Spontaneous antibodies against Engrailed-2 (EN2) protein in patients with prostate cancer

Short title: Spontaneous antibodies against Engrailed-2

Nicola E Annels[1], Guy R Simpson[1], Mick Denyer[1], Sophie E McGrath[1], Giulia Falgari[1], Emma Killick[2], Ros Eeles[2], Justin Stebbing[3], Dmitri Pchejetski[3], Ramsey Cutress[4], Nick Murray[4], Agnieszka Michael[1], Hardev Pandha[1]

1. Oncology, Faculty of Health and Medical Sciences, Leggett Building, University of Surrey, Guildford, Surrey, GU2 7WG, UK.

2. Institute of Cancer Research, Sutton, Surrey, SM2 5NG, UK.

3. Charing Cross Hospital, Fulham Palace Road, London, W6 8RF, UK.

4. University Hospital Southampton, Somers Cancer Research Building, Southampton, Hampshire, SO16 6YD, UK.

Corresponding author: Hardev Pandha

Tel: 01483 688602

Fax: 01483 688558

E-mail: h.pandha@surrey.ac.uk

Keywords: Autoantibodies, Engrailed-2, Prostate Cancer.

Abbreviations

EN2	Engrailed 2
IgG	Immunoglobulin G
NY-ESO-1	New York Esophageal Squamous Cell Carcinoma 1
LREC	Local Research Ethics Committee
PSA	Prostate Specific Antigen
LHRH	Luteinizing-hormone-releasing hormone
CRRPSA	castrate resistant, rising PSA
BRCA	Breast Cancer
IMPACT	Identification of Men with a genetic predisposition to Prostate Cancer
ELISA	enzyme-linked immunosorbent assay
PBS	Phosphate Buffered Saline
BSA	Bovine Serum Albumin
AMACR	alpha-Methylacyl-CoA Racemase
DHFR	Dihydrofolate reductase

Summary

We reported the expression of the homeodomain-containing transcription factor Engrailed-2 (EN2) in prostate cancer and showed that the presence of EN2 protein in the urine was highly predictive of prostate cancer. This study aimed to determine whether patients with prostate cancer have EN2 auto-antibodies, what the prevalence of these antibodies is and whether they are associated with disease stage. The spontaneous IgG immune response against EN2 and for comparison the tumour antigen NY-ESO-1, were tested by ELISA in three different cohorts of prostate cancer patients as well as a group of men genetically predisposed to prostate cancer. 32/353 (9.1%) of the SUN cohort representing all stages of prostate cancer demonstrated EN2 IgG responses, 12/107 patients (11.2%) in the advanced prostate cancer patients showed responses, whilst only 4/121 patients (3.3%) with castrate resistant prostate cancer showed EN2 auto-antibodies. No significant responses were found in the predisposed group. Anti-EN2 IgG responses were significantly higher in patients with prostate cancer compared to healthy control males and similarly prevalent to anti-NY-ESO-1 responses. Whilst EN2 autoantibodies are not a useful diagnostic or monitoring tool EN2 immunogenicity does provide the rationale to pursue studies using EN2 as an immunotherapeutic target.

Introduction

Prostate cancer is the most common cancer in men in the UK, accounting for a quarter of all new cases of cancer in males [1]. A large proportion of patients with localised small volume disease are either cured by treatment or do not require intervention (i.e. are managed by active surveillance). There are currently intense worldwide efforts to establish reliable biomarkers both for the early detection of prostate cancer and also to identify which cancers are likely to progress. We recently reported the diagnostic potential of Engrailed-2 (EN2) protein, a homeodomain-containing transcription factor secreted into the urine of men with prostate cancer [2]. In a follow-up study we further demonstrated that urinary engrailed-2 (EN2) levels predict tumour volume in men undergoing radical prostatectomy for prostate cancer [3] and thus may be useful in deciding which patients to treat and which to monitor closely. Moreover, tissue expression (cytoplasmic, nuclear and membrane) of EN2 was found exclusively in prostate cancer and not normal prostatic epithelium in tissue microarrays and patient specimens. EN2 was expressed equally at all stages of prostate cancer (T stage 1-4) as well as in metastatic disease (lymph nodes) (Morgan personal communication).

Antibodies fulfil their adaptive immune response commonly to prevent infection. However, the function of tumour-associated autoantibodies, which bind self-antigens and have escaped self-tolerance, is generally unknown. Autoantibodies can mediate antibody-dependent cell mediated cytotoxicity and complement-dependent cytotoxicity. They may also enhance antigen cross-presentation and activation of T lymphocytes. It has been shown that IgG responses to tumour-associated proteins exist in cancer patients, making it possible to use human sera to identify immunologically-recognized potential tumour antigens [4]. IgG responses to tissue associated antigens may have diagnostic value, aid in risk stratification in terms of tumour progression or provide a tool for monitoring patients response to treatment. Previous studies have clearly shown

a multitude of antibody responses to prostate associated antigens [5-8]. We proposed that *de novo* expression of EN2 in prostate cancer could potentially elicit an autoantibody response, and that this could be quantified and potentially used as an adjunct to current diagnostic and monitoring biomarkers.

In this study we assessed the spontaneous antibody response to EN2 in men with prostate cancer, both treatment naive at different stages but also patients who had received optimal treatment. We detected a significantly higher prevalence of IgG responses to EN2 in prostate cancer patients compared to healthy control males, with positive antibody responses being found predominantly in patients with higher volume and more advanced stage prostate cancer.

Materials and Methods

Patient Populations

The study evaluated blood samples from cancer patients, individuals at high risk of developing prostate cancer and healthy volunteer controls. In all cases, written informed consent was obtained and the donation of blood samples for autoantibody evaluation had received approval by the local ethics committee.

Our control groups consisted of men who were unlikely to harbour prostate cancer based on the absence of symptoms, no family history of cancer, a PSA <2 ng/ml (Av age:65; range 40-89). In a similar way, the female controls for the breast and ovarian cancer study were women with no past history of any cancer, no symptoms and no family history of any malignancy. Patients with histological (on routine biopsy) evidence of benign prostatic hypertrophy, prostatic intra-epithelial neoplasia and prostatitis were excluded from the study. These patients have, generally, an abnormal PSA and even with saturation biopsies an underlying malignancy cannot be excluded. In the case of prostatitis the PSA is often markedly raised, and may remain so for 6-9 months. Despite our large cohorts of patients and controls in the study, the total number of patients with these 3 conditions would have been too small for meaningful statistical comparison.

Blood was taken from prostate cancer patients from the Royal Surrey County Hospital (Guildford, UK). We evaluated four specific cohorts:

(1) Samples from a comprehensive prostate cancer biobank, consisting of the pre-treatment sample of 353 men with all stages of prostate cancer (SUN cohort) LREC: 08/H1306/115.This cohort of patients consisted of men with newly diagnosed cancer patients prior to starting any form of treatment. The median

age of the cohort was 71 years old, the median PSA at diagnosis was 9 and the median Gleason score was 6.

(2) Patients with rising PSA despite LHRH agonist hormone treatment with normal radiological evaluation and no symptoms, designated CRRPSA (castrate resistant, rising PSA). This was a cohort of patients recruited to a clinical trial of allogeneic whole cell vaccine EUDRACT number 2006-001619-31. The median age of the patients was 71, the median PSA was 5.5 and the median Gleason score was 7.

(3) Men with castrate resistant disease with radiological evidence of metastatic disease LREC: 08/H1306/115. This was a cohort of patients presenting to oncology clinics and receiving chemotherapy. All patients had metastatic prostate cancer with either bone or soft tissue disease.

(4) Patients at high risk of cancer. Samples from the IMPACT cohort (Identification of Men with a genetic predisposition to Prostate Cancer: Targeted Screening in BRCA1/2 mutation carriers) were used. To be eligible for this cohort, men had to be either a BRCA1 or BRCA2 mutation carrier or from a family harbouring a BRCA1/2 mutation but had tested negative themselves (LREC: 05/MRE07/25) [9].

For control comparison of unrelated cancers, we tested two independent female breast cancer sample sets of sera taken from women prior to first definitive treatment for their breast cancer from Charing Cross Hospital (LREC:07/MRE09/54) and Southampton General Hospital (LREC: 05/Q1702/13) [10]. We also evaluated a further control group of women with ovarian cancer, at the Royal Surrey County Hospital (LREC: 09/H1103/50**)**. Most women had advanced ovarian cancer (FIGO Stage 3c or 4) and had received 3 cycles of neoadjuvant chemotherapy prior to their blood test. In a few instances, blood was obtained prior to any treatment. Blood samples from each cohort

were taken in accordance with the protocol associated with each study. In all patients, sera/plasma was stored in 0.5ml aliquots at −80°C until used for analysis.

Expression of recombinant EN2 and NY-ESO-1 proteins within non-fusion and fusion E coli expression vectors.

EN2 cDNA with optimised codon usage was synthesised and cloned by GenScript, USA. This *EN2* cDNA was re-cloned into the pQE 31 plasmid (Qiagen, Manchester, UK) placing it under the control of a T7 promoter as well as adding aminoterminal histidine tag. The NY-ESO-1 cDNA was obtained from pLPCX NY-ESO-1 (a gift from Beatrice Yin, Sloan Kettering Institute, NY, USA). To obtain high expression of NY-ESO-1 protein a fusion partner (mouse dihydrofolate reductase) was needed. To achieve this expression, the cDNA of NY-ESO-1 was re-cloned into the pQE 40 plasmid (Qiagen, Manchester, UK) which resulted in the fusion NY-ESO-1 to the carboxyterminal end of mouse dihydrofolate reductase (plus histidine tag). As a non-specific E coli protein control, mouse dihydrofolate reductase was produced from the pQE 40 plasmid containing no insert. All plasmids were transformed into E coli host strain (M15 (Qiagen, Manchester, UK) which contain multiple copies of the pREP4 plasmid which constitutively expresses the lac repressor protein, resulting in regulated efficient and controlled expression of E coli proteins. The histidine tags allow purification of recombinant proteins by affinity chromatography on Ni-NTA resin (Qiagen, Manchester, UK) under denatured conditions.

ELISA to detect circulating auto-antibodies to EN2

ELISA MaxiSorp 96-well plates (Nunc, UK) were coated with recombinant EN2, NYESO-1 or control DHFR protein produced in E.coli at a concentration of 4ug/well in 0.1M carbonate buffer (33.5 mM Na_2CO_3 0.1M $NaHCO_3$, pH9.6) and incubated

overnight at 4°C. Plates were blocked with 3% BSA/PBS for 2 hours at room temperature on a rapid shaker. All washes were performed with PBS/0.1%Tween-20. Following 4 washes, plates were incubated with patients and control plasma diluted 1:100 in 3%BSA/PBS for 1 hour at room temperature on a rapid shaker. All plasma/sera were assayed in triplicate. Plates were washed and incubated with goat anti-human IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, UK) or mouse anti-human IgG1, IgG2, IgG3 or IgG4-HRP (Life Technologies) in 3%BSA/PBS for 1 hour on a shaker at room temperature. The plates were then incubated with 100μl of 3,3',5,5'-tetramethylbenzidine solution (TMB) (Sigma, UK) for colour development. The reaction was stopped by addition of 2M H_2SO_4. Optical density (OD) was measured at 450nm by using a plate reader (Beckman Coulter, UK). The mean value plus 3 standard deviations calculated from the test results for healthy controls for plasma IgG was used as a cut-off value for evaluation of each assay. This ELISA was standardized using rabbit (EN2, Abcam, UK) and goat Abs (NY-ESO-1, Lifespan BioSciences, Seattle, WA) with specificity for each of the antigens.

Immunoblot Analysis

To confirm ELISA results EN2 detection was performed by running 1μg of purified EN2 protein or the control protein DHFR on a NuPAGE gel followed by semi-dry western blotting, blocking using 5% Marvel and chemiluminescent detection. EN2 detection was achieved using 1:1000 dilution HRP-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, UK). To show that plasma or sera antibodies also recognize our recombinant EN2 protein, blots were incubated with patient sera or plasma diluted 1:100 for 24hrs, washed and detected using HRP conjugated goat anti-human IgG (Jackson ImmunoResearch, UK) diluted 1:10,000.

Statistical Analyses

The chi-squared test with a 95% confidence interval was performed using MedCalc to evaluate the statistical significance of differences in the proportion of EN2 or NY-ESO-1 antibody responders between patient cohorts and healthy controls. Differences were considered significant when the probability values obtained from the statistical tests were 0.05 or less.

Results

In order to determine whether circulating EN2-specific antibodies were present in patients and healthy controls, an ELISA was developed using recombinant EN2 protein produced by expression in E.coli. Samples from patients and healthy controls were screened on rEN2 and as a comparison on one of the most immunogenic cancer testis antigens, rNY-ESO-1-coated microplates. In addition samples were also screened against a non-immunogenic murine protein (DHFR) similarly produced in E.coli to eliminate sera/plasma that non-specifically reacted with bacterial contaminants in the preparation. Western blotting experiments were performed to verify ELISA results in selected patient samples compared to a commercial antibody. The commercial antibody only detected a ~45kDa protein in the lane containing the purified recombinant EN2 protein (Fig1, goat anti-EN2 Ab, Abcam). Similarly patient sera and plasma samples that were shown to be EN2 Ab positive by ELISA also gave a positive signal by western blotting (Fig 1, patient 1, 2 and 3) and conversely those patients that were negative for EN2 Abs by ELISA gave a negative result by western blotting (Figure 1, patient 4 and 5).

Patients with prostate cancer demonstrate spontaneous IgG anti-EN2 responses in plasma/serum

(1) We first evaluated IgG anti-EN2 and anti-NY-ESO-1 autoantibody responses in our SUN cohort. These were pre-treatment samples from men with all stages of prostate cancer (Fig 2A). To be considered positive for an IgG response the cut-off values were calculated as the mean value plus three standard deviations from the data pool of the control sera/plasma samples. We found 32/353 (9.1%) of the entire SUN cohort taken together as one group demonstrated EN2 IgG responses, compared to a 4.8% response to NY-ESO-1. The proportion of EN2

IgG responders was significantly different between patients and controls ($p<0.001$). Fig 2B shows titration curves of EN2 antibody-positive and negative sera from the SUN cohort. Similar titration curves were also obtained from NY-ESO-1antibody-positive and negative sera (data not shown). If patients were subdivided according to Gleason score or clinical stage, Fig 2C and D, there were no significant differences in the proportion of EN2 IgG responders with respect to disease stage (T1 to T4 individually) or Gleason score. T4 stage patients were not considered statistically because of the low numbers. In addition there was also a significant difference in the proportion of NY-ESO-1 IgG responders from the whole SUN cohort compared to healthy controls ($p<0.05$). However, similar to the EN2 IgG response there were no significant differences in the proportion of NY-ESO-1 IgG responders with respect to disease stage (T1 to T4 individually) or Gleason score.

(2) We next evaluated samples from men with a rising PSA level despite optimal hormone treatment, but with no disease radiologically (CRRPSA). We found no significant increase in the proportion of EN2 IgG responders in this group of patients compared to healthy controls. However the proportion of patients within this group responding to NY-ESO-1 was statistically different from healthy controls ($p<0.01$) (Fig 3A).

(3) We found that in men with castrate resistant advanced metastatic disease 12/107 patients (11.2%) were confirmed as positive for IgG responses to EN2. This proportion of EN2 IgG responders was statistically different compared to healthy controls ($p<0.001$). None of these patients were receiving chemotherapy at the time the blood was drawn (Fig 3B).

(4) In men at high risk of developing prostate cancer (IMPACT study participants) no significantly increased IgG responses to EN2 or NY-ESO-1 were detected compared to the age matched healthy control group (Fig 3C).

Of the patients that demonstrated a positive IgG response to EN2 and/or NYESO-1, from the SUN study cohort only 2 out of the 47 patients showed a response to both antigens, from the CRRPSA cohort none of the Ab positive patients responded to both antigens and from the men with castrate resistant disease with radiological evidence of metastatic disease only 2 out of 19 patients showed a response to both antigens.

Dominant IgG1 and IgG3 response against EN2

21 serum/plasma samples reacting with EN2 were additionally subjected to IgG subclass testing (IgG subclass 1-4). The level of the IgG subclass of the EN2 antibody was determined in each patient using the same ELISA as before but for detection we used subclass-specific secondary antibodies. We predominantly found antibodies from subclass IgG1 and/or IgG3 in all patients. 11/21 patients tested showed both an IgG1 and IgG3 response, 9/21 patients gave only an IgG1 response and one patient showed only an IgG3 response. No significant IgG2 or IgG4 response was observed in any of the patients (Figure 4).

EN2 antibody response remains stable in seropositive patients regardless of treatment

From the SUN study cohort sequential follow-up samples were available which allowed us to study the EN2 autoantibody response in a longitudinal manner. From those patients that had demonstrated a positive auto-antibody response to EN2, sequential samples were available from 5 patients under active surveillance, 3 patients who had received radiotherapy and 5 patients who had received a radical prostatectomy. ELISA results on the sequential samples from these patients showed that the antibody response remained stable in all but one of these patients regardless of treatment and over a period of up to 3 years 11 months. In only one of the patients (SUN 147) who had received a radical prostatectomy did the EN2 antibody response fall below the cut-

off level for positivity following surgery and remained negative for 2 years 10months post-surgery (Figure 5).

No significant detection of anti-EN2 IgG responses in female oncological patients

The assessment of serum/plasma IgG anti-EN2 antibodies in patients with malignancies was extended to female patients with breast or ovarian cancer, cancer types which we have found to have a high prevalence of EN2 expression at the protein level (data not shown). As shown in Figure 6, autoantibody was rarely detected and was not higher in patients with breast or ovarian cancer compared to female healthy controls. This was in contrast to IgG anti-NY-ESO-1 responses which were clearly found in both female cancer groups at a frequency previously published in the literature [11].

Discussion

Serum autoantibodies directed against tumour associated antigens have been frequently detected in the sera of patients with different types of cancer [4], including prostate cancer. A multitude of autoantibodies to various antigens such as PSA, prostatic acid phosphatase, HER-2/neu, p53, glucose-regulated protein 78 and alpha-Methylacyl-CoA Racemase (AMACR) have been observed in the sera of prostate cancer patients [12-15]. Several groups have tried to identify antibody responses to such prostate tissue antigens as a means of prostate cancer detection. Maricque et al. showed that IgG responses to a panel of commonly recognized prostate antigens were as frequently detected in patients with clinical prostatitis as in patients with prostate cancer thus demonstrating the need to define those antigens which are clinically relevant for prostate cancer [7]. Other studies investigating the antibody responses to individual proteins, including huntingtin-interacting protein 1 (HIP1) [16] and alpha-methylacyl-CoA racemase (AMACR) [15] have shown that antibody responses to these proteins are highly specific for prostate cancer. Thus the identification of new immunogenic antigens specific for prostate cancer will help to build up a panel of commonly recognized prostate cancer antigens that could either be used to detect prostate cancer or could be used to evaluate immune responses to the prostate. Indeed Smith et al. used a panel of 126 prostate tissue-associated antigens common in patients with prostate cancer to investigate whether changes in IgG responses to this panel might be used as a measure of immune response, and potentially antigen spread, following prostate cancer-directed immune-active therapies [17]. They found that different antigens were recognized following androgen deprivation compared with vaccine therapies.

In the current study we assessed the levels of auto-antibodies against EN2 in sera from patients with prostate cancer, other cancers and from individuals at high risk groups.

This is, to our knowledge, the first assessment of spontaneous immune responses to EN2 in prostate cancer. We have demonstrated a significant increase in anti-EN2 IgG antibodies in patients with prostate cancer as compared to control subjects. Furthermore the frequency of anti-EN2 IgG responses in our prostate cancer patient cohorts was similar to the number of seropositive patients specific for the highly immunogenic tumour antigen, NY-ESO-1. NY-ESO-1 is a cancer/testis antigen which is expressed in a number of human cancers including prostate cancer [18]. NY-ESO-1 appears to be one of the most immunogenic tumour antigens inducing a humoral immune response and specific CD8 T-cell reactivity in ~50% of patients with advanced NY-ESO-1-expressing tumours [19, 20]. Due to its high immunogenicity and broad expression in a variety of cancers, monitoring of antibody responses to NY-ESO-1 has already been used as a selection criterion for patients enrolling in NY-ESO-1 cancer vaccine trials and as a tool to assess the immunologic and clinical responses of the treatment ([19, 20, 21]. Notably an elevated EN2 IgG response was not related to an elevated NY-ESO-1 response indicating that a high EN2 response is highly specific and not related to a general autoimmune phenomenon. The prevalence of humoral immune responses to EN2 within our prostate cancer patient cohorts was similar to what has previously been reported in prostate cancer patients for other prostate cancer associated proteins (11% PSA and 15.5% Her-2/neu) [12].

We first evaluated pre-treatment sera from men representative of stages 1-4 prostate cancer from a prospective biobank. We found a significant prevalence of IgG responses to EN2 in the cancer patients versus controls and this was comparable to the response frequency to NY-ESO-1 measured in the same samples. As this group contained patients at different stages of prostate cancer we then evaluated whether IgG responses to EN2 were more frequent at a particular disease stage. We observed no statistical difference in the frequency of EN2 IgG responses between groups of

patients based either on Gleason score or T stage. This is in keeping with studies by Maricque et al. who also reported that immune responses to multiple antigens were detectable as frequently in patients with early stage disease as in patients with advanced metastatic disease [7].

We then went on to test two groups of patients with castrate resistant disease. Here we found that there were a significantly lower proportion of EN2 IgG responders in those patients with undetectable cancer but a rising PSA (CRRPSA). In contrast the group with metastatic disease displayed EN2 IgG responses comparable to the SUN cohort. This suggests that detectable disease needs to be present in order for a measurable EN2 IgG response to be present. This is further supported by our finding that there were no significantly elevated anti-EN2 IgG responses or indeed anti-NY-ESO-1 IgG responses in the IMPACT high risk group compared to healthy controls. A number of studies have reported that circulating autoantibodies to tumour associated antigens have been found to be detectable several years before radiographic detection or incidence screening was able to identify the tumours [22-24]. However, the mechanism underlying their emergence and regulation of their production is not fully understood. Previous reports have described the detection of specific serum autoantibodies in pre-diagnostic sera and hence their potential use as a screening tool in asymptomatic high risk populations [22-24]. Due to the availability of sequential samples from patients within the SUN study cohort we were able to address this by performing a longitudinal assessment of the EN2 autoantibody response in seropositive patients and the effects of different types of treatment on the antibody response. Interestingly in all patients the EN2 autoantibody response persisted over the entire sampling period (up to nearly 4 years in some patients) regardless of the type of treatment received. The only exception was one patient who became EN2 sero-negative following radical prostatectomy. This finding is perhaps not that surprising as it is known that low-level

antibodies can persist up to a life-time in an individual following vaccination or infection. This can be attributed to on-going production from a rapid-turnover of memory B-cell pools [25] or long-lived plasma cells [26] which can be maintained by antigenic stimulation (e.g. persistent antigenic re-exposure) and/or antigen-independent activation mechanisms [27]. One can speculate whether the persistence of the antibody response is due to low levels of residual tumour or simply due to long-lived plasma cells. It is tempting to conclude that the single patient where the antibody response disappeared following surgery was actually cured given the stability of the antibody levels in the majority of patients studied. However, the persistence of the EN2 antibody response in most patients precludes its use as a monitoring tool for response to treatment.

In this study we went on to further characterize the humoral response to EN2 by determining the subclass of antibody induced. It is known that human B cells produce 4 subclasses of IgG (IgG1, IgG2, IgG3, IgG4), with each subclass having different biological functions [28, 29]. The dominant subclasses induced in the antibody response against EN2 were IgG1 and IgG3. Although the clinical significance of the different IgG1 and IgG3 patterns of response observed in our patients cannot be addressed due to the small numbers of patients studied, it is known that the distribution of IgG subclasses within an antibody response is affected by a number of factors including nature of antigen, dose, route of entry and also the host genotype [30]. This was demonstrated in a vaccination study using recombinant CEA where a gradual shift from a predominant IgG1 response at 6 months to an IgG4 response at 15 months was seen [31]. Similar to our findings the humoral response to NY-ESO-1 has also been reported to consist of antibodies of the IgG1 and IgG3 subclass [32]. IgG1 induction results from Th1 type CD4 T-cell activation [33], whilst immune-complexes consisting of the antigen and IgG1 or IgG3 mainly bind to FcγRIIa [34, 35] which is a potent

leukocyte activator and stimulates the release of high levels of inflammatory cytokines. Thus the induction of IgG1 and IgG3 provide favourable conditions for effector T-cell activation in the immune response to EN2. Our group are currently investigating cellular immune responses to EN2.

Interestingly the present work showed that the increased anti-EN2 IgG levels appeared to be more significant in male cancer patients than in female cancer patients. This was in contrast to the auto-antibody response to NY-ESO-1 which was significant in both male and female cancer patients. This gender difference in the humoral immune response has been reported before and further supports the suggestion that sex hormones may significantly affect the humoral immune response to some tumour associated antigens [36, 37].

In summary, this study has shown that the homeodomain-containing transcription factor Engrailed-2 (EN2) is immunogenic as evidenced by the significant prevalence of IgG responses to EN2 in prostate cancer patients. Antibody immunity to this tumour antigen was observed at different stages of the disease. The confirmation of its immunogenicity provides the rationale to pursue studies using EN2 as an immunotherapeutic target.

Furthermore, our findings may have additional clinical relevance as it is unlikely that any single marker for prostate cancer will have the desired high specificity and sensitivity for initial diagnosis. Combined analysis of different autoantibodies have already increased diagnostic sensitivity and specificity in breast cancer (p53, HER2neu, IGFBP-2 and TOPO2a [38] and lung cancer (p53, NY-ESO, CAGE, GBU4-5, Annexin 1 [39]. Identification of antibodies to further novel tumour associated

proteins may thus help build up a ‘panel’ which may also include non-protein biomarkers and which in combination may be sufficiently predictive.

Acknowledgments

We would like to thank The Prostate Project Charity for their financial support.

NE designed the study, carried out experiments and wrote the manuscript, GS produced recombinant proteins and performed western blotting…MD carried out ELISAs….. SM carried out ELISAs….GF carried out ELISAs…EK helped in acquiring patient samples….RE helped in acquiring patient samples…… JS helped in acquiring patient samples……..DP helped in acquiring patient samples…… RC helped in acquiring patient samples……NM helped in acquiring patient samples….AM helped in acquiring patient samples…, HP involved in design of study…..

All authors declare no conflicts of interest

References

1. (Cancer Research UK. Prostate Cancer Incidence statistics:www.cancerresearchuk.org/cancerinfo/cancerstats/types/prostate/incidence/).
2. Morgan R, Boxall A, Bhatt A, Bailey M, Hindley R, Langley S, et al. Engrailed-2 (EN2): a tumor specific urinary biomarker for the early diagnosis of prostate cancer. Clin Cancer Res 2011; 17:1090-8.
3. Pandha H, Sorensen KD, Orntoft TF, Langley S, Hoyer S, Borre M, et al. Urinary engrailed-2 (EN2) levels predict tumour volume in men undergoing radical prostatectomy for prostate cancer. BJU Int 2012; 110:E287-92.
4. Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmits R, et al. Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci U S A 1995; 92:11810-3.
5. Dunphy EJ, Eickhoff JC, Muller CH, Berger RE, McNeel DG. Identification of antigen-specific IgG in sera from patients with chronic prostatitis. J Clin Immunol 2004; 24:492-502.
6. Hoeppner LH, Dubovsky JA, Dunphy EJ, McNeel DG. Humoral immune responses to testis antigens in sera from patients with prostate cancer. Cancer Immun 2006; 6:1.
7. Maricque BB, Eickhoff JC, McNeel DG. Antibody responses to prostate-associated antigens in patients with prostatitis and prostate cancer. Prostate 2011; 71:134-46.
8. Smith HA, Maricque BB, Eberhardt J, Petersen B, Gulley JL, Schlom J, et al. IgG responses to tissue-associated antigens as biomarkers of immunological treatment efficacy. J Biomed Biotechnol 2011; 2011:454861.

9. Mitra AV, Bancroft EK, Barbachano Y, Page EC, Foster CS, Jameson C, et al. Targeted prostate cancer screening in men with mutations in BRCA1 and BRCA2 detects aggressive prostate cancer: preliminary analysis of the results of the IMPACT study. BJU Int 2011; 107:28-39.
10. http://www.cancerresearchuk.org/cancer-help/trials/a-study-to-develop-a-blood-test-for -use-in-breast-screening.).
11. Stockert E, Jager E, Chen YT, Scanlan MJ, Gout I, Karbach J, et al. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. J Exp Med 1998; 187:1349-54.
12. McNeel DG, Nguyen LD, Storer BE, Vessella R, Lange PH, Disis ML. Antibody immunity to prostate cancer associated antigens can be detected in the serum of patients with prostate cancer. J Urol 2000; 164:1825-9.
13. Mintz PJ, Kim J, Do KA, Wang X, Zinner RG, Cristofanilli M, et al. Fingerprinting the circulating repertoire of antibodies from cancer patients. Nat Biotechnol 2003; 21:57-63.
14. Nilsson BO, Carlsson L, Larsson A, Ronquist G. Autoantibodies to prostasomes as new markers for prostate cancer. Ups J Med Sci 2001; 106:43-9.
15. Sreekumar A, Laxman B, Rhodes DR, Bhagavathula S, Harwood J, Giacherio D, et al. Humoral immune response to alpha-methylacyl-CoA racemase and prostate cancer. J Natl Cancer Inst 2004; 96:834-43.
16. Bradley SV, Oravecz-Wilson KI, Bougeard G, Mizukami I, Li L, Munaco AJ, et al. Serum antibodies to huntingtin interacting protein-1: a new blood test for prostate cancer. Cancer Res 2005; 65:4126-33.
17. Smith HA, Maricque BB, Eberhardt J, Petersen B, Gulley JL, Schlom J, et al. IgG responses to tissue-associated antigens as biomarkers of immunological treatment efficacy. J Biomed Biotechnol 2011; 2011:454861.

18. Chen YT, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc Natl Acad Sci U S A 1997; 94:1914-8.
19. Jager E, Gnjatic S, Nagata Y, Stockert E, Jager D, Karbach J, et al. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancers. Proc Natl Acad Sci U S A 2000; 97:12198-203.
20. Jager E, Karbach J, Gnjatic S, Neumann A, Bender A, Valmori D, et al. Recombinant vaccinia/fowlpox NY-ESO-1 vaccines induce both humoral and cellular NY-ESO-1-specific immune responses in cancer patients. Proc Natl Acad Sci U S A 2006; 103:14453-8.
21. Davis ID, Chen W, Jackson H, Parente P, Shackleton M, Hopkins W, et al. Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4(+) and CD8(+) T cell responses in humans. Proc Natl Acad Sci U S A 2004; 101:10697-702.
22. Lu H, Ladd J, Feng Z, Wu M, Goodell V, Pitteri SJ, et al. Evaluation of known oncoantibodies, HER2, p53, and cyclin B1, in prediagnostic breast cancer sera. Cancer Prev Res (Phila) 2012; 5:1036-43.
23. Li Y, Karjalainen A, Koskinen H, Hemminki K, Vainio H, Shnaidman M, et al. p53 autoantibodies predict subsequent development of cancer. Int J Cancer 2005; 114:157-60.
24. Trivers GE, De Benedetti VM, Cawley HL, Caron G, Harrington AM, Bennett WP, et al. Anti-p53 antibodies in sera from patients with chronic obstructive pulmonary disease can predate a diagnosis of cancer. Clin Cancer Res 1996; 2:1767-75.

25. Macallan DC, Wallace DL, Zhang Y, Ghattas H, Asquith B, de Lara C, et al. B-cell kinetics in humans: rapid turnover of peripheral blood memory cells. Blood 2005; 105:3633-40.
26. Manz RA, Hauser AE, Hiepe F, Radbruch A. Maintenance of serum antibody levels. Annu Rev Immunol 2005; 23:367-86.
27. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science 2002; 298:2199-202.
28. Jefferis R. Isotype and glycoform selection for antibody therapeutics. Arch Biochem Biophys 2012; 526:159-66.
29. Steplewski Z, Sun LK, Shearman CW, Ghrayeb J, Daddona P, Koprowski H. Biological activity of human-mouse IgG1, IgG2, IgG3, and IgG4 chimeric monoclonal antibodies with antitumor specificity. Proc Natl Acad Sci U S A 1988; 85:4852-6.
30. Papadea C, Check IJ. Human immunoglobulin G and immunoglobulin G subclasses: biochemical, genetic, and clinical aspects. Crit Rev Clin Lab Sci 1989; 27:27-58.
31. Ullenhag GJ, Frodin JE, Strigard K, Mellstedt H, Magnusson CG. Induction of IgG subclass responses in colorectal carcinoma patients vaccinated with recombinant carcinoembryonic antigen. Cancer Res 2002; 62:1364-9.
32. Mischo A, Bubel N, Cebon JS, Samaras P, Petrausch U, Stenner-Liewen F, et al. Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad antibody responses in humans, a RAYS-based analysis. Int J Oncol 2011; 39:287-94.
33. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996; 383:787-93.

34. van de Winkel JG, Capel PJ. Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. Immunol Today 1993; 14:215-21.
35. Houghton AN, Scheinberg DA. Monoclonal antibody therapies-a 'constant' threat to cancer. Nat Med 2000; 6:373-4.
36. Liu L, Liu N, Liu B, Yang Y, Zhang Q, Zhang W, et al. Are circulating autoantibodies to ABCC3 transporter a potential biomarker for lung cancer? J Cancer Res Clin Oncol 2012; 138:1737-42.
37. Cheng Y, Xu J, Guo J, Jin Y, Wang X, Zhang Q, et al. Circulating autoantibody to ABCC3 may be a potential biomarker for esophageal squamous cell carcinoma. Clin Transl Oncol 2013; 15:398-402.
38. Lu H, Goodell V, Disis ML. Humoral immunity directed against tumor-associated antigens as potential biomarkers for the early diagnosis of cancer. J Proteome Res 2008; 7:1388-94.
39. Murray A, Chapman CJ, Healey G, Peek LJ, Parsons G, Baldwin D, et al. Technical validation of an autoantibody test for lung cancer. Ann Oncol 2010; 21:1687-93.

Titles and legends to figures

Figure 1. Western blotting experiments for verification of ELISA results shown for individual patients.

The first two lanes denotes the control experiment using a goat anti-EN2 antibody and donkey anti-goat IgG-HRP secondary antibody to detect the purified recombinant EN2 in lane 1 whereas lane 2 that contained the control protein DHFR remained negative. Sera from patient 1 and plasma from patients 2 and 3 were EN2 antibody positive in ELISA whereas plasma from patients 4 and 5 were negative in ELISA.

Figure 2. IgG responses to EN2 can be detected in serum of patients with early and late stages of prostate cancer.

Shown are scatter graphs of individual values of serum/plasma immunoglobulin IgG anti-EN2 and anti-NY-ESO-1 antibodies tested by enzyme-linked immunosorbent assay (ELISA) in patients with prostate cancer (SUN study cohort includes men at all stages of prostate cancer, n=353) versus healthy controls (n=214) (A). Serially diluted sera from 4 EN2 antibody-positive patients (■) and sera from 4 healthy volunteers (□) are shown (B). Patients were then grouped according to their combined Gleason score (C) or their T stage (1-4) (D). The solid black lines indicate the mean. The blue lines indicate the cut-off values calculated as the mean value plus three standard deviations from the data pool of the control sera/plasma samples. Values above the cut-off were considered as positive. The levels of antibodies against the recombinant proteins EN2 and NY-ESO-1 are expressed in optical density (OD). The proportion of IgG responders to each antigen were compared between patients and healthy controls using a chi-squared test. The proportion of patients responding to EN2 and NY-ESO-1 were found to be statistically different from the healthy controls ($p<0.001$; *** and $p<0.05$;* respectively).

Figure 3. Detectable tumour burden needs to be present in order for a measurable EN2 IgG response to be present.

Shown are scatter graphs of individual values of serum/plasma immunoglobulin IgG anti-EN2 and anti-NY-ESO-1 antibodies tested by enzyme-linked immunosorbent assay (ELISA) in patients with A: castrate resistant disease with rising PSA despite LHRH agonist hormone treatment with normal radiological evaluation and no symptoms, designated CRRPSA (castrate resistant, rising PSA) LREC (n=121) , B: castrate resistant disease with radiological evidence of metastatic disease (n=107) and C: individuals at high risk of cancer (IMPACT cohort) (n=448). In each cohort the proportion of EN2 or NY-ESO-1 IgG responders was compared to that found in healthy control males. The solid black lines indicate the mean. The blue lines indicate the cut-off values calculated as the mean value plus three standard deviations from the data pool of the control sera/plasma samples. Values above the cut-off were considered as positive. The levels of antibodies against the recombinant proteins EN2 and NY-ESO-1 are expressed in optical density (OD). The proportion of patients responding to each antigen was compared between patients and healthy controls using a chi-squared test. The proportion of patients responding to NY-ESO-1 within the CRRPSA cohort were found to be different from the healthy controls ($p<0.01$; **) (A). In the castrate resistant disease with radiological evidence of metastatic disease group (B) the proportion of IgG responders to both EN2 and NY-ESO-1 were found to be different from healthy controls ($p<0.001$; *** and $p<0.05$;* respectively). No statistical difference in the proportion of IgG responders to either EN2 or NY-ESO-1 were found within individuals at high risk of cancer (IMPACT cohort) compared to healthy controls.

Figure 4. IgG subclass analysis of EN2 antibody response reveals both IgG1 and IgG3 responses

Plasma/sera from EN2 seropositive prostate cancer patients were firstly diluted to detect full-length EN2 coated wells in a standard ELISA. After blocking, diluted murine monoclonal antibodies specific to various human Ig isotype (IgG1-IgG4) were added to determine the Ig subtypes of EN2 specific antibodies in these plasma/sera. Representative results are shown from six patients demonstrating different patterns of IgG1 and/or IgG3 responses.

Figure 5. Persistence of EN2 antibody responses in prostate cancer patients

The longitudinal EN2 antibody response measured by ELISA was determined in EN2 sero-positive patients. Sera were obtained at various time points over a period ranging from 34 to 45 months in different patients. The O.D. value was measured at wavelength 450nm. Values obtained with serum dilutions of 1:100 are shown. Representative results of this longitudinal assessment of the EN2 autoantibody response are shown for patients who were under active surveillance, patients who had received radiotherapy and patients who had undergone radical prostatectomy. All patients studied remained EN2 seropositive (above the cut-off values calculated as the mean value plus three standard deviations from the data pool of the control sera samples, blue line) over the time period studied except patient SUN147 who became sero-negative following radical prostatectomy.

Figure 6. No significant detection of EN2 IgG responses in female oncological patients
Shown are scatter graphs of individual values of serum/plasma immunoglobulin IgG anti-EN2 and anti-NY-ESO-1 antibodies tested by enzyme-linked immunosorbent assay (ELISA) in patients with breast cancer (A) or ovarian cancer (B) versus healthy sex and age-matched controls. The solid black lines indicate the mean. The blue lines

indicate the cut-off values calculated as the mean value plus three standard deviations from the data pool of the control sera/plasma samples. Values above the cut-off were considered as positive. The levels of antibodies against the recombinant proteins EN2 and NY-ESO-1 are expressed in optical density (OD). The proportion of IgG responders to each antigen were compared between patients and healthy controls using a chi-squared test. The proportion of IgG responders to NY-ESO-1 were found to be different in both the breast cancer and ovarian cancer patient cohorts compared to healthy controls ($p<0.001$; *** and $p<0.01$;** for the breast cancer cohorts and $p<0.001$;*** for the ovarian cancer cohort).