Basic Investigations

STUDIES ON THE DETECTION KIT FOR THE COLORECTAL CARCINOMA ASSOCIATED LARGE EXTERNAL ANTIGEN

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ABSTRACT

Objective: To investigate the detecting method and diagnostic value of the tumor marker colorectal carcinoma associated large external antigen (LEA) for colorectal carcinoma. Methods: Monoclonal antibody ND-1, which can recognize LEA, was labeled with biotin aminocaproylhydrazide (BACH) and horseradish peroxidase (HRP) respectively. One step sandwich ELISA Kit for detecting LEA in serum was developed by the biotinylated antibody and enzyme conjugation. The validity and reliability of the kit were evaluated by sera selected from clinic. Results: the OD_{405} of healthy group was 0.056±0.038, and that of patients was 0.553±0.441. The difference between the two groups was significant ($P<0.01$). The cutoff value, which was 0.248, was determined by analysis of ROC. The diagnosis sensitivity, specificity and validity of the kit were 89.29%, 87.5%, 88.54% respectively. The ROC value was 0.95. The intra-CV was less than 5%, and the internal-CV was less than 10%. There was no significant difference between the results obtained by the kit and those obtained by pathological diagnosis ($P<0.01$). The expression of LEA was associated with the differentiated degree of colorectal carcinoma and had no relation to the Dukes' phase of the disease. Conclusion: LEA is practically useful tumor marker for the diagnosis of colorectal carcinoma. The kit developed can be used for the qualitative diagnosis for the disease.

Key words: Large external antigen; Colorectal carcinoma; Tumor marker; Kit

The colorectal carcinoma associated large external antigen (LEA) was first reported by Jin-dan Song, et al. in 1986.[1] Studies in serology and histology indicated that the antigen, with excellent specificity and sensitivity for colorectal cancer diagnosis, was clinically useful in the detection of colorectal carcinoma. Using anti-LEA monoclonal antibody ND-1, we attempted to establish one step sandwich ELISA kit for detecting LEA and investigated the diagnostic value of LEA for colorectal carcinoma.

MATERIALS AND METHODS

Sera

Fifty-six patients were studied with defined colorectal carcinoma, 22 of them were well differentiated, 29 moderately differentiated and 5 poorly differentiated; according to Dukes' phase, 8 in Dukes' I, 20 in Dukes" II, 15 in Duckes' III, 13 in Dukes' IV. In addition, 40 non-colorectal carcinoma sera were included. 34 control sera, matched for age and sex, were obtained from normal healthy blood bank donors.

Purification of Monoclonal Antibody

The mouse ascite was prepared by common procedure. The antibody ND-1 was purified by HiTrap Protein G column (AmPharmacia Inc.). The

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purity and activity were determined by SDS-PAGE and indirect ELISA respectively. The product was transformed into powder by freeze dry in vacuum.

Biotinylation of Antibody

The antibody ND-1 was biotinylated via sugar moieties by biotin aminocaproylhydrazide (BACH) according to the procedure described by O' Shannessy.[7] The label reaction was carried out with an input molar Biotin/IgG ratio of 200. the activity of the product was determined by indirect ELISA. The biotinylated antibody took effect of capturing antibody in ELISA.

Preparation of the Enzyme-antibody Conjugate

The conjugation of horseradish peroxidase (HRP) to antibody ND-1 was performed according to a modified mehtod reported by S. Madersbacher[8] with an input molar HRP/IgG ratio of four. The conjuugate was purified by a combined chromatography included HiTrap ConA and HiTrap Protein G column (AmPharmacia Inc.).[9] The molar ratio of bound enzyme per antibody was determined by OD_{405} and OD_{208}. The activity of the product was determined by ELISA. The conjugate was performed with detecting antibody in ELISA.

Coating the Plates with Streptavidin

Streptavidin was coated to plates by the procedure of Hylkema.[10] The biotin binding capacity of the plates was determined by indirect ELISA.

Development of One Step Sandwich ELISA

The ELISA conditions in this study were optimized and standarized according to experimental design techniques,[11] which mainly included (a) the optimum straptavidin concentration for coating the plates; (b) the optimum, detecting antibody and capture antibody concentrations and (c) variation of assay temperature and differing incubation times for each step. The assay was carried out in triplicate and the positive, and negative control were performed by supernatant of cultured CCL187 and healthy serum respectively. The ELISA protocol is as following: Streptavidin (50μl) at a concentration of 10μ g/ml was coated onto the plate surface. Serum or control (50 μl mixture of capture antibody (1.25 μ g/ml) and detecting antibody (0.6 μ g/ml) diluted in 50 mmol/l MES, pH6.1. The plates were incubated for 1.5 hours at 37℃. After the incubation, each swell was washed three times with 0.05% Tween-20 in PBS and filled with 100 μl of ABTS solution. After 30 min at 37 ℃ , the development of colour was terminated by the addition of 2M H_2SO_4 (50 μl), and the color was read on the microtiter plate reader at 405 nm.

Statistical Methods

The mean value and standard error of different groups were calculated. The data were analyzed by parametric statistical methods.

RESULTS

Difference of the Quantity of LEA in Sera between Healthy Group and Colorectal Carcinoma Patients Group

The OD_{405} of LEA in healthy persons was 0.056±0.038, while the patients was 0.553±0.441. The difference was significant (t'=8.42, df=44, P<0.01).

ROC Analysis for the Diagnostic Validity for Colorectal Carcinoma Patients of the Kit

The receiver operating characteristic curve (ROC) was worked out by setting the cutoff value at $\bar{x}+2s$, $\bar{x}+4s$, $\bar{x}+6s$, $\bar{x}+8s$, $\bar{x}+10s$, respectively. The results, shown in Table 1 an Figure 1, indicated that the sensitivity, specificity and validity of the test were best balanced, when the cutoff value was $\bar{x}+6s$, which was 0.248. At this point, the ROC value was 0.95. This proved that the kit was specific and valid for the colorectal carcinoma diagnosis.

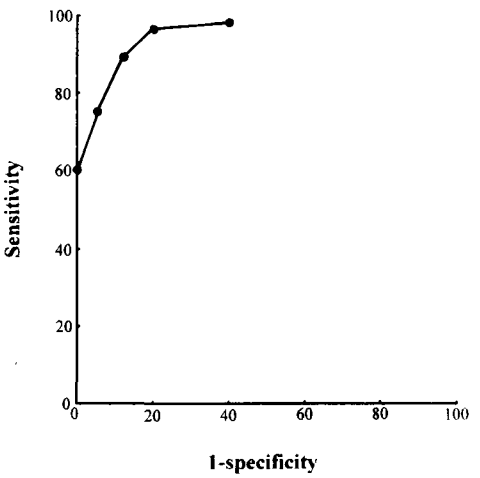

Fig. 1. The ROC curve of the diagnostic validity of the kit

Compare with Pathologic Diagnosis

Compared with pathologic diagnosis, there were 50 true positive, 35 true negative, 5 false positive, 6 false negtive among the diagnostic results acquired by the kit. As proved by χ^2 test, the two methods have no significanct difference (χ^2=53.13, P<0.01).

Table 1. ROC analysis for the diagnostic validity of the kit

Parameters	Cut off				
	$\bar{x}+2s$	$\bar{x}+4s$	$\bar{x}+6s$	$\bar{x}+8s$	$\bar{x}+10s$
Sensitivity(%)	98.21	96.43	89.29	75.00	60.71
Specificity(%)	65.00	80.00	87.50	95.00	100.00
Validity(%)	82.29	89.58	88.54	83.33	77.08
Positive anticipate (%)	77.46	87.10	90.90	95.45	100.00
Negative anticipate (%)	96.00	94.12	85.37	73.08	64.52

Repetition

Inter-CV (in quadruple) and intra-CV (during 4 continuous day) of the kit, obtained by two sera samples, were 3.3%, 2.7% and 8.2%, 9.1% respectively.

Diagnosis for Different Dukes' Phase and Differentiated Degree of Coloectal Carcinoma

The diagnostic sensitivity to different Duckes' phase and differentiated degree of colorectal carcinoma were investigated. Results are shown in Table 2. The diagnostic sensitivity of LEA for well-to-moderately differentiated colorectal carcinoma was obviously stronger than that for poorly differentiated one (u=2.662~3.234, P<0.05). In addition, the sensitivity value did not vary according to different Dukes' phase (u=0.177~0.776, P>0.05

Table 2. Analysis to the diagnosis for different Dukes' phase and differentiated degree of colorectal carcinoma of the kit

	Samples	Sensitivity(%)	Samples	Validity(%)
Dukes' Phase	56			
I	8	87.50	7	48.96
II	20	90.00	18	59.38
III	15	93.30	14	56.25
IV	13	84.20	11	52.08
Differentiated degree	56			
Well	22	90.90	20	61.46
Moderately	29	79.31	23	60.42
Poorly	5	20.00	1	39.58

DISCUSSION

Large external antigen, a novel colorectal carcinoma associated antigen, has unique physicochemical characteristics. Compared with cacinoma embryonic antigen (CEA), the major differences were: 1. LEA can not be analyzed by standard 7.5% SDS-PAGE and it could be eluted in the void-volume of a Sepharose 4B column and it was resistant to extraction from the cell surface of colorectal cell lines. 2. ^{125}I-CEA could not be immunoprecipitated with the ND-1 antibody. Purified CEA was not recognized by the ND-1 antibody with semiquantittive blot assay. This indicated that antigenity of LEA and CEA were evidently different. 3. Indirect immunofluorescence showed that LEA and CEA had different distributive patterns; researches of immunological electromicroscopy showed LEA and CEA had different tissue specificity. LEA was expressed most strongly on well-to-moderately differentiated colocarcinoma.[2-4]

Currently, some authors have carried out comparative studies of LEA and CEA to identify their diagnosis value in clinic by pathological and serological methods. In pathological diagnosis, the sensitivity of LEA for colorectal carcinoma was 86.3%, the specificity was 53.5%, while the CEA was 88.5% and 41.5% respectively. Comparing with CEA, the sensitivity of LEA was similar with CEA (P>0.05), but the specifivity was stronger than that of

CEA ($P<0.05$). In addition, LEA preferred high-to-well differentiated carcinoma to poorly differentiated ones. The positive rates in well, moderate and poor ones were 100%, 78.9% and 33.3% respectively.[5] In serological diagnosis, the positive rate of LEA for colorectal carcinoma was 68.7%, the specificity was 96.8%, while that of CEA was 56.6% and 93.&% respectively. The differences were significant ($P<0.05$).[6] These results indicated that LEA is more valid than CEA for the diagnosis of colorectal carcinoma and is promising in clinic application.

Studies of the diagnostic value of LEA to different differentiated colorectal carcinoma indicated that the diagnostic sensitivity of LEA for well and moderately differentiated carcinoma was stronger than that for poorly differentiated ones. As shown in the investigation of different Dukes' phase patients, the diagnostic sensitivity of LEA did not vary according to different Dukes' phase. This character of LEA is remarkablely distinguished from other tumor markers, such as CEA, CA19-9, CA242 and so on.[12] We can infer from this discovery that the expression of LEA may be constant once the colorectal carcinoma occurs. It is worth further investiging whether LEA can be applied to screen the early colorectal carcinoma.

In order to improve the sensitivity and specificity of the detecting kit, we applied following techniques. First, biotinylated the antibody via sugar moities. The sugar compent of IgG mainly distributes on the Fc region, and they were far from the antigen binding region of the antibody, so the immunoactivity and specificity of IgG were all most not affected by the biotinylation. Second, enzyme conjugate was purified by a combined chromatography method. This eliminated the affection of free antibody and enzyme on the sensitivity and background of the detection. Third, capture antibody was immobilized onto the solid support by streptavidin-biotin system. This can avoid the lose of antigen capture capacity caused by traditional absorbance method.[13] Furthermore, due to its unique characters, streptacdin can greatly reduce the background of immunoassay. Finally, experimental design techniques was applied to obtaine optimal and standard ELISA conditions. Owing to this, the performance of the kit was highly improved.

To sum up, LEA is practically useful tumor marker for the diagnosis of colorectal carcinoma; the kit developed, with rather well reliability and validity, can be used for the qualitative dignosis for colorectal carcinoma.

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