

OPTIMIZATION OF DIAGNOSTIC ELISA - BASED TESTS FOR THE DETECTION OF AUTO-ANTIBODIES AGAINST TUMOR ANTIGENS IN HUMAN SERUM

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ABSTRACT

Colorectal cancer is one of the most common cancer types worldwide and it continues to be a serious public health problem. Early detection and diagnosis are of great importance in cancer management. At present, diagnostic blood tests are based on the detection of tumor-associated markers such as carcino-embryonic antigen (CEA), the cancer antigen CA19-9 for gastrointestinal cancer, CA15-3 for breast cancer or CA125 for ovarian cancer. The lack of sensitivity and specificity of these markers prevents their general use in cancer screening of an average risk population. Therefore, new cancer biomarkers or better screening methods are necessary to improve the diagnostics of the disease. This study was directed to the optimization of a diagnostic, enzyme linked immunosorbent assay (ELISA) based test to identify and validate new serum markers, such as extracellular Protein Kinase A (ecPKA) and Nicotinamide *N*-Methyltransferase (NNMT). In this type of assay, the cancer antigens are quantified indirectly - by detecting the presence of auto-antibodies against tumor proteins in human serum. The result of the optimization and validation process was in the case of ecPKA a reproducible and stable assay. In case of NNMT the assay was probably not sensitive enough.

KEY WORDS: Auto-antibody, tumor marker, ELISA, colorectal cancer.

INTRODUCTION

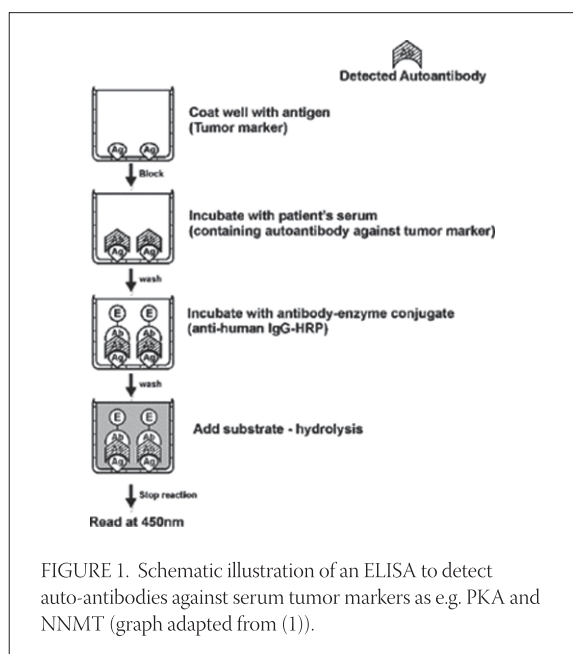
Serum tumor markers have been the most widely used approach for cancer detection. Currently, the majority of the available tumor markers represent cancer antigens. For example, the PSA represents a marker for prostate cancer, the CEA for colorectal cancer, the cancer antigen CA15-3 for breast cancer, the cancer antigen CA19-9 for gastrointestinal cancer, the cancer antigen CA125 for the diagnosis of ovarian cancer, AFP is a liver cancer marker or human hCG, a breast cancer marker (1, 2). Quantification of all markers is based on the antigen determination method and generally exhibits limited specificity and sensitivity. Therefore, new cancer biomarkers or better screening methods are necessary to improve the diagnostics of the disease. There is increasing evidence that the immune system of patients with cancer responds to tumor antigens with the production of antibodies. The observed accumulation of such "auto-antibodies" in cancer patients suggests them to be of high diagnostic/prognostic value (1, 3). Therefore, specifically developed auto-antibody detecting ELISAs could be used to quantify tumor markers, indirectly. As shown in Figure 1, the serum auto-antibodies that will be measured are bound between the coating antigen (tumor marker) and the enzyme labeled antibody, directed against human IgG. An increase in signal indicates the presence of auto-antibodies in the serum samples. It has been shown previously that the auto-antibody ELISAs exhibit enhanced sensitivity and specificity for some of the tumor markers, compared to ELISAs detecting the tumor antigen. In general, the auto-antibody ELISA is superior to antigen-determining kits concerning rapidity

and reproducibility, is easy to perform and represents a cost-saving method (4, 5). In this context, two interesting candidates for tumor markers are ecPKA and NNMT. In normal mammalian cells, the cAMP-dependent PKA is located strictly intracellular. In cancer cells of various cell types, however, PKA has been shown to be secreted into the medium. The speculation is that ecPKA excretion might elicit the induction of serum auto-antibodies against ecPKA, indicating that ecPKA might be a cancer antigen, and that the presence of such auto-antibodies could serve as a diagnostic marker. This has already led to the development of a novel enzyme immunoassay method that measures the level of IgG auto-antibodies generated against ecPKA (1), which we intended to confirm and optimize with our antibodies (Figure 1).

NNMT has recently attracted much interest in the field of different types of cancer. In humans, NNMT is predominantly expressed in the liver and is predicted to be located in the cytoplasm (6, 7). It could represent a new marker for colorectal cancer (CRC) that will further enhance detection of the disease and trigger a follow-up colonoscopy, as CRC patients showed elevated NNMT levels in serum. Furthermore, NNMT showed higher sensitivity than the established CRC-tumor marker CEA, indicating that NNMT could be more suitable to discriminate between patients with CRC and apparently healthy individuals (8, 9, 10, 11). But further studies are needed to address the potential correlation of NNMT-serum levels with the presence of CRC. As NNMT was described to be secreted into serum, we tested whether it elicited the development of auto-antibodies as well. All these facts indicate the probable utility of this new auto-antibody assay as a cancer screening tool without the false positives often associated with conventional testing. It could also provide a novel technology for cancer detection (1).

MATERIALS AND METHODS

The serum samples used for the optimization of the detection method were obtained from subjects with CRC (n = 8, mean age 59 years ± 21 years). Tested cancer patients were of different age and sex, with a wide range of stages of malignancy and different therapies. Control serum was obtained from healthy volunteers (n = 7, mean age 30 years ± 27 years). The serum samples were kept at -80°C until use and were thawed only once before use. The anti-ecPKA auto-antibodies and NNMT-levels were measured by solid phase ELISA. For this purpose, flat bottom polystyrene 96-well



Immunolon-4 HBX microtiter plates were used (binding capacity 100-200 ng IgG/cm²). Each single ELISA was repeated at least twice and standards, samples, blanks and/or controls were analyzed in duplicates.

Detection system for anti-PKA auto-antibody ELISA.

The microtiter plates were coated with 2 µg/cm² of the purified recombinant human PKA Cα subunit in 100 mm³ coating buffer (phosphate buffered saline (PBS). The plate was sealed with adhesive sealing films for microplates (EXCEL Scientific, Inc.) and was incubated overnight, in the dark at room temperature (RT). The plates were then washed once with PKA-buffer I (20 mmol/dm³ Hepes, 0,9% NaCl, 30 mmol/dm³ sucrose, plus 0,1% bovine serum albumin (BSA), pH 7,0), blocked for 2 h at RT with 100 µl of a four-fold dilution of BlockAce (AbD Serotec) and washed two times with PKA-buffer II (50 mmol/dm³ sodium citrate, 0,3 mol/dm³ NaCl, 0,5% Tween 20, pH 5,0-5,2). Then 100 mm³ of 10.000-fold diluted serum samples were added and incubated for 1 h at 37°C. Sera were diluted in sample PKA-buffer III (PBS pH 7,4; 0,1% Tween 20 and plus 0,25% BSA). After three washes with PKA-buffer II, 100 mm³ of 20.000-fold diluted anti-human IgG-HRP (Jackson ImmunoResearch Laboratories Inc., USA; Cat. Nr. 109-035-003) conjugate were added in buffer IV (PBS, 0,9% NaCl and plus 1% BSA), incubated for 1 h at RT. Then the plate was washed five times in washing PKA-buffer II, and incubated with 100 mm³ of the prestained TMB PLUS substrate solution (BOTREND) for 30 minutes at RT in the dark. The enzyme reaction was stopped with 100 mm³ of 0,45 mol/dm³ H₂SO₄. The absorbance was read within 30 minutes at 450 nm with an ELISA reader (SPEKTRA MAX).

Anti-NNMT auto-antibody ELISA.

The ELISA steps, the buffer system, incubation time and temperature for this detection system are identical to anti-PKA auto-antibody ELISA. Coating was carried out with 2 µg/ml of the NNMT human recombinant protein (GenWay Inc.; Cat. Nr.10-288-22087F). A two-fold serial dilution of chicken α-human polyclonal NNMT IgY (starting at 500-fold; GenWay Inc.; Cat. Nr.15-288-22087) was used as standard reagent and serum samples were diluted 10.000 – or 25.000-fold. As crude controls for the specificity of the assay, 100 mm³ of pure and ten-fold diluted mouse universal negative control (Dako Cytomation; Code Nr. N1698) were added. Anti-human IgG-HRP conjugate (Jackson ImmunoResearch Laboratories, Inc., USA; Cat. Nr. 109-035-003) was (two-fold) serially diluted in buffer IV (PBS, 0,9% NaCl, 1% BSA; from 10.000-

fold dilution to 40.000-fold dilution) to determine the most suitable concentration. Mouse α-human polyclonal NNMT (Abnova Inc.; Cat. Nr. H00004837-A01) was also tested as a standard reagent in the optimization process but showed less sensitivity and specificity compared to chicken α-human polyclonal NNMT IgY.

Detection and analyses.

Measurements were carried out on the SPECTRA MAX-ELISA plate reader, with standard quantification software for ELISAs. All optical density (OD) values represent means of duplicates +/- standard deviation.

RESULTS

The anti PKA auto-antibody assay.

First, the coating antigen and HRP-labeled antibody concentrations of the published ecPKA auto-antibody assay were tested for their applicability, using positive (CRC) and negative (healthy) serum samples. Wells coated with antigen showed a mean absorbance of 0,126 ± 0,0076 with coefficient of the variation (%CV) of 6,0% for the positive samples and 0,043 ± 0,0035, CV = 8,1% for the negative samples. When the plates were used uncoated (in absence of coating antigen), the mean value for the positive samples was 0,074 ± 0,0043, CV = 5,8% and 0,067 ± 0,004, CV = 5,9% for the negative. In both cases the %CV was below 10% and therefore acceptable. For purposes of optimization, four different dilutions of two different HRP-labeled IgG antibody preparations, the whole molecule (H+L) and IgG-Fcγ (specific fragment), were tested (Jackson ImmunoResearch Laboratories Inc.,USA; Cat. Nr. 109-035-003 and 109-035-008). When positive samples (patients with CRC) were tested, the 5.000-fold dilution of the IgG-HRP (H+L) antibody showed the best performance with a mean OD value of 0,183 ± 0,009 (CV 4,9%) which was significantly higher compared to the IgG-HRP Fcγ specific fragment (OD = 0,143 ± 0,001, CV 0,6%). Therefore the IgG-HRP whole molecule was chosen for further tests. In addition, six different concentrations (50; 25; 12,5; 6,25; 3,125 and 1,5625 ng/cm³) of the purified polyclonal rabbit PKA/C alpha/beta antibody were tested for the applicability as standard-reagent (STD). The distribution of the absorbance values vs. the concentrations of one representative experiment is shown in Figure 2. Two serum-dilutions were also tested to determine the best signal to noise ratio (s/n ratio). The 10.000-fold dilution resulted in a higher s/n ratio than the 25.000-fold dilution. The mean s/n ratio of the 10.000-fold diluted serum was 1,27 ± 0,08 (CV 6,3%), while the mean s/n ratio of the 25.000-

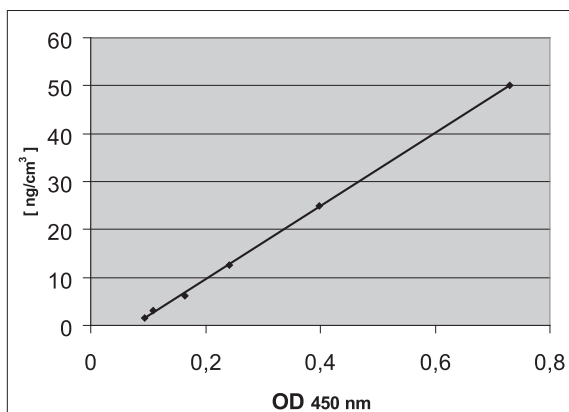


FIGURE 2. Standard curve with anti-PKA IgG, determined by anti-ecPKA auto-antibody ELISA. Linear distribution of the absorbance values for the anti-PKA STD-dilutions, measured with an ELISA reader; on x-axis: optical density values measured at 450 nm; on y-axis: anti-PKA IgG in ng/cm³. The concentrations of the STD were: 1,5625; 3,125; 6,25; 12,5; 25 and 50 ng/cm³. The coefficient of determination (R²) was 0,9997.

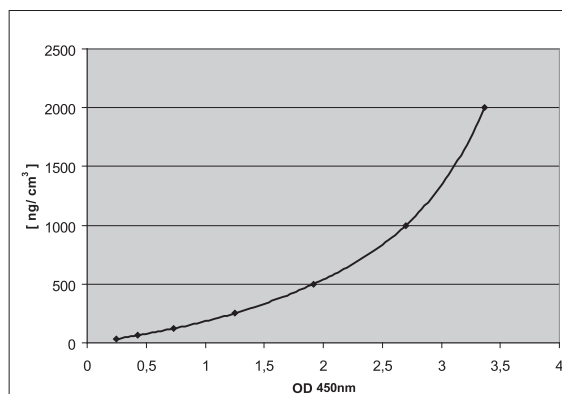


FIGURE 3. Standard curve with anti-NNMT IgY, determined by NNMT auto-antibody ELISA. Polynomial (5. order) distribution of the absorbance values for anti-NNMT STD-dilutions, measured with an ELISA reader; on x-axis: optical density values measured at 450 nm; on y-axis: anti-NNMT IgY in ng/cm³. Tested concentrations: 31,25; 62,5; 125; 250; 500; 1000 and 2000 ng/cm³. The coefficient of determination (R²) was 1.

fold diluted serum was $1,07 \pm 0,02$ (CV 2,5%). Therefore, the 10.000-fold dilution was used for serum samples analyzed with the anti-ecPKA auto-antibody assay. In this case, the OD for the blank was $0,068 \pm 0,0085$.

The NNMT auto-antibody assay. Analogue to the anti-ecPKA auto-antibody assay, the goal was to establish an ELISA that could detect potentially existing auto-antibodies against NNMT in the serum of patients with CRC. Several concentrations were tested for the STD, in this case the chicken α -human polyclonal NNMT IgY and four different dilutions of anti-human IgG-HRP antibody. As human STD-preparations were not commercially available, the STD of different species (e.g. mouse, chicken) was used. Fortunately, the anti-human IgG exhibited the expected cross-reactivity towards these non human STD. These tests showed on the one hand whether the absorbance values were in the desired range ($OD < 2$) and on the other hand which dilution of the enzyme conjugate was the most suitable one. STD concentration of 500 ng/cm³ (and lower) in combination with the 5.000-fold diluted IgG-HRP showed the best signal to noise (s/n) ratio and absorbance signals were in desired range (Figure 3). Additionally, mouse α -human polyclonal NNMT was also tested as STD, but showed a lower s/n ratio indicating lower sensitivity. The mean s/n ratio of the mouse α -human polyclonal NNMT was 1.44 and the mean s/n ratio of the chicken α -human polyclonal NNMT IgY was 2.13 (determined with a low STD concentration of 62,5 ng/cm³). The mouse universal negative control showed lower absorbance with a mean OD value of $0,066 \pm 0,002$; CV = 3,0%. Serum samples (10.000-fold

dilution) from a small set of patients with CRC showed the mean OD value of $0,078 \pm 0,003$, CV = 3,8% and the healthy controls $0,072 \pm 0,004$, CV = 5,5% which resulted in no significant differences between the healthy controls and the patients with cancer. The OD value for the blank was $0,08 \pm 0,003$, CV = 3,8%, indicating the possibility that auto-antibodies against NNMT don't exist or a too low sensitivity of the assay system.

DISCUSSION

The diagnosis of cancer is one of the most important and critical steps in cancer management. It has been shown that in the early phases of carcinogenesis, an increase of ecPKA- and NNMT expression occurs and that the presence of auto-antibodies generated against ecPKA highly correlate with cancer. The anti-ecPKA auto-antibodies serum levels were shown to be markedly up-regulated in patients with cancer (12, 13, 14). For NNMT, auto-antibodies have not been analyzed so far. Nevertheless it is assumed that the detection of ecPKA- and potentially also of NNMT auto-antibodies could serve as a diagnostic method for cancers of various cell types. Regarding auto-antibody measurement, the ecPKA and the NNMT protein could represent two new, interesting "indirect" biomarkers for early cancer detection. The auto-antibody biomarker ELISA represents a good alternative to the antigen determining ELISA because it saves time and costs of the currently available antigen diagnostic kits. Critical factors in ELISA development are antibodies/antigens, liquid and solid phase and of course the samples. The choice of antibodies is particularly important. Monoclonal antibodies (MAb) recog-

nize a single epitope and therefore, the use of MAbs results in a higher specificity of the detecting method but at the costs of lower sensitivity. Polyclonal antibodies (PAb) show higher sensitivity because of the possibility that more antibodies bind to a single antigen molecule, but they have a higher risk for unspecific cross-reactivity (15). There is no empirically correct choice and therefore, all candidate antibodies/antigens have to be tested to determine the optimal components. In this study, PABs were used for the ELISAs, because the adequate MAB was not commercially available at the time of ELISA optimization. Therefore, a higher specificity of the assays couldn't be established. The advantage of using PABs for these ELISAs is that they can be generated in a variety of species (e.g. rabbit, goat, mouse, chicken, donkey, etc.) thus giving rise to more options in experimental design. Sometimes cross-reactivity is a desired phenomenon, as in our case, the anti-human IgG fortunately exhibited positive cross-reactivity towards the non human NNMT - STD. PABs generally make the detection more robust and are more tolerant to small changes in the nature of the detected analyte. Sample type (e.g. serum or tissue extract) can also have a great effect on assay performance and on the choice of the assay components. Serum samples are complex mixtures and the auto-antibodies which are to be measured can be present in various concentrations. They can also be similar to other molecules which can lead to unwanted cross-reactivity with molecules that are not relevant for this test. All factors together can have an effect on the sensitivity and the specificity of the method. The results of the NNMT auto-antibody ELISA suggested the absence of NNMT

auto-antibodies in human serum. But the negative result could also have been due to a lack of assay sensitivity or due to the limited sample number. The assay parameters that could still be adapted for a better assay sensitivity are e.g. the coating antibody concentration, incubation time and sample dilution buffer. For the detection systems used in course of this study, a lower coating concentration was chosen. This should ensure that the antibody is the limiting factor. High concentrations of coating antigen can lead to less binding (the so-called "hook effect"). Furthermore, the addition of detergents or salt to the reaction mixture should reduce low affinity interactions indicating that the optimal composition and pH value of the fluid phase has to be determined for better removal of unbound and excessive components. All these factors have an influence on the sensitivity as well as on the specificity (16, 17). In course of this study several conditions were tested for the washing- and incubation buffers. Lower concentration of the detergent Tween 20 and higher salt concentration was tested for the washing buffer. An increased detergent concentration was used in the case of the incubation buffer. After establishing the best (optimal) protocol, the respective buffers were used for serum sample analysis. Still, the interassay absolute values were not completely identical, therefore a standard dilution series has to be included with every plate. The antibody/antigen and buffer optimization experiments performed in this pilot study, using a two and three dimensional serial dilution system (CTA), led to the development of an assay that was successful in distinguishing CRC patients from healthy volunteers using the anti-ecPKA auto-antibody ELISA.

CONCLUSION

The detection of auto-antibodies against tumor antigens in human serum certainly represents a promising approach in the field of cancer diagnostics. In fact, each tumor antigen that is shed into the blood stream is capable of inducing auto-antibody formation. Both developed tests successfully detect antibodies against the two tumor proteins that were used for the generation of the standard curve. In serum samples, however only PKA auto-antibodies were detectable. All together, both tests still need to be validated and have to be tested on a larger number of samples. In case of NNMT to finally prove the absence of NNMT auto-antibodies and in case of ecPKA to further confirm the correlation with CRC.

List of Abbreviations

C α	-	catalytic subunits of PKA
CRC	-	colorectal cancer
CTA	-	checkerboard titration assay
ecPKA	-	extracellular Protein Kinase A
F γ	-	IgG specific fragment γ
H+L	-	hard and light chain of an immunoglobuline
HD	-	healthy donors
NNMT	-	nicotinamide <i>N</i> -Methyltransferase
OD	-	optical density
PBS	-	phosphate buffered saline
s/n ratio	-	signal to noise ratio
STD	-	standard reagent
TMB	-	3,3',5,5'- tetramethylbenzidine base

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