Abstract

Purpose: The purpose of the present study was to screen the autoantibody signature of colon cancers to develop serum markers for colon cancer detection.

Experimental Design: A phage cDNA expression library of colon cancer was built. The library was sequentially screened by a pool of 10 colon cancer sera, goat antihuman IgG, and a pool of two healthy sera to identify phage-expressed antigens recognized by tumor-associated antibodies. The clones picked out by these screening were subjected to a training set with 24 colon cancer sera and 24 healthy sera. The antigen combination, which got the most satisfactory classification, was tested by an independent set of 24 colon cancer sera with equal number of sera from normal donors. The carcinoembryonic antigen (CEA) level of these sera was detected for the additional classification analysis with or without the antigen combination.

Results: A cDNA expression library consisting of $2^{15}$ primary clones was prepared. After three turns of screening, 24 antigens recognized by tumor-associated antibodies were picked out for serum marker identification. The training set showed that a six-marker combination got the most satisfactory classification in a logistic regression model; leave-one-out validation achieved 91.7% sensitivity and 91.7% specificity. In a testing set with this marker panel, we correctly predicted 85% of the samples. Although according to CEA level alone, we correctly predicted 75% of the samples with 42% of cancer patients misclassified. When CEA was combined with the six markers, the sensitivity and specificity increased to 91.7% and 95.8%, respectively. The six antigen sequences in the phage display system are relatively short peptides. Only two of them showed homology to known protein sequences.

Conclusions: Autoantibodies against phage-expressed antigens derived from colon cancer tissues could be used as serum markers for the detection of colon cancer.

Early detection and diagnosis are of utmost importance in cancer therapies. The immunoassay testing serum biomarkers in body fluid samples has been widely used in cancer detection, such as carcinoembryonic antigen (CEA) for colon cancer (1), prostate-specific antigen for prostate cancer (2), CA15-3 for breast cancer (3), CA19-9 for gastrointestinal cancer (4), and CA125 for the diagnosis of ovarian cancer (5). However, these available markers, which based on cancer antigen determinations, have not been recognized as ideal cancer biomarkers due to the limited sensitivity and specificity (2).

Multiple studies have shown that cancer patients produce detectable autoantibodies to certain tumor-associated antigens. And these immunoreactions may be indispensable for cancer development (6). Detection of the humoral immune response to tumor-associated antigens, which are recognized as “foreign” by the immune system, provides information about the tumor status (7–11). Profiling cancer antigen–related autoantibodies have shown potential in early detection of cancers. Zhong et al. (12) showed that autoantibody measurements successfully predicted lung cancer before radiographic detection on incidence screening. Detection of autoantibodies may also help the metastasis prediction. Antiprostasome autoantibodies have been reported to indicate a high tendency of metastasis in prostate cancer (13). By serologic analysis of recombinant cDNA expression libraries of human tumors with autologous serum (SEREX), several colon cancer–related autoantibodies have been identified, some of which frequently existed in cancer patients’ sera (14, 15).

This study describes an approach to detect serum autoantibodies, which based on a modified SEREX screening to clone and evaluate the potential of these tumor antigens as biomarkers for a serum-based screening test to detect the presence of colon cancers.
Materials and Methods

Tumor and serum samples. Fresh colon carcinoma tissue was obtained from a 72-yr-old man of a high grade colon adenocarcinoma with multiple liver and lymph node metastasis. The primary cancerous tissue was procured by an experienced pathologist from the surgical resection specimen of Cancer Hospital, Chinese Academy of Medical Science, Beijing and was frozen immediately in liquid nitrogen until used for library construction. Serum was also collected from the patient as autologous serum for the library screening. Nine allogeneic sera, collected from two high-grade cancers patients, four middle-grade cancers patients, and three low-grade cancers patients were pooled with the autologous serum for primary immune screening. Sera of two healthy donors were collected and pooled for further negative immune screening. Additional serum samples for validation and test were obtained from 48 patients with colon cancer and 48 healthy donors. Cancer sera and healthy sera were collected from department of abdominal surgical oncology and department of cancer prevention, Cancer Hospital Chinese Academy of Medical Science, respectively. All sera were collected before any treatment procedure and stored at -80°C until use. All the donors signed informed consent forms for sample collection.

RNA extraction and construction of cDNA expression library. The cDNA library was constructed from the cancerous tissue of colon cancer. An amount of 5 μg of mRNA were used for reverse transcription, and the cDNA was ligated into the λ-ZAP expression vector (Stratagene). After in vitro packaging, a library containing 2 × 10⁶ primary recombinants was obtained. The cDNA library was not amplified before immuno-screening.

Immunoscreening of cDNA expression library. All the serum samples were preabsorbed to reduce antibodies reactive with antigens related to the vector system as described by Lee et al. (16). Absorbed sera were finally diluted to 1:50 in 1% bovine serum albumin, 1 × TBS, and 0.02% NaN₃ and stored at 4°C before use. Library screening was done as described (6, 17). Plaques expressing positively immunoreactive proteins were picked for a second round screening to exclude clones directly reacted with goat anti-human IgG antibody. The remaining clones were restested with the pooled patients’ sera by the same screening method to confirm the primary seroreactivity. For further negative immune screen, the positive clones were screened by pooled healthy donors’ sera. Those did not react with the healthy sera were subjected to following analysis.

Nucleotide sequencing and bioinformatics analysis. Serum-positive clones were subcloned and excised in vivo to phagemid form using the ExAssist helper phage (referred to instruction manual of ZAP-cDNA Gigapack III Gold Cloning kit; Stratagene). Plasmid DNA was then purified and subjected to DNA sequencing using an ABI Prism automated sequencer (Genecore Biotechnologies Corporation). When possible, phage identity was made based on significant nucleotide and translated peptide matches with a single gene in the Genbank database using nucleotide BLAST and protein BLAST search engines.

Phage plaque assay. To investigate the seroreactivity of isolated clones in colon cancer patients and healthy donors, a phage plaque assay was done according to the standard procedure (17). λ-ZAP phage without insert was used to transform exponentially growing Escherichia coli, with XL1-Blue as a negative control. Nitrocellulose membranes with isopropyl-l-thio-B-D-galactopyranoside – induced Plaque-derived proteins were cut to squares with 4 to 8 plaques on them for reactivity with 48 cases of colon cancer and 48 cases of healthy serum samples. Assay was scored positive only when reactive color of tested clones was clearly distinguishable from control clones.

Statistical analysis. Statistical analysis was done with Statistical Package for the Social Sciences software, version 10.0. Antibody reactions to SEREX-defined antigen in the sera of colon cancer patients and normal controls were analyzed by t-tests to determine if they were correlated with the presence of colon cancer. In training course, 48 samples were used to build up classifiers that were able to distinguish patients from normal samples using individual or a combination of markers. Receiver operating characteristics curves were generated to compare the predictive sensitivity, specificity, and the area under the curve. The classifiers were then examined by using leave-one-out cross-validation within all the 48 samples (18). For test course, this set of classifiers was then used to predict the probability of disease in an independent set of 48 cases. Sample set was analyzed by t test, and no significant difference was found in gender and age between patient groups and control groups (Table 1). The CEA level was also introduced for the classification of the 48 test cases. In the 48 colon cancer samples and control samples of the test set, the median levels of CEA were 51.63 ng/mL (range, 0.6-1,000) and 1.62 ng/mL (range, 0.6-4), respectively.

Results

High throughput screening and marker selection. A cDNA expression library consisting of 2 × 10⁶ primary clones was prepared from the colon cancer tissues. Recombinant phages at a concentration of 2 × 10³ plaque-forming unit per 18-cm plate were cultured until the plaques were visible for antigen induction and transferring to nitrocellulose membranes. Together, we cultured 500 plates for antigen display and screening. About 1 × 10⁶ recombinants were displayed for the screening. These phage clones were primarily screened with a pool of autologous and allogeneic sera of 10 colon cancer patients. One hundred forty-nine clones positively reacted with the autoantibodies in the sera of colon cancer patients. These clones were picked out to directly react with anti-human IgG secondary antibody. Those directly reacted with anti-human IgG secondary antibody were excluded for further screening. Then we got 79 clones that really reacted with autoantibodies in patients’ sera. Repeated screening with the pool of colon cancer patients sera defined 53 phage clones that could stably reacted with sera of colon cancer patients. The remaining 53 clones then underwent negative immune screen with a pool of sera from two healthy donors. After this screening, we got 24 clones that positively reacted with sera of colon cancer patients but did not react with the pooled sera of healthy donors. The screening procedure is illustrated in Fig. 1.

Serologic detection of autoantibodies for diagnostic assay of colon cancer using the panel of positive clones. These 24 clones were then subjected to serologic analysis with 24 cases of colon cancer patients and 24 cases of healthy donors for the training set. Thirteen of these candidate markers showed statistically significant differences between cases and controls (P < 0.05), six of which had a P value of <0.01; three markers offered the highest level of discrimination (P < 0.001). Logistic regression analysis was then done to calculate the sensitivity and specificity of individual and combinations of these candidate markers. A combination of six markers got the most satisfactory sensitivity and specificity. Leaving-one-out validation revealed the sensitivity and specificity of the six-marker combination were 91.7% and 91.7%, respectively.

The test course was done with an independent set of 48 cases, including 24 cases of colon cancer patients and 24 cases of healthy donors. Using the panel of six markers, the diagnostic assay could correctly classify 20 of 24 patients with 3 healthy donors misclassified. The sensitivity and specificity were 83.3% and 87.5%, respectively. We next calculated receiver operating characteristic curve for the six markers in the test set. The ability of the six-marker panel to discriminate colon cancer samples
from control samples was significant \( (P < 0.001) \), with an area under the curve equal to 0.933 (95% confidence interval, 0.853-1.014). The CEA levels of these samples were also examined. According to CEA level, 10 of 24 colon cancer patients were misclassified. The area under the curve for CEA was 0.727 \( (P < 0.01; 95\% \text{ confidence interval, } 0.58-0.875; \text{Fig. 2}) \). If CEA level was combined with the six-marker panel, only two patients and one healthy donor were misclassified (Fig. 3). We failed to distinguish clinicopathologic features of tumors by the marker combination in the total 48 cancer samples.

**Sequence analysis of phage-expressed proteins.** The six clones were sequenced and the nucleotide sequences of the six predictive phage-expressed proteins were compared with the Genbank database to obtain possible identities. According to the insert nucleotide sequence, the phage clone–expressed polypeptide was identified. Two of these polypeptides (clone 9 and clone 42) showed great homology to human LGR6 and C6orf192, respectively. The other four polypeptides (clone 24, clone 114, clone 55, and clone 50) do not show obvious homology to any genes. Then, we predicted the epitopes of these four polypeptides according to Jameson-Wolf antigenicity index by DNAstar software. The sequences of the epitopes were again analyzed by protein blast engine for homology. These markers were identified homologous to hCG1989951, AGBL5, PDE4A, and hypothetical protein FLJ23556 (Table 2).

**Discussion**

Autoantibodies in the sera of cancer patients can be used to isolate new tumor antigens by SEREX (19, 20). However, these antigens have limited potential for translation into clinical use, for they were identified by autologous serum and less reacted with allogenic sera. In our study, pooled sera of 10 colon cancer patients were used for cDNA expression library screening. Autoantibodies that commonly existed in different cases of cancer sera would have stronger reactivity in SEREX screening than those only existed in an individual cancer serum. Therefore, antigens identified by our study might have higher reactive frequencies with sera of colon cancer population and might be serum markers for colon cancer detection. Consistent with our studies, several groups have identified tumor-related autoantibodies by pooled allogenic sera (7, 12, 21).

In healthy population, natural antibodies are commonly existed, which are present in the serum of healthy individuals in the absence of deliberate immunization with any antigen. A vast majority of natural antibodies react with one or more self-antigens (22). By interacting with the large number of self-constituents present in an organism, natural antibodies establish an extensive dynamic network that contributes to the general homeostasis of the organism (23). Although immunoreactive patterns of natural autoantibodies are not identical among different sera, there are some cellular components that cause natural autoreactivity in most individuals (24). Therefore, positive clones identified by SEREX screening may include those reacted with natural antibodies. To exclude these antigens, we preformed a negative selection by screening clones

### Table 1. Characteristics of the patient and control samples

<table>
<thead>
<tr>
<th>Sample set</th>
<th>No. of patients*</th>
<th>Age (y)</th>
<th>Gender</th>
<th>No. of controls</th>
<th>Age (y)</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>Training</td>
<td>24</td>
<td>43-68 (mean, 57.17)</td>
<td>Male (9)</td>
<td>24</td>
<td>43-69 (mean, 56.58)</td>
<td>Male (10), Female (14)</td>
</tr>
<tr>
<td>Test</td>
<td>24</td>
<td>31-74 (mean, 55.63)</td>
<td>Female (15)</td>
<td>24</td>
<td>34-72 (mean, 55.96)</td>
<td>Male (12), Female (12)</td>
</tr>
</tbody>
</table>

*The 48 colon cancer samples are all adenocarcinomas; differentiation: well (7), moderate (31), and poor (10); stage: I-II (13), III-IV (35).*

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![Fig.1. Experimental design of cancer sera reactive marker screening. The flowchart delineates serologic screening of the cDNA expression library, as well as the positive clones picked out at each step of screening.](image)
reacted with healthy sera. In a total of 53 clones that positively reacted with cancer sera, 29 (55%) could react with healthy sera. As we considered that these antigens might react with some commonly existed natural antibodies, these clones were then excluded for training set. Even so, the remaining clones might also react with part of normal sera. In fact, we observed that two of the 24 remaining clones showed similar reactivity between cancer and control sera in the training set.

We statistically identified a six-autoantibody marker combination for colon cancer detection. Forty-four samples (91.7%) were correctly classified in a leave-one-out validation of 48 samples in training set. In the test set, 41 samples (85.4%) were successfully predicted. Whereas according to CEA level, only 36 cases were correctly classified in the 48 tested samples, with 10 of 24 (42%) patients misclassified. These results indicated a promising clinical use of the six autoantibody markers in colon cancer detection. Autoantibody signatures may be useful in combination with initial CEA detection. Combining the six markers with CEA, 44 of 48 samples (91.7%) were correctly classified in the test set. These data showed that the six autoantibody detectors significantly added to the diagnostic power of CEA \((P < 0.001)\) by 33.4% for colon cancers. A similar result was reported by combination autoantibody signatures with prostate-specific antigen (7). As these serologic markers are antibodies, we are trying to prepare antigen protein chip for future large-scale screening and convenient clinical detection.

The intensity of each immunoreaction between the autoantibody and its corresponding antigen should be considered as a whole to evaluate the malignant status.

The peptides of each inset were translated according to the nucleotide sequences. The sequences in the phage display system are probably those of relatively short peptides rather than full-length proteins. The phenomenon was also reported by other groups (7,12,21). Of the six phage peptides identified in this study, four are not present in peptide stretches of known human proteins. These peptides may be weakly homologous to known proteins or may have no distinct homology to the primary sequences of known proteins and, thus, may be "mimotopes" (i.e., stretches of amino acids that mimic an antigen but are not homologous at the sequence level; ref. 7). In our studies, the phage peptides that do not show homology to any human proteins were further analyzed to predict their epitope sequences. Additional homologous matching was done with the epitope sequence. The matched proteins may be the possible proteins these peptides mimic. Clone 9 is the only phage peptide that shows completely homology to a known human protein LGR6, which belongs to the leucine-rich repeat-containing subgroup of the G protein-coupled 7-transmembrane protein superfamily. The members of this
subgroup are conserved in evolution (25). Some members of LGR subfamily have been shown to be essential for mouse kidney development (26, 27).

In summary, our studies suggest that autoantibody signatures of colon cancer may be useful for the screening and early diagnosis of colon cancer. However, whether the set of six-autoantibody panel is colon cancer-specific still need to be evaluated by additional validation using sera from colon cancer patients with or without colonitis, patients with autoimmune conditions, and patients of other diseases or cancers. The value of this technique remains to be determined by multinstitutional studies of large population screening.

**Table 2.** Sequence identity of six diagnosis related humoral immune response targets

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Translated peptide sequence</th>
<th>Size of translated peptides (aa)</th>
<th>BLASTp Protein ID (first hit)</th>
<th>BLASTp Protein name (first hit)</th>
<th>BLASTp Identities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 9</td>
<td>GPVPLPP</td>
<td>7</td>
<td>EAW91410.1</td>
<td>LGR6</td>
<td>7/7 (100%)</td>
</tr>
<tr>
<td>Clone 24*</td>
<td>GSSSYAKVELFSKAAAEEKHPSCWWVQG-VIFA-VMLF5LYS1LYQ1YSQSLTIWCA</td>
<td>55</td>
<td>EAW50231.1</td>
<td>hCG1989951</td>
<td>6/12 (50%)</td>
</tr>
<tr>
<td>Clone 42</td>
<td>GMLVIYCVYF</td>
<td>10</td>
<td>EAW48012.1</td>
<td>C6orf192</td>
<td>6/10 (60%)</td>
</tr>
<tr>
<td>Clone 50*</td>
<td>GLVPNRRHEMVRVYCCFLKTI</td>
<td>21</td>
<td>EAW49518.1</td>
<td>hypotetical protein</td>
<td>6/11 (55%)</td>
</tr>
<tr>
<td>Clone 55*</td>
<td>GPVPSRVGLGAENGPCPCHQGDVFPCP-VLSARMSEQPEAEEAALBAHVGHDCV-GGQVLPHHRANNL</td>
<td>70</td>
<td>AAD34217.2</td>
<td>PDE4A</td>
<td>9/20 (45%)</td>
</tr>
<tr>
<td>Clone 114*</td>
<td>GGPLRPLRSAGLKRCLSTITTIRFSHPTTR-NMFKQQ</td>
<td>36</td>
<td>NP_068603.4</td>
<td>AGBL5</td>
<td>7/11 (64%)</td>
</tr>
</tbody>
</table>

*The peptides of these clones do not show homology to any known proteins. The homology was done with their epitope sequence (italic font).

**References**

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