Human IgG antibody profiles differentiate between symptomatic patients with and without colorectal cancer


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Human IgG antibody profiles differentiate between symptomatic patients with and without colorectal cancer

G Kijanka,1 S Hector,1 E W Kay,2 F Murray,3 R Cummins,4 D Murphy,1 B D MacCraith,5 J H M Prehn,1 D Kenny4

ABSTRACT

Objective: Patients with cancer have antibodies against tumour antigens. Characterising the antibody repertoire may provide insights into aberrant cellular mechanisms in cancer development, ultimately leading to novel diagnostic or therapeutic targets. The aim of this study was to characterise the antibody profiles in patients whose symptoms warranted colonoscopy, to see if there was a difference in patients with and without colorectal cancer.

Methods: Patients were recruited from a colonoscopy clinic. Individual serum samples from 43 patients with colorectal cancer and 40 patients with no cancer on colonoscopy were profiled on a 37 830 clone recombinant human protein array. Antibody expression was evaluated by quantitative reverse transcription-PCR and by immunohistochemistry on tissue microarrays.

Results: Using a sex- and age-matched training set, 18 antigens associated with cancer and 4 associated with the absence of cancer (p<0.05) were identified and confirmed. To investigate the mechanisms triggering antibody responses to these antigens, antigen expression was examined in normal colorectal mucosa and colorectal carcinoma of the same patients. The identified antigens showed cellular accumulation (p53), aberrant cellular expression (high mobility group B1 (HMGB1)) and overexpression (tripartite motif-containing 28 (TRIM28), p53, HMGB1, transcription factor 3 (TCF3), longevity assurance gene homologue 5 (LASS5) and zinc finger protein 346 (ZNF346)) in colorectal cancer tissue compared with normal colorectal mucosa.

Conclusions: It is demonstrated for the first time that screening high-density protein arrays identifies unique antibody profiles that discriminate between symptomatic patients with and without colorectal cancer. The differential expression of identified antigens suggests their involvement in aberrant cellular mechanisms in cancer.

Colorectal cancer is the leading cause of cancer-related mortality in the Western world. Deaths from colorectal cancer can be prevented through effective screening.1–3 Recent studies have defined specific antibody responses to tumour-related antigens in patients with cancer.4–7 Since these antibodies are often triggered by changes in the structure or expression of self proteins in tumour cells,8,9 they may serve as potential immunological markers of cancer.10,11 Various technologies have been used to identify cancer-specific antibodies. Phage display offers a powerful platform to identify antibody signatures. However, phage display technology is labour intensive, and peptides expressed by bacteriophage often do not correspond to native antigens, thus limiting identification of molecular targets in cancer.12 Proteomics is a novel approach to identify tumour antigens. This method largely relies on tumour cells as a source for antigens. Detection of low abundant proteins and membrane proteins is problematic with this approach. Large protein arrays provide a unique opportunity to profile antibody signatures from libraries containing thousands of different proteins. A significant advantage of large protein arrays is that complex antibody repertoires from cohorts of patients can be easily identified.

In this study, we set out to characterise the antibody profile of patients presenting to hospital whose symptoms warranted colonoscopy. We screened >1800 patients until a cohort of 40 sex- and age-matched patients, with and without colorectal cancer, was identified. We characterised the antibody profile in this training set and then in an additional extended set of 23 patients with cancer and 20 patients without cancer. In patients with colorectal cancer, we identified a unique antibody signature consisting of 18 markers. We demonstrate disease-associated alterations in the expression of corresponding antigens in tissue from patients with colorectal cancer, which may serve as new diagnostic markers or new therapeutic targets. For symptomatic patients without cancer, we also defined an antibody signature consisting of four antigens, which we termed “antimarkers”.

Serum antibodies to p55, a known marker for cancer, were identified as part of the cancer signature. The expression of p55 in tissue from patients with cancer was confirmed, showing the utility of this screening approach. Serum antibodies to the antigen high mobility group B1 (HMGB1) were found significantly more often in patients with cancer. Immunohistochemistry of colorectal cancer samples demonstrated aberrant HMGB1 expression in tissue. Other antigens identified as a part of the colorectal cancer antibody signature showed upregulation of their corresponding genes in cancer tissue, foremost the tripartite motif-containing 28 (TRIM28) gene with overexpression in the majority of analysed colorectal carcinomas. This study demonstrates the utility of screening serum samples with high-density protein arrays in appropriate cohorts of patients. Our results confirm the presence of antigens known to be expressed in colorectal cancer and identify novel...
antigens that may be important in the diagnosis and treatment of cancer.

MATERIAL AND METHODS

Patients and samples

This study was approved by the Ethics (Medical) Research Committee at Beaumont Hospital, Dublin. Informed consent was obtained from all patients. A total of 1820 patients undergoing colonoscopy were screened daily by the clinical research nurses. Patients with a history of cancer, systemic inflammatory or autoimmune disease and patients taking immunosuppressive medication were excluded from the study. The colonoscopy findings were reviewed with the consultant physician and, if a diagnosis of cancer or normal colonoscopy was made, then patients were eligible to participate. Subjects were then asked to provide a blood sample. In all patients with a clinical diagnosis of cancer, histology findings were reviewed to confirm the diagnosis. In total, 43 Caucasian patients with newly diagnosed colorectal cancer and 40 Caucasian patients with no neoplasm of the colon (non-cancer controls) fulfilled the inclusion criteria and were recruited consecutively. From this cohort the 20 patients with cancer and 20 sex- and age-matched non-cancer controls were used as a training set to identify cancer- and non-cancer-associated antibody profiles. The diagnosis of cancer was independently verified on tissue samples by a pathologist. Blood was obtained from all patients prior to chemotherapy, radiation and surgical treatment. Serum was prepared and stored at −80°C. Serum samples were coded and sent blinded for antibody profiling.

Protein arrays

High-density protein arrays were used to screen serum samples (hEx1, Imagenes, Berlin, Germany). The hEx1 human cDNA library was directionally cloned in an Escherichia coli vector that allows expression of His6-tagged fusion proteins in the presence of isopropyl-β-D-1-thiogalactopyranoside (IPTG). A total of 198 556 hEx1 clones were screened for expression of His6-tagged fusion proteins. In total, 57 850 clones expressing recombinant human proteins were identified and used for generation of high-density protein arrays. The array has a minimum of 10 000 unique proteins.

Serum screening

To identify immunoglobulin G (IgG) antibodies in serum of patients, the hEx1 protein arrays were prepared as described. Briefly, hEx1 protein arrays were incubated with diluted serum (1:100) for 16 h. Mouse antihuman IgG antibody (GG-7, Sigma-Aldrich, St Louis, Missouri, USA), alkaline phosphatase (AP)-conjugated goat antimouse IgG antibody (A1418, Sigma-Aldrich) and the substrate Attrophos (JBL Scientific, San Luis Obispo, California, USA) were used as detection reagents (online supplementary method 1). Images were captured with a Fuji LAS 3000 imager.

Protein array analysis

High-resolution images of protein arrays incubated with serum and detection antibodies were analysed using Visual Grid software (GPC Biotech, Martinsried, Germany). Each clone on the array is arrayed in duplicate; only clones showing distinct signals in both positions on the array were defined as positive (fig 1). Analysis of each protein array gave a list of positives which corresponds to a unique antibody profile for every subject. The unique antibody profile from each subject was compared with that of all other subjects. The clones identified in this analysis were then compared between patients with and without cancer by Fisher exact test. False-positive signals caused by binding of the detection antibodies (antihuman IgG and antimouse AP antibodies) directly to proteins on the array were identified by probing the protein array with the detection antibodies in the absence of human serum. These clones were excluded from the analysis.

Gene expression by real-time quantitative reverse transcription-PCR (RT-PCR)

Gene expression of antigens identified in the serum profiling was measured in tissue samples using real-time quantitative RT-PCR (Taqman assay) with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Tumour and normal tissue were available for 16 of the 43 patients with colon cancer. All samples for gene expression analysis were stored in RNAlater at −20°C. Total RNA was extracted using Qiagen RNeasy spin columns (Qiagen, Hilden, Germany) and cDNA was synthesised using Superscript II reverse transcriptase (Invitrogen, Carlsbad, California, USA) followed by PCR with Applied Biosystem’s 7500 Real-Time PCR System. The mRNA levels of the gene of interest and that of the internal standard (β-actin) were measured concurrently from the same cDNA preparations. The comparative CT method was used to quantitate gene expression. Briefly, the normalised value of expression for the target gene with reference to β-actin was obtained by 1/2^(-ΔΔCT). 2^(-ΔΔCT) is derived from Xn = X0 (1+E)n, where X0 is the initial number of target molecules, E the efficiency of amplification, and n the number of cycles (supplementary methods online). All mRNA expression values are ratios relative to β-actin and are expressed as x10^-3. For each gene, n = 1 or 2, each consisting of three separate PCRs.

ZNF700 (zinc finger protein 700) primers and probes were purchased as a “made to order” gene expression assay. β-Actin primers and probes were purchased as a ready to use predeveloped assay reagent (PDAR, Applied Biosystems) with a 5’ FAM (6-carboxy-fluorescein) reporter dye and 3’ TAMRA (6-carboxy-tetramethylrhodamine) quencher. All other primers and probe sets were purchased as ready to use inventoried gene expression assays (Applied Biosystems). Both “made to order” and inventoried probes are MGB (minor groove binding) with a 5’ FAM reporter dye and 3’ NFQ (non-fluorescent quencher).

Immunohistochemistry on tissue microarrays (TMAs)

TMAs were constructed from formalin-fixed, paraffin-embedded tissue as previously described. Tissue from 43 patients with colorectal cancer and 19 patients with no neoplasm of the colon, from surgical resections or biopsy samples, was examined for antigen expression. All staining was conducted on the BondMax automated immunohistochemical staining platform (Vision BioSystems, Newcastle, UK). Sections were incubated with primary antibody against p53 (DO-7, Dako, Glostrup, Denmark) and HMGB1 (M02, Abnova, Heidelberg, Germany). Detection and visualisation of stained cells was achieved using the Bond Polymer Refine Kit (Vision BioSystems) with DAB (diaminobenzidine) as the chromogen.

Statistical analysis

Fisher two-sided exact test was employed to compare frequencies for each identified antigen in patients with colorectal cancer.
and in non-cancer control subjects. A p value <0.05 was considered statistically significant. Statistically significant antigens identified in the training set were reassessed in the extended set. To correct for multiple testing of statistically significant antigens, we applied the Bonferroni correction at a level of \( \alpha = 0.05 \) and the false discovery rate (FDR) method at a level of \( q = 0.05 \), for both markers and antimarkers separately. Sensitivity and specificity were calculated according to Altman and Bland.\(^{21}\) For gene expression studies, mean ratios of tumour to normal mRNA levels were shown with the standard error (SE), and p values were determined using Wilcoxon signed-rank test.

**RESULTS**

**Patient samples**

A total of 1820 patients attending the colonoscopy clinic were screened. Initially, a training set of 20 patients with colorectal cancer and 20 sex- and age-matched patients with no cancer was identified. To identify a cancer-specific antibody signature in patients with colorectal cancer, we analysed the frequencies of antibody detection in this sex- and age-matched training set (table 1). An additional 23 patients with colorectal cancer and 20 patients with no cancer were further characterised. All 20 patients with colorectal cancer and 20 patients without cancer of the training set and the additional 23 patients with colorectal cancer and 20 patients with no cancer of the extended set were screened on high-density protein arrays, and individual profiles were identified. The resulting antibody profiles identified in the training set were further characterised in the extended cohort. We identified an antibody signature for colorectal cancer including antibodies against p53, HMGB1 and TRIM28. We then characterised the protein and RNA expression of the known cancer antigen p55 in tissue from this patient cohort to validate our approach. In addition, we further characterised the expression of HMGB1 by both protein and RNA expression. The expression of TRIM28 and other identified antigens was characterised by RNA levels alone to assess their potential as molecular markers of cancer. The algorithm for analysis is summarised in fig 2.

**Antibody profiles**

The antibody profiles for all 83 subjects were characterised blinded to any clinical data. The antibody profile for each subject was characterised. When two identical clones on the array were recognised by serum antibodies, this was defined as positive (fig 1). An average of 833 positives was identified for each subject. Although individual profiles were unique, many of the positives were shared by different individuals. Combining the antibody profiles for the 83 subjects showed that 19 645 different clones were positive in one or more individuals. Thus 52% of the 37 830 clones present on the hEx1 protein array were recognised by antibodies from the total cohort of subjects. Of these clones, 11 703 were positive in two or more sera. When the protein array data were analysed, the clinical information was unblinded and the data were analysed as patients with and without cancer (fig 3). The diagram illustrates the heterogeneous distribution of antibodies among

**Table 1** Clinical characteristics of the training set

<table>
<thead>
<tr>
<th></th>
<th>Patients with colorectal cancer (n = 20)</th>
<th>Non-cancer controls (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>10 (50%)</td>
<td>10 (50%)</td>
</tr>
<tr>
<td>Male</td>
<td>10 (50%)</td>
<td>10 (50%)</td>
</tr>
<tr>
<td>Age at diagnosis, years</td>
<td>Median (IQR)</td>
<td>62 (51–67)</td>
</tr>
<tr>
<td></td>
<td>61 (50–69)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
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<td>Never</td>
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<td>15</td>
</tr>
<tr>
<td>Current</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
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<td>2</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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<td>6</td>
<td>4</td>
</tr>
<tr>
<td>&lt;10 units per week</td>
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<td>14</td>
</tr>
<tr>
<td>10–20 units per week</td>
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</tr>
<tr>
<td>21–30 units per week</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>&gt;30 units per week</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Aspirin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Yes</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>
patients with colorectal cancer and non-cancer controls. Antibodies to >9000 antigens were identified exclusively in either group. Additionally, antibodies to >9000 antigens were identified in both patients with and without cancer.

Analysis of the antibody repertoire

All positives identified on the array were ranked according to statistical significance to discriminate between patients with cancer and controls, using Fisher exact test. Antibodies to 43 antigens in the cancer group and 9 antigens in the non-cancer control group, identified in the training set, correlated significantly with the presence or absence of colorectal cancer. To characterise the total patient cohort we then expanded the sample size and re-analysed the p values for the entire patient cohort. Of the antigens identified in the training set, 18 antigens from the cancer group and 4 from the non-cancer group were confirmed in this extended set (table 2).

We then set out to determine combinations of antigens that could discriminate between patients with and without colorectal cancer. All 18 antigens (table 2) derived from the training set and the extended set were examined separately. Starting with the antigen most significantly associated with cancer versus the non-cancer group (lowest p value), a predictive ability was calculated based on specificity and sensitivity. By adding next ranked antigens stepwise, the predictive ability of the combined antigens was reassessed until the optimal combination was found. The analysis resulted in a subset of 12 antigens discriminating between 43 patients with colorectal cancer and 40 control patients without cancer with 80% specificity and 83.7% sensitivity.

Identified cancer antigens

Analysis of the array data in the training set and the extended set identified 18 antigens in the cancer group and 4 antigens associated with absence of cancer (table 2). These antigens range from membrane proteins (TSLC1 (tumour suppressor in lung cancer 1)), cytoplasmic proteins (SNP29), nuclear proteins (p53) to hypothetical proteins (FLJ10154). Apart from clone G19547 expressing p53, all markers and antimarkers listed in table 2 were expressed by one cDNA clone. An additional p53 clone (E16563) expressed a shorter, 178 amino acid p53 fragment immunoreactive with four colorectal cancer sera. The length of identified antigens varied from large fragments to full-length cDNA clones (online supplementary table S1).

Accumulation of p53 protein in tumour cells is known to trigger p53 antibody production in patients with colorectal cancer. In our study, antibodies to p53 were identified in 19% of patients with colorectal cancer and were absent in non-cancer controls. The expression of p53 is a well recognised marker of cancer; thus we characterised p53 protein expression in our patient cohort to confirm the utility of our approach. Tissue samples from excised cancers were examined. TMAs were constructed from the same group of patients used in the serum screening. Immunohistochemistry results showed that p53 stained weakly in normal colorectal tissue, whereas 51% (22/43) of colorectal cancer tissues stained strongly for p53 (fig 4A), correlating with advanced tumour stages (fig 4B). Anti-p53 antibodies were found to be present in seven cancer patients strongly expressing the protein in the tumour tissue (fig 4C). One patient, who was positive for p53 antibody in serum, did not stain for p53 in tissue. At the gene expression level, p53 mRNA was elevated in tumour tissue compared with adjacent normal tissue in 15 of the 16 (81%) patients examined (p = 0.002) (fig 4D).

We assessed the 18 identified cancer markers in relation to Dukes stages in patients. Of the 8 patients with serum antibody to p53, 1 patient was diagnosed Dukes A and 1 patient Dukes B, while 2 patients were Dukes C and 4 were Dukes D. These data suggest that the presence of serum p53 antibodies may correlate with advanced stage in colorectal cancer. While p53 antibodies may correlate with advanced Dukes stage, the other 17 antigens do not show such a trend.

Molecular targets in colorectal cancer

p53 and other proto-oncogenes such as HER-2/neu are known to trigger antibody responses in patients with cancer and are associated with tumour pathogenesis. HMGB1 is postulated to be an oncoprotein and has been implicated in the pathogenesis of colorectal cancer, but there is a paucity of literature on the antibody profile in patients with colorectal cancer. In our study, antibodies to HMGB1 were found to be present in seven cancer patients strongly expressing the protein in the tumour tissue (fig 4C). One patient, who was positive for HMGB1 antibody in serum, did not stain for HMGB1 in tissue. At the gene expression level, HMGB1 mRNA was elevated in tumour tissue compared with adjacent normal tissue in 73 of the 76 (97%) patients examined (p < 0.0001) (fig 4D).

Therefore, we investigated the expression of HMGB1 in tissue. Figure 5A shows representative images of HMGB1 staining in tumour tissue. We identified nuclear staining for HMGB1 in 93% (40/43) of tumour tissue samples and in 97% (37/38) of normal colorectal tissue from the same patients (fig 5B). The nuclear localisation of HMGB1 was in concordance with previous studies. We did, however, detect cytoplasmic staining in subpopulations of tumour cells. Sixty-seven percent (29/43) of tumours showed cytoplasmic staining for HMGB1, whereas...
only 5% (2/38) of normal tissue samples from the same patients showed cytoplasmic HMGB1 localisation (p < 0.0001). We also analysed the expression of HMGB1 in tissue of patients without cancer. All 19 cases showed nuclear staining and 11% (2/19) showed an additional cytoplasmic staining for HMGB1. These findings suggest that cytoplasmic expression of HMGB1 occurs preferentially in malignant cells and may have implications in tumour pathogenesis. At the gene expression level, similar results were found for HMGB1 as for p53, with 13 of 16 patients (81%) showing elevated expression of HMGB1 mRNA in tumour compared with matched normal tissue (p = 0.001).

In addition to p53 and HMGB1, we also examined mRNA levels of TSLC1, a marker identified in the screen that has been implicated in cancer.30 We also examined a number of these markers whose role in cancer is unclear. Gene expression of TSLC1, TRIM28, LASS5 (longevity assurance gene homologue 5), TCF3 (transcription factor 3; E2A immunoglobulin enhancer-binding factors E12/E47) and zinc finger motif genes ZNF346, ZNF638, ZNF700 and ZNF768 were examined in matched tumour and normal tissue. The majority of patients also showed elevated mRNA expression levels of TRIM28, LASS5, TCF3 and ZNF346 in tumour tissue compared with matched normal colonic tissue (fig 6). mRNA levels of TSLC1, ZNF638, ZNF700 and ZNF768 were elevated in a small number of patients, but not to a statistically significant level (online supplementary fig S1).

**DISCUSSION**

The aim of this study was to determine if large-scale protein arrays could identify antibodies that discriminate between symptomatic patients who warranted colonoscopy with and without cancer. We show that the antibody profile between these two sets of patients is different. By examining tissue from these cohorts we confirmed that there was a correlation between p53 antibodies and expression in tissue in patients with colorectal cancer. To validate the relevance of the antibodies detected in the colorectal cancer group further we investigated the tissue expression of identified antigens. The aberrant tissue expression of the novel antigen HMGB1 and the upregulation of mRNA levels of TRIM28 and several other
genes confirm that our approach of screening high-density protein arrays identifies biologically relevant antigens discriminating between two cohorts of patients. Thus our results show that antibody detection discriminates between these cohorts of patients.

Several technologies have been used to identify cancer-specific antibodies. Peptide libraries derived from tumour tissue using phage display have revolutionised the search for cancer antigens. However, technological difficulties have hampered the identification of complex antibody repertoire. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires. In the present study, protein arrays represent a powerful alternative to the identification of complex antibody repertoires.

The finding that several known antibodies identified in this study were previously described in other cancers shows that many of the pathways of tumourogenesis may be shared by different cancers. Moreover, screening the arrays used in this study with samples from patients with inflammatory bowel disease to see if the antigens identified are similar to those with colorectal cancer may well identify new pathways in the pathogenesis of colon cancer.

Our results demonstrate previously described antigens to colorectal cancer such as the tumour suppressor p53. Correlation of p53 antibodies with p53 protein expression in cancer tissue further validates this approach. Anti-p53 serum antibodies have been successfully used as predictors of other cancers. However, despite their specificity, p53 antibodies are unsuitable for population screenings, as only 20–30% of patients with colorectal cancer have these antibodies. The combination of several antigens with high specificity may prove effective in clinical decision making such as referral for colonoscopy, or potentially may assist in treatment or diagnosis of recurrent disease. We performed a stepwise procedure to assess the predictive ability of the marker set and identified a highly specific and sensitive set of markers. The predictive ability of the marker set was also corrected using statistical methods. A Bonferroni correction at a level of $\alpha = 0.05$ was applied to our set of 18 markers and 4 antimarkers, this resulted in a set of 7 markers. The combination of several antigens with high specificity may prove effective in clinical decision making such as referral for colonoscopy, or potentially may assist in treatment or diagnosis of recurrent disease. We performed a stepwise procedure to assess the predictive ability of the marker set and identified a highly specific and sensitive set of markers. The predictive ability of the marker set was also corrected using statistical methods. A Bonferroni correction at a level of $\alpha = 0.05$ was applied to our set of 18 markers and 4 antimarkers, this resulted in a set of 7 markers and 2 antimarkers with statistical significance for multiple testing. The Bonferroni correction is, however, a conservative method and, when applied to our marker set, it excluded markers such as HMGB1 or TRIM28 which we show to be of biological relevance. Using the less restrictive FDR, we found 14 of 18 markers and all 4 antimarkers with statistical significance for multiple testing.

Table 2  Markers and antimarkers identified in serum screening

<table>
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<tr>
<th>Protein name</th>
<th>Swiss-prot ID</th>
<th>RZPD clone ID</th>
<th>Colorectal cancer (n = 43)</th>
<th>Non-cancer controls (n = 40)</th>
<th>p Value</th>
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<td></td>
<td></td>
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<tr>
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<td>L03527</td>
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<td>LASS5</td>
<td>M17513</td>
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<td>0</td>
<td>0.006</td>
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<tr>
<td>Cellular tumour antigen p53</td>
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<td>G19547</td>
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<td>N24509</td>
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<td>E2A immunoglobulin enhancer-binding factors E12/E47</td>
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The analysis of serum from 43 patients with colorectal cancer and 40 non-cancer controls identified these 18 antigens associated with colorectal cancer ($p < 0.05$) and 4 antigens associated with non-cancer controls ($p < 0.05$). p values were calculated using two-sided Fisher exact test. Corrected for multiple testing: *Bonferroni; *false discovery rate (FDR); *stepwise analysis.
Figure 4  Overexpression of tumour suppressor p53 in colorectal cancer tissue correlates with advanced Dukes stages and p53 serum antibodies in patients with colorectal cancer.  (A) Representative examples of tissue microarray-based immunohistochemical (IHC) staining using monoclonal antibody to p53 (DO-7) (×20). (a) Normal colorectal mucosa with weak nuclear p53 staining. (b) Colorectal carcinoma with strong nuclear p53 staining. (B) The intensity of p53 staining in tumour tissue increases with advanced Dukes stage as identified by immunohistochemical analysis of all 43 examined cases. (C) Serum antibodies to p53 correlate with expression of p53 protein in cancer tissue. (D) p53 gene expression is significantly elevated in colorectal tumours compared with adjacent normal tissue.  (A) Ratios between p53 expression at the mRNA level in tumour tissue compared with adjacent normal tissue in 16 patients, mean (SE).  (B) The graph represents the gene expression at the mRNA level in colorectal tumour and matched normal colonic tissue; the line represents the average expression in either tumour or normal tissue.  The p value was calculated using Wilcoxon rank-sum.

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The humoral immune response may be used for diagnosis but may also elucidate mechanisms of the disease. The HMGB1 protein identified in this study may have a role in suppression of antitumour immunity. HMGB1 is a non-histone chromatin-associated protein with a variety of roles including transcriptional regulation and DNA repair, and it functions as a cytokine. It has been implicated in chemotherapy resistance and in tumour metastasis. HMGB1 is postulated to be an oncoprotein and its protein expression has been shown to be altered in a variety of human cancers. We show that HMGB1 mRNA is elevated in colorectal cancer tissue compared with matched normal colon tissue, a finding also found by Yu et al. At the protein level, HMGB1 has been shown to be overexpressed in colorectal cancer. While HMGB1 has been implicated in the pathogenesis of colorectal cancer, there is a scarcity of literature on the antibody profile in patients with colorectal cancer.

Using immunohistochemistry, we showed nuclear localisation of HMGB1, which was in concordance with previous studies. Moreover, we also found that HMGB1 was more frequently present in cytoplasm of cancer cells than in cells from normal colorectal tissue from the same patients. The predominant cytoplasmic expression of HMGB1 in malignant cells suggests a potential mechanism in eliciting antibody production to this antigen.

Examination of mRNA levels of markers identified in this study indicates that some of these markers are elevated in colorectal cancer tissue. Of those genes that were found to be differentially expressed at the mRNA level, little is known about their relationship to cancer. The transcriptional co-repressor TRIM28 was previously linked with coordination of cellular DNA damage responses and differentiation of embryonal carcinoma cells and a transcriptional regulator of the interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) signalling pathway. The transcription factor TCF3 is a transcriptional repressor of E-cadherin, implicated in epithelial to mesenchymal transition, and may be linked to tumour aggressiveness. Additionally it is involved in the Wnt signalling cascade, which is linked to colorectal cancer progression. LASS5 is a C16 ceramide synthase; ceramides play a role in apoptosis and may act in modulating response to chemotherapeutic agents. Members of the LASS family have been shown...
to be involved in cell growth regulation, cancer differentiation and response to chemotherapy. ZNF346 may play a role in cell growth and survival, and when ectopically expressed induces apoptosis. The differential increase in the expression of these genes in colorectal cancer tissue suggests that these proteins may play a role in the pathogenesis of colorectal cancer.

Our screening studies demonstrate the complexity of antibody responses in patients with and without cancer. The process by which these and other self proteins become immunogenic is not yet understood. Progression of cancer correlates with enhanced deregulation of key signalling pathways and therefore different stages of cancer may trigger different antibody profiles. In this study we showed that p53 antibodies correlated with advanced Dukes stage, while other antibodies were found in patients with all stages of cancer. Since early detection of cancer is a key factor in patients' outcome, a combination of markers encompassing all stages of cancer may aid in earlier detection of the disease. Another interesting feature of our study was the detection of four antibodies in symptomatic patients associated with absence of cancer. Such antimakers may help decide if colonoscopy procedures are required or may suggest mechanisms that are downregulated in the pathogenesis of cancer.

In summary, we show for the first time that screening serum from symptomatic patients with large protein arrays identifies both known and new antibodies that may have a role in the diagnosis and treatment of patients with colorectal cancer.

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**Competing interests:** None.

**Ethics approval:** This study was approved by the Ethics (Medical) Research Committee at Beaumont Hospital, Dublin.

**Provenance and peer review:** Not commissioned; externally peer reviewed.

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Colon cancer

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