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Full Length Research Paper

Evidence of a positive correlation between accelerated oestrogen receptor phosphorylation and breast cancer progression

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Estrogen receptor beta (ERβ) is predicted to play an important role in prevention of breast cancer development and metastasis. Phosphorylation of Estrogen receptor alpha (ERα) has been proven to be involved in the progression of breast cancer and it is believed oestrogen receptor beta too phosphorylated at multiple sites within the protein upon ligand binding although the exact function of this site-specific phosphorylation is unknown. Nevertheless it is assumed that the site-specific phosphorylation of ERβ may be involved in the progression of human breast cancer. To test this hypothesis we developed novel monoclonal antibodies using synthetic peptide specific for putative serine phosphorylation site in human ERß (S87). These antibodies tested on human cancerous breast tissue samples provided clear evidence of phosphorylation of ERβ at S87 progressively as cancer advanced. These antibodies could be used in targeting the phosphorylation site which could help in treatment strategies and control of cancer progression.

Key words: Oestrogen receptor beta, breast cancer, monoclonal antibodies, phospho specific anti-peptide antibodies.

INTRODUCTION

Oestrogen conducts its mechanism of action through one of the oestrogen receptors oestrogen receptor alpha (ERα) or oestrogen receptor beta (ERβ). The two subtypes of oestrogen receptors belong to the superfamily of nuclear receptors and they are predominantly found in the nucleus with a fraction of receptors found as cell membrane bound determined by post-translational modification (Jiang and Hart, 1997). Each subtype consists of six different functional domains (A-F) (Figure 1) and the genes coding for both ERα and ERβ are located in different chromosomes and have 8 exons (Palmieri et al., 2002). This interaction between oestrogen and receptors depend not only on the availability of oestrogen but also on the types of ERs and their levels in these cells. ER levels are low in normal breast tissue and high levels have been directly correlated with an increased risk of breast cancer (Khan et al., 1994). Studies show that phosphorylation of ERα can be important in regulating gene expression and it has been proposed that this could contribute to cancer progression (Joel et al., 1998; Sun et al., 2001; Lannigan, 2003; Shah and Rowan, 2005). Currently several investigations are being conducted in the phosphorylation of oestrogen receptor beta (ERβ) and it is relevance in pathological conditions including cancers.

Post-translational modifications of proteins are of a common feature of any protein and are vital to their activities. ERα protein is known to be phosphorylated in multiple sites but little is known about the phophorylation of ERβ (Lannigan, 2003; Tremblay et al., 1999). However the precise function of phosphorylation of ERα is controversial; nevertheless, it has been reported that both ligand-dependent and ligand-independent regulation of ERα phosphorylation may have roles in physiological and pathophysiological activities (Ali and Coombes, 2002). More specifically, the cross-talk between ERα and growth factor pathways is mediated through phosphorylated

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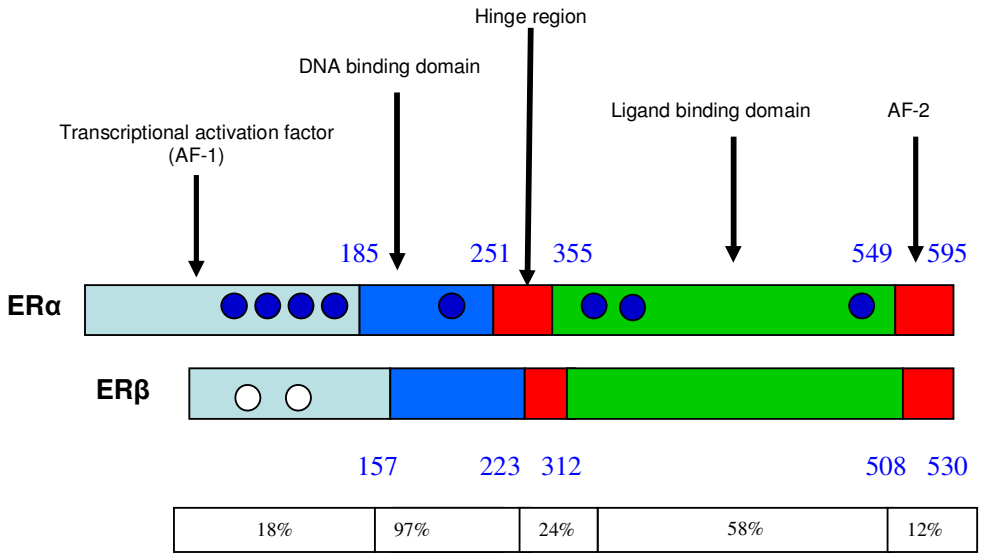

Figure 1. Genetic structure of oestrogen receptors. Structure of oestrogen receptor alpha (595 aa) and beta (530 aa) genes. Homology of domains are presented in percentage (adopted from Gennari et al. (2004).
● Putative phosphorylation sites in oestrogen receptor alpha (S104, S106, S118, S167, S236, S305, T311 and Y537),
○ Putative phosphorylation sites in oestrogen receptor beta (S87 and S105),
* S- serine; T- theronine; Y-tyrosine; (Lannigan, 2003; Chen et al., 2002; Kato et al., 1995).

ERα. These altered activities are closely associated with progression of human breast cancer and the development of resistance to endocrine therapies (Murphy et al., 2006).

Several phosphorylation sites in human ERα gene have been identified (Figure 1). However, Serine^{118} in the N-terminus of ERα phosphorylation has been studied widely (Lannigan, 2003; Chen et al., 2002; Kato et al., 1995) and it reveals that both oestrogen and growth factors such as EGF and IGF-1 can result in phosphorylation of Serine^{118} on ERα (Joel et al., 1998; Lannigan, 2003; Chen et al., 2002). Nevertheless, oestrogen increases phosphorylation by two to three folds more than with EGF (Joel et al., 1998). Several other studies also indicate that kinases are involved in the direct phosphorylation of Serine^{118} on ERα (Kato et al., 1995; Bunone et al., 1996; Park et al., 2005). The persistent activation of kinases (for example Erk1/2) induces phosphorylation and is believed to be part of the mechanism by which ERα positive human breast cancer cells develop oestrogen-independent growth in culture (Martin et al., 2003; Jeng et al., 2000). Further, over-expression of growth factor receptors such as HER2 and/or EGF-receptor, in breast cancer is often associated with resistance to anti-oestrogen (tamoxifen) treatment (Dowsett et al., 2005; Osborne et al., 2005).

These findings indicate that resistance to tamoxifen is due to persistent ligand-independent activation of ERα via phosphorylation of Serine^{118} due to uncontrolled activation of kinases (Murphy et al., 2006). Until recently these hypotheses have not been proven due to lack of research tools and the development of phosphor specific monoclonal antibodies to ERα now allows exploration of the potential relevance of phosphorylation of Serine^{118} in ERα in human breast cancer cells.

Similarly, ERβ play an important role in the mechanism of oestrogen activity and in the development and progression of human breast cancer. Although the precise role of ERβ in breast cancer is unknown, it is believed that phosphorylation of serine molecules in the N-terminus (Serine^{87} and Serine^{105}) is involved in the progression of breast cancer (Palmieri et al., 2002; Lannigan, 2003) (Figure 2). Targeting ERβ phosphorylation pathways would be an exciting new approach in the direction of advanced endocrine therapy and ERβ could be used as a better biomarker of prognosis and treatment response in breast cancer. The development of highly specific monoclonal antibodies to putative phosphorylation sites of oestrogen receptor beta would be of highly important for the advancement of the understanding of the role of oestrogen receptor beta in the progression of breast cancer.

MATERIALS AND METHODS

Selection of antigens

An immunogenic peptide was selected from the ER beta protein sequence (putative serine phophorylation site at position 87) to be used as immunogens. Phosphor peptide (includes phosphorylated

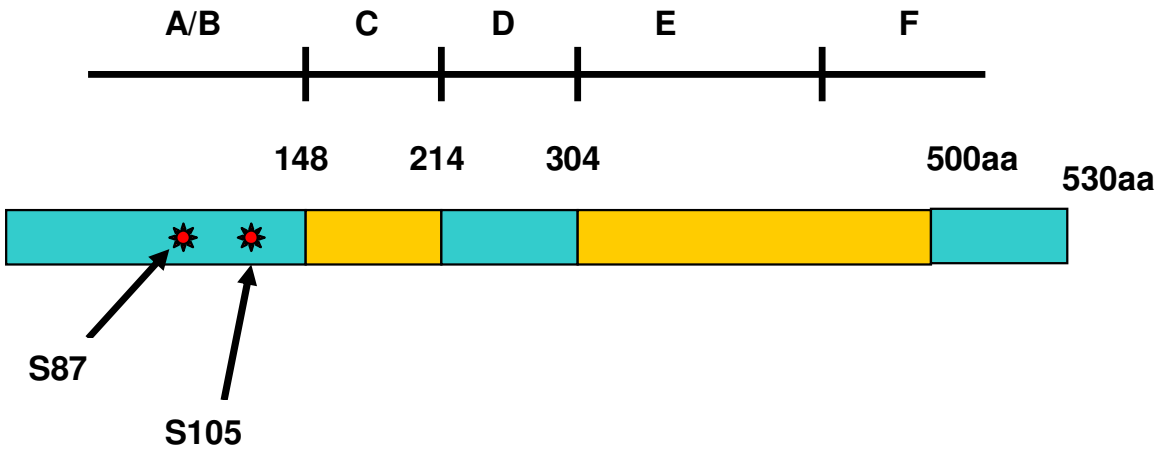

Figure 2. Predicted phosphorylation sites of oestrogen receptor beta Putative phosphorylation sites of oestrogen receptor beta (ERβ) *S87 and S105 (Serine position 87 and 105) (Lannigan, 2003; Chen et al., 2002; Kato et al., 1995).

Serine molecule) was commercially synthesised (Biomol International UK) with an addition of cystien molecule at the C-terminal end.

Peptide -GHL- Ser (PO_3H_2)-PLVVHRQLC

Beneath is the amino acid sequence of human oestrogen receptor beta protein. The position of the peptide chosen as immunogen is shaded.

MDIKNSPSSL NSPSSYNCSQ SILPLEHGSI YIPSSYVDSH HEYPAMTFYS PAVMNYSIPS
61 NVTNLEGGPG RQTTSPNVLW PTP GHLSPLV VHRQLSHLYA EPQKSPWCEA RSLEHTLPVN
121RETLKRKVSG NRCASPVTGP GSKRDAHFCA VCSDYASGYH YGVWSCEGCK AFFKRSIQGH
181NDYICPATNQ CTIDKNRRKS CQACRLRKCY EVGMVKCGSR RERCGYRLVR RQRSADEQLH
241 CAGKAKRSGG HAPRVRELLL DALSPEQLVL TLLEAEPPHV LISRPSAPFT EASMMMSLTK
301 LADKELVHMI SWAKKIPGFV ELSLFDQVRL LESCWMEVLM MGLMWRSIDH PGKLIFAPDL
361 VLDRDEGKCV EGILEIFDML LATTSRFREL KLQHKEYLCV KAMILLNSSM YPLVTATQDA
421 DSSRKLAHLL NAVTDALVWV IAKSGISSQQ QSMRLANLLM LLSHVRHASN KGMEHLLNMK
481 CKNVVPVYDL LLEMLNAHVL RGCKSSITGS ECSPAEDSKS KEGSQNPQSQ

Peptide coupling

Mal-sac-HNSA (10 mg) (N-Maleimido-6-aminocaproyl ester of 1-hydroxy-2-nitro-4-benzenesulfonic acid) was dissolved in purified protein derivative (PPD) (10 mg of tuberculin dissolved in 0.1M phosphate buffer pH 7.4) and was stirred for 30 min on a shaker at room temperature. This mixture was then loaded onto a Sephadex G25 column, which was equilibrated with 0.1M phosphate buffer (pH 6.0). Activated tuberculin was collected in a polypropylene tube. The peptide (10 mg) was dissolved in 2 ml of 0.01 M hydrochloric acid (HCl) and then 0.5 ml of 0.5 M phosphate buffer (pH 7.4) was added to this peptide solution and mixed well with the activated tuberculin. This coupled tuberculin was agitated overnight on a shaking platform before making up the volume to 10 ml in physiological saline.

Immunization

Female mice (T/O or Balb/C strain mice) aged 8 to 12 weeks were primed with BCG (Bacillus of Calmette and Guérin) (human BCG vaccine/diluent-0.2 - 0.3 ml/mouse). Three weeks into priming, these mice were then immunised subcutaneously with the peptide immunogen emulsified with an equal amount of Freund's incomplete adjuvant (FICA,GibciBRL, Grand lisland, NY). Mice were then boosted three times at an interval of three to four weeks.

Preparation of oestrogen receptor beta nuclear extracts

Nuclear extracts were obtained from transiently transfected HEK293 cells expressing ERβ1 and ERβ2, this was then stimulated with EGF to phosphorylate the oestrogen receptors. The following steps were undertaken in the preparation of phosphorylated form of oestrogen receptor beta protein.

1) Determination of ligand-dependent phosphorylation of ERβ
2) Preparation of nuclear extracts
3) Immunoprecipitation
4) Immunoblotting to determine the presence of phosphorylated oestrogen receptor beta protein

Determination of ligand-dependent phosphorylation of ERβ

Initially we investigated whether ERβ isoforms would be phosphorylated at serine residues in response to growth factors by transfecting HEK 293 cells with ERβ1 or ERβ2 GFP tagged plasmids and using an anti GFP antibody to immunoprecipitate GFP tagged proteins. This would allow only GFP tagged proteins to be run on a western blot which we could then use a non specific phosphor serine antibody to detect any phosphorylated serines. By comparing EGF (epidermal growth factor) stimulated and un-stimulated cells we could determine if the expression of phosphor serine was increased in the stimulated cells.

Preparation of nuclear extracts

HEK 293 cells (ECACC no 85120602) obtained from the European collection of cell cultures (Porton Down, UK) were maintained in Dulbecco's modified essential medium (DMEM, Sigma, Poole, UK) supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 100U penicillin, streptomycin and 0.25ug/ml fungizone (Invitrogen, Paisley, UK) at 37°C in 5% CO_2.

For nuclear extracts, cells were plated in 6 well tissue culture plates at $1x10^6$ cells/ml in phenol red free DMEM with 10% charcoal stripped FBS (CSFBS) for 24 h prior to transfection. Cells were transiently transfected with 1 ug of a plasmid expressing full length cDNA to ERbeta 1 tagged with GFP (pERb1GFP) or ERbeta 2 tagged with GFP (pERb2GFP using 2ul of JetPEI (Polyplus transfection Inc, New York, USA) in phenol red, serum and antibiotics free DMEM for 4 h at 37°C in 5% CO_2. The media was removed and fresh media containing 10% CSFBS was added. The cells were incubated for 48 h. Serum free media was added 24 h prior to stimulation. Cells were stimulated with 10^{-8}M epidermal growth factor (EGF, Sigma) for 30 min in order for the ERβ1 to be phosphorylated. Media was removed and the cells were washed in cold PBS before adding 0.8 ml of ice cold NP-40 lysis buffer. Cells were scraped using a cell scraper into the buffer and transferred to an ice cold tube, vortexed for 10 s followed by centrifugation at 13000 rpm for 15 min at 4°C.

Immunoprecipitation

Samples in NP-40 lysis buffer were boiled for 5min before the addition of 75 ul protein G plus/protein A-agarose (Calbiochem) and incubated overnight at 4°C. The lysates were centrifuged at 12000 g for 20 s at 4°C and supernatants transferred to a new cold tube. One ul of anti GFP antibody, (molecular probes cat no. A11122) was added to the lysate and incubated for 1 h followed by the addition of 20 ul of protein G plus/protein A and a further incubation overnight at 4°C. The samples were centrifuged at 12000 g for 20 s followed by careful removal of the supernatant so as not to disturb the beads. The beads were re-suspended in 1 ml of wash buffer 1 and incubated for 20 min on a rocker at 4°C.

The beads were again pelleted by centrifuging at 12000 g for 20 s at 4°C. The pellet was re-suspended in 1 ml of wash buffer 2 and incubated for 20 min on a rocker for 20 min at 4°C. A further spin at 12000 g for 20 s at 4°C was carried out with the pellet being re-suspended in 1 ml of wash buffer 3 and incubated on rocker for 20 min at 4°C. A final spin at 12000 g for 20 s was carried out and the supernatant removed to a new tube. Loading buffer was added and the samples heated at 70°C for 10 min before loading onto a 4 to 12% polyacrylamide gel.

Immunoblotting to determine the presence of phosphorylated oestrogen receptor beta protein

Gels were run at 120 volts in 1x SDS loading buffer for 50 min. The separated proteins were transferred to Immobulin membrane and blocked in blocking buffer (5% milk in TBS/tween) for 1h at room temperature on shaker. A 1:500 dilution of anti mouse phosphor serine antibody (Sigma, UK) was added and the blots were incubated overnight at 4°C on a shaker. Membranes were washed 4x 5 min in TBS tween at room temperature on shaker. Anti mouse HRP antibody (Sapu) was added at 1:5000 in 5% milk/TBS/Tween for 1 h at room temperature on shaker. Membranes were washed 3x in TBS/Tween for 5 min each at room temperature before developing using ECL Plus (Amersham) and exposing to X-ray film. Membranes were stripped and re-probed with the anti mouse GFP antibody (1:500) or 1:50 dilution of anti mouse ER beta (Figure 3).

Serum screening by ELISA

Two weeks from the last boost, blood samples were collected through the tail vein from each immunised mouse (5 µl of blood into 1 ml of 1% BSA (w/v) containing 0.1% sodium azide (w/v)). This was centrifuged for 10 min at 1600 rpm and serum was aspirated and stored at 4°C. Subsequently these serum samples were screened on ELISA for antibody presence against uncoupled peptide (YAEPQK-Ser(PO_3H_2)-PWCEAR). All five serum samples showed strong reaction to the phosphorpeptide demonstrating strong response against the immunisation (Figure 4). Among those five mice, three highly responded mice were chosen for further boost of the immunogen. After three further boosts, mice were sacrificed and spleen cells were removed and stored in 1 ml aliquots in liquid nitrogen.

Step 1: Coating a microplate with the peptide (uncoupled peptide)

Peptide was dissolved in 0.2 M sodium carbonate-sodium bicarbonate buffer, pH 9.4 which was then added to a microplate (96 well-Nunc Maxisorb plates-SLS, UK) at a concentration of 1µg/ml (50µl/well) and incubated at room temperature on a shaking platform for two hours. The plate was dried by inverting vigorously onto a soft tissue and blocked with ELISA blocking buffer (1%BSA (w/v) in PBS) (100µl/well) and agitated for 30 min at room temperature.

Step 2: Addition of primary antibody

Serial dilutions of tail bleed-serum samples were made in Tris conjugate (25 mM Tris buffer pH 7.4, 0.05% w/v Tween-20 and 0.5% w/v BSA-stored at –20°C). Before the addition of serum samples, plates were washed with wash solution (0.05M Tris-HCl buffer containing 0.15 M NaCl and 0.05% (w/v) Tween-20 (Sigma) for 3 to 5 times in order to remove unbound and loosely bound proteins. Serum samples (50 µl/well) were added to duplicate wells on the ELISA plate and incubated at room temperature on a shaking platform for an hour.

Step 3: Addition of secondary antibody (anti-mouse antibody)

After a thorough wash with wash solution (3 to 5 times), the secondary antibody was added at 50 µl/well (peroxidase conjugated goat anti mouse I gG –1 in 1000 dilution in Tris conjugate) and incubated for 30 min at room temperature.

Step 4: Addition of substrate

Plates were washed again thoroughly with wash solution five times and this was followed by washes with distilled water for another five times. The plates were dried and equal amounts of substrate solution A and B (hydrogen peroxidase substrate and tetramethyl benzidine -Insight biotechnology) was mixed and added to the wells (50 µl/well) and left in the dark for 5 to 10 min for the reaction to develop. The reaction was stopped by the addition of ortho-phosphoric acid (6% v/v; 50µl/well). Finally, the absorbance reading at 450 nm was measured by using a plate reader (Benchmark-Bio-Rad). Serum samples from two unimmunised mice were used as controls in these experiments.

Development of hybridoma and screening of supernatants

Hyper immunised spleens were harvested and placed in a sterile

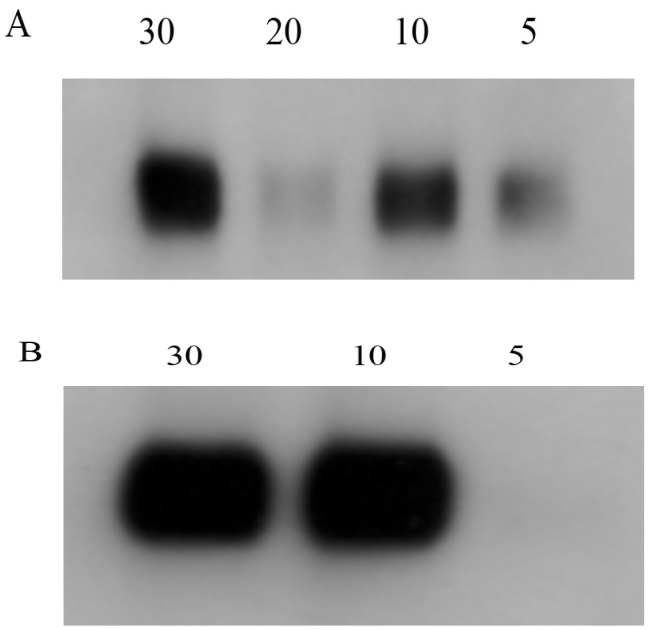

Figure 3. Immunoblotting of phosphorylated oestrogen receptor beta. Each lane was loaded with 20ug of total protein extracted from HEK 293 cells that had been transiently transfected to express either (A. ERβ1) or (B. ERβ2) and had been stimulated with EGF for 5, 10, 20 or 30 mins. The black band correspond to where the anti phosphor serine antibody has detected a phophorylated serine.

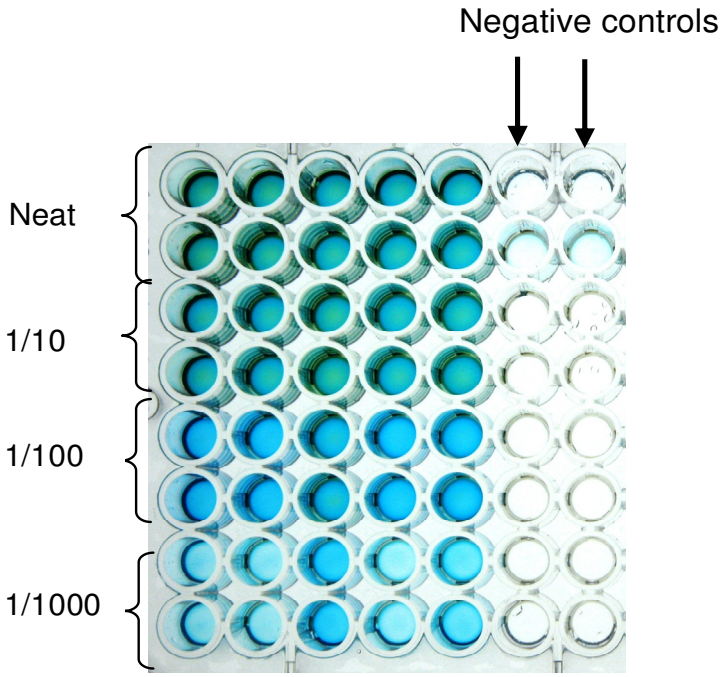

Figure 4. Serum screening against phosphorylated oestrogen receptor beta peptide on ELISA. Test shows that the serial dilutions of all five serum samples reacted strongly against oestrogen receptor phosphor-peptide. Serum samples from two unimmunised mice were used as negative control in this experiment.

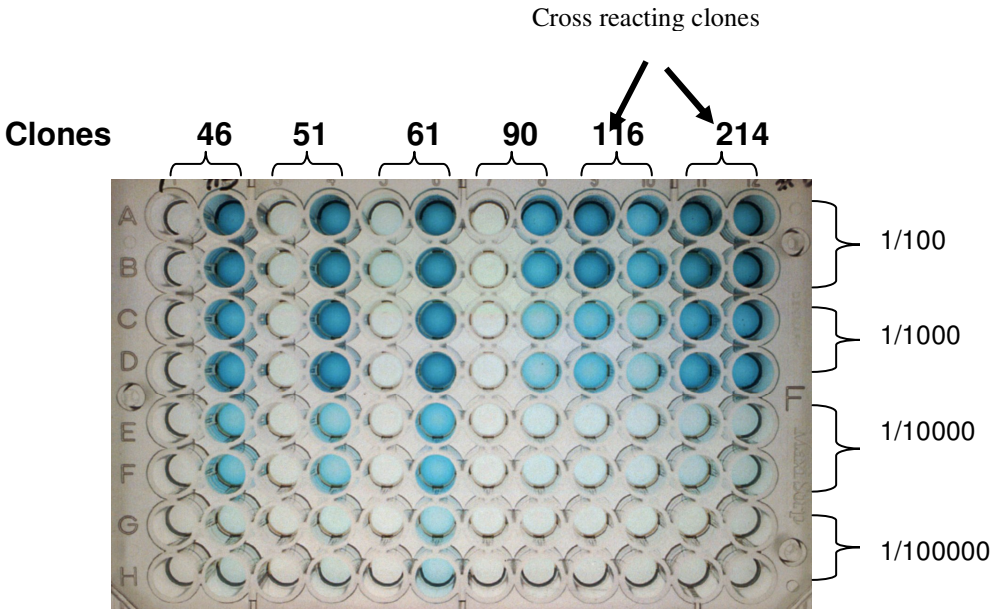

Figure 5. Titration of supernatants.Titratration of selected clones (hybridoma supernatants derived from specific clones 46, 51, 61, 90, 116 and 214) against unphosphorylated and phosphorylated (alternative columns respectively) nuclear extracts (contained oestrogen receptor beta protein) coated side by side. Plate shows cross reaction of clones 116 and 214 against both phosphorylated and unphosphorylated proteins.

petridish with IMDM cell culture medium. Each spleen was processed separately in the following manner. Each spleen was washed three times in IMDM cell culture media in separate petridishes and then placed in a Petridish containing 5 ml of IMDM cell culture medium. Spleen was teased apart using sterile forceps to produce a single cell suspension which then centrifuged at 2000RPM to collect immune cells. These cells were then mixed with myeloma cells (non-antibody producing mouse B cells) (SP2/0) at a ratio of 1:2 in a falcon tube to which 1.5 ml of polyethylene glycol (PEGMW1500 Roche, USA) was added drop-wise while mixing over a period of 1 min in a water bath at 37°C. The cell suspension was continuously mixed for another minute to avoid cell aggregation and then 9 ml of IMDM cell culture media was added drop-wise over a period of 3 min (1 ml in 60 s, 3 ml in 60 s and 5 ml in 60 s). Finally, the total volume was made up to 40 ml and incubated at 37°C for 10 min (5% CO_2) after which period; cell suspension was centrifuged at 1000RPM for 10 min to separate the fused cell mixture. After discarding the IMDM, the palette was re-suspended in 3 ml of HAT (HAT-hypoxanthine-aminopterin, thymidine) hybridoma culture medium (supplemented with 0.1% gentamycine and 15% of bovine fetal calf serum) and added to 90 ml of methylcellulose medium (Clonacell-HY, StemCells Technologies UK) After mixing thoroughly this cell mixture was then transferred to a flask and incubated (incubator supplied with 5% CO_2 at 37°C) for an hour. Finally the cell mixture was plated out in Petri dishes and placed in the incubator (7 to 10 days) for hybridoma clones to develop. Subsequently individual colonies of clones were picked (1100 clones) and grown in HAT hybridoma culture medium in tissue culture plates in order for collecting supernatants. After four days into culturing hybridoma clones, 30 µl of supernatants from each clone was screened on ELISA against phosphorylated oestrogen receptor beta peptide, nuclear extract that contained phosphorylated oestrogen receptor beta and nuclear extract with unphosphorylated oestrogen receptor beta protein. These test produced several hybridoma clones that secrete high affinity novel monoclonal antibodies to phosphoryated region of the oestrogen receptor beta protein.

Enzyme-linked immunosorbent assay (ELISA) screening of hybridoma supernatant on phosphorylated and un-phosphorylated nuclear extracts

Hybridoma supernatants were tested on oestrogen receptor beta protein in its native form. Serial dilutions of supernatants tested on ELISA against un-phosphorylated and phosphorylated (alternative columns respectively) nuclear extract which contained oestrogen receptor beta protein reacted only with phosphorylated oestrogen receptor protein and did not show any reaction with unphosphorylated protein (Figure 5). This is a clear evidence of raised antibodies are specific to the phosphorylated region of the protein and they react with a specific epitope. Out of several clones tested over 20 of them showed high titre antibody presence. Some of the clones showed some positive reaction to the lowest dilution tested.

Selection of highly specific clones

Highly positive clones identified in the initial screening of supernatants were grown in 24 well cell culture plates in HAT media supplemented with methyl cellulose. Each well contained several hundreds of cells derived from a single clone in 1.5 ml of media. Conditioned media were harvested from each well when medium turned orange/yellow (acidic) colour and these wells were replenished with fresh media for continued cell growth. Serial dilutions [(10^{-2}, 10^{-3}, 10^{-4}, $10^{-}5$, 10^{-6} and10^{-7}) of each conditioned media was made with Tris conjugate buffer ((25 mM Tris buffer pH 7.4, 0.05% w/v Tween-20 and 0.5% w/v BSA) and added to microplate coated with nuclear extracts that contained

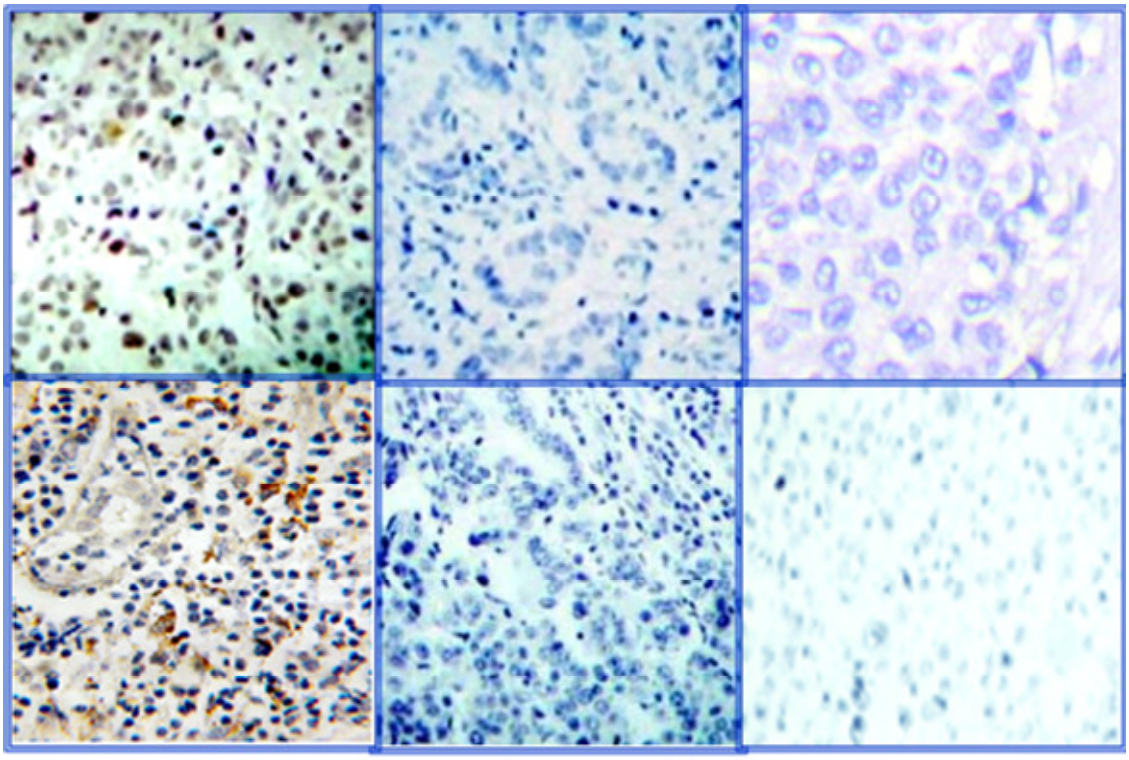

Figure 6. Immunohistochemistry of cancerous breast tissues; Immunolocalisation of phosphorylated oestrogen receptor beta in cancerous breast tissue of various clinical stages. Slides shows the novel phosphor specific antibodies (clones 24C and 99C respectively) bind to denatured form of phosphorylated oestrogen receptor beta protein on tissue samples. F- Control tissue (non cancerous); E- Tissue section derived from a tumor less than 2cm diameter (cancer at Stage 2A); D- Tissue section derived from a tumor bigger than 5 cm across (stage 2B); C- Tissue section derived from a tumor from the chest wall (Stage 3B) larger than 5cm in diameter (cancer had not spread to the lymph nodes of the armpit or collar bone); B-: Tissue section derived from a tumor from the chest wall (Stage 3C - cancer had spread to lymph nodes in the armpit and under the breast bone); A-: Tissue section derived from a tumor at an advanced stage of cancer (stage 4 - metastasized).

phosphrylated oestrogen receptor beta protein (Figure 5). Finally ELISA was developed as described earlier and absorbance readings were taken at 450 nm using a microplate reader (Benchmark-Bio-Rad, UK).

Immunolocalisation of novel phosphor-specific monoclonal antibodies

Cancerous breast tissue samples of diverse clinical stages (purchased from Bioserve, Beltsville USA) were serially sectioned at 5 µm thick and mounted on poly-l-lysine (VWR International Ltd, Lutterworth, UK) coated slides. These slides were then de waxed twice in Histoclear (National Diagnostic, East Riding, UK) for 5 min each and rehydrated through an alcohol series (BDH Analar; 100, 100, and 70%, 2 min each), followed by antigen retrieval in sodium citrate buffer (pH 6.0) at high temperature for 20 min. Then, sections were quenched for 30 min in 3% hydrogen peroxide in methanol to block endogenous peroxidases activity. Sections were incubated with 20% (v/v) normal goat serum in PBS supplemented with 4% (w/v) BSA to reduce non specific antibody binding. After washing with PBS, sections were incubated with tissue culture supernatants of mouse anti phosphor antibodies (oestrogen receptor beta) overnight at 4°C. Next day, sections were washed with PBS and a secondary antibody, goat anti-mouse immunoglobulin (1:200 in PBS; DakoCytomation Limited, Ely, UK) was added and incubated at room temperature for 30 min. After which stage, sections were washed with PBS and incubated for 60 min with peroxidase-conjugated avidin biotin complex (ABC; diluted 1:200 in PBS; Vector Labs, Peterborough, UK). Following washing with PBS, the peroxidase activity was developed with 0.07% 3,3'-diaminobenzidine tetrahydrochloride (ABC; Zymed labs, Cambridge, UK). Finally, sections were counterstained for 2 min with Erlich's haematoxylin. One section served as a negative control (omitting the addition of supernatants). Slides were examined using an E600 microscope (NikonUK Ltd, Kingston-Upon-Thames, UK) (Figure 6).

RESULTS

Since the main goal of the project was to prepare monoclonal antibodies specific for phosphorylated oestrogen receptor beta protein, our initial objective was to select the best responding mice to the peptide immunogen. Mouse serum screening (ELISA) on both phosphorylated and unphosphoryalted peptides were carried out after the second boost of immunogen and all five serum samples collected from these immunised mice distinctly showed strong reaction only to the phosphorylated

peptide demonstrating an active immune response against the immunogen (Figure 4). The serum samples (two) collected from unimmunised mice used as controls in this experiment provided further confidence of the presence of antibodies to oestrogen receptor beta peptide in the serum of immunised mice.

In the advanced stage of the procedure fusion of spleen cells with myeloma cells produced large number of hybridoma clones. The primary screening was carried out on the phosphorylated and unphosphorylated peptides in order to select clones that are specific to the phosphorylated peptide. Out of 1100 supernatant screened, 876 (79.6%) displayed specificity to the phosphorylated peptide and 54 (4.9%) clones reacted against both phosphorylated and unphosphorylated peptides. The secondary screening was designed to select the clones which are specific to the native protein. This was carried out using nuclear extracts which contained of phosphorylated oestrogen receptor beta. We determined the presence of phosphorylated form of oestrogen receptor beta in the nuclear extract preparation through immunoblotting, which showed a strong black band corresponding to the site where anti phosphor serine antibody, detected a phophorylated serine (Figure 3). This outcome is a clear demonstration that the serine molecule at position 87 of the oestrogen receptor beta protein contained in the nuclear extract preparation is phosphorylated.

The positive clones identified in the primary screening, the 876 (79.6%) of clones were tested on phosphorylated and unphosphorylated nuclear extracts. There were 792 (90.4%) supernatants positively reacted exclusively to the nuclear extract which contained phosphorylated oestrogen receptor protein. This is clear evidence that the novel antibodies raised are specific to the epitope that contained a phosphorylated serine molecule. The antibody used as positive control (an antibody to unphosphorylated oestrogen receptorbeta-1 C-terminal) against unphosphorylated peptide reacted strongly demonstrating this experiment free from any significant error. This test also identified 179 clones (20.4%) that cross reacted with both phosphorylated and unphosphorylated nuclear extracts. Overall, 613 (55.72%) clones (out of 1100 clones) were identified as specific to the phosphorylated oestrogen beta receptor protein.

After the preliminary assessment of the supernatants as described earlier, high titre antibody secreting hybridomas (39 clones) were selected for further characterisation. Through repeated titration on microplates coated with nuclear extract that contained phosphorylated version of oestrogen receptor beta protein, 12 highly sensitive, highly specific hybridomas with titres of up to 10-6 were selected. These 12 clones were again tested on a selection of negative controls. No cross reaction was observed with any of the supernatant on unrelated phosphoserine containing peptides (VTA-Ser(PO_3H_2)-RTWAAVLC or ATW-Ser(PO_3H_2)-VTAWLWC) or an unrelated phosphorylated protein (histone).

To further establish the specificity, the novel monoclonal antibodies were subjected to immunohistochemical tests on formaldehyde fixed paraffin wax embedded sections of cancerous human breast tissue sections of various clinical stages. This immunolocalisation test showed strong binding of these antibodies against the putative phosphorylation sites of oestrogen receptor beta protein (Figure 6). The test further demonstrated that the oestrogen receptor beta phosphorylation in breast cancer varied according to the severity of the clinical stage of breast cancer.

DISCUSSION

The importance of Serine phosphorylation has been studied by a number of different investigators and there is significant evidence to show their role in physiology and in cancer. Estrogen receptor beta (ERbeta) is a transcription factor that regulates expression of target genes in a ligand-dependent manner. Activation of gene expression is mediated by two transcription activation functions AF-1 and AF-2, which act in a promoter- and cell-specific manner. Whilst AF-2 activity is regulated by estrogen (E2) binding, the activity of AF-1 is additionally modulated by phosphorylation at several sites and one of these phosphorylation sites, serine 87 (S87).

The hybridoma technology facilitates in developing monoclonal antibodies of pre-determined specificity. To test the hypothesis of oestrogen receptor phosphorylation at serine position 87 (a putative phosphorylation site), a peptide of 12 as in length (Peptide (GHLSPLVVHRQLC) was selected from the oestrogen receptor beta protein to be used as immunogen. and synthesised commercially (serine molecule was phosphorylated) (GHL-Ser(PO_3H_2)-PLVVHRQLC). Phosphor specific monoclonal antibodies to oestrogen receptor beta could be an important research tool that can help in the investigation of breast cancer progression. In this study all five serum samples collected from immunised mice reacted very strongly with the phosphor peptide, demonstrating active immune response to the immunisation. Further, serum samples collected from un-immunised mice used in this experiment as negative controls did not show any reaction to the peptide (Figure 4). Therefore, the peptide is highly immunogenic and has triggered an immune response in all five mice used in this procedure. This can be attributed to the adjuvants used in this procedure that have helped in the rapid expansion and maturation of antigen-specific B lymphocytes.

The fusion of affinity matured IgG secreting B cells with myeloma cells produced large number of hybridoma clones. The culture supernatants collected form these several hybridoma clones tested on ELISA showed a

strong positive reaction to phosphor peptide. In the same test positive control (an antibody to unphosphorylated oestrogen receptorbeta-1 C-terminal) reacted strongly with the coating of unphosphorylated form of oestrogen receptor beta nuclear extract, providing a strong conformation of the presence of antibodies in the supernatants are only for putative phosphorylation sites of the protein.

When the supernatants were screened side by side with phosphorylated and unphosphorylated nuclear extracts, a strong reaction was observed against the phosphorylated form of nuclear extract and conversely small fraction of clones cross reacted with unphosphorylated form of nuclear extract (Figure 5). These cross reaction could be ascribed to an epitope or part of an epitope that lies outside of the phosphorylated serine molecule of the peptide immunogen used (GHL or PLVVHRQLC of the immunogen). Further, no cross reaction was observed when tested on any unrelated phosphor-serine containing peptides or an unrelated phosphorylated protein tested against these novel monoclonal antibodies. This is further demonstration of specificity of the novel antibodies developed.

Conclusion

Immunolocalisation tests conducted on human cancerous breast tissue sections of various clinical stages showed very clear distinction between tissue sections in that binding of novel antibodies increased as the stage of the cancer advanced (Figure 6). Studies by Lannigan (2003); Shah and Rowan (2005) proposed that phosphorylation of oestrogen receptor could contribute to cancer progression; and our study is a clear evidence of the hypothesis proposed. The novel phosphor-serine antibodies reacted exclusively to cancerous tissues where high amount of phosphorylated form of oestrogen receptors were found, on the other hand, control tissue used in this experiment (non cancerous breast tissue of various ages) did not show any reaction on immunohistochemical tests. This result is another clear evidence of the specificity of the novel antibodies to phosphorylated oestrogen receptor beta protein. The outcome of the result is a clear proof yet to confirm the hypothesis that there is a positive correlation between the phosphorylation of oestrogen receptor beta and progression of breast cancer.

Among the panel of highly specific monoclonal antibodies developed some of the clones were identified as most suited to immunohistochemical tests than on ELISA experiments. This is because antibodies that are developed to linear peptides are able to bind easily to denatured protein on the tissue sections. In this study we have developed monoclonal antibodies to phosphorylated serine at position 87 in the oestrogen receptor beta protein to demonstrate that there is a positive correlation between serine phosphorylation and advancement of breast cancer. We believe that these antibodies could be used in further understanding of the role of oestrogen receptor beta phosphorylation in breast cancer progression.

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