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We proposed to develop a new approach for tumor-specific antibody detection based on Random Peptide Phage Display (RPPD) technology and verify whether such assays could be applied in the context of breast cancer, to determine the immunogenic spectrum of these patients. The relevance of our study was based on the obvious burden represented by this disease in terms of frequency and morbidity and the potential benefit that the development of new diagnostic, prognostic and therapeutic strategies can provide. During this grant period we have developed RPPD assays that effectively select *mimotopes* specifically present in sera from cancer patients. Even more exciting is the fact that homology searches have revealed that most of the selected peptides are present in known cancer-related proteins, thus unveiling potential new candidate tumor-antigens. We have also observed cross-reactivity of certain peptides with other patients hosting the same tumor type. These results set an ideal scenario for 1) The application of this methodology in the individual analysis of breast cancer patients' immune response and 2) the pursuit of large breast cancer series screening in order to detect new tumor antigens commonly present in these patients, as anticipated in TASKS 2 and 3 of our proposal.

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# A NEW APPROACH FOR THE IMMUNODIAGNOSTICS OF BREAST CANCER BY RANDOM PEPTIDE PHAGE DISPLAY

**Manuel Perucho, PhD. Principal Investigator**

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## INTRODUCTION

In our initial proposal we intended to test a new approach for the development of diagnostic tools suitable for the early detection of breast cancer. Our approach was based on the assumption that the immune system detects at very early stages of the disease, the aberrant proteins associated with neoplastic transformation (1, 2, 3, 4). The immune system is responsible for targeting and elimination of alien elements including infectious agents and tumor cells developing a highly specific and complex response against its targets. The antibody spectrum present in the serum of a patient represents a pool of information that, once deciphered, becomes useful data in the development of cancer diagnostic, therapeutic, and prognostic solutions. We proposed to use peptide sequences compiled in RPPD libraries (5) as *mimotopes* for the identification of protein motifs recognized by antibodies present in the sera of breast cancer patients. We hypothesized that random peptide phage display (RPPD) libraries could be an optimal method for the isolation of cancer related epitopes from sera. This approach had proven successful in identifying ligands specific for autoimmune and infectious diseases (6, 7). RPPD libraries had also been proven useful for the characterization of proteins critical for the cell to cell and cell to extracellular matrix interactions (8, 9, 10, 11) and for the development of selective organ targeting (12, 13). If succesful, the high sensitivity, specificity and speed of our approach should represent a remarkable breakthrough in the field of cancer immunodiagnostics. Our aim was to apply this strategy to test the hypothesis that tumor specific antigens of breast cancer patients can be detected by RPPD. After a first stage of collecting the necessary sera from breast cancer patients and optimizing the experimental conditions for such assays, as prerequisites for the isolation of epitopes recognized by sera antibodies of breast cancer, we planned to undertake an extensive study in order to determine if some of these tumor-specific antigens were shared by a significant number of cancer patients. Such achievement should justify the initiation of translational studies in order to evaluate the clinical significance of detecting such antibodies.

## BODY

***TASK 1.*** *To assemble a small panel of sera from breast cancer patients and design a method for the detection of phage displaying peptides with high affinity for immobilized human antibodies.*

***MILESTONE 1.*** *The establishment of the experimental conditions for the enrichment of phage specifically binding to breast cancer patient sera. The demonstration of success in the task will be shown by measuring the input/output ratio after biopanning of phage on positive and negative sera.*

**Panel of sera**

We have collected a series of 48 breast cancer, 28 colorectal cancer and 24 normal healthy individuals' sera, procured by the Midwestern and Eastern divisions of the Cooperative Human Tissue Network (CHTN) of the NCI. All sera were drawn from patients at the time of surgery. Because of the previous availability of sera from colorectal cancer (CRC) patients at the start of the project, the first experiments that we carried out to determine the experimental conditions to achieve specific binding to the sera antibodies were done with the CRC sera. However, the principle of the method, once optimized, should be generalizable to breast cancer patients. During the period of these experiments, we have received and accumulated 48 sera from breast cancer patients to continue with the next steps of the project in the next year. Accumulation of a sufficient number of sera facilitated investigation of the last step of the project (Task 3). We have already used the breast cancer sera as controls to show the specificity of phage binding to the cancer patient serum.

**Isolation of phage displayed peptides selected by high affinity human antibodies.**

In order to select peptides specific for antibodies from each patient serum, we developed a four-round procedure of phage panning. We used 7-mer, 8-mer, 9-mer cyclic and a 12-mer linear peptide libraries (14) of variable peptide length or a mixture of several libraries for the affinity selection of IgG and IgM immunoglobulins from cancer patients sera. Each round of the protocol consisted of an initial step of preadsorption of the phage libraries on immunoglobulins from healthy individuals in order to minimize further selection of non-cancer related antibodies, followed by affinity selection of the remaining antibodies present in the patient serum. Phage particles were eluted from the bound antibodies and subsequently amplified in bacteria. The amount of IgG or IgM immunoglobulins from healthy individuals used for preadsorption in each round of selection was fourfold higher than that from cancer patients. The binding results were quantified as the number of colony-forming units (cfu) produced by bacteria infected with the phage bound to normal and cancer patient sera antibodies. The panning procedure was considered successful when the number of cfu obtained after the fourth round of selection was at least twofold higher than those derived from healthy individuals' control antibodies (Figure1, Appendix 1).

**Characterization of selected peptides**

The colonies derived from the patient antibodies were grown overnight and phage were purified by double precipitation with polyethylene-glycol. Phage DNAs were isolated and sequenced.

**Assessment of the specificity of selected peptides.**

In order to verify whether the isolated peptides were specific for CRC, we cloned the cDNAs encoding two of them, TGVRGQRISQ (patient A) and MKQSGHHRSE (patient B), in GST fusion vectors. We then purified the proteins

expressed in bacteria in order to screen a large number of sera by ELISA. Patient A peptide was present in 9 of the 18 phage particles isolated after selection on his own IgG. Likewise, patient B peptide was selected on IgG antibodies. This peptide had a KXGHH motif, which was also found in 9 of the 18 phage isolated in this case. Randomly selected peptides from unselected libraries were also expressed as GST fusions to be used as controls for non-specific binding. Only peptides isolated from phage libraries by affinity selection on cancer sera, when expressed as GST fusion proteins, bound IgG antibodies from their corresponding cancer sera in the ELISA (Figure 2 Appendix 1).

Next, we screened sera from carriers of different types of cancer and from healthy individuals to test whether they would bind to the GST-peptide fusion proteins. Nineteen of these patients had CRC, 48 had breast cancer, and 24 additional sera were obtained from healthy individuals. Three out of 19 sera from CRC patients bound to either GST-fusion protein derived from patient A or B peptides in the ELISA. Two CRC sera bound patient A peptide and one bound patient B peptide. ELISA results were considered positive whenever the binding signal to either peptide was at least twofold stronger than the signal obtained from the control peptides. None of the breast cancer or control sera reacted positively, thus showing that peptides TGVRGQRISQ (patient A) and MKQSGHHRSE (patient B) isolated from phage libraries are specific for CRC ($p=0.049$; *two tailed Fisher exact test*). These results provide solid evidence for the specificity of the epitopes represented by the RPPD selected peptides for the autologous cancer type.

**Identification of tumor related protein sequences containing domains identical to RPPD selected peptides.**

We performed a homology search with the peptide sequences isolated from patients A and B through library panning on IgG and IgM antibodies. For this purpose we used the publicly available protein database resources from the NCBI and the EMBL. We utilized the BLAST and FASTA algorithms. The first was accomplished using the Advanced BLAST module (15, 16) available on-line from the NCBI (URL: http://www.ncbi.nlm.nih.gov/BLAST/) which performs the search against the non-redundant database (GenBank Complementary DNA translations, PDB, SwissProt, PIR and PRF databases). For FASTA searches we chose the FASTA3 (17, 18) module from the EMBL outstation at EBI (Hinxton) also available on-line (URL:http://www2.ebi.ac.uk/fasta3/). This tool browses Swissprot, Trembl (translated EMBL database), and TremblNew (translated EMBL new database). The parameters of both modules were adjusted for optimal short-motif homology search. With BLAST we were able to limit the search to human sequences. In a typical homology search with the cancer specific peptides, some of the retrieved protein sequences corresponded to well known tumor-associated antigens recognized by cytotoxic T lymphocyte (CTL) response (Table 1, Appendix 2).

Among the 18 phage clones isolated on IgM antibodies of patient A, four contained the sequence QDLYSSA. An additional phage displayed a similar sequence, QDIFSSS (Table 1, Appendix 2). A BLAST search for the sequence QDLYSSA retrieved 5 different protein sequences. Among the highest scored sequences were those of the SART-1 tumor antigen and IgE autoantigen. These two protein sequences are 81% identical (GenBank acces # GI:2723284 and GI:2342526) and contain the motif QDLYS. Another sequence, FQSPK, represented by two of the isolated phage was identical to a fragment present in three components of the fucosyltransferases family, FUT3, FUT5 and FUT6 (19), as shown in the FASTA search. These three genes share more than 80% of sequence identity. They are involved in the synthesis of the Lewis histo-blood group of antigens. Two members of this group, sialyl Lewis x (sLex) and sialyl Lewis a (sLea), are well characterized tumor-antigens. Different carcinoma and leukemic cells display a substantial increase of sLex and/or sLea expression (20, 21, 22, 23).

A large body of evidence supports the role of these antigens in gastrointestinal cancer as early tumor markers and indicators of prognosis, in terms of tumor metastasis, recurrence and patient survival (24, 25). The relevance of FUT genes in the synthesis and expression of selectin ligands is corroborated by the abrogation of the metastatic behavior of CRC cells after stable transfection of antisense FUT3 sequences into these cells followed by a dramatic drop of the transcript (26, 27). In the same line of evidence, sLex antigen is reportedly overexpressed in breast tumor cells and this overexpression is positively correlated with the amount of FUT6 messenger RNA (28) (27). It is also noteworthy that an excessive overexpression of sialyl Lex elicits NK cell response against tumor cells rather than facilitate their hematogenous disemination (29)

Among the peptide sequences isolated on patient D, peptide QSLDHSSC matched with the highest score to a fragment of the MUC2 protein. Another well known cancer antigen, the carcinoembrionary antigen (CEA) (30), was among several equally scored sequences homologous to peptide YSWRAT. Peptide PQGWLGV showed homology with the melanoma antigen gp100 among other related sequences of this family. The peptide sequence NERSEAR matched P120 catenin. Surprisingly, the similarity search for two unrelated peptide sequences PGHVRGTLGR and YVDTLSKL recognized by patient D IgM antibodies, produced matches with two different domains within the same protein, neogenin (NGN), albeit not with exceptionally high scores.

The homology search for sequences binding to IgG antibodies from cancer patient sera yielded MUC4 (peptide QNPGETSKMN), a putative tumor suppressor gene, FAT, and two different cancer/testis antigen group members, MAGE C1 and CT7 (peptide TNVISYTPSSLY). We finally performed homology searches with each of 10 peptide sequences isolated from healthy individuals.

Two peptide sequences showed homology to portions of the variable regions of IgG and IgM immunoglobulins. This result could reflect the fact that these peptides represent idiotypic determinants present on normal immunoglobulins. The eight other peptide sequences yielded no biologically relevant hits.

The considerable amount of tumor related, and particularly, CRC associated antigenic proteins retrieved from our computational analysis, provides an unanticipated strong support for the biological relevance of the RPPD selection assay. Moreover, the presence within the retrieved tumor antigens of previously characterized tumor antigens such as CEA, MUC2, MAGE, etc, supports the suitability of this methodology in the assessment of the specific tumor-induced immune response in a particular patient. In the same line of thought, it suggests the potential of this methodology for the identification of new tumor antigens in diverse tumor models.

**Experimental validation of computational results.**

In order to experimentally verify our computational findings, we elected to test whether the products of genes unveiled through homology search of sequences obtained from RPPD libraries screening are overexpressed by the corresponding tumors. Multiplex RT-PCR of RNA samples from patient A showed overexpression of FUT5 mRNA in tumor cells (Figure 3, Appendix 1). This result makes it unlikely for this particular protein to be a random result retrieved in the homology search.

Conversely, analysis of the expression of SART 1 antigen, yielded no significant increase in the amount of mRNA in the corresponding tumor. Secondary structure analysis of SART-1 protein sequence showed that the fragment QDLYS, which matches the sequence of our selected peptide, represents a coiled region that is likely to be located on the surface of the protein and to constitute an antigenic determinant. In order to overcome the potential lack of exposure of the antigenic sequence due to conformational alterations of SART-1 in the construct, we cloned in frame a 75 aminoacid fragment of the SART-1 protein in a GST vector, with the QDLYS motif located in its central portion. IgM antibodies from patient A serum bound the purified fusion protein as shown by ELISA, while serum from patients B, C, D and 4 normal individuals did not (Figure 4, Appendix 1). This finding strongly suggests that SART-1 protein triggered an IgM response in patient A, despite the lack of overexpression at the RNA level.

Tumor-associated antigens often induce a host immune response because they are overexpressed in tumor cells. HER-2/neu (31), CEA (30), and PSA (prostate-specific antigen) (32) illustrate this phenomenon. Similarly, we have evidence of the overexpression of FUT5 at the transcription level in the autologous tumor. This finding further supports the RPPD CRC-related epitope selection and minimizes the possibility of a random retrieval of this protein

sequence from the database. SART-1 showed no altered mRNA in our study. While this might represent a limitation of our method, in terms of validation of the homology search results, the binding of autologous IgM to a recombinant fragment of SART-1, as evidenced by ELISA, strongly suggests that in this particular case, the candidate tumor antigen indeed induced the immunity of the patient despite not being overexpressed at the time of serum collection. Interestingly, an independent report of an otherwise identical sequence categorized it as an IgE autoantigen. Autoantigens may induce the production of autoantibodies without being overexpressed or mutated (33).

The homology detected between two different peptides selected from the same patient with different regions of the same molecule, NGN, and the cross-reactivity observed in the specificity assessment of peptides, TGVRGQRISQ (patient A) and MKQSGHHRSE (patient B) with other CRC patient sera, provide additional support for the biological relevance of the RPPD selection.

These two peptides are among a subset of sequences that showed no homology with any relevant proteins compiled in databases. However, these peptide sequences were specific for CRC, as shown by ELISA. Antibodies raised against native proteins frequently recognize discontinous epitopes (33). Thus those peptides might well contain inserts that either mimic discontinuous antigenic determinants of known tumor-associated antigens or represent yet unidentified tumor-associated antigens. Most of the peptides associated with IgG antibodies from CRC patients in this study probably were sequences mimicking discontinuous epitopes. In contrast, most of the peptides corresponding to IgM antibodies from our CRC patients represented linear or continuous antigenic determinants present in proteins known to induce humoral and CTL immune responses.

The validity of our method is further supported by the lack of homology with tumor associated antigens observed among 10 peptide sequences selected after one round of panning on normal serum. Remarkably, some of these sequences were similar to fragments of the variable regions of the heavy chains of IgG and IgM immunoglobulins. This suggests that the phage display approach can also detect anti-idiotypic antibodies present in normal human serum.

**RPPD as a method for tumor antigen detection.**

The data obtained upon accomplishment of TASK1 from our original proposal, conclusively shows that RPPDL and protein databases can be used for the identification of tumor associated antigens that induce humoral or cellular immune response in cancer patients. RPPDL assay provides the researcher with a list of peptide sequences or immunoprints, recognized by antibodies present in the serum of a patient. The time span required to complete the process is short (~ 1 week). This represents a considerable improvement compared to other existing methods like the serological analysis of recombinant cDNA expression libraries (SEREX). In

this approach the patient sera is used to screen prokaryotically expressed cDNA libraries prepared from autologous tumor specimens. This approach requires the construction of good quality cDNA expression libraries from the autologous tumors, what makes it inefficient for the screening of large series of sera samples. The RPPD method is faster, less laborious and in addition does not require the natural antigen to be present.

***TASK 2.*** *To screen a number of currently available random peptide phage display (RPPD) libraries and characterize peptide consensus sequences that show specific binding to antibodies present in breast cancer patients.*

***MILESTONE 2.*** *The isolation of phage specifically binding to antibodies present in the sera of breast cancer patients but not in the sera of healthy individuals. The characterization of the sequence of common oligopeptides that bind to two initial breast cancer patient sera, but not to sera from healthy donors.*

As a preliminary requisite for the accomplishment of the main results contemplated in task 1, we have developed a suitable method based on RPPD library screening for the characterization of the specific immune response of colorectal cancer patients. The efficiency, specificity and speed of our method should enable us to run screening studies on different tumor models.

In addition to the 7-mer, 8-mer, 9-mer and 10-mer cyclic random peptide libraries and the 12-mer linear phage diplayed peptide library used in our studies (Pasqualini and Ruoslahti, 1996; Pasqualini et al, 1997), we also conducted biopanning of a human bone marrow cDNA library on patient antibodies from two individuals, one gastric cancer and one colon cancer patient, to determine whether the same affinity selected peptides could be identified using a cDNA library and additional sera. The procedures carried out were the same as have been described above. Six rounds of biopanning amplified a number of phage with binding affinity toward either IgG or IgM antibodies. 58 clones from the various rounds were sequenced and a homology search of the sequences performed (Table 2). The homology search proved unsuccessful as most identified sequences corresponded to abundant cellular proteins, such as alpha and beta globin-like peptides and heat shock proteins, but not any known tumor antigens. Although there were many anonymous sequences that potentially could represent the sought tumor antigens, we concluded that further detailed analysis of these sequences was too kisky and the experiments were not pursued. It was later determined that the cDNA library used in biopanning was degenerate and unstable. cDNA libraries are now commercially available and can be used in future studies.

We gathered a collection of 48 sera withdrawn from breast carcinoma patients, and these were used as controls in the assay for the initial two colon cancer patients positive sera. Optimization of the experimental conditions for CRC enabled us to apply the same approach for breast cancer. As shown for the specific antigens for CRC detected by the RPPDL approach, we expected to detect differences in the spectrum of immunogens in breast cancer. However, in the initial experiments with these sera we were unable to identify tumor specific antigens for breast cancer. The small amount of sera obtained precluded to pursue these experiments, as the sera was mostly used up. Fortunately, the positive results obtained from the CRC experiments enabled us to carry out additional experiments for the planned final stages of the project (Task 3) to test the potential crossreactivity between circulating antibodies against tumor antigens. However, the experiments were not as initially planned for breast cancer patients, but for colon cancer patients. Nevertheless, we reasoned that the findings obtained from these experiments should be useful for an understanding of the extent of cancer patient antigen-antibody crossreactivity.

***TASK 3.*** *To study the generality of the occurrence of these breast tumor specific antibodies by searching for their presence in a large panel of sera from breast cancer patients.*

***MILESTONE 3.*** *The identification of phage displaying peptides specifically recognized by the antibodies from a majority of breast cancer patients.*

In addition to the ELISA assays performed previously with the GST-peptide fusion proteins, additional ELISA assays were carried out using synthesized peptides with homology to SART-1, a known colon tumor antigen and a protein with sequences homologous to a number of selected phage peptides. The synthesized peptides, with sequence CQDLYSSAC, were biotinylated and analyzed by mass spectrometry to confirm biotinylation of the peptides. After optimal conditions were determined, ELISA assays were performed using the following protocol: 1 microM biotinylated peptide was bound to neutravidin coated wells on polystyrene plates (Pierce, Rockford, IL) for 2 hours on a shaker at 4oC. After removing unbound biotinylated peptide with 1x PBST, the wells were incubated with 0.3% BSA blocking buffer for 30 min and again washed with 1x PBST. Next, the wells were incubated with 1:5000 dilutions of sera from either colon or gastric cancer patients or healthy controls for 30 min on a shaker at 4oC. Unbound sera was washed with 1x PBST and the wells incubated with a 1:20K dilution of primary anti-human IgG-horseradish peroxidase (HRP) conjugate for 30 min at 4oC, followed by a wash step. Sera bound to the SART 1-like peptide was measured by detection with chemiluminescent substrate for HRP.

Maximal binding of SART-1-like peptide CQDLYSSAC to cancer patient sera was 51% above control healthy sera and binding above controls was detected in 5 out of 14 cancer sera tested, approximately selecting for one-third of the cancer cases tested. Therefore, there appears to be present in the sera from a significant proportion of CRC patients antibodies directed against the SART-1 antigen, as predicted by the hypotesis. However, the streng of the binding affinity was weak, and threfore, the assays were not sufficiently robust for a generalized testing. The low affinity of peptide to sera antibodies was disappointing, but there are a number of possible reasons for this observed weak binding. Separation of biotinylated peptide from free biotin through HPLC techniques might decrease background signal which could shadow the true binding signal. It is also possible that in the optimal concentration of sera used in the ELISA assays, there is not enough target antibody present, thus accounting for the absence of or weak signal. The peptide may also not be in the proper conformation for optimal binding to antibody again causing a false-negative detection of binding.

Similarly, of the peptide sequences homologous to known tumor antigens identified through screening of random peptide phage libraries, it could be that other sequences are better targets for serum antibody binding than those homologous to SART-1. Carcinoembrionary antigen or CEA and the mucin genes are such examples. We will attempt to further study these alternate binding peptide sequences through ELISA assays with the full-length antigens as well as the identified peptide sequences if subsequent funding is acquired.

## KEY RESEARCH ACCOMPLISHMENTS

- Development of a method for selection of peptides with high affinity for human antibodies by means of RPPD libraries.
- Characterization of selected peptides isolated from cancer patients sera.
- ELISA evidence for the specificity of the isolated peptides for cancer sera and for the autologous tumor type.
- Computational confirmation of the mimotope nature of isolated peptides.
- Identification of proteins, some of them already known tumor antigens, that contain domains homologous to the selected peptides.

## REPORTABLE OUTCOMES

- A publication in preparation to be submitted to *Nature Biotechnology* reporting the assay as an efficient method for retrieval of tumor antigens from cancer patients.
- Application for a second term of financial support in order to fulfill the application of the method to breast cancer. Considering the so far attained results, we should be also able to select, isolate and characterize peptides

specific for breast cancer. Some of these peptides are expected to be homologous to domains of proteins compiled in public databases. Among these, some should be already known breast-cancer related tumor-antigens. Others will unveil potentially new tumor antigens. Screening of larger series of sera should ensue in order to establish which of these antigens are present in a significant proportion of patients. Future projects should address the physiological relevance and potential application in diagnostic, therapeutic or preventive strategies of these immunogens.

## CONCLUSIONS

We have now successfully accomplished the initial stage of our project. The attained results show that, as hypothesized in our original proposal, RPPD is a suitable method for the individual characterization of the specific immune response unleashed against autologous tumors. We have optimized a new assay that upon implementation in the context of breast cancer, should enable us to decode the tumor-related immune response in a particular breast cancer patient. However, problems of weak affinity need to be addressed before a screening of large series of patients can be implemented. Although additional projects should verify the physiological relevance of those findings, both aims unequivocally share clinical interest.

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# APPENDICES

## Appendix 1

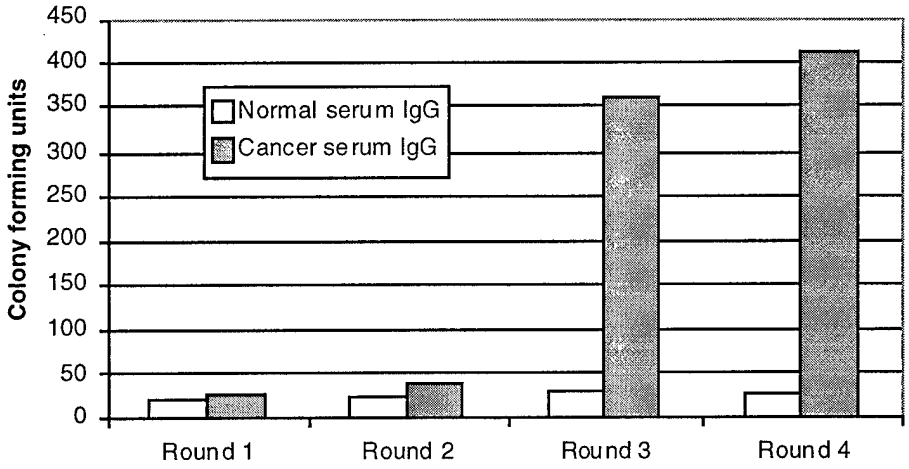


**Figure 1**. The graph shows the progressive enrichment of a RPPD library with phage particles that bind specifically to IgG antibodies from the serum of cancer patient A after four rounds of biopanning. After each round of preadsorption on excess of normal serum IgG and affinity selection on patient A IgG, amplified phage particles were purified and quantified. Equal amounts of phage (107 cfu) were incubated for 1 hour with normal serum IgG and cancer patient A IgG immobilized on protein G agarose. After washing out unbound phage, the remaining phage were eluted and used to infect bacteria. Aliquots of infected bacteria were spread on LB plates containing tetracycline. The next day, the number of colonies on plates corresponding to normal IgG and cancer patient A IgG were quantified.

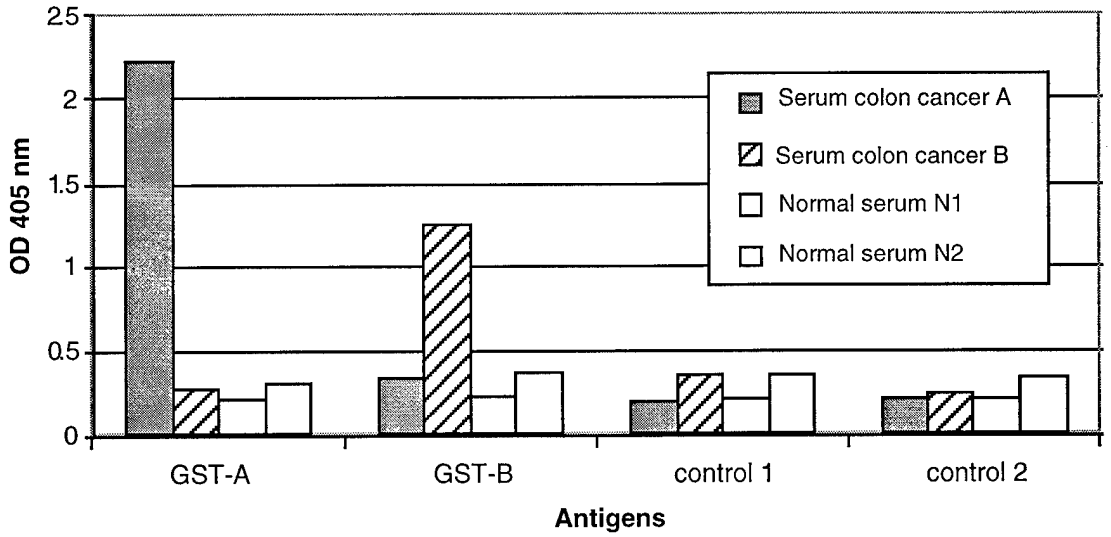


**Figure 2**. The graph depicts the specificity of binding of RPPD selected peptides to serum from autologous patients as evidenced by ELISA. Conversely, the sera from normal individuals display no affinity for these mimotopes. Purified GST-peptide fusion antigens were used to coat ELISA microplates. After blocking with 3% bovine serum albumin (BSA) in PBS the antigens were incubated overnight with cancer patient and normal individual sera diluted 1:200 with 3% BSA in PBS. The binding was detected using goat anti-human IgG antibodies conjugated with alkaline phosphatase and p-NPP as a substrate. GST-A and GST-B are TGVRGQRISQ and MKQSGHHRSE peptides expressed as GST fusion proteins. As control peptides we used arbitrarily chosen peptides isolated from phage libraries and expressed as GST fusion proteins. The average of three measurements is shown.

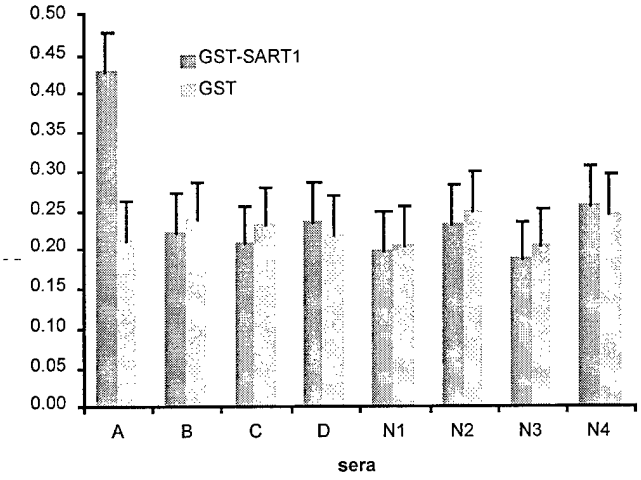


**Figure 3.** The graph illustrates the significant reactivity of patient A serum to a SART-1 antigen recombinant construct as shown in ELISA. Binding to the SART-1 GST fusion protein of serum IgM antibodies from cancer patients A, B, C and D and from four healthy individuals (N1-N4) is charted. Averages and the standard error of the mean of three measurements are shown.

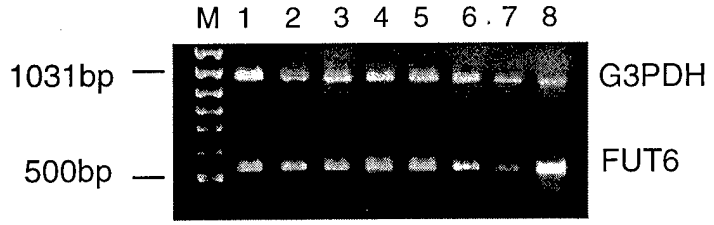


**Figure 4**. The dramatic difference in expression of FUT5 in patient A CRC tumor sample (lane 14) correlates with the selection of a mimotope from this patient that is contained in FUT5 protein sequence. This finding supports the biological relevance of our method. Simultaneous RT-PCR amplification of FUT5 and G3PDH gene fragments from normal colon tissue and colon tumor tissue mRNA. PCR products were electrophoresed on a 1% agarose gel. Numbers 1, 3, 5, 7, 9 and 11 are RT-PCR products from normal colon samples mRNA. Numbers 2, 4, 6, 8, 10 and 12 are RT-PCR products from corresponding colon tumor samples. Number 13 is the PCR product obtained from a cDNA of normal colon obtained from Clontech. Number 14 is the RT-PCR product obtained from the tumor of colon cancer patient A.

# APPENDIX 2

**Table 1. Peptide sequences selected from RPPD libraries and corresponding homologous proteins**

| Patient | Ab | Peptide sequences from "biopanning" selected phages | | | Homology search results<br>Acc. # Sequence name |
|---|---|---|---|---|---|
| A | IgG | TGVRGQRISQ(9) | *QNPGETSKMN*(6)[a] | KYRWYK(3) | [a]3551821 **Mucin 4** |
| A | IgM | AVHFPD (1)<br>DLITPGD (2)<br>AEPPFEF (2)<br>PSKAAYVV (3) | *QDLYSSA* (3)[b]<br>QDIFSSA (3)<br>MSSVMTY(1) | *FQSPK(1)*[c]<br>ASHQNRP(1)<br>FRQAAS(1) | [b] 2723284 **SART-1**<br>[c]17.30136 **FUT6 (Fucosyltransferase)** |
| B | IgG | FSRRAQQVGAK(3)<br>DHNRSM<br>SHNRVSN<br>KKGM**GHH**GNG | K**AYGHHLS**AE(2)<br>GLGVGHK<br>SYSGYWHSWI<br>FGAKSH**GHH**R | KSNKCF<br>MKQS**GHHRS**E<br>WTRRPYDELIV | |
| C | IgG | KENGR**SPTHS**(10)<br>GRSNKSG(1) | ***SPTHP***(5)[d] | GRRNKSG(1) | [d]422832 **Mucin 6** |
| D | IgG | V**PWSK**PWWTQ(1)<br>GHNNHNRHHP(1)<br>**SNVRSF**DNPI(1)<br>ANT**PWSK**TL(2)<br>IPLPPPSRPF(2)<br>HNTRNWTLPP(1) | **SNVISY**PDV(1)<br>GN**PWSK**QINI(1)<br>VNTTSYNMRP(2)<br>L**PWSK**LSSPS(1)<br>SNVKNYMAIP(1)<br>QLHPHNLHSP(1) | TLHTTHSPFK(1)<br>NYEPVPRGAR(1)<br>TDAA**PWSK**VT(2)<br>GKSLHGSHHP(1)<br>***SNVISF***RHAS(1)[e]<br>*TNVISYTPLY*(3)[e] | [e]32.52909 **Cancer/testis antigen CT7** |
| D | IgM | LVYPQQ(1)<br>*QSLDHSSC*(5)[f]<br>LNPQSPRD(4)<br>*YSWRAT*(4)[g]<br>*NERSEAR*(1)[h]<br>HFHHLAVRGR(1)<br>*PQGWLGW*(1)[i]<br>GTVEPD(1) | PTRWGARLVK<br>GGRWNR(2)<br>PETTDK(2)<br>RTGRMWR(1)<br>RGQSLA(1)<br>*PGHVRGTLGR*(2)[j]<br>AVRRPD(4)<br>QRLAAGFH(1) | QLAETLF(1)<br>GRKTELF(1)<br>YLASPFE(1)<br>FRVARAA(1)<br>*YVDTLSKL*(1)[k]<br>VVGLVP(2)<br>QIQLSGG(1)<br>VKNRGR(1)[l] | [f]186398 **Mucin 2**<br>[g]180241 **Carcinoembryonic antigen**<br>[h]3152863 **p120 catenin isoform 4ABC**<br>[i]547230 **Melanoma antigen gp100**<br>[j]1621607 **Neogenin**<br>[k]1621607 **Neogenin**<br>[l]553465 **IgG heavy chain V region** |
| Normal | IgG | *DIRLSAQL*(1)[m]<br>*SWSGYYTY*(1)[n]<br>TNGVHHGR(1)<br>GLYNFMGK(1) | RRTDYLLNGI(1)<br>DPTVSESS(1)<br>APQGYLFK(1)<br>ESSTKSE | NQHLILSVG(1)<br>*SIAAAVH*(1)[o]<br>RANKEPAT(1) | [m]542874 **Ig kappa chain V region L13**<br>[n]346202 **IgG heavy chain V region**<br>[o]1914757 **IgG heavy chain V region** |

1. All databases retrieved proteins represented in the table are of *homo sapiens* origin
2. Numbers in parenthesis indicate the number of identical copies of the same phage after selection
3. Highlighted in bold are conserved motifs present in different phages
4. Highlighted in italics represent those peptide sequences yielding biologically relevant and cancer related sequences
5. Superscript letters relate peptide sequences with their relevant homologous sequences
6. Accession numbers provided correspond to the genbank (NCBI) entry reference of each sequence

**Table 2. Peptide sequences selected from a phage library containing sequences from a human bone marrow cDNA library and corresponding homologous proteins.**

| Patient | Serum Antibody | Sequence homology search results |
|---|---|---|
| 16184 B1A | IgG | anonymous chrom 14<br>mitochondrial DNA<br>alpha-globin 1 (HBA1)<br>alpha-globin 2 (HBA2)<br>hepatoma-derived growth factor<br>anonym. chrom Xq27.1-27.3<br>talin mRNA<br>anonym. chrom 20p12<br>ribonuclease 6 precursor |
| 16184 B1A | IgM | S164 gene<br>anonym. chrom 1p36.11-36.33<br>HBA1<br>HBB<br>Genbank Clone DJ0756H11<br>anonym. chrom 16<br>anonym. chrom 6q22-23<br>anonym. chrom 6q16.1-16.3<br>anonym. chrom 7q11.23 |
| S18 | IgG | beta globin (HBB)<br>anonym. chrom 22q11.2<br>anonym. chrom 20p12.2-13<br>anonym. chrom 12q24.1-116.6-118.9<br>7SL gene<br>cDNA DKFZp566C114<br>mito. DNA<br>hsp89-a-d-N, hsp90<br>anonym. chrom Xq23 |
| S18 | IgM | PolyA bindingprotein-like 1<br>vaccinia-relatedkinase 1<br>HBA2, HBA1<br>anonym. chrom 22q11.2<br>hsp89-a-d-N, hsp90<br>Genbank clone YZ83B08<br>anonym. chrom 20p12.2-13<br>anonym. chrom 6q24.1-25.2 |

1. All databases retrieved proteins represented in the table are of *homo sapiens* origin

# PAPER DRAFT

## PEPTIDE PHAGE DISPLAY LIBRARIES AS TOOLS FOR CANCER IMMUNODIAGNOSTICS.

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**Keywords:** colon, cancer, phage display libraries, tumor antigens

## ABSTRACT

A strategy to identify cancer-specific epitopes using random peptide phage-displayed libraries and cancer patient sera is described. We used peptide libraries to decode the repertoire of antibodies present in sera of colorectal cancer patients. Through biopanning on antibodies from these patients, we selected from peptide libraries the peptides recognized by cancer-specific IgG or IgM antibodies. By screening of a large number of sera for antibody binding to selected peptides with ELISA we have shown that these peptides were colon cancer specific. A search of protein databases revealed that many peptide sequences selected from peptide libraries were identical to fragments of well-known tumor associated antigens. To rule out the possibility that identity between selected peptide sequences and the fragments of proteins found through databases homology search was due to chance we carried out the multiple RT-PCR analysis of tumor RNA of one cancer patient. We have shown that FUT-6 mRNA corresponding to peptide sequence selected on cancer patient's IgM antibody was dramatically overrepresented in his tumor tissue RNA in comparison with the same mRNA transcript in 13 other tumor and normal tissue RNA. Our results show that for some cancer patients an array of peptide sequences recognized by their serum antibodies (immunofootprint) can be easily defined by using random peptide libraries. One possible application of these immunofootprints could be the designing of simple individual immunoassays for detecting tumor recurrences or metastases.

## INTRODUCTION

Tumor cells induce specific humoral and cellular immune responses in the autologous host [Old, 1981 #20][Knuth, 1984 #1][Sahin, 1995 #2][Disis, 1996 #3][Boon, 1996 #22][Rosenberg, 1996 #4]. The identification of tumor associated antigens recognized by cytotoxic T lymphocytes (CTL) is essential for the development of cancer immunotherapy, and the design of preventive cancer vaccines. In addition, tracing of tumor-specific or tumor-associated antigens recognized by circulating antibodies in cancer patients has an inherent diagnostic and prognostic value. Random Peptide Phage Displayed libraries (RPPDL) have been widely utilized to map protein interacting sites. The role of protein inhibitors has been also assessed by this method (Renata ref). More recently, in vivo screening of RPPDL have evidenced the organ- and tissue-specific molecular heterogeneity of vasculature, unveiling a potential molecular address-code based on homing receptors unmasked by phage-displayed peptides. This approach is proving to be an effective strategy for organ specific targeting [Arap, 1998 #151][Arap, 1998 #148] [Rajotte, 1998 #163] [Pasqualini, 1996 #156]. Peptide libraries are also efficient tools for the assessment of antibody specificity [Scott, 1990 #5]. In this assay, a RPPDL is incubated with a target antibody. Subsequently, phage bound to the antibody are eluted and amplified in host bacteria. This process, termed "biopanning", can be repeated several times in order to attain an enriched population of best binders. Bound peptides are later identified upon phage DNA sequencing (REFERENCE). Depending on the nature of the antibody, the sequences of best binding peptides can represent either linear antigenic determinants or discontinuous antigenic determinants [Stephen, 1995 #6]. Both, monoclonal antibodies and polyclonal antibodies from sera can be used for biopanning. Screening of RPPDL with sera from subjects immunized against the human hepatitis-B virus envelope protein (HBsAg), led to the isolation of peptide sequences that mimic disease-specific epitopes in the absence of the natural antigen [Folgori, 1994 #7]. Likewise, screening with sera from pre-diabetic patients, identified a diabetes-specific epitope that mimics a

pancreatic-islet related autoantigen [Mennuni, 1997 #8]. Yet, screening of peptide libraries with cancer patients sera for the identification of tumor antigens has not been reported.

In this work, we identified tumor-specific peptide sequences recognized by antibodies present in colorectal cancer (CRC) patients sera with the RPPDL method. IgG and IgM antibodies from patients sera were independently screened. We isolated several peptide sequences that bind IgG or IgM antibodies, some of them specific for CRC. Protein database homology search revealed that some of these sequences are found in known tumor-associated antigens. Significantly, some of these tumor-associated antigens are reportedly recognized by CTL.

## RESULTS

### Panning of peptide libraries on IgG or IgM immunoglobulins from colorectal cancer patient sera.

Sera from seven CRC patients were used for panning of RPPDL on IgG antibodies. Four serum samples were positive, implying that they contributed to the enrichment of phage libraries as the phages bound antibodies from the cancer patient but not from a control pooled serum from 24 healthy individuals. Two sera positive for enrichment on IgG antibodies were also tested for enrichment on IgM antibodies. In both instances, each serum contributed to enrich the libraries, as the phages bound their own IgM antibodies but not IgM antibodies present in the control. Figure 1 shows the enrichment of phage particles that specifically bind to IgG antibodies from a CRC patient serum after four rounds of successful selection. We also performed library panning with sera obtained from four healthy individuals. No enrichment of the libraries was observed when compared to the control serum.

For each positive serum, the associated enriched libraries typically contained a group of phage with identical inserts and an additional number of peptides with

inserts similar to this group. Several single represented inserts were also present. Interestingly, the peptide sequences derived from phage binding to IgG antibodies were different from those binding to IgM antibodies, from the same serum. We also sequenced ten inserts from phage isolated after one round of panning with IgG from a healthy individual. All ten sequences were different. Table 1 shows the sequences of peptides identified for each positive serum.

**ELISA Screening of sera against identified peptides.**

In order to verify whether the isolated peptides were specific for CRC, we cloned the cDNAs encoding two peptides, TGVRGQRISQ (patient A) and MKQSGHHRSE (patient B), in GST fusion vectors. We then purified the proteins expressed in bacteria in order to screen a large number of sera by ELISA. Patient A peptide was present in 9 of the 18 phage particles isolated after selection on his own IgG. Likewise, patient B peptide was selected on IgG antibodies. This peptide had a KXGHH motif, which was also found in 9 of the 18 phage particles isolated in this case. Randomly selected peptides from unselected libraries were also expressed as GST fusions to be used as controls for non-specific binding. As seen in figure 2, only peptides isolated from phage libraries by affinity selection on cancer sera, when expressed as GST fusion proteins, bind IgG antibodies from their corresponding cancer sera in the ELISA.

Next, we screened sera from carriers of different types of cancer and from healthy individuals to test whether they would bind to the GST-peptide fusion proteins. Nineteen of these patients had CRC, 48 had breast cancer, and 24 additional sera were obtained from healthy individuals. Three out of 19 sera from CRC patients bound to either GST-fusion protein derived from patient A or B peptides in the ELISA. Two CRC sera bound the patient A peptide and one bound patient B peptide. ELISA results were considered positive whenever the binding signal to either peptide was at least twofold stronger than the signal obtained from the control peptides. None of the breast cancer or control sera reacted positively, thus showing that peptides TGVRGQRISQ (patient A) and

MKQSGHHRSE (patient B) isolated from phage libraries are specific for CRC ($p=0.049$; *two tailed Fisher exact test*).

**Search for proteins domains identical to selected peptides.**

In a typical homology search with the cancer specific peptides, some of the retrieved protein sequences corresponded to well known tumor-associated antigens recognized by CTL (Table 1). Among the 18 phage clones isolated on IgM antibodies of patient A, four identical ones contained the sequence QDLYSSA. An additional phage displayed a similar sequence, QDIFSSS (Table 1). A BLAST search for the sequence QDLYSSA retrieved 5 different protein sequences. Among the highest scored sequences were those of SART-1 tumor antigen and IgE autoantigen. These two protein sequences are 81% identical (GenBank acces # GI:2723284 and GI:2342526) and contain the motif QDLYS. Another sequence, FQSPK, represented by two of the isolated phage was identical to a fragment present in three components of the fucosyltransferases family, FUT3, FUT5 and FUT6, as shown in the FASTA search.

Among the peptide sequences isolated on patient D, peptide QSLDHSSC matched with the highest score to a fragment of the MUC2 protein. Another known cancer antigen, the carcinoembrionary antigen (CEA) [Tsang, 1995 #12], was among several equally scored sequences homologous to the peptide YSWRAT. Peptide PQGWLGV showed homology with the melanoma antigen gp100 among other related sequences of this family. The peptide sequence NERSEAR matched P120 catenin. Surprisingly, the similarity search for two unrelated peptide sequences PGHVRGTLGR and YVDTLSKL recognized by patient D IgM antibodies, produced matches with two different domains within the same protein, neogenin (NGN), albeit not with exceptionally high scores.

The homology search for sequences binding to IgG antibodies from cancer patient sera yielded MUC4 (peptide QNPGETSKMN), a putative tumor suppressor gene, FAT, and two different cancer/testis antigen group members,

MAGE C1 and CT7 (peptide TNVISYTPSSLY). We finally performed homology searches with each of 10 peptide sequences isolated from healthy individuals. Two peptide sequences showed homology to portions of the variable regions of IgG and IgM immunoglobulins. This result could reflect the fact that these peptides represent idiotypic determinants present on normal immunoglobulins. The eight other peptide sequences yielded no biologically relevant hits.

**Relevance of the database search findings.**

We elected to test whether the products of genes unveiled through homology search of sequences obtained from RPPD libraries screening are overexpressed by the corresponding tumors. Multiplex RT-PCR of RNA samples from patient A showed overexpresion of FUT5 mRNA in tumor cells (Figure 3). This result makes it unlikely for this particular protein to be a random result retrieved in the homology search. Conversely, analysis of the expression of SART 1 antigen, yielded no significant increase in the amount of mRNA in the corresponding tumor (data not shown). Secondary structure analysis of SART-1 protein sequence showed that the fragment QDLYS, which matches the sequence of our selected peptide, represents a coiled region that is likely to be located on the surface of the protein and to constitute an antigenic determinant. In order to overcome the potential lack of exposure of the antigenic sequence due to conformational alterations of SART-1 in the construct, we just cloned in frame a 75 aminoacid fragment of the SART-1 protein in a GST vector, with the QDLYS motif located in its central portion. IgM antibodies from patient A serum bound the purified fusion protein as shown by ELISA, while serum from patients B, C, D and 4 normal individuals did not (Figure 4). This finding strongly suggests that SART-1 protein triggered an IgM response in patient A, despite the lack of overexpression at the RNA level.

## DISCUSSION

Our aim was to test RPPD technology as a new method for the identification of tumor-associated antigens. We have been able to isolate several peptides specific for CRC from autologous sera. Most of the isolated peptide sequences share homology with motifs present in previously characterized tumor-associated antigens. Since these antigens are known to trigger CTL response in cancer patients, RPPD technology is likely to prove useful in the rapid identification of tumor-associated antigens.

The list of proteins with domains homologous to peptides (immunoprint) obtained from patient A IgM, included SART-1 and a group of fucosyltransferases. SART-1 was originally identified by expression-gene cloning [Guilloux, 1996 #121] using a CTL cell line previously known to recognize squamous cell carcinoma tumor-antigens[Shichijo, 1998 #9]. The largest of the two proteins coded by this gene (125Kd), contains the QDLYS peptide and is widely expressed in the nuclei of normal and malignant proliferating cells [Shichijo, 1998 #9]. SART-1 protein function is yet to be determined, however, several peptides within its sequence perform as tumor-antigens specifically recognized by CTL in different cancers [Shichijo, 1998 #9; Kawamoto, 1999 #126; Kikuchi, 1999 #123; Matsumoto, 1998 #125].

Tumor-associated antigens often induce a host immune response because they are overexpressed in tumor cells. HER-2/neu [Fisk, 1995 #17], CEA [Tsang, 1995 #12], and PSA (prostate-specific antigen)[Correale, 1997 #18] illustrate this phenomenon. SART-1 showed no altered mRNA in our study, however, the binding of autologous IgM to a recombinant fragment of SART-1 as evidenced by ELISA, strongly suggests that this molecule induced an immune response in patient A. This apparent contradiction might be explained by yet unreported post-transcriptional or even post-translational modulation of SART-1 expression. Interestingly, an independent report of an otherwise identical sequence categorized it as an IgE autoantigen. Autoantigens may induce the production of autoantibodies without being overexpressed or mutated.[Janeway, 1997 #167].

FUT3, FUT5 and FUT6 form a gene cluster at chromosome 19p encoding three components of the fucosyltransferase family [Cameron, 1995 #112] that share more than 80% of sequence identity and contain the FQSPK motif. These proteins have not been categorized as tumor-antigens yet. However, they are involved in the synthesis of the Lewis histo-blood group of antigens. Two members of this group, sialyl Lewis x (sLex) and sialyl Lewis a (sLea), are E and P selectin receptor ligands found to be expressed on the surface of activated endothelial cells. The selectin-sLex interaction mediates the recruitment and extravasation of leukocytes in physiological conditions [Rosen, 1996 #113] and is postulated to be similarly involved in the hematogenous spread of tumor cells. Hence sLex and sLea are well characterized tumor-antigens. Different carcinoma and leukemic cells display a substantial increase of sLex and/or sLea expression [Fukushima, 1984 #114][Fukuda, 1985 #115][Kim, 1988 #30][Shimodaira, 1997 #119]. A large body of evidence supports the role of these antigens in gastrointestinal cancer as early tumor markers and indicators of prognosis, in terms of tumor metastasis, recurrence and patient survival [Nakamori, 1993 #116][Renkonen, 1997 #94]. The relevance of FUT genes in the synthesis and expression of selectin ligands is corroborated by the abrogation of the metastatic behavior of CRC cells after stable transfection of antisense FUT3 sequences into these cells followed by a dramatic drop of the transcript [Weston, 1999 #120] [Majuri, 1995 #80]. In the same line of evidence, sLex antigen is reportedly overexpressed in breast tumor cells and this overexpression is positively correlated with the amount of FUT6 messenger RNA [Matsuura, 1998 #10] [Majuri, 1995 #80]. It is also noteworthy that an excesive overexpression of sialyl Lex elicits NK cell response against tumor cells rather than facilitate their hematogenous disemination [Ohyama, 1999 #102]. Our results show that patient A had developed IgM response against the FQSPK domain present in FUT3,5 and 6. As expected, one of these proteins, FUT5 is overexpressed at the mRNA level as shown in our RT-PCR assay (Figure 3) suggesting that one or more FUT proteins are behaving as tumor-antigens in this patient.

Three high molecular weight mucins, MUC2,4 and 6, were present in the immunoprint of three of our patients. MUC2 is a tumor-antigen overexpressed in mucinous carcinomas of colon, breast, ovary and pancreas. MUC2 induces CTL immune response, particularly in patients with mucinous carcinomas [Bohm, 1998 #11]. Moreover, a recent postulate implicates this protein in metastasis of CRC. Antisense transfection of MUC2 gene into metastatic CRC cells, shows a reversion of this phenotype, correlated with an 80% drop of the transcript. Interestingly, there is also a decrease of sLex and sTn antigens in this cells and their binding to E-selectin is likewise inhibited [Sternberg, 1999 #128][Bresalier, 1996 #131]. MUC6 is another mucin originally isolated from a gastric carcinoma cDNA library. Neither MUC6 nor MUC4 immunogenicity have been elucidated yet. However, MUC6 is aberrantly expressed in carcinomas and preneoplassic lesions of colon, stomach and breast [Bartman, 1999 #133][de Bolos, 1998 #134][Ho, 1995 #136]. Similarly, MUC4 is reportedly overexpressed in colon [Zhang, 1998 #146], pancreas [Balague, 1994 #141][Hollingsworth, 1994 #139] and ovarian cancers [Giuntoli, 1998 #137].

Patient D IgG immunoprint included CT7 [Chen, 1998 #16] and MAGE C-1 [Lucas, 1998 #143]. These proteins share 66% of identity among their sequences and the region of homology with the same selected peptide. Both CT7 and MAGE C1 are components of the cancer/testis (CT) antigen family, a well characterized group of tumor antigens known to be expressed in diverse malignancies. CT7 tumor-associated antigen was originally cloned after screening of an expression library from a melanoma cell line with an allogenic melanoma patient serum. The CT family of antigens were originally identified by serological analysis of recombinant cDNA expression libraries (SEREX), another method used for cloning of tumor-associated antigens recognized by circulating antibodies [Sahin, 1995 #2]. This approach requires the laborious construction of cDNA expression libraries from the autologous tumors. The recently cloned human-homologue of drosophilas' FAT tumor-suppressor gene was also listed in this immunoprint. Yet, little is known about the precise role or the immunogenic

potential of this new member of the cadherin superfamily [Dunne, 1995 #166]. FAT gene particularly illustrates the compelling need of high complexity phage libraries in order to maximize the accuracy of the information provided by this assay. FAT attained the highest score in the homology search of peptides selected from patient D serum (TNVISYTPSSLY), however, the region of homology within the protein (ISYTPS) was only represented by one of the selected peptides. In contrast, CT7 and MAGE C1 homologous region (SNVIS), within the same peptide was present in three additional independent peptides selected from the same serum. Hence, CT proteins become better immunogen candidates for patient D.

In addition to the above mentioned MUC2, patient D IgM immunoprint listed another well established tumor-antigen, carcinoembrionary antigen (CEA) [Tsang, 1995 #12], together with P120 catenin, a protein related to the tumor-associated antigen β-catenin [Rosenberg, 1999 #165] and three other MAGE-related proteins, gp100, PMEL 17 and ME20 antigens, that share more than 90% identity. gp100protein was recently reported as a tumor rejection antigen recognized by tumor-infiltrating lymphocytes [Kawakami, 1994 #164]. Also in this immunoprint was NGN a protein expressed on gastrointestinal and other embryonic tissues of vertebrates, suggesting a role in development. NGN is almost 50% identical to the product of DCC gene (Deleted in Colorectal Cancer), a candidate tumor-suppressor gene. The pattern of expression during embryogenesis is also similar between these molecules, suggesting a potential relation in their function [Meyerhardt, 1997 #15][Vielmetter, 1997 #132]. The homology detected between two different peptides selected from the same patient and different regions of the same molecule, NGN, provides additional support for the biological relevance of the RPPDL selection.

In our similarity searches, some peptide sequences showed no homology with any relevant proteins compiled in databases. However, these peptide sequences

were specific for CRC, as shown by ELISA. Antibodies raised against native proteins frequently recognize discontinous epitopes [Janeway, 1997 #167]. Thus those peptides might well contain inserts that either mimic discontinuous antigenic determinants of known tumor-associated antigens or represent yet unidentified tumor-associated antigens. Most of the peptides associated with IgG antibodies from CRC patients in this study probably were mimotopes (sequences mimicking discontinous epitopes). In contrast, most of the peptides corresponding to IgM antibodies from our CRC patients represented linear or continuous antigenic determinants present in proteins known to induce humoral and CTL immune responses.

Strictly, we cannot exclude the possibility that some of the tumor antigens retrieved based on their homology with selected peptides are random hits. However, the high level of FUT5 gene mRNA detected in the autologous tumor compared to 12 other normal and tumor tissues from colon cancer patients (Figure. 3) provides experimental evidence for the relevance of the data retrieved from protein databases. Also in support of the relevance of de homology search results is the fact that two different peptide sequences recognized by antibodies from the same patient serum matched different fragments of the same molecule (NGN). Conversely in certain cases, like SART-1 antigen, the apparent lack of transcript overexpression by the tumor, might represent a limitation for validation of the computational results. However, if the tumor specific-antigens yielded by the homology search are relevant, the cancer serum antibody that binds a phage-displayed peptide should bind the identical peptide sequence in the context of the retrieved protein. We have shown that antibodies present in the serum used for the selection of SART-1 homologous peptide, specifically bind a fragment of SART-1 protein containing the target sequence, while antibodies from other sera do not.

The validity of our method is furtherly supported by the lack of homology with tumor associated antigens observed among 10 peptide sequences selected after one round of panning on normal serum. Remarkably, some of these sequences

were similar to fragments of the variable regions of the heavy chains of IgG and IgM immunoglobulins. This suggests that the phage display approach can also detect anti-idiotypic antibodies present in normal human serum.

Our data conclusively shows that RPPDL and protein databases can be used for the identification of tumor associated antigens that induce humoral or celular immune response in cancer patients. RPPDL assay provides the researcher with a list of peptide sequences or immunoprint, recognized by antibodies present in the serum of a patient. The time span required to complete the method is short (~ 1 week). Thus for those patients whose sera achieve selection of cancer-specific peptides, individualized or generic immunoassays can be rapidly accomplished for diagnostic, therapeutic or preventive purposes following this method.

## MATERIALS AND METHODS.

**Cancer patient sera**. The sera from colon cancer patients were procured by the Midwestern and Eastern divisions of the Cooperative Human Tissue Network (CHTN) of the NCI. All sera were drawn from patients at the time of surgery.

**Random peptide phage displayed libraries**. We used 7-mer, 8-mer, 9-mer, and 10-mer cyclic peptide libraries and a 12-mer linear phage displayed peptide library. The libraries were constructed as described (Smith and Scott, 1993) (Renata reference).

### Isolation of phage binding antibodies from colon cancer patient sera.

We used linear and cyclic peptide libraries of variable peptide length or a mixture of several libraries for the affinity selection of IgG and IgM immunoglobulins from cancer patients sera. In order to select peptides specific for antibodies from each serum, we developed a four-round procedure of phage panning. Each round of the protocol consisted of an initial step of preadsorption of the phage libraries on normal immunoglobulins, followed by affinity selection of the antibodies present in the patient serum and amplification of the phage particles eluted from the antibodies in bacteria as described (REFERENCE). In more detail, IgG or IgM

antibodies from a CRC patient were immobilized on protein G (Gibco BRL) or anti human IgM (Sigma) agarose, respectively The inmobilized Ab were then washed in PBS and preblocked with 3% bovine serum albumin (BSA) in PBS for 30 min. 50 µl of colon cancer patient serum was incubated with 50 µl of BSA-preblocked protein G agarose beads for 30 min. In the control experiment, 50 µl of serum from a healthy individual were used instead. Likewise, 200 µl of pooled human sera from 24 healthy individuals were incubated with 200 µl of BSA-preblocked protein G agarose beads for 30 min to be used to preadsorb the phage libraries on normal IgG. After incubation, the beads were washed in PBS 4 times. We resuspended $10^{11}$ colony forming units (cfu) of phage in 200 µl of 3% BSA / 0.05% Tween 20 / PBS solution. The suspensions were incubated with immobilized IgG antibodies from the pool of healthy individuals sera for 2h at 4°C. The phages unbound to normal IgG antibodies were split into equal volumes. One volume was transferred to protein G agarose beads with immobilized IgG antibodies from the serum of a CRC patient and the other volume was transferred to the same amount of protein G agarose beads with immobilized IgG antibodies from control healthy individuals. These phages were incubated for 2h at 4°C. The unbound phage were removed after 10 washes with 3% BSA / 0.05% Tween 20 / PBS buffer solution. The bound phage were eluted with 100 µl of 0.1M glycine buffer, pH 2.2, containing 1 mg/ml BSA and 0.1 mg/ml of phenol red indicator. The eluted phage solution was neutralized by addition of 1/10 volume of 1M Tris buffer, pH 9. The neutralized phage were incubated with 1 ml of starved, competent *E. coli* K91kan ($OD_{600}$=1-2) for 20 min at room temperature without shaking. Then 10 ml of LB medium containing 0.2 µg/ml of tetracycline were added. These mixtures were incubated at RT for 20 min, and 10 to 100 aliquots from each sample were spread on agar plates containing 40 µg/ml of tetracycline. The colonies were counted after 12 h. The remaining infected bacteria were grown overnight at 37°C in the presence of 40 µg/ml of

tetracycline. The amplified phages were purified from the bacterial supernatant, quantified and subjected to further rounds of selection. The amount of IgG or IgM immunoglobulins from healthy individuals used for preadsorption in each round of selection was fourfold higher than that from cancer patients. The binding results were quantified as the number of colony-forming units (cfu) produced by bacteria infected with the phage bound to normal and cancer patient sera antibodies. The panning procedure was considered successful when the number of cfu obtained after the fourth round of selection was at least twofold higher than those binding to control antibodies from healthy individuals.

**Sequencing of phage DNA inserts.**

The four-round biopanning process was considered successful when the number of colonies on agar plates originating from cancer antibodies was at least twofold higher than the number of colonies derived from normal IgG. The colonies derived from the patient antibodies were grown overnight and the phage was purified by double precipitation with polyethylene-glycol. The phage DNAs were purified using Strataclean Resin (Stratagene) and later sequenced with the primer 5' CCCTCATAGTTAAGCGTAACG. The sequencing method used was the D-Rhodamine ABI prism automatic sequencing system (Perkin Elmer), as described in the manufacturer protocol.

**Expression of recombinant peptides and protein fragments**

PCR amplifications of the peptide-encoding DNA inserts were performed with phage ssDNA and the primers 5' AGGCTCGAGGATCCTCGGCCGACGGGGCT 3' (sense) and 5' AGGTCTAGAATTCGCCCCAGCGGCCCC 3' (antisense). The template was purified from phage with Strataclean resin (Stratagene). The PCR conditions included 35 cycles of 1 minute at 95$^{O}$C for denaturation, 1 minute at 53$^{O}$C for annealing, and 1 minute at 72$^{O}$C for extension. The PCR products were ethanol-precipitated in the presence of glycogen (1 µl of a 20 mg/ml stock solution; Boehringer-Mannheim) and then digested with BamHI and EcoRI. The fragments were then ligated into the same restriction sites of the pGEX2TK

vector (Pharmacia). Bl-21 *E. Coli* cells were transformed with the constructs and the fusion proteins were purified using gluthatione coupled beads (SIGMA) following the manufacturer's protocol (Pharmacia NJ).

A cDNA encoding a 75 amino acid fragment of SART-1 protein with the QDLYS sequence in its central region was amplified by PCR from a plasmid containing the full length SART-1 gene using primers 5' AGTCATGGATCCAGGAGCCGGCAGCTGCAGAA 3' (sense) and 5' AGTCATGAATTCGCCTTTGTCCTTGAGGGTAAG3' (antisense). The PCR conditions were as described above, except that the annealing temperature was $55^{o}$C. The PCR product was digested with BamHI and EcoRI and ligated into the same restriction sites of the pGEX2TK vector.

**ELISA.**

A 10 μg/ml solution of purified GST-fusion proteins in 0.1M $NaHCO_3$ was used to coat multiwell plates (50μl per well). After washing with 0.05% Tween 20 / PBS (washing buffer) and blocking with 3% BSA /0.05% Tween 20 / PBS (blocking buffer), the plates were incubated overnight at 4°C with cancer patients' and healthy individuals' sera diluted 1/200 in blocking buffer. The plates were then washed and incubated for 2 h at 4°C with anti-human alkaline phosphatase-conjugated antibodies (GIBCO) diluted in blocking buffer. The plates were later washed and developed using p-NPP (SIGMA) as a substrate.

**Protein database search**.

We performed a homology search with the peptide sequences isolated through library panning on IgG and IgM antibodies. For this purpose we used the on-line available protein database resources from the NCBI and the EMBL. We utilized the BLAST and FASTA algorithms. The first was accomplished using the Advanced BLAST module[Altschul, 1990 #159][Altschul, 1997 #158] available on-line from the NCBI (URL: http://www.ncbi.nlm.nih.gov/BLAST/) which performs the homology search against the non-redundant (nr) database (includes

all GenBank Complementary DNA translations, PDB, SwissProt, PIR and PRF databases). For FASTA searches we chose the FASTA3[Pearson, 1988 #161][Pearson, 1990 #162] module from the EMBL outstation at EBI (Hinxton) also available on-line (URL:http://www2.ebi.ac.uk/fasta3/). This tool browses Swissprot, Trembl (translated EMBL database), and TremblNew (translated EMBL new database). The parameters of both modules were adjusted for optimal short motif homology search. With BLAST we were able to limit the search to human sequences.The PROTEAN module of DNASTAR suite (Lasergene) was utilized to predict secondary structure as well as the surface and antigenicity probability of certain sequences.

## RT-PCR

Multiplex RT-PCR assay was performed using the Clontech RT-PCR kit. 2μg of DNAase-treated RNA from normal and colon tumor tissues were used with random hexamers to synthesize the first strand. Control G3PDH and FUT5 or SART-1 gene fragments were amplified in a single PCR reaction. The primers used for amplification of the FUT5 cDNA fragment were: 5' ATGGATCCCCTGGGCCCGGCCA 3' (sense) and 5' CCGTAGGGCGTGAAGATGT 3' (antisense). The primers used for amplification of the SART-1 cDNA fragment were 5' ATGGGGTCGTCCAAGAAGCATC 3' (sense) and 5' AGGGCAGGTAGTCAGGCTTCTTCT 3' (antisense). The primers used for amplification of a 983 bp fragment of control G3PDH gene were supplied with the RT-PCR kit. PCR conditions were 35 rounds of 95°C for 1 min, 58°C for 1 min, and 72°C for 4 min, followed by a 10 min at 72°C final extension step. PCR products were visualized by electrophoresis on a 1% agarose gel.

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LEGENDS

**Figure 1**. Enrichment of RPPD library with phage particles that bind specifically to IgG antibodies from the serum of cancer patient A after four rounds of biopanning. After each round of preadsorption on an excess of normal serum IgG and affinity selection on patient A IgG, amplified phage particles were purified and quantified. Equal amounts of phage (107 cfu) were incubated for 1 hour with normal serum IgG and cancer patient A IgG immobilized on protein G agarose. After washing out unbound phage, the remaining phage were eluted and used to infect bacteria. Aliquots of infected bacteria were spread on LB plates containing tetracycline. The next day, the number of colonies on plates corresponding to normal IgG and cancer patient A's IgG were quantified.

**Figure 2**. ELISA assay for binding of serum IgG antibodies to peptides isolated from RPPD libraries. Purified GST-peptide fusion antigens were used to coat ELISA microplates. After blocking with 3% bovine serum albumin (BSA) in PBS the antigens were incubated overnight with cancer patient and normal individual sera diluted 1:200 with 3% BSA in PBS. The binding was detected using goat anti-human IgG antibodies conjugated with alkaline phosphatase and p-NPP as a substrate. GST-A and GST-B are TGVRGQRISQ and MKQSGHHRSE peptides expressed as GST fusion proteins. As control peptides we used arbitrarily chosen peptides isolated from phage libraries and expressed as GST fusion proteins. The average of three measurements is shown.

**Figure 3**. Simultaneous RT-PCR amplification of FUT5 and G3PDH gene fragments from normal colon tissue and colon tumor tissue mRNA. PCR products were electrophoresed on a 1% agarose gel. Numbers 1, 3, 5, 7, 9 and 11 are RT-PCR products from normal colon samples mRNA. Numbers 2, 4, 6, 8, 10 and 12 are RT-PCR products from corresponding colon tumor samples. Number 13 is the PCR product obtained from a cDNA of normal colon obtained from Clontech. Number 14 is the RT-PCR product obtained from the tumor of colon cancer patient A.

**Figure 4.** ELISA assay of binding to the SART-1 GST fusion protein of serum IgM antibodies from cancer patients A, B, C and D and from four healthy individuals (N1-N4). Averages and the standard error of the mean of three measurements are shown.