Identification of heat shock protein 90 and other proteins as tumour antigens by serological screening of an ovarian carcinoma expression library

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Serological screening of recombinant cDNA expression libraries has been widely used for the identification of tumour antigens in various cancer types. Identification of tumour antigens in ovarian cancer may facilitate the development of vaccine-based therapies and of disease biomarkers. The purpose of our investigation is to identify tumour antigens in ovarian cancer by using the serological analysis of recombinant cDNA expression libraries method. A recombinant ovarian carcinoma cDNA expression library was screened with ascites fluid, pooled from five ovarian cancer patients. Twelve tumour antigens encoded by known genes were isolated, including ribosomal protein S18, heat shock protein 90, J-K-recombination signal binding protein, ribonucleoprotein H1, RAN binding protein 7, TG-interacting factor, eukaryotic translation initiation factor p40 subunit, human amyloid precursor protein-binding protein 1, ribosomal protein L8, CDC23, IQ motif containing GTPase activating protein 1, and ribosomal protein L3. Heat shock protein 90 was chosen for further investigation. The prevalence of hsp90 autoantibodies in ovarian cancer was determined with immunoassay. Sera from 22 normal females, 32 from ovarian cancer (22 stage III/IV, 10 stage I/II), 37 colorectal cancer, 13 breast cancer, 10 lung cancer, 20 benign gynaecologic diseases, and 10 benign breast lesions were screened. Seven (32%) stage III/IV ovarian cancer, 1 (10%) stage I/II ovarian cancer, 1 (3%) colorectal cancer, 1 (8%) breast cancer, and 1 (5%) benign gynaecologic disease sera were found to contain hsp90 autoantibodies. These data support the view that hsp90 autoantibodies are frequently found in late stage ovarian cancer. Hsp90 may, therefore, represent a novel biomarker for ovarian cancer and a candidate ovarian cancer vaccine target.

Keywords: tumour antigens; ovarian cancer; serological analysis of recombinant cDNA expression libraries (SEREX); heat shock protein 90 (hsp90); autoantibodies; cancer autoimmunity

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MATERIALS AND METHODS

Immunoscreening of an ovarian carcinoma cDNA expression library with ascites fluid from ovarian cancer patients

The Uni-ZAP™ premade ovarian carcinoma cDNA expression library was purchased from Stratagene, La Jolla, CA, USA. Ovarian cancer ascites fluids were pooled from five different primary ovarian cancer patients. The cDNA library was plated on NZY agar plates at a density of 500 clones/15 cm plate. The plates were incubated at 42°C for 4 h to allow plaques to develop. Nitrocellulose filters soaked with isopropyl β-D-thiogalactopyranoside (IPTG) were then laid on top of the plaques and incubated at 37°C for 4 h to transfer the plaques onto the membranes. Subsequently, the filters were blocked with 5% non-fat dry milk/PBS-T (80 mM sodium orthophosphate, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, and 0.1% Tween-20) overnight, at 4°C. To screen the library, the ascites were diluted 1:100 in 5% non-fat dry milk/PBS-T. The diluted ascites were first incubated with E. coli phage lysate (from Stratagene) for 2 h at room temperature to minimize the cross-reaction between the autoantibodies and the bacterial/phage proteins. Nitrocellulose filters were then incubated with this preabsorbed ascites for 2 h at room temperature to identify cellular proteins that react with the autoantibodies in the ascites. Following probing with the ascites, the filters were washed with PBS-T for three times and further treated with alkaline phosphatase-conjugated goat anti-human IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) diluted 2000-fold in 5% non-fat dry milk/PBS-T for 1 h at room temperature. The filters were washed again as above and then proceeded to chemiluminescence detection with a dioxetane-based substrate (Diagnostic Products Corporation, Los Angeles, CA, USA). The plaques exhibiting immunoreactivities were excised from the plates and the phages were converted into plBluescript phagemids by in vivo excision with Exassist™ helper phage, following the manufacturer’s instructions. The excised phagemids were purified and subjected to automated DNA sequencing with M13 forward and reverse primers. The insert sequences were compared to the known sequences in the Genbank database with the BLASTN alignment algorithm (Altschul et al, 1997).

Identification of autoantibodies against heat shock protein 90 in various cancer sera with immunoassay

Pure heat shock protein 90, isolated from HeLa cells, was purchased from Stressgen, Victoria, BC, Canada. For the immunoassay, 100 μl/250 ng well⁻¹ of hsp90 diluted in the coating buffer (0.1 M ethanalamine in phosphate buffer saline (PBS), pH 7.2) were first directly coated on the 96-well white polystyrene plate (Greiner Labtechnik, Germany). 100 μl well⁻¹ of 6% bovine serum albumin (BSA) was also coated as a background control. After overnight incubation at room temperature, the plate was washed three times with washing buffer (containing 9 g l⁻¹ NaCl and 0.5 g l⁻¹ Tween 20 in 10 mmol l⁻¹ Tris buffer, pH 7.40), then blocked with 200 μl well⁻¹ coating buffer for 1 h. Subsequently, the plate was washed again as above. 100 μl of human sera, diluted 50-fold in assay buffer (containing 60 g l⁻¹ BSA, 50 mmol l⁻¹ Tris, pH 7.80, 2.5% normal mouse serum, 15% normal goat serum, 1% bovine IgG, and 0.5 g l⁻¹ sodium azide) were then pipetted into both BSA and hsp90-coated wells, incubated for 1 h and washed six times. Finally, 100 μl well⁻¹ of alkaline phosphatase-conjugated goat anti-human IgG, Fc fragment specific antibody (Jackson Immunoresearch, West Grove, PA, USA) diluted 5000-fold in assay buffer was added, incubated for 1 h and washed. The signal was detected with a time-resolved fluorometric procedure as follows: 100 μl of difluorosulphate phosphate substrate, diluted 10-fold in substrate buffer (0.1 M Tris, pH 9.1, 0.1 M NaCl and 1 mM MgCl₂) were pipetted into each well and incubated for 10 min. 100 μl of developing solution (1 M Tris base, 0.4 M NaOH, 2 mM TβCl₃ and 3 mM EDTA) were then added on top. The fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 Immunolinker (MDS Nordion, Kanata, ON, Canada). The details of this procedure have been published elsewhere (Christopoulos and Diamandis, 1992). All serum samples were analysed in duplicate.

Patient sera

The samples used in this study are residual sera of routine testing from healthy blood donors and patients with various malignancies and benign diseases. We included sera from 22 normal females and the following patients: 32 ovarian cancer (22 stage III/IV, 10 stage I/II), 13 breast cancer, 10 prostate cancer, 10 lung cancer, 20 benign gynecologic diseases, and 10 benign breast lesions. All sera were stored at −80°C until analysis.

RESULTS

Isolation of tumour antigens by immunoscreening of an ovarian carcinoma cDNA expression library with ascites fluid from ovarian cancer patients

A recombinant ovarian carcinoma cDNA library was screened with ascites pooled from five ovarian cancer patients. In total, 50,000 clones were screened. Twelve clones were found to have immunoreactivities. An example of a positive clone is shown in Figure 1. In order to confirm the identities of these positive clones, they were isolated and converted to phagemids. The inserts were then sequenced and compared to the GenBank database with the BLASTN program. All 12 positive clones are known proteins (Table 1). Most of these proteins are involved in gene transcription, protein translation, and mitosis. Heat shock protein 90 was selected for further evaluation because this protein is commercially available in pure form, allowing development of an ELISA-type screening assay.

Determination of the prevalence of heat shock protein 90 autoantibodies in ovarian cancer

SEREX technology identifies tumour antigens by screening with sera which are usually obtained from a small number of patients. The immunogenicities of the identified tumour antigens should therefore be tested in a larger patient population, to determine their prevalence in various types of cancer. Since the proteins expressed in the prokaryotic recombinant cDNA expression libraries may have different conformation or post-translational modifications compared to their native human forms, the autoantibodies could recognize the recombinant proteins, but not the native forms. Hsp90 was chosen for further evaluation because it was the only commercially available antigen from the list of Table 1. Purified
human hsp90 directly coated on the microtiter plate was used to examine the presence of autoantibodies in the sera from normal controls, various cancers, and benign diseases. In total, sera from 22 normal female, 32 ovarian cancer (22 stage III/IV, 10 stage I/II), 37 colorectal cancer, 13 breast cancer, 10 lung cancer, 20 benign gynecological diseases, and 10 benign breast lesions were screened. For the 22 normal sera, fluorescence count ratios between hsp90-coated wells vs BSA-coated wells had a mean of 1.42 and a standard deviation of 0.28. Thus, the upper limit of the fluorescence ratio was selected as 2.00, which represents the mean plus two standard deviations (95% percentile). Sera with ratios $>2.00$ were defined as hsp90 autoantibody-positive. With this method, 7 (32%) stage III/IV ovarian cancer, 1 (10%) stage I/II ovarian cancer, 1 (3%) colorectal cancer, 1 (8%) breast cancer, and 1 (5%) benign gynecological disease sera were found to contain hsp90 autoantibodies (Table 2). Antibody titers for all sera tested are shown in Figure 2. At the level of 100% specificity (fluorescence ratio $>2.3$), there are still six sera from late stage ovarian cancer, one serum from early ovarian cancer, one serum from colorectal cancer, and one serum from a patient with breast cancer are positive for hsp90 autoantibodies. Our results indicate that hsp90 autoantibodies are mostly present in late stage ovarian cancer.

**DISCUSSION**

In our immunoscreening of the ovarian carcinoma cDNA expression library, twelve known proteins were identified as putative tumour antigens (Table 1). To the best of our knowledge, these proteins have not been previously reported as ovarian tumour antigens. However, some of these proteins or their homologues have already been recognized as tumour antigens by SEREX in other types of cancer, such as the JK recombination signal binding protein, eIF2B (a homologue of eIF3) (Gure et al., 2000), and hsp70 (a homologue of hsp90) (Scanlan et al., 1998). One feature of many proteins that we identified is their involvement in gene transcription, protein translation, and mitosis. Among the 12 proteins of Table 1, 10 belong to these categories. This pattern is consistent with the SEREX-defined tumour antigens reported in the literature (Chen, 2000). Although it seems that the immune responses to these housekeeping proteins may be nonspecific and likely related to the high turnover and necrosis associated with cancer, new experimental evidence indicates that these immunogenicities actually result from specific structural changes or aberrant expression of these proteins. For example, CDC27, which...

### Table 1

| Clone name | Identity | Genbank Accession Number | Functional significance |
|------------|----------|--------------------------|------------------------|
| 841        | Ribosomal protein S18 | X69150 | Protein translation |
| 816–3      | Heat shock protein 90 kDa | M16660 | Molecular chaperone |
| 816–4      | JK-recombination signal binding protein | L07872 | Transcription factor |
| 816–5      | Ribonucleoprotein H1 | L22009 | mRNA processing |
| 816–6      | RAN binding protein 7 | AF098799 | Mediate nuclear import of ribosomal proteins |
| 816–7      | TG-interacting factor | X89750 | Transcription factor |
| 816–8      | Eukaryotic translation initiation factor 3 (eIF3), p40 subunit | U54559 | Protein translation |
| 816–9      | Human amyloid precursor protein-binding protein 1 | U50939 | Cleave β amyloid protein precursor |
| 1210–1     | Ribosomal protein L8 | NM000973 | Protein translation |
| 1210–2     | CDC23 (Cell division 23) | NM004661 | Cell division |
| 1210–3     | IQ motif containing GTPase activating protein 1 (IQGAP1) | NM003870 | Cell–cell adhesion |
| 1210–4     | Ribosomal protein L3 | NM000967 | Protein translation |

### Table 2

| Patient group | Number of patients screened | Number of positive patients | Positivity rate (%) |
|---------------|-----------------------------|-----------------------------|---------------------|
| Normal female | 22                          | 0                           | 0                   |
| Ovarian cancer|                             |                             |                     |
| Stage III/IV | 22                          | 7                           | 32                  |
| Stage I/II   | 10                          | 1                           | 10                  |
| Lung cancer  | 10                          | 0                           | 0                   |
| Prostate cancer | 20                        | 0                           | 0                   |
| Colorectal cancer | 37                      | 1                           | 3                   |
| Breast cancer | 13                          | 1                           | 8                   |
| Benign gynaecologic diseases | 20                   | 1                           | 5                   |
| Benign breast lesion | 10                | 0                           | 0                   |
participates in mitosis, is an MHC class II-restricted tumour antigen in melanoma. It has been shown that a mutation within this gene causes protein structure change, and hence, induction of immune responses (Wang et al., 1999). In addition, eukaryotic translation initiation factor 4 (eIF4), which is involved in protein translation, is a SEREX-defined antigen in lung cancer. It has been shown that its immunogenicity is due to overexpression (Brass et al., 1997). We speculate that the proteins identified here generate autoimmune reactions through similar mechanisms. It is noteworthy that eIF3 and CDC23, which are the homologues of eIF4 and CDC27, respectively, were identified in our screening. Some of the proteins that we have identified here are likely to be overexpressed in various types of cancer, including ribosomal protein S18 (Chassin et al., 1994), RAN binding protein 7 (Li et al., 2000), and eIF3, p40 subunit (Nupponen et al., 1999). Therefore, given the aberrant expression of these proteins in various malignancies, it is not surprising that they elicit immune responses.

To examine the prevalence of the hsp90 autoantibodies in ovarian cancer, purified human hsp90 was used in an ELISA-type screening assay. In our initial SEREX library screening, we utilized ascites fluid pooled from five ovarian cancer patients with late stage disease. Hsp90 autoantibodies have the highest frequency (32%) in late stage ovarian cancer, which is consistent with the presence of hsp90 autoantibodies in the initial ascites pool. Hsp90 autoantibodies were also observed in colorectal cancer (3%) and breast cancer (8%). It appears that hsp90 is a tumour antigen shared by various types of cancer. The correlation of hsp90 autoantibodies and late stage ovarian cancer implies that it may have utilities as a novel prognostic biomarker for ovarian cancer. In osteosarcoma, hsp90 autoantibody positivity has been found to be associated with response to chemotherapy, whereas, its absence correlates with metastasis (Trieb et al., 2000). In contrast, Conroy et al. (1998) reported that the presence of hsp90 autoantibodies indicated metastasis in breast cancer. What hsp90 autoantibodies could predict in ovarian cancer awaits further detail investigations to analyse the relationship of hsp90 autoantibodies and various clinicopathological variables.

Hsp90 has been investigated as a cancer vaccine target in animal models. It has been shown that hsp90 purified from autologous tumours has a protective effect in mouse (Granter et al., 2000; Ullrich et al., 1986). It is now believed that such a protective effect of hsp90 is mainly due to the tumour-derived antigenic peptides that bind to it (Ishii et al., 1999; Srivastava et al., 1998; Wells and Malkovsky, 2000). In our study, since the prokaryotic expression library and purified hsp90 from HeLa cells are unlikely to harbour such antigenic peptides, it seems that the immunoreactivities that we observed are hsp90 specific. Our results indicate that hsp90 alone could be used as a cancer vaccine target.

In summary, we show here that many candidate tumour antigens could be identified in SEREX screening of ovarian cancer. Our findings warrant further investigations on these proteins, aiming to elucidate their immunogenicity in ovarian cancer. Hsp90 is immunogenic in 32% of late-stage ovarian cancer, suggesting that it is a potential target for a therapeutic vaccine in ovarian cancer.

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REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402

Angelopoulou K, Diamandis EP (1997) Detection of the TP53 tumour suppressor gene product and p53 auto-antibodies in the ascites of women with ovarian cancer. Eur J Cancer 33: 115–121

Angelopoulou K, Diamandis EP, Sutherland D, Kellen JA, Bunting PS (1994) Prevalence of serum antibodies against the p53 tumour suppressor gene protein in various cancers. Int J Cancer 58: 480–487

Angelopoulou K, Rosen B, Stratis M, Yu H, Solomon M, Diamandis EP (1996) Circulating antibodies against p53 protein in patients with ovarian carcinoma. Correlation with clinicopathologic features and survival. Cancer 78: 2146–2152

Angelopoulou K, Stratis M, Diamandis EP (1997) Humoral immune response against p53 protein in patients with colorectal carcinoma. Int J Cancer 70: 46–51

Boon T, van der Bruggen P (1996) Human tumor antigens recognized by T lymphocytes. J Exp Med 183: 725–729

Brass N, Heckel D, Sahin U, Pfreundschuh M, Sybrecht GW, Meese E (1997) Translation initiation factor eIF-4gamma is encoded by an amplified gene and induces an immune response in squamous cell lung carcinoma. Hum Mol Genet 6: 33–39

Chinin C, Diamandis EP, Sutherland D, Kellen JA, Bunting PS (1994) Identification of genes overexpressed in tumors through preferential expression screening in trophoblasts. Cancer Res 54: 5217–5223

Chen YT (2000) Cancer vaccine: identification of human tumor antigens by SEREX. Cancer J Sci Am 6: S208–S217

Christopoulos TK, Diamandis EP (1992) Enzymatically amplified time-resolved fluorescence immunoassay with terbium chelates. Anal Chem 64: 342–346

Conroy SE, Sasiendi PD, Fentiman I, Latchman DS (1998) Autoantibodies to the 90 kDa heat shock protein and poor survival in breast cancer patients. Eur J Cancer 34: 942–943

Duffy MJ (1999) CA 15-3 and related mucins as circulating markers in breast cancer. Ann Clin Biochem 36: 579–586

Frasca L, Piazza C, Piccolella E (1998) CD4+ T cells orchestrate both amplification and deletion of CD8+ T cells. Crit Rev Immunol 18: 569–594

Greiner M, Raymond A, Romney D, He L, Whitesell L, Katsanis E (2000) Immunoprotective activities of multiple chaperone proteins isolated from murine B-cell leukemia/lymphoma. Clin Cancer Res 6: 909–915

Butts CS (1999) Vaccines for ovarian carcinoma. Cancer Control 6: 335–342

Chassin D, Benilla JL, Delattre C, Fernandez H, Ginisty D, Janneau JL, Prade M, Contesso G, Caillou B, Tournaire M et al. (1994) Identification of genes overexpressed in tumors through preferential expression screening in trophoblasts. Cancer Res 54: 5217–5223

Chen YT (2000) Cancer vaccine: identification of human tumor antigens by SEREX. Cancer J Sci Am 6: Suppl 3: S208–S217

Conroy SE, Sasiendi PD, Fentiman I, Latchman DS (1998) Autoantibodies to the 90 kDa heat shock protein and poor survival in breast cancer patients. Eur J Cancer 34: 942–943

Duffy MJ (1999) CA 15-3 and related mucins as circulating markers in breast cancer. Ann Clin Biochem 36: 579–586

Frasca L, Piazza C, Piccolella E (1998) CD4+ T cells orchestrate both amplification and deletion of CD8+ T cells. Crit Rev Immunol 18: 569–594

Greiner M, Raymond A, Romney D, He L, Whitesell L, Katsanis E (2000) Immunoprotective activities of multiple chaperone proteins isolated from murine B-cell leukemia/lymphoma. Clin Cancer Res 6: 909–915
Gure AO, Stockert E, Scanlan MJ, Keresztes RS, Jager D, Altorki NK, Old LJ, Chen YT (2000) Serological identification of embryonic neural proteins as highly immunogenic tumor antigens in small cell lung cancer. Proc Natl Acad Sci USA 97: 4198 – 4203

Ishii T, Udono H, Yamano T, Ohta H, Uenaka A, Ono T, Hizuta A, Tanaka N, Srivastava PK, Nakayama E (1999) Isolation of MHC class I-restricted tumor antigen peptide and its precursors associated with heat shock proteins hsp70, hsp90, and gp96. J Immunol 162: 1303 – 1309

Li SR, Gyselman VG, Dorudi S, Bustin SA (2000) Elevated levels of RanBP7 mRNA in colorectal carcinoma are associated with increased proliferation and are similar to the transcription pattern of the proto-oncogene c-myc. Biochem Biophys Res Commun 271: 537 – 543

Luo LY, Soosaipillai A, Dimandis EP (2001) Molecular cloning of a novel human gene on chromosome 4p11 by immunoscreening of an ovarian carcinoma cDNA library. Biochem Biophys Res Commun 280: 401 – 406

Molina R, Jo J, Filella X, Zanon G, Pahisa J, Munoz M, Farrus B, Latre ML, Escriche C, Estape J, Ballesta AM (1998) c-erbB-2 oncoprotein, CEA, and CA 15.3 in patients with breast cancer: prognostic value. Breast Cancer Res Treat 51: 109 – 119

Nupponen NN, Porkka K, Kakkola L, Tanner M, Persson K, Borg A, Isola J, Visakorpi T (1999) Amplification and overexpression of p40 subunit of eukaryotic translation initiation factor 3 in breast and prostate cancer. Am J Pathol 154: 1777 – 1783

Pardoll DM, Topalian SL (1998) The role of CD4+ T cell responses in anti-tumor immunity. Curr Opin Immunol 10: 588 – 594

Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmitz R, Stenner F, Luo G, Schobert I, Pfreundschuh M (1995) Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci USA 92: 11810 – 11813

Scanlan MJ, Chen YT, Williamson B, Gure A, Stockert E, Gordan JD, Tureci O, Sahin U, Pfreundschuh M, Old LJ (1998) Characterization of human colon cancer antigens recognized by autologous antibodies. Int J Cancer 76: 652 – 658

Srivastava PK, Menoret A, Basu S, Binder RJ, McQuade KL (1998) Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. Immunity 8: 657 – 665

Trieb K, Gerth R, Holzer G, Grohs JG, Berger P, Kotz R (2000) Antibodies to heat shock protein 90 in osteosarcoma patients correlate with response to neoadjuvant chemotherapy. Br J Cancer 82: 85 – 87

Ullrich SJ, Robinson EA, Law IW, Willingham M, Appella E (1986) A mouse tumor-specific transplantation antigen is a heat shock-related protein. Proc Natl Acad Sci USA 83: 3121 – 3125

Wang RF, Wang X, Atwood AC, Topalian SL, Rosenberg SA (1999) Cloning genes encoding MHC class II-restricted antigens: mutated CDC27 as a tumor antigen. Science 284: 1351 – 1354

Wells AD, Malkovsky M (2000) Heat shock proteins, tumor immunogenicity and antigen presentation: an integrated view. Immunol Today 21: 129 – 132