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Serological immune response to cancer testis antigens in patients with pancreatic cancer

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Serological screening approaches have allowed for the identification of a large number of potentially relevant tumor antigens in cancer patients. Within this group, cancer testis antigens represent promising targets for cancer immunotherapy, since they are widely expressed in a variety of human cancer entities. In pancreatic cancer, however, there are only few data available about the expression pattern and serological response to cancer testis antigens and other serological-defined tumor antigens. Therefore, we investigated the IgG antibody response against 11 cancer testis antigens (SCP-1, GAGE, LAGE-1a,-1b, CT-7, NY-ESO-1, SSX-1-5) recombinantly expressed on yeast surface (RAYS) in patients with pancreatic cancer ($n = 96$), chronic pancreatitis ($n = 18$) and healthy donors ($n = 48$). We found in 14% of all patients antibody responses to SCP-1, but not to other cancer testis antigens (GAGE, LAGE-1a,-1b, CT-7, NY-ESO-1, SSX-1-5). Antibody response correlated with the expression of SCP-1 in the primary tumor of the respective patient as shown by RT-PCR, immunohistochemistry and Western blot. In contrast, no serological response to cancer testis antigens was observed in healthy donors. The humoral immune response against SCP-1 was associated with the size of tumor, but not with other clinico-pathological parameters such as histology, stage, presence of lymph node metastases, grading, age, gender or gemcitabine treatment. In conclusion, antibody response to cancer testis antigen SCP-1 is found in a proportion of pancreatic carcinoma patients. These results indicate that identification of additional tumor antigens by serological screening of tumor cDNA expression libraries by RAYS is a promising goal in pancreatic cancer.

Key words: cancer testis antigens; neoplasm antigens; SCP-1; pancreatic neoplasm; RAYS; SEREX; autoantibodies; immunoglobulin G

The recent progress in surgery,[1] radiotherapy[2] and chemotherapy[3] of pancreatic cancer has not changed the 5-year survival rate of this tumor entity, which remains 3–5% for all stages.[4] Therefore, efforts to find therapeutic alternatives for pancreatic cancer patients have to be enforced. Immunotherapy has the potential to provide anti-tumor activity that can be integrated into conventional surgical, radio- and chemotherapeutic treatment without significantly adding to therapy-associated toxicity.

A prerequisite for the development of tumor-specific immunotherapeutic strategies is the existence of tumor antigens, i.e., genes that are either exclusively or preferentially expressed in malignant tissues, compared with normal tissues. According to their expression pattern and the specificity of the immune responses they evoke, antigens expressed by human neoplasms can be classified into different groups.[5] These include the cancer testis antigens (CTA), also called cancer germline antigens (e.g. the MAGE family), the differentiation antigens (e.g. tyrosinase), the products of viral genes (e.g. HPV-16), mutated genes (e.g. mutated p53), differentially spliced genes (e.g. LDH C[6]), overexpressed genes or amplified genes (e.g. eIF-4γ [7]) as well as common autoantigens that are expressed by the malignant cells of a tumor (e.g. U1 snRNP).[5] In pancreatic cancer, only few tumor antigens are known and most of them are overexpressed in tumor cells, but will also be found at a certain level in normal human cells (e.g. CEA, Her-2/neu, MUC-1, CA19-9, CA 242, 17-1A and mesothelin).[8,9] The expression of these genes in normal tissue limits their use as targets for specific immunotherapy. Therefore, the identification of more, especially more specific, tumor antigens is warranted.

In recent years, a powerful serological technique using high-titred immunoglobulin G antibodies obtained from the sera of cancer patients has been developed for the identification of tumor antigens. By means of SEREX, the serological identification of antigens by recombinant expression cloning, it was possible to identify a multitude of new cancer antigens in different tumor entities, which are documented in the international SEREX database (www2.licr.org/Cancer ImmunomeDB/).[10] For example, several new cancer testis antigens were defined by this approach (i.e., NY-ESO-1, SSX-1-5, SCP-1 and CT-7).[11] Cancer testis antigens are characterized by their expression in a variety of malignant tumors but not in normal tissue, with the exception of testis. They were identified by the screening of recombinant expression of tumor tissue from non-pancreatic origin (i.e. melanoma, esophagus carcinoma),[12] but were found to be present in pancreatic carcinoma samples.[13] For some of these antigens, it has been demonstrated that they do not only elicit a humoral B-cell response but also a cellular CD8+/CD4+ T cell response.[14] This means that SEREX-defined tumor antigens might be appropriate target antigens for either antibody- or vaccine-based approaches.

However, there is growing evidence that the antigen repertoire detectable by the conventional SEREX approach is limited.[5] This might be attributed in part to the fact that potential antigens that are subject to posttranslational modifications remain undetected in bacterial expression systems, which generally are not capable of post-translationally modifying recombinantly expressed proteins. As a consequence, we recently established a eukaryotic cDNA expression system in yeast (RAYS) to overcome this SEREX-inherent problem.[15,16] Yeast has the advantage that recombinant proteins can be expressed on the cell surface as part of the cell wall in a more naturally folded and partially glycosylated manner.[16–18] The ability of the yeast display system to detect potentially relevant tumor antigens not recognized by conventional SEREX demonstrates the power of this new tool.[15]

Considering these facts, it seems to be promising to use SEREX and/or RAYS in combination with a cDNA expression library of pancreatic adenocarcinoma tissue to identify novel pancreas carcinoma-associated antigens. The prerequisite for such an approach would be a sufficiently strong humoral immune response against pancreatic carcinoma antigens. However, at the present time there are only few data available about the extent of antibody responses

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TABLE I – PRIMER PAIRS USED FOR AMPLIFICATION OF CDNA FRAGMENTS

5′ GAGE-2	GAATTCATGAGTTGGCGAGGAAGATCG
3′ GAGE-2	CTCGAGACACTGTGCTTGCTTTTGACCACC
5′ NY-ESO-1	GAATTCCAGGCCGAAGGCCGGGGGCAC
3′ NY-ESO-1	CTCGAGTTAGCGCCTCTGCCCTGAGGG
5′ LAGE-1a	GAATTCCAGGCCGAAGGCCAGGGCACAGG
3′ LAGE-1a	CTCGAGTTAGCGCCTCTGCCCTGAG
5′ LAGE-1b	GAATTCCAGGCCGAAGGCCAGGGCACAGG
3′ LAGE-1b	CTCGAGCTAAATGTGAGGGGCAGAGAACATCAC
5′ SSX-1	GAATTCAACGGAGACGACACCTTTGCAAAG
3′ SSX-1	CTCGAGCTCGTCATCTTCCTCAGGGTCAC
5′ SSX-2,4	GAATTCAACGGAGACGACGCCTTTG
3′ SSX-2,4	CTCGAGCTCGTCATCTTCCTCAGGGTC
5′ SSX-3	GAATTCAACGGAGATGACACCTTTGCAAGG
3′ SSX-3	CTCGAGCTCATCATCTTCCTCAGGATCGCTG
5′ SSX-5	GAATTCAACGGAGACGATGCCTTTGTACGG
3′ SSX-5	CTCGAGCTCGTCATCTTCCTGAGGGTCG
5′ SCP-1.1	GGATCCAGTGTGGTGATGGAAAAGCAAAAGCCCTTTGC
3′ SCP-1.1	ATACCTGCTTTTCCTTGTCATTTATTTCC
5′ SCP-1.2	GGATCCAGTGTGGTGGTGCAAGCTGAGAATTCCAGACTGG
3′ SCP-1.2	AGTGTGAAGTTAATTCAGTATTCTTAAGC
5′ SCP-1.3	GGATCCAGTGTGGTGAGTGAACAGTATTATTCAAAAGAG
3′ SCP-1.3	ATTCTTTTGCCTCTCTTTTGAGTTTTTCC
5′ SCP-1.4	AGAGCATCTTTGGAGATTGAACTATCC
3′ SCP-1.4	AACAAATAACTTTTCAGCTTCTTTTAG
5′ CT-7	CCAAAGAATTCGGCACAATTGCCTCAGGGG
3′ CT-7	CTCGAGTCACTCAGAAGAGAAGCTGGGGGA

against tumor antigens in pancreatic cancer,[19] and it cannot be excluded that humoral immune response is suppressed by pancreatic carcinoma-related factors.[20] Against this background, the objectives of this study were (a) to examine systemically the humoral immune response to cancer testis antigens as representative tumor antigens in pancreatic cancer and (b) to test the applicability of RAYS for the screening of antibody responses in this tumor entity.

Material and methods

Tissues and cell lines

This study was approved by the local ethical review board (''Ethikkommission der Ärztekammer des Saarlandes'') and performed according to the Declaration of Helsinki. After informed consent, serum samples were collected from 100 patients with inoperable or operable adenocarcinoma of the pancreas. In a proportion of patients (76%), tumor samples were obtained from pancreaticoduodenectomy (Whipple operation) or pancreatectomy at the Saarland University Medical School (Homburg, Germany), at the Rostock University Medical School (Germany), at the University of Heidelberg Medical School (Mannheim, Germany) and at the University of Hamburg Medical School (Germany) between January 1997 and November 2000. Tumor stage and grading were determined according to the UICC (Union International contre Cancer).[21]

Reverse transcriptase-PCR for the detection and amplification of cancer testis antigens in tumor samples

cDNA was obtained from different tissues and RT-PCR was performed as previously described using primer pairs given in Table I.[13] cDNA plasmids coding for the SSX and GAGE family members were kindly supplied by Dr. Y. Chen/Dr. A. Gure (Weill Medical College of Cornell University, New York) and by Dr. B. van den Eynde (Ludwig Institute for Cancer Research, Brussels, Belgium), respectively.

Immunohistochemistry

Immunohistochemical staining of SCP-1 protein was done in paraffin-embedded sections with mouse monoclonal anti-SCP-1-antibody (SC554, IgG_1), as described earlier.[13]

Expression of cancer testis antigens on yeast surface (RAYS)

The amplified codogen regions of the CT-antigens GAGE-2, NY-ESO-1, SSX-1, SSX-2, SSX-3, SSX-4, SSX-5, SCP-1.1, SCP-1.2, SCP-1.3, SCP-1.1-2, SCP-1.2-3, SCP-1.2-4, LAGE-1a, LAGE-1b and CT-7 were cloned into the multiple cloning site of the pYD1 plasmid (Invitrogen, Leiden, The Netherlands; Table I).[15] Plasmids were introduced into *Saccharomyces cerevisiae* EBY100, using a commercially available transformation kit (EasyComp, Invitrogen, The Netherlands), according to the manufacturer's recommendations. Thereafter, yeast were spread on MD-Leu plates (0.67% yeast nitrogen base, 2% dextrose, 0.01% leucine, 1.5% agar) for the selection of transformed clones. Colonies were picked and cultured [in YPD/CAA-Glu: 1% yeast extract, 2% polypeptone and 2% dextrose or in YNB/CAA-Glu: 2% dextrose, 0.67% yeast nitrogen base and 0.5% Casamino acids (Difco, Livonia, Minnesota, USA)] with constant agitation to an approximate OD (600 nm) of 4 (30°C, 4 h). To induce surface protein expression, yeast were pelleted by centrifugation, resuspended to an OD (600 nm) of 0.6 in YNB/CAA-Gal and grown with agitation for additional 48 h (20°C).

Immunofluorescence staining for flow cytometry

Recombinant antigen expression on yeast surface (RAYS) was used for serological analysis as described (Fig. 1).[16] In brief, 10^4–10^5 transformed and induced yeast cells containing tumor cDNA fragment—pYD1 plasmids—were collected by centrifugation (2000 *g*, 5 min), washed with PBS and incubated with 100 µl of preabsorbed serum (1:100 dilution, RT, 30 min) with occasional agitation. Parental control yeast transformed with empty pYD1 vector (Ctrl pYD1) served for human sera preabsorption (Fig. 3*b*). Then, cells were washed, secondary biotinylated anti-human-IgG Fcγ specific serum (Dianova, Hamburg, Germany) was added and the mixture incubated was with occasional agitation (dilution 1:200, RT, 30 min). Finally, R-Phycoerythrin-conjugated Streptavidin (Dianova, Hamburg, Germany) was added (dilution of 1:200, RT, 15 min) and washed twice with PBS (0.1% Tween) before analyzing the sera reactivity by flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany). Expression control for the cloned antigens was performed by the detection of expressed His-tag or X-press-tag, using the specific biotinylated anti-penta-His antibody (1:500, Qiagen, Hilden, Hamburg) or the anti-X-Press antibody (1:500, Invitrogen, Leiden, The Netherlands), respectively

(Figs. 3*a* and 3*b*). Thirty thousand cells were counted and the ratio between the intensity of the signal measured on antigen expressing and non-expressing (Ctrl-pYD1) yeast was calculated for each individual sample.

Western blot analysis

Serum antibody responses against endogenous SCP-1 protein were tested by standard Western blot analysis.[15,22] Briefly, 1.5, 0.5 and 0.15 mg of testis and 2 mg of the pancreatic tissues (pancreatic carcinoma, chronic pancreatitis and normal pancreas) were diluted in SDS-loading buffer and electrophoresed on a 8% SDS polyacrylamide gel. Then, separated proteins were blotted with a semi-dry blotter (Bio-Rad, Munich, Germany) on a PVDF-membrane (Bio-Rad), and SCP-1 protein was recognized by a previously determined optimal dilution of mouse monoclonal anti-SCP-1-antibody 554 (6 μg/ml). Anti-mouse conjugated peroxidase antibody (1:3,000, Bio-Rad) and enhanced chemoluminescence kit (Amersham Pharmacia, Freiburg, Germany) were used for detection (Fig. 6*b* and 6*c*).

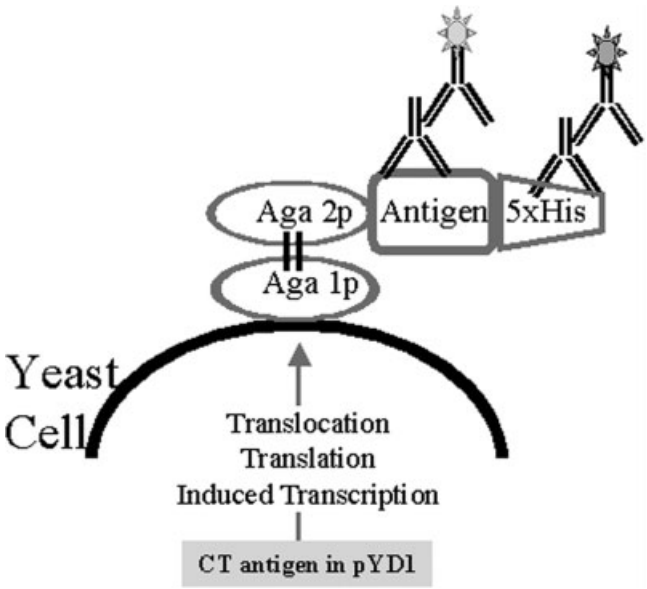

Figure 1 – Schematic diagram of surface expression of CT antigens on yeast cell wall. The vector pYD1 with inducible promoter was used for high yield expression of CT antigens on the surface of *S. cerevisiae* as a tightly regulated fusion protein to Aga2p. Antigens expressed in this way can be utilized for detection of reactive IgG serum antibodies derived from cancer patients and healthy donors. Correct expression was confirmed by staining of the C-terminal penta-his epitope.

Statistical analysis

Crosstables were tested by χ^2- or Fisher exact test where appropriate. To interpret the significance of differences observed, p values <0.05 were considered as significant.

Results

Study population

In total, we investigated the humoral immune response against 11 cancer testis antigens in 96 sera of patients with pancreatic carcinoma (SCP-1, GAGE2, LAGE-1a, LAGE-1b, SSX-1-5, NY-ESO-1, CT-7). In a certain number of cases ($n = 24$), tumor tissue of the primary tumor was available for investigation of protein and mRNA expression by immunohistochemistry and RT-PCR. The average age of the involved patients was 62 years and the women to men ratio were 1:1.2. Half of the patients (47%) had metastatic disease, and the other half (53%) was submitted to diagnostic or therapeutic laparotomy. Inoperable pancreatic carcinoma was detected in 13% of the patients and 40% had been treated by pancreaticoduodenectomy (Whipple operation) or pancreatectomy. The majority of operable patients (72%) suffered from locally advanced pancreatic cancer, i.e., UICC tumor stages II (pT3N0M0) or III (pT1-3N1M0).[21] The complete clinico-pathological characteristics of the patients, including grading, were available for 63 cases (65%, Table II).

Expression of cancer testis antigens on yeast surface

The concept for the expression of cDNA fragments on the yeast surface and the serological detection by human immunoglobulin has been described before[15,16] and is illustrated in Figure 1. In summary, the expression of cancer-specific antigens is induced on the surface of *S. cerevisiae* as fusion proteins to Aga2p, which are his-tagged at the carboxy terminus. Correct expression of the respective antigen is demonstrated by flow-cytometric detection of

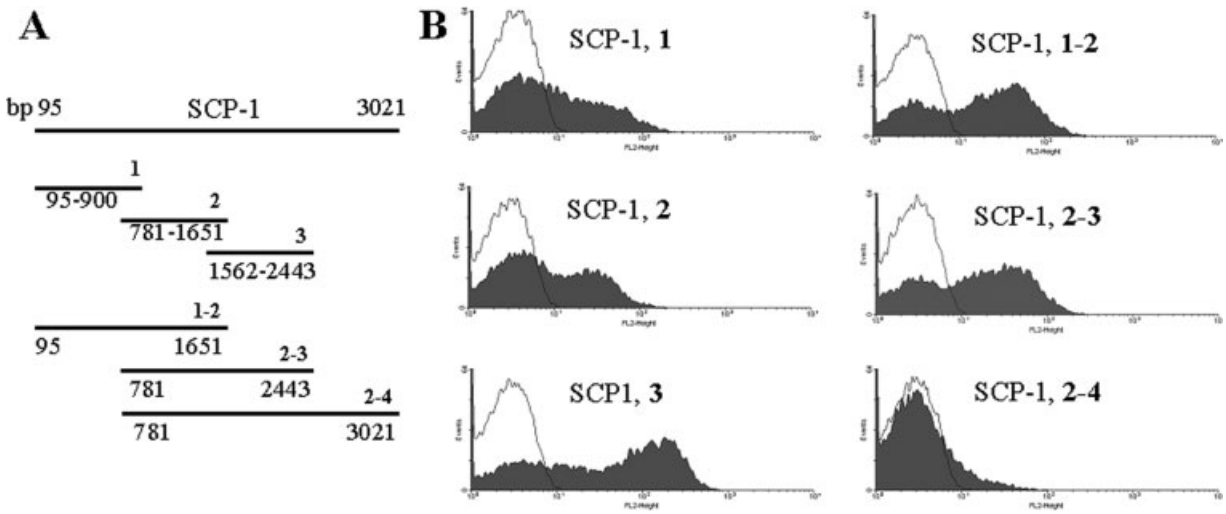

Figure 2 – Recombinant antigen expression of the SCP-1 protein fragments 1, 2, 3, 1-2, 2-3 and 2-4 on yeast surface. (*a*) Schematic diagram of the SCP-1 fragments cloned into pYD1 expression plasmid (bp, base pair). (*b*) Correct protein induction and cell surface expression was analyzed for SCP-1 protein fragments 1, 2, 3, 1-2, 2-3 and 2-4, as described. Antigen expression on induced (closed curve) and noninduced (open curve) yeast was detected using an anti-Histidin antibody.

TABLE II – PATIENTS CHARACTERISTICS AND SERUM REACTIVITY AGAINST SCP-1

Characteristic	Number of patients per group	Number of patients with seroreactivity against SCP-1
Age		
>60 years	43	8 (19)
≤60 years	20	3 (15)
Gender		
Male	32	7 (22)
Female	31	4 (13)
pT status		
pT_{1-2}	15	0 (0)[1]
pT_{3-4}	48	11 (23)
pN status		
pN_0	25^2	3^2 (12)
pN_1	25^2	5^2 (20)
Stage		
I/II	23	3 (13)
III/IV	40	8 (20)
Grading		
Well/moderately differentiated	35	6 (17)
Poor	28	5 (18)
Total	***63***	***11 (17)***

Values in parentheses are in percentages.
[1] $p = 0.041$, T_{1-2} versus T_{3-4} by χ^2-test.–[2] $n = 50$ for pN.

the His-tag by an anti-His-antibody (Figs. 2*b* and 3*a*, closed curve). If no human cDNA fragment is inserted in the pYD1 vector (control pYD1 yeast), Aga2p is expressed on the yeast surface and can be detected by the anti-His-antibody (Fig. 3*b*, closed curve). Non-induced yeast cells do not express the carboxy-terminal His-tag (Figs. 2*b*, 3*a* and 3*b*, open curve). In this way, strong antigen expression on the yeast surface was observed for the tumor antigens GAGE-2, LAGE-1a, LAGE-1b, CT-7, NY-ESO-1 and SSX-1 up to SSX-5. However, we failed to express the full length protein of SCP-1 on yeast surface. To include SCP-1 in the analysis, the protein was divided into different fragments (SCP-1 fragment 1, 1-2, 2, 2-3, 3, 2-4) (Fig. 2*a*), which revealed satisfactory expression levels of SCP-1 fragments 1, 2, 3, 1-2 and 2-3 on yeast surface (Fig. 2*b*), whereas the expression of SCP-1 fragment 2-4 was low (Fig. 2*b*). An explanation for the insufficient expression of the C-terminal SCP-1 fragment 2-4 on yeast surface could be the basic character of this protein domain, which may influence transport or fixation to the cell wall.

Serological analysis of 11 cancer testis antigens with pancreatic cancer sera

Importantly, correctly expressed cancer specific antigens can be detected by IgG antibodies present in the sera of cancer patients. As an example, the reactivity of such a serum with the cancer testis antigen SCP-1 (fragment 2-3) is demonstrated in Figure 3*c*

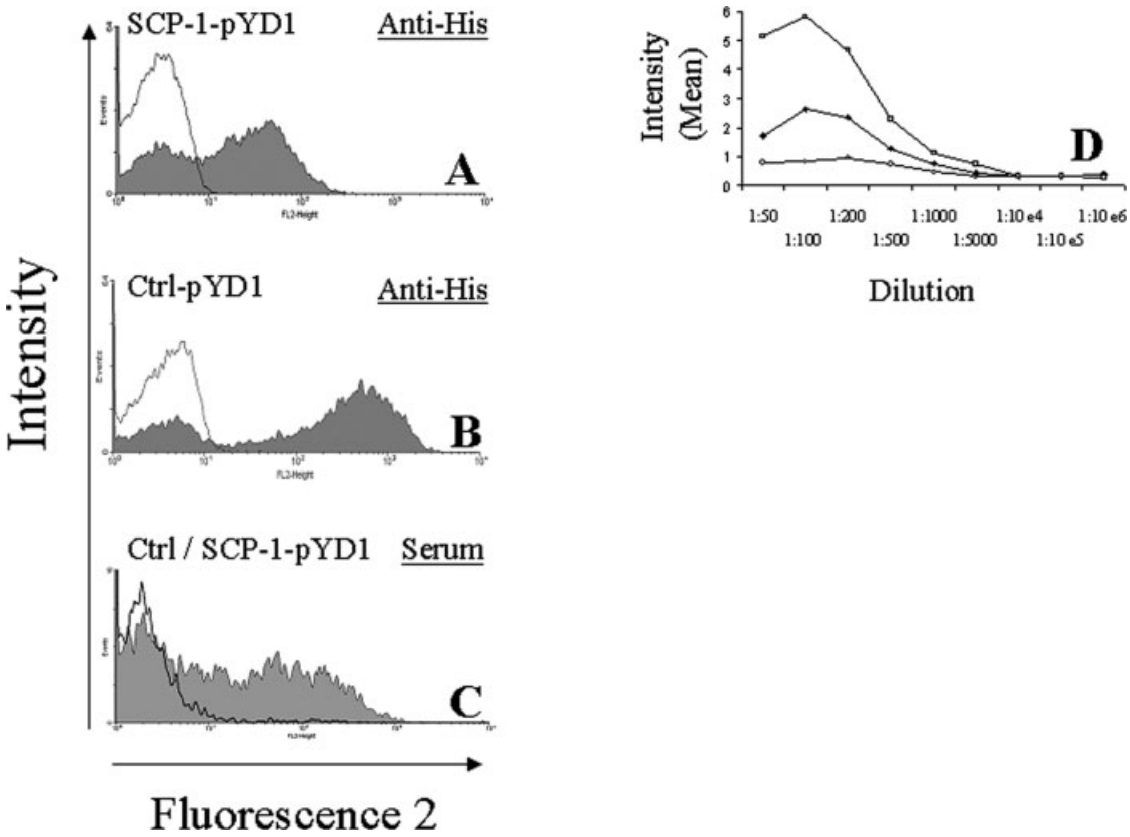

FIGURE 3 – Quality control of antigen-expression on yeast cell wall exemplary for the cancer-testis-antigen SCP-1 (fragment 2-3). (*a*) Correct expression of antigen is demonstrated by flow-cytometric detection with anti-Histidine antibody on induced yeast (closed curve) containing the SCP-1-pYD1 vector. Non-induced yeast serves as negative control (open curve). (*b*) Correct expression of Aga2p on induced parenteral yeast transformed with empty pYD1 vector (Ctrl pYD1) detected by anti-Histidin antibody (closed curve). This yeast served as negative control in comparison to SCP-1-pYD1 vector containing yeast to exclude cross reactions of sera against yeast or vector epitopes [(*c*), open curve]. Non-induced yeast serves as negative control (open curve). (*c*) Reactivity of pancreatic cancer serum diluted 1:100 with SCP-1-pYD1 (closed curve). Ctrl pYD1 serves as negative control (open curve). (*d*) Strength of the humoral immune response against SCP-1-pYD1 examined by serum dilution. Positive reaction can be observed up to a dilution of 1:5000 in highly reactive sera (upper line) and up to a dilution of 1:1000 in moderately reactive sera (middle line), whereas negative sera did not show significant dilution effects (lower line).

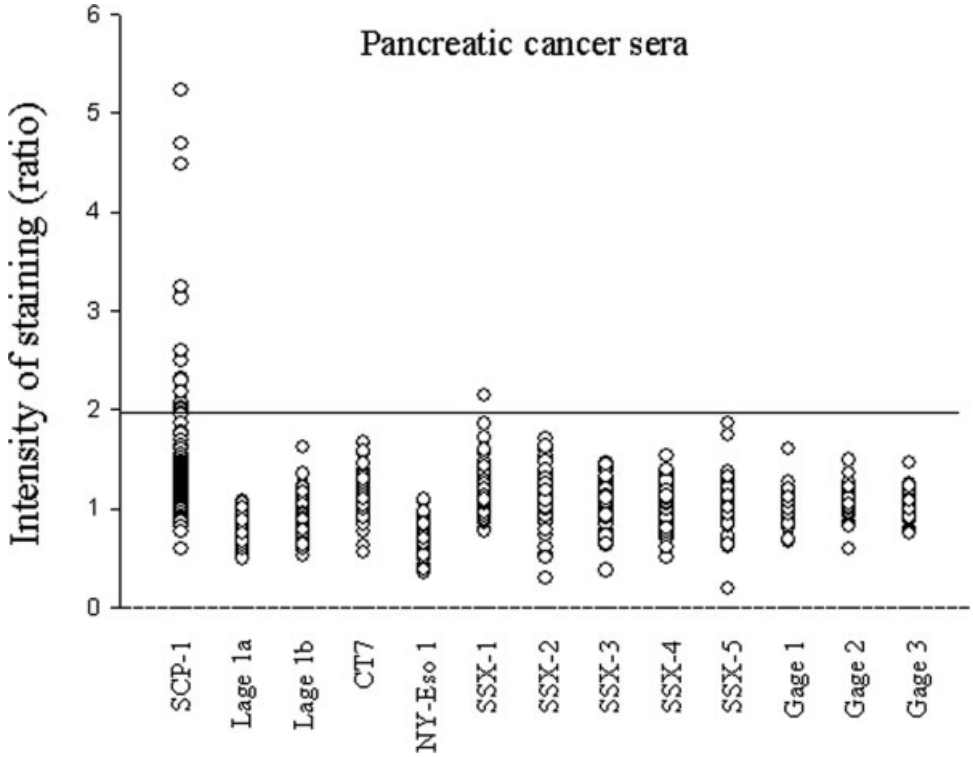

FIGURE 4 – Serological response against CT antigens in pancreatic carcinoma. Dot plot diagram of 96 pancreatic cancer sera incubated with yeast expressing CT antigens as listed in the X axis. Patients sera were diluted 1:100 and analyzed by flow cytometry for binding as described. The ratio between the intensity of staining of test yeast and control pYD1 yeast was calculated. Values above 2 were considered as positive signals (line).

(closed curve). As negative control, serum was incubated with yeast containing empty pYD1 vector (control pYD1 yeast = ctrl pYD1) and did not demonstrate a positive reaction (Fig. 3*c*, open curve). In this way, we analyzed the humoral immune response against 11 different cancer testis antigens in 96 sera from pancreatic adenocarinoma patients. For this purpose, a final panel of 16 yeast clones expressing cancer testis antigens (SSX-1-5, LAGE-1a, LAGE-1b, NY-ESO-1, CT-7, GAGE-2, SCP-1 fragment 1, 1-2, 2, 2-3, 3, 2-4) was produced. In order to evaluate the specificity of the antibody responses for cancer patients, tumor sera were compared to 46 sera from healthy donors and 18 sera from patients with acute or chronic pancreatitis. As described earlier, all sera were diluted 1:100 and were tested in parallel on induced yeast expressing the Aga2 fusion protein alone (control pYD1 yeast) or fused to the respective cancer testis antigen (test yeast, p.e. SCP-1-pYD1) (Fig. 3*c*). The ratio between sera reactivity on test yeast and control pYD1 yeast was calculated. Values above 2 were considered as positive signals. Humoral immune response against cancer testis antigen SCP-1 was found in 14 out of 96 pancreatic cancer patients (14.6%, Fig. 4). For SSX-1, one positive antibody response was detected in the total of 96 cases. A positive serum response against other cancer testis antigens was not observed (Fig. 4). A detailed analysis of the serum response against different SCP-1 fragments demonstrated that most of the reactive sera are directed against fragments 2, 3 and 2-3, which represent the central part of the SCP-1 protein, including amino acids 781-2443 (Fig. 5). However, it is important to note that analysis of serum reactivity against the C-terminal region of SCP-1 was limited by the low expression of fragment 2-4 on yeast surface (Fig. 2*b*), which may lead to false negative results. Sera from normal donors ($n = 48$) and from patients with acute ($n = 3$) or chronic pancreatitis ($n = 15$) did not react with any SCP-1 protein fragments expressed on the yeast surface (Fig. 5). To analyze the strength of the immune response against SCP-1, reactive sera were diluted in different concentrations (1:50, 1:100, 1:200,1:500, 1:1,000, 1:5,000, $1:10^4$, $1:10^5$, $1:10^6$). Reactivity in lower dilutions correlated with high values concerning the ratio between sera reactivity on test yeast and control pYD1 yeast. For example, a positive reaction can be observed up to a dilution of 1:5,000 in highly reactive sera (Fig. 3*d*, upper line) and up to a dilution of 1:1,000 in moderately reactive sera (Fig. 3*d*, middle line), whereas negative sera did not show significant dilution effects (Fig. 3*d* lower line).

Serum response and mRNA expression of cancer testis antigens

The analysis of tissue samples of pancreatic adenocarcinoma revealed SCP-1 mRNA expression in 62.5% (15/24) cases (selected samples in Fig. 6*c*). In 9 out of these 15 cases (60%) a humoral immune response was detected by RAYS, whereas no serum reactivity was found for RT-PCR negative cases ($p = 0.05$ by Fisher exact test). Cancer testis antigens GAGE-2 and SSX-4 were found to be expressed in 16% (4/24) and 5% (1/24) of the cases, respectively, but no humoral immune response was observed. Neither serum reactivity nor mRNA expression was shown for CT7 (0/24), SSX-1, SSX-2, SSX-3 (0/24), NY-ESO-1 (0/24) and CT-7 (0/24). The mRNA-expression of LAGE-1a, -1b and SSX-5 was not investigated. Cancer testis antigen mRNA expression was not found in normal pancreas ($n = 4$, Fig. 6*c*). Despite the detection of SCP-1 mRNA in 2 out of 8 and GAGE mRNA in 1 out of 9 patients with chronic pancreatitis, we did not observe any humoral immune response against SCP-1 or GAGE in 15 sera derived from patients with chronic pancreatitis.

Serum response and protein expression of SCP-1

In a limited number of pancreatic carcinoma patients who were mRNA- and seropositive for SCP-1 ($n = 4$, Fig. 6*c*), tumor tissue samples were available as paraffin-embedded sections and were investigated for the expression of SCP-1 by immunohistochemistry, using a monoclonal anti-SCP-1-antibody. SCP-1 positive tumor cells were demonstrated in 2 of 4 patients with seroreactivity against SCP-1, whereas we could not detect SCP-1 protein expression by immunohistochemistry in 4 samples from SCP-1 seronegative patients. As demonstrated previously,[13] SCP-1 positive tumor cells showed a fine granular cytoplasmatic or a spotted nuclear staining. A typical example is shown in Figure 6*a*. SCP-1

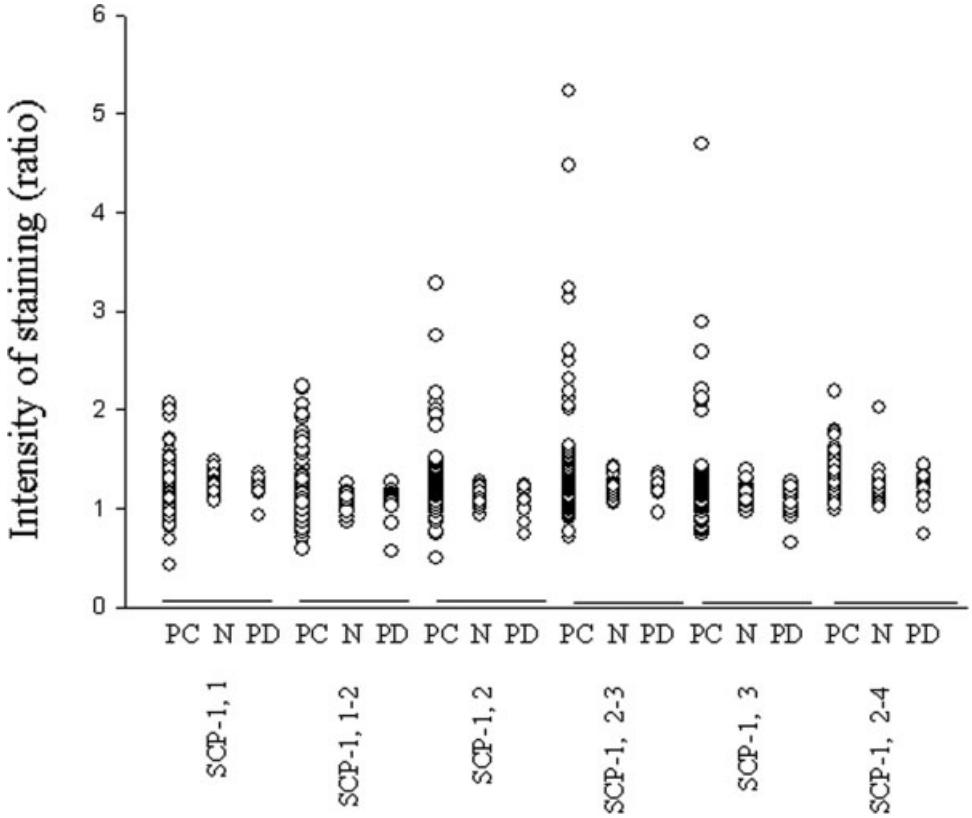

FIGURE 5 – Serological response against different SCP-1 protein fragments in 96 patients with pancreatic carcinoma (PC), 18 patients with chronic pancreatitis (PD) and 46 healthy individuals (N). Dot plot diagram of sera incubated with yeast expressing SCP-1 fragments 1, 1-2, 2, 2-3, 3 and 2-4. Patients sera were diluted 1:100 and analyzed by flow cytometry as described.

expression in pancreatic carcinoma samples derived from patients with SCP-1 seroreactivity was confirmed by Western blot analysis after the correct staining procedure had been established on testis tissue (Fig. 6*b*). Detection of a protein with molecular weight of 114 kDa was found in protein extracts of SCP-1-RT-PCR-positive tumor probes, but not in SCP-1-RT-PCR-negative probes or extracts obtained from chronic pancreatitis or normal pancreas (Fig. 6*c*).

Serum response to SCP-1 and correlation with clinicopathological parameters

Pancreatic carcinoma patients with pT status 3 or 4 demonstrated seroreactivity against SCP-1 in 23% of the cases, whereas none of the patients with pT status 1 or 2 had a humoral immune response against SCP-1 ($p = 0.041$ by χ^2-test, not significant if a significance level adjusted to multiple testing is used). This indicates that patients with larger tumor and antigen masses tend to develop more frequently a serological response against SCP-1 than patients with smaller tumors. Seroreactivity against SCP-1 did not correlate with age, gender, pN-status, stage or grading (Table II). In addition, seroreactivity was found in almost the same portion of patients not treated or treated with gemcitabine [(18% (7/40) versus 17% (4/23), respectively)]. This means that seroreactivity was not affected by the treatment of patients with gemcitabine, which was reported to compromise humoral immune responses.[23]

Discussion

Despite some evidence for a tumor-specific immune response in patients with pancreatic cancer, its extent and prognostic importance is still a matter of debate. In this study, we investigated the serological response to different cancer testis antigens by means of a novel system for recombinant expression of tumor antigens in eukaryotic yeast (RAYS). In a proportion of patients, we noticed an antibody response to the cancer testis antigen SCP-1, whereas no seroreactivity was found against this antigen in patients with acute or chronic pancreatitis or in healthy donors. This demonstrates that a strong tumor-specific humoral immune response to a SEREX-defined tumor antigen is present in patients with pancreatic carcinoma. Moreover, it might be an indication that other so far unknown tumor antigens may be identified by techniques based on the screening of sera obtained from patients with pancreatic carcinoma.

SCP-1 is the most frequently found antigen in pancreatic carcinoma (60%), regarding the mRNA expression.[13] The observed humoral immune response to this cancer testis antigen also suggests a relevant protein expression of SCP-1 in pancreatic carcinoma. This is additionally supported by the simultaneous detection of SCP-1 mRNA (by RT-PCR) and SCP-1 protein (by Western Blot and immunohistochemistry) in tumor samples of SCP-1-seropositive cases. Moreover, we could recently demonstrate a HLA-class I[24] and HLA-class-II-restricted[25] T cell response against SCP-1, indicating an integrated immune response involving T and B cells specific for this antigen in cancer patients. With the restriction that data are only available at the level of mRNA expression, the second most frequently expressed cancer testis antigen is GAGE (16%). Nevertheless, we could not detect antibody responses against this antigen, a finding which is, to our knowledge, in agreement with the published literature. GAGE was originally defined by reverse immunology using a GAGE-specific T cell clone,[26] which could indicate that GAGE elicits a cellular but possibly not a humoral immune response.[27] Another rather methodical reason might be that RAYS is not sensitive enough to detect anti-GAGE antibodies, although its sensitivity for other CT antigens is at least equivalent to Western blotting or ELISA.[16] Other cancer testis antigens are very rarely (SSX-4, 2%) or not at all (SSX-1, SSX-2, NY-ESO-1 and CT-7) found to be expressed in pancreatic adenocarcinoma, as shown in this present and a more

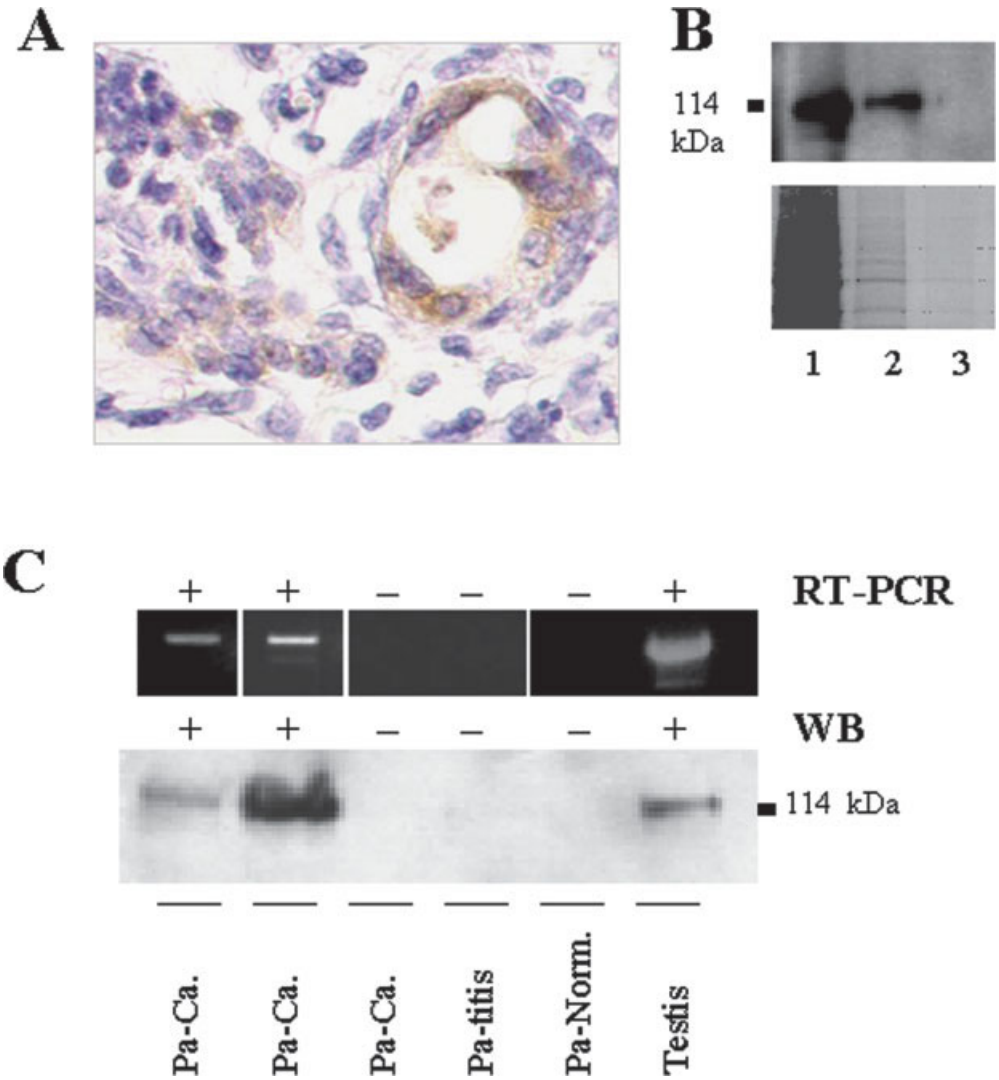

FIGURE 6 – Detection of SCP-1 at mRNA and protein level in pancreatic cancer of seropositive patients. (*a*) Immunohistochemical detection of SCP-1 by monoclonal antibody SC554 in the primary tumor of a patient with pancreatic adenocarcinoma. Staining was performed as described in Material and Methods. Picture was taken with a final magnification of ×800. (*b*) Detection of SCP-1 protein in testis by Westen Blot. Descending amounts of protein extract (lane 1, 1.5 mg; lane 2, 0.5 mg; lane 3, 0.15 mg) separated in SDS gel electrophoresis were analyzed for the presence of SCP-1 protein by SC554 monoclonal antibody (upper picture). Coomassie staining indicates different amounts of loaded tissue sample (lower picture). A protein with the molecular weight of ~114 kDa was demonstrated and the strength of bands decreased in accordance to the amount of protein. Theoretic MW of pSCP1:114 kDa. (*c*) Detection of SCP-1 protein in pancreatic tissue by Western Blot. Samples of pancreatic cancer ($n = 3$), chronic pancreatitis and normal pancreas were analyzed for the expression of SCP-1 protein by SC554 antibody. SCP-1 was found in SCP-1-RT-PCR-positive tumor samples, but not in SCP-1-RT-PCR-negative samples or extracts obtained from chronic pancreatitis or normal pancreas (lower picture). All SCP-1-RT-PCR tumor samples were obtained from patients with seroreactivity against SCP-1 (upper picture).

extensive prior investigation.[13] In line with these expression data, we could not discover a serological immune response against these antigens.

The humoral immunogenicity of SCP-1 had originally been demonstrated in a patient with renal carcinoma.[28] SCP-1 (or SYCP-1/HOM-TES-14) is involved in meiotic chromosome pairing[29] and is frequently found in other neoplasms (between 30 and 50%), including breast carcinomas[30] and gastrointestinal cancers.[11,31,32] In breast carcinoma, we found a similar frequency of patients with serum reactivity against SCP-1 (12%) as in pancreatic carcinoma. In pancreatic cancer, serological responses against other tumor antigens like MUC-1 or mutated p53 are reported to be present in a similar range, i.e. 7–22% of patients.[33–36] Notably, responses against both MUC-1 and p53 were found in healthy donors and inflammatory processes like chronic pancreatitis.[35,36] In contrast, we did not observe any humoral immune response to SCP-1 in healthy donors and patients with acute or chronic pancreatitis. This observation may be related to the fact that MUC-1 and p53 are present in many or even all normal tissues. SCP-1 expression, in contrast, is almost exclusively restricted to cancer cells and germline cells, which usually are not accessible to the immune system. Surprisingly, SCP-1 expression was demonstrated in rare cases of chronic pancreatitis.[13] This is in line with previous reports of similarities in gene expression shared by adenocarcinoma of the pancreas and chronic pancreatitis, i.e. Ki-ras, and supports the concept that alterations such as K-ras mutation or CT antigen expression might transform into overt adenocarcinoma if additional genetic changes occur.[37] The lack of antibody response as to this disease in our study may be due to the limited number of samples tested.

It is unclear why a humoral immune response against a particular antigen can be found only in a subset of patients with pancreatic carcinoma or other cancers. Immunogenicity may depend on the level of expression, posttranslational modification or other types of protein processing, the extent of which may be varying among tumors even of the same origin.[38] Other factors that may influence the immune response include the variability of tumors and individuals as to MHC molecules and antigen presentation.[39]

This study demonstrates that recombinant antigen expression on yeast surface is valuable for the detection of humoral immune responses in pancreatic cancer. Therefore, this technique can also be used to identify novel tumor antigens so far not detectable by conventional SEREX analysis, which was previously performed for pancreatic cancer by Nakatsura et al.[40,41] We have recently shown that in contrast to RAYS, recombinant bacterial protein expression as performed by conventional SEREX approaches does not display the entire spectrum of epitopes recognized by serological immune responses against tumor antigens.[15] Therefore, the use of a eukaryotic expression system widens the potential spectrum of antigens detected by the humoral immune system. This opens the perspective that RAYS will enable the identification of a new quality of tumor antigens in pancreatic carcinoma, antigens that have escaped detection to date because they elicit immune responses against posttranslationally modified or conformational epitopes. With respect to therapeutic vaccine- or antibody-based approaches, there is growing evidence that it is important to have more than just one or two target antigens available. This is underlined by the heterogeneous expression of cancer testis and other antigens in pancreatic cancer.[13] Therefore, the next step will be a systematic screening for new target antigens by means of the RAYS technology. Hopefully, this will lead to the discovery of a more extended list of appropriate targets to broaden our antigen repertoire for the treatment of pancreatic cancer, because multiepitope immunotherapeutic approaches targeting two or more tumor antigens may represent the most promising strategy for the successful treatment of cancer.

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