from the index patient was studied by using a microtiter assay (Mintz *et al*., 2003). Antibodies from the index patient or control donors (purified as described above) were used to coat MaxiSorp 96-well plates (Nalge-Nunc, Naperville, IL, USA) at a concentration of 1 $\mu$g/well. Plates were coated at 4°C overnight. The plates were then blocked with phosphate-buffered saline (PBS) containing 3% BSA. For binding reactions, $10^9$ TU of each phage clone were individually added to the coated and blocked plates. After binding at RT for 2 h, the wells were washed four times. Finally, adding log-phase *E. coli* K91 and incubating at RT for 30 min rescued the bound phage. The mixture was then diluted in 10 ml of LB culture media supplemented with 0.2 $\mu$g/ml of tetracycline and incubated for another 30 min at RT. Serial dilutions of the culture were plated on LB plates containing 40 $\mu$g/ml of tetracycline. Plates were incubated at 37°C overnight before counting phage TU of either selected phage or negative control insertless phage, as described (Arap *et al*., 2002).

*Construction and purification of recombinant peptides*

Selected peptides were produced as a recombinant GST-fusion protein. Peptide coding sequences were PCR-amplified and modified to contain the *Bam*HI and *Eco*RI sites. The amplified sequence, containing the peptide coding sequence, was cloned into the *Bam*HI–*Eco*RI sites of the vector pGEX-2TK (Amersham Biosciences, Piscataway, NJ, USA) and checked by restriction digestions and PCR. Positive clones were electroporated into the bacterial expression host strain BL21 (DE3) pLys (Stratagene, La Jolla, CA, USA). Expression of the recombinant GST-fusion proteins was induced by 200 $\mu$M isopropylthiogalactoside (IPTG) and compared to the negative control (empty vector pGEX-2TK). Recombinant GST-fusion proteins expression was confirmed in the selected clones. Recombinant proteins were purified from bacterial lysates by affinity chromatography by using a glutathione Sepharose 4B resin (Amersham Biosciences) as described (Arap *et al*., 2002; Mintz *et al*., 2003).

*Enzyme-linked immunosorbent assays and competitive inhibition assays*

Binding of antibodies purified from ascites of the index patient (or from ascites of a panel of other unrelated ovarian cancer patients) to the selected peptide and its corresponding mimic antigen were studied by enzyme-linked immunosorbent assay (ELISA) and by competitive inhibition assays. Briefly, we used solutions of purified GST, GST peptide, or recombinant HSP90 (Stressgen, Victoria, Canada) to coat MaxiSorp 96-well plates (Nalge Nunc International Corporation) at a concentration of 500 ng/well. Plates were coated at 4°C overnight and blocked for at least 2 h at RT. Serial dilutions of the affinity-purified antibodies were added to the coated and blocked plates and binding was performed at RT for 2 h. After the binding reaction, the wells were washed four times with blocking solution containing 0.01% Tween 20 (v/v). Secondary antibodies conjugated to horseradish peroxidase were added at a concentration of 1 : 5000, incubated for 1 h at RT, and then washed. After washing, we measured the bound enzymatic activity by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB; Calbiochem, San Diego, CA, USA). The reaction was stopped after 15 min by the addition of 0.5 M $H_2SO_4$. Absorbance at 450 nm was determined in an automated ELISA plate reader (Bio-Tek, Winooski, VT, USA). For peptide inhibition assays, synthetic cyclic peptides were used; cyclic peptides with unrelated sequences were used as negative controls. Increasing concentrations of each synthetic peptide were pre-incubated with corresponding ascites-derived antibodies for 30 min and then on the immobilized recombinant GST-fusion-coated wells. Competitive inhibition assays for HSP90 were carried out in a similar manner using the recombinant GST-fusion protein to inhibit the interaction between affinity-purified antibodies in solution and immobilized HSP90.

*Immunohistochemistry*

Archived formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene, rehydrated in ethanol, and nonspecific sites were blocked with protein-blocking buffer (Dako, Carpinteria, CA, USA). Antibodies from the ascites of ovarian cancer patients were affinity-purified, coupled to biotin (Vector Laboratories, Burlingame, CA, USA), and analysed by SDS-gel electrophoresis (Mintz *et al*., 2003). A biotinylated antibody was applied to the tissue sections, followed by a rinsing step and addition of streptavidin conjugated to horseradish peroxidase. Positive staining cells were visualized by the addition of diaminobenzidine (DAB) and viewing the sections with phase-contrast microscopy by using an inverted optical microscope (Olympus IX70). All tissue sections were counterstained with hematoxylin. For immunohistochemistry inhibition, the affinity-purified antibodies were pre-incubated with either the corresponding recombinant GST-fusion protein or GST alone for 30 min at RT (1 mg each). A polyclonal antibody against HSP90 (Lab Vision Corporation, Fremont, CA, USA) was used at a dilution of 1 : 300 for sections stained for the identified antigen. After the antigen retrieval process in a microwave oven, a streptavidin peroxidase procedure was used to immunostain HSP90.

*Protein purification and identification*

The HEY human ovarian cancer cell line (Buick *et al*., 1985) was used as a source for protein purification. Cells were grown to subconfluence, harvested in PBS, treated with TM buffer (100 mM Tris–HCl, 2 mM $MgCl_2$) containing 1% Triton X-100 (v/v), sonicated, and centrifuged to separate the cytoplasmic/membrane fraction from the nuclei. The supernatant was collected, resolved on a 4–20% gradient SDS–PAGE, and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was probed with anti-peptide antibodies affinity-purified from the ascites of the index patient and detected by enhanced chemiluminescence (ECL; Amersham Biosciences). The band containing the protein recognized

by the antipeptide antibodies was excised and sent for protein identification. Identification was carried out by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry. Molecular masses of peptide fragments were generated by tryptic digestion and compared to NCBI protein databases.

*Tissue arrays*

Both high-grade ovarian cancer tissue arrays and ovarian cancer progression tissue arrays were used in this study. In each type of array, searching the large SPORE Ovarian Tumor Database at UTMDACC for patients with epithelial ovarian carcinoma identified subjects for whom pathologic material (formalin-fixed, paraffin-embedded tissue blocks) was available. An expert reference gynecologic oncology pathologist (JL) individually reviewed all hematoxylin and eosin (H&E)-stained sections to select representative areas of each tumor from which to acquire suitable cores for microarray analysis. Tissue microarray blocks were constructed by using a precision instrument (Beecher Instruments; Silver Spring, MD, USA). Core samples were taken from morphologically representative areas of paraffin-embedded tumor tissues and by assembling them on a recipient paraffin block. For each case, two replicate cores were collected and each was placed on a separate recipient block. A summary of array contents is presented in Table 2.

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