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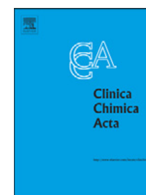

Serum anti-p53 antibody detection in carcinomas and the predictive values of serum p53 antibodies, carcino-embryonic antigen and carbohydrate antigen 12–5 in the neoadjuvant chemotherapy treatment for III stage non-small cell lung cancer patients

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ABSTRACT

Background: The serum p53 antibody (s-p53 Ab) is a valuable prognostic factor for carcinomas, but its common detection method, based on enzyme linked immunosorbent assay (ELISA), needs to be improved due to low sensitivity. Although neoadjuvant chemotherapy (NACT) is widely used in the treatment of non-small cell lung cancer (NSCLC) in China, forecasting chemoresistance is still a pressing problem.
Methods: Hybrid phage and wild-type p53 protein (wt p53 protein) were produced before the establishment of phage-ELISA and p53-ELISA. S-p53 Abs of 829 patients with various types of cancer was detected by a double ELISA system. 47 III stage NSCLC patients treated with mitomycin, vindesine and cisplatin (MCV)-based NACT were chosen for s-p53 Abs, carcino-embryonic antigen (CEA) and carbohydrate antigen (CA) 12-5 predictive value analysis.
Results: Through the combination of p53-ELISA and phage-ELISA (p53-phage ELISA), the sensitivity of s-p53 Abs in lung, breast, colorectal, gastric, esophageal, liver and ovarian cancer increased to 39.0%, 33.3%, 41.7%, 32.1%, 30.9%, 23.1% and 43.2% respectively. S-p53 Abs proved to correlate with nodal involvement, TNM stage, histological type (in lung cancer) or tumor size (in gastric cancer). As for the 47 III stage NSCLC treated with NACT, s-p53 Abs and CA12-5 remarkably decreased after NACT treatment ($P = 0.034$ and $P = 0.007$) and pre-NACT low s-p53 Abs correlated with high objective chemoresponse rate ($P = 0.016$).
Conclusions: p53-phage ELISA system has an edge over single p53-ELISA. S-p53 Abs level correlates with cancer patients' clinicalpathological parameters and can predict the chemoresponse of III stage NSCLC patients during MCV-based NACT treatment.

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1. Introduction

Neoplasia is always accompanied by gene mutations. The *p53* gene, as a critical tumor suppressor gene, was found mutated in >50% cancers [1,2]. Mutant *p53* gene encodes aberrant p53 protein whose half-life is longer than wt p53 protein. This aberrant protein is considered to be the antigen eliciting p53 autoantibodies due to a quite weak tolerance to endogenous p53 in the organism [3].

In 1982, s-p53 Abs was first reported by Crawford et al. [4] in the serum of 9% of breast cancer patients. Afterwards, antibodies of this sort were found in various types of tumor and cancer including lymphoma, leukemia, sarcoma, lung, prostate, thyroid, bladder and pancreatic cancer [5–7]. Many trials have demonstrated that high titer s-p53 Abs correlates with bad prognosis as well as short survival time [8,9]. Furthermore, in our last paper, we initially reported that s-p53 Abs had a predictive value for chemoresponse in the NACT treatment of III stage breast cancer patients [10].

Although s-p53 Abs can be easily detected by ELISA, the traditional ELISA approach has a prohibitively low sensitivity [7,11,12] and produces false negatives. As such, an improved, efficient and accurate ELISA system is hoped for.

Currently, hybrid phage displayed the peptide of p53 N-terminus immunodominant epitope SQAMDDLMLS (SQ) and wt p53 protein was produced and phage-ELISA as well as p53-ELISA was accordingly

Abbreviations: s-p53 Abs, serum p53 antibodies; wt, wild-type; NACT, neoadjuvant chemotherapy; SQ, SQAMDDLMLS; NSCLC, non-small cell lung cancer; CEA, carcino-embryonic antigen; CA12-5, carbohydrate antigen 12–5; MVC, mitomycin, vindesine and cisplatin; STBM, serum tumor biomarker; TNM, tumor, node and metastasis; AJCC, American Joint Committee on Cancer; CT, computed tomography; WHO, World Health Organization; ECOG, Eastern Cooperative Oncology Group; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; OR, objective response; NR, no response.

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constructed. S-p53 Abs of 829 patients with lung, breast, colorectal, gastric, esophageal, liver and ovarian cancer were detected by both phage-ELISA and p53-ELISA. The positive rates of the two ELISAs were jointly compiled and the result was designated as the positive rate of p53-phage ELISA.

NACT is commonly used as a systemic treatment for NSCLC in China. In this study, 47 III stage NSCLC patients who were treated with MVC-based NACT were examined both before and after NACT treatment. The change of the three serum tumor biomarkers (STBMs) after chemotherapy and their predictive values for chemoresponse were investigated.

2. Materials and methods

2.1. Patients, volunteers, sera, and the NACT scheme

Eight-hundred twenty-nine newly diagnosed cancer patients including 349 lung, 213 breast, 60 colorectal, 56 gastric, 55 esophageal, 52 liver and 44 ovarian cancers were enrolled in this study; all of them were from the Jilin Provincial Tumor Hospital between April 2005 and March 2008. Four hundred healthy volunteers were from the Northeast Normal University Attached Hospital. Patients and volunteers' recruitment and the sera sample collection were performed under the guidelines of protocols approved by the institutional review board. Informed consent was obtained from all persons.

Forty-seven III stage NSCLC patients who prepared to accept NACT treatment were chosen for further study. For each patient: age, sex, histological type, tumor size and lymph node situation were recorded before NACT treatment was begun. Tumor, node and metastasis (TNM) staging of tumor was defined according to the International Tumor Node Metastasis classification proposed by the American Joint Committee on Cancer (AJCC). Patients' histological types were determined by the examinations of bronchoscopic biopsy, brushing, or percutaneous needle aspiration specimen. Before NACT treatment, all patients underwent physical examination, imaging diagnostics with chest X-ray, computed tomography (CT) of the brain and the abdomen, bone scan and blood biochemical analysis. The following medical conditions were determined to be the eligibility criteria for recruitment: World Health Organization (WHO) Eastern Cooperative Oncology Group (ECOG) performance status 0–2, leukocytes $\geq 4.0\times10^9$/l, thrombocytes $\geq$100,000/μl, hemoglobin $\geq$10 g/l, serum creatinine $<$1.5 mg/dl, total bilirubin $<$26 μmol/l and serum transamines value$<$3 times of normal values. All patients within the group eligible under the above conditions were administered three 3-week cycles of neoadjuvant. The standard dosage of mitomycin was 8 mg/m^2 (day 1), 3 mg/m^2 vindesine (day 1) and 45 mg/m^2 cisplatine (days 1 and 2).

All patients' sera samples were obtained and stored at −80 °C until used. For the 47 NSCLC patients, sera samples were obtained 10 days before and after NACT. Four hundred healthy volunteers' sera samples were used to determine the cut-off value and reliability of the ELISA methods for detection of cancer patients' s-p53 Abs.

2.2. Immobilized antigens: wt p53 protein and phage-displayed peptide

Human recombination of wt p53 proteins with 6-His tag at N-termini were produced by M15 (kept by our lab) *Escherichia coli* (*E. coli*) and purified by Ni-NTA agarose (GE Healthcare, Pittsburgh, PA, USA). The phage-displayed peptide was also prepared according to literatures [13,14].

Two complementary DNA fragments of p53 protein N-terminus 37–46 amino acids SQAMDDLMLS were synthesized: 5′-GGAGGGTTCTCAAGCTATGGATGATTTAATGTTATCTCCAT-3′; 5′-CGATGGAGATAACATTAAATCATCCATAGCTTGAGAACCCTCCGC-3′ (Sangon, Shanghai, China). An oligonucleotide coding for the peptide SQ was cloned into pfd88 plasmid (kept by our lab); then, hybrid phage was produced by infecting TG_1 cells (kept by our lab) with the modified bacteriophages pfd88-SQ and purified.

2.3. SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of wt p53 protein and hybrid phage protein

A proper amount of purified wt p53 protein, hybrid phage protein and protein markers (Blue Plus, TransGen, Beijing, China) were separated by 15% and 20% SDS-PAGE respectively. Then the gels were visualized by Coomassie brilliant blue staining or silver staining. In Western blot, proteins were electrophoretically transferred to nitrocellulose membrane and blocked overnight at 4 °C with 5% non-fat dried milk in TBST [TBS (10 mmol/l Tris pH 7.5, 150 mmol/l NaCl) containing 0.1% Tween 20]. Afterwards, the membrane was incubated by monoclonal antibody against p53 (DO-7, Santa Cruz Biotechnology, Santa Cruz, CA) or cancer patients' sera (1:200 diluted in blocking buffer) for 1 h at 37 °C. Washed 3 times with TBST, the membrane was incubated for 1 h at room temperature with HRP-conjugated antibodies (Sigma-Aldrich, St. Louis, MO). It was then washed another 3 times with TBST and the membrane was stained with 3-amino-9-ethylcarbozole (AEC, AMRESCO, Solon, OH, USA), used as a chromogen. Blank controls and negative controls were carried out in parallel.

2.4. Establishment of ELISA procedures for detection of s-p53 antibodies

The wt p53 protein and the hybrid phage described at Section 2.2 were used as 2 immobilized antigens in p53-ELISA and phage-ELISA.

2.4.1. p53-ELISA

Polystyrene 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 50 μl wt p53 protein at a concentration of 5 μg/ml dissolved in 0.05 mol/l carbonate buffer, pH 9.6. Plates were subsequently washed three times with PBST [PBS containing 0.05% (v/v) Tween 20] and then twice with PBS. Excess binding sites were blocked using 100 μl of the blocking buffer (5% non-fat dried milk dissolved in PBS, pH 7.5). Wells were washed and 50 μl of serum diluted to 1/200 in the blocking buffer were added and incubated for 1 h at 37 °C. Plates were washed, incubated for 45 min at 37 °C with 50 μl of HRP-conjugated antibodies, diluted at a ratio of 1/10,000, and washed again. The peroxidase activity retained in the wells was assayed by the addition of 100 μl of tetramethylbenzidine (TMB, AMRESCO, Solon, OH, USA) solution. The reaction was stopped by adding 50 μl 2 N H_2SO_4 per well and the absorbance in each well was measured at 450/620 nm in a microtiter plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). All samples were measured in duplicate and the mean of the duplicate values was taken as the final read-out.

2.4.2. Phage-ELISA

Procedures followed in phage-ELISA are identical to the p53-ELISA which was stated at 2.4.1 except: the immobilized antigen was hybrid phage at a concentration of 60 μg/ml and the sera were pre-incubated by 10 μg/ml wt phage overnight at 4 °C.

2.4.3. Determination of cut-off value for p53-ELISA and phage-ELISA

Four hundred healthy volunteers' sera were investigated under the optimal conditions of p53-ELISA and phage-ELISA format to determine the cut-off values. Two sera were chosen as the controls and were correspondingly added to each plate. All results were expressed as p53 index: p53 index = $OD_{450/620nm}$ absorbance of a sample/$OD_{450/620\ nm}$ absorbance of the control serum. With specificities of 95% and 95.25% respectively, serum specimens with the p53 index of $>$1.7 were considered positive in p53-ELISA, and $>$1.1 were considered positive in phage-ELISA.

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