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ภาคผนวก

ตารางการปฏิบัติงาน

	2541		2542												2543			
	พ.ย.	ธ.ค.	ม.ค.	ก.พ.	มี.ค.	เม.ย	พ.ค.	มิ.ย.	ก.ค.	ส.ค.	ก.ย.	ต.ค.	พ.ย.	ธ.ค.	ม.ค.	ก.พ.	มี.ค.	เม.ย.
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. การศึกษาเตรียมงาน	•	•																
2. ดำเนินการวิจัยและรวบรวมข้อมูล			•	•	•	•	•	•	•	•	•	•	•	•	•	•		
3. วิเคราะห์ข้อมูล															•	•	•	•
4. รายงานผลการวิจัย																	•	•

Case record form

Serum p53 protein as Predictive Factor for Unfavorable Response to TOCE in Thai patients with HCC

Date of record..... Identification Number.....

Name..... Occupation.....

Address.....

.....

Tel.....

Age.....yrs Birthdate..... Sex.....

1. Performance status ☐0 ☐I ☐II ☐III ☐IV

2. Risk Factors	Yes	No	unknown
2.1 Alcohol	☐	☐	☐
2.2 HBV	☐	☐	☐
2.3 HCV	☐	☐	☐

2.4 Others.....

3.Previous Therapy ☐ Yes ☐ No

3.1 Chemotherapy Dates of Rx.....

Agents.....

3.2 Surgery Date.....

Type of Surgery.....

3.3 Others.....

4. Diagnosis

4.1 Diagnosis date .....

4.2 Criteria diagnosis

☐Histological Diagnosis hepatocellular carcinoma

☐Clinical Diagnosis + AFP more than 400 IU/L

5.Roegenographic Findings

Methods	Details
☐US of Liver ☐CT ☐MRI	
Date.....	
	Cirrhosis ☐Yes ☐No
	Mass
	Single ☐Yes ☐No
	Multiple ☐Yes ☐No
	Location(lobe) ☐R ☐L ☐Both
	Size specify.....
	.....
	Ascites ☐Yes ☐No
	Regional nodes
	Enlargement ☐Yes ☐No
	Detatils.....
	Portal vein inavastion
	☐Yes ☐No

6.Laboratory Findings

Date			
TB			
DB			
AP			
SGOT			
SGPT			
Gamma-GT			
Albumin			
Globulin			
PT			
FPG			
HbsAg			
Anti HCV			
AFP			
Serum P53			

7.Staging 7.1 แสดงการแบ่งระยะของของโรคมะเร็งตับชนิด HCC อาศัยการแบ่งตามระบบของ TNM.

Primary tumor	
TX	Primary tumor ไม่สามารถวัดได้
T0	ไม่พบ primary tumor
T1	ขนาดก้อนไม่เกิน 2 ซม. และไม่มีการลุกลามหลอดเลือด
T2	ขนาดก้อนไม่เกิน 2 ซม. แต่มีการลุกลามหลอดเลือด ขนาดก้อนเกิน 2 ซม. และไม่มีการลุกลามหลอดเลือด มีหลายก้อนแต่ก้อนใหญ่ที่สุดมีขนาดไม่เกิน 2 ซม., เป็นที่ตับกลีบเดียวและไม่มีการลุกลามหลอดเลือด
T3	ขนาดก้อนเกิน 2 ซม. และมีการลุกลามหลอดเลือด มีหลายก้อนแต่ก้อนใหญ่ที่สุดมีขนาดไม่เกิน 2 ซม., เป็นที่ตับกลีบเดียวแต่มีการลุกลามหลอดเลือด มีหลายก้อนแต่ก้อนใหญ่ที่สุดมีขนาดเกิน 2 ซม., เป็นที่ตับกลีบเดียว, อาจมีหรือไม่มีการลุกลามหลอดเลือด
T4	มีหลายก้อนและมากกว่า 1 กลีบ ลุกลามแขนงใหญ่ของหลอดเลือดดำ portal หรือ hepatic

Regional lymph node		Distant metastasis	
NX	ไม่ทราบลักษณะของต่อมน้ำเหลืองข้างเคียงได้	MX	ไม่ทราบการแพร่กระจาย
N0	ไม่มีการกระจายไปต่อมน้ำเหลืองข้างเคียง	M0	ไม่มีการแพร่กระจาย
N1	มีการกระจายไปต่อมน้ำเหลืองข้างเคียง	M1	มีการแพร่กระจาย

Stage grouping

I	T1	N0	M0
II	T2	N0	M0
IIIA	T3	N0	M0
IIIB	T1-3	N1	M0
IVA	T4	Any N	M0
IVB	Any T	Any N	M1

7.2 การแบ่งระยะตาม Okuda

Stage	Tumor size		Ascites		Albumin		Bilirubin	
	>50%	<50%	+	-	< 3 g/dL	>3 g/dL	>3 mg/dL	< 3 mg/dL
I	(-)		(-)		(-)		(-)	
II	1 หรือ 2 (+)							
III	3 หรือ 4 (+)							

*(+) : sign of advanced disease

8.Treatment TOCE

Number of procedures	Date	Complications
1		
2		
3		
4		

9.Evaluation of response of TOCE

	First TOCE	Second TOCE
9.1 Tumor size		
9.2 Percentage of lipioidal staining		

10.Result of response to TOCE

☐Response O Tumor size decrease more than 50 %

O Percentage of lipiodal more than 50 %

☐Non-response

O Not reach criteria respose

O Progressive disease(tomor size increase > 25 % or new lesions)

☐Can't evaluate

10.Status of patients Evaluation Date .....

☐ Alives

☐Dead ..... Date.....

Cause of dead.....

****

P53 pan ELISA

One-step-immunoassay

Photometry one-step-enzyme-immunoassay for the quantification of p53 (human, mouse, rat) in cell homogenates, plasma or serum.

Cat. Nr. 1 828 789

96 tests

Contents

6. Preparation of working solutions

7. Assay procedure
    - 7.1 General recommendations
    - 7.2 Pipetting scheme for the microtiter plate
    - 7.3 Working instructions
8. Related products
9. Quick reference protocols
    - 9.1 Required solutions
    - 9.2 Working procedure flow sheet

Kit contents

1. Anti-human-p53 pan-peroxidase (POD),
   Polyclonal antibody from sheep, lyophilizable (bottle 1, red cap)
2. Human p53 standards,
   Six concentrations between 0 pg/ml and 1200 pg/ml (see lot-specific label),
   Lyophilizates (botle 2a-f, orange caps)
3. Incubation buffer/Sample diluent,
   50 ml, ready-to-use solution (bottle 3, white cap)
4. 10x washing buffer, 100 ml (bottle 4, blue cap)
5. TMB substrate solution, 26 ml ready-to-use solution (bottle 5)
6. TMB stop solution, 8 ml ready-to-use (bottle 6)
7. Streptavidin coated microtiter plate,
   Precoated with anti-p53-biotin, monoclonal from mouse,
   8-well modules in a frame, 96 wells total
8. Self adhesive plate cover foils, 2 foils
9. Pack insert (blooklet)

Stability: The kit stable at 4 C until expiry date (see lot-specific lable imprint). For stability and storage conditions of working solutions see chapter 6. Microtiter plate strips, that have not been used, can be kept at least 4 weeks if stored in the dark and tightly closed at 4 C.

Advantages of the p53 pan ELISA

1.detects wild-type and mutant p53 (pantropic capture antibody and polyclonal detection antibody)

2.high sensitivity (10 pg/ml)

3.fast performance (2.5 h)

4.easy handling (one-step-immnoreaction only 3 steps total)

5.low background (streptavidin-coated microtiter plate)

6.easy and reliable calibration (6 pre-diluted standards)

7.function tested

1. Introduction

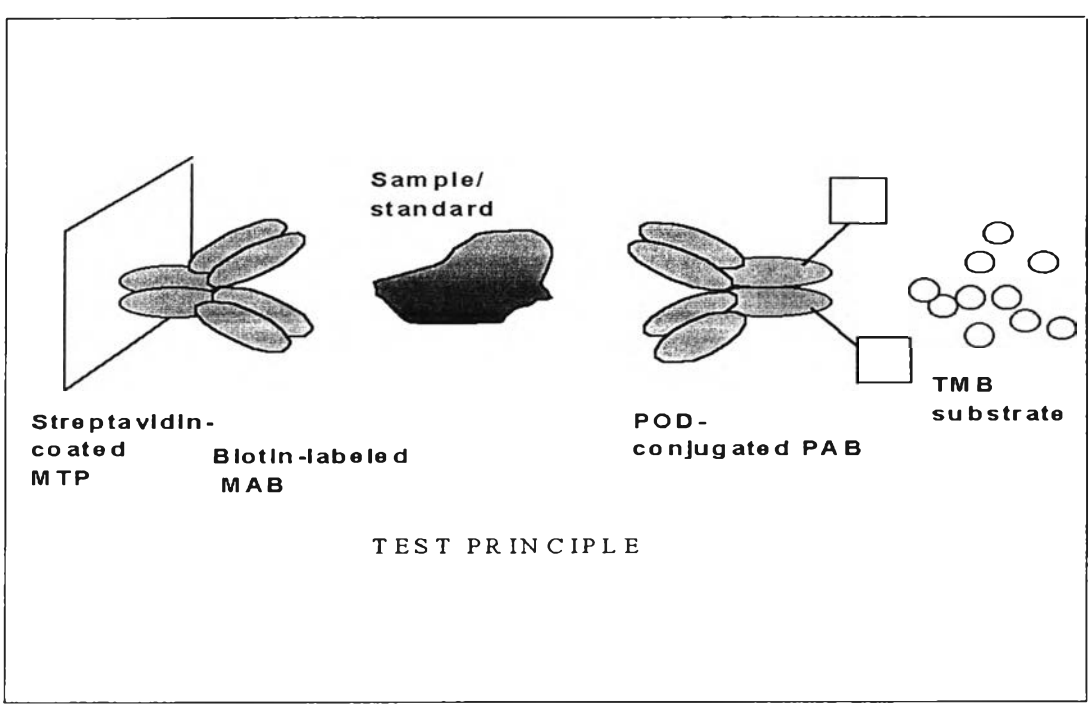
The p53 tumor suppressor is involved in the control of cell growth and programmed cell death. Inactivation of p53 by mutation, deletion or certain viral and cellular proteins increases the susceptibility to malignant transformation. Wild-type p53 levels in normal cells and tissues were found to be very low whereas in mammalian tumors and tumor cell lines mutant p53 polypeptide is often detectable in high concentrations. This accumulation of mutant p53 protein is the result of a conformational change in the protein, with consequent prolonged half-life and stability. Mutations in the p53 gene are among the most common genetic alterations in human malignancies.

2. Application

Research kit for the quantification of p53 (human, mouse, rat) in serum, plasma or homogenates from tumor-tissue or tumor cell lines. This assay is intended for use as a research tool in further uncer-staining the meaning of p53 levels in plasma, serum and cell homogenates.

3. Test principle (one-step-immunoassay)

The assay is based on a quantitative "sandwich ELISA" principle. The biotin-labeled capture antibody is pre-bound to the streptavidin-coated microtitier plate. During on single incubation step the p53-containing sample (specimen or standard) reacts with capture antibody and peroxidase-labeled detection antibody to form a stable immuno-complex. Subsequent to the washing step, the peroxidase bound in the complex is developed by tetramethylbenzidine (TMB) as a substrate. The photometrically determined color is proportional to the concerntration of p53.



รูปที่ 7 แสดง Test principle ของการตรวจหาโปรตีน p53 ด้วยวิธี ELISA

4. Assay characteristics

4.1 Sensitivity

The lower limit of detection (LLD) of the assay, which I commonly used to express analytical sensitivity has been determined from four independent experiments. In this study the mean signal of zero-standard plus two standard deviations was calculated from the standard curve to be 9 pg/ml.

A p53 concentration of 50 pg/ml will result in a signal of approximately two-times the signal of the zero-standard.

4.2 Measuring range

The linear measuring range of the ELISA is between 50 pg/ml and 1000 pg/ml

4.3 Specificity

The biotin-labeled capture antibody from mouse recognizes a conserved, pantropic, denaturation stable antigenic determinant of the p53 protein (human, mouse, rat). The peroxidase-labeled detection antibody is highly specific for wild-type and mutant p53 from different species.

4.4 Precision

4.4.1 Intra-assay variance

To determine the intra assay variance, three different concentrations of p53 were added to each of three different sera. The resulting of p53 concentrations were then determined in 5-fold measurements. A variance of <= 4.5% was established.

4.4.2 Inter-assay variance

To determine the inter assay variance, three different concentrations of p53 were added to each of three sera. The resulting p53 concentrations were then determined in 5-fold measurements of four successive days. In this experiment a variance of <= 12.5% was established.

4.4.3 Recovery

For the evaluation of the recovery, each of three different sera were spiked with three different known concentrations of p53 and one serum sample was left

untreated. The concentrations in the spiked and untreated samples were measured in 5-fold determinations. The recovery is defined as the difference between the measurements of the spiked and unspiked samples in serum, a recovery of 80-115% of the added p53 was found over the whole measurement range.

4.5 Human p53 standards

The p53 standards (mutant) derived the human epidermoid carcinoma cell line A431 is lyophilized in an artificial stabilizing matrix.

4.6 Sample material

Tissue homogenates, cell lysates, serum, plasma may be used as sample.

4.7 Assay time

2.5 hours at room temperature.

4.8 Warnings/Precautions.

- Use always the same assay procedure to minimize inter-assay variances
- Do not substitute kit reagents with those from other lots.
- Do not use kit reagents beyound expiration date.
- Do not expose kit reagents to strong light during storage or incubation time.
- Avoid contact of kit reagents with oxidizing agents and metal.
- Exposure to sodium azide will inactivate the conjugate.
- Avoid contact of skin with kit reagents.
- If the substrate stop solution (1M sulfuric acid) comes into contact with skin or eyes, wash immediately with sufficient amount of water and contact a physician.
- Strictly perform the assay at the recommended incubation times and temperature.
- Use plastic disposable, avoid glassware.
- Use polypropylene test tubes for sample dilutions.

4.9 Storage instructions

Store kit reagents, if not otherwise stated, at 4 C when not in use. Bring kit reagents to room temperature before starting the assay.

5. Sample preparation

Store material must not contain azide!

5.1 Serum

Use a serum separator or clot tube and allow samples to coagulate at room temperature. Within 30 min, after coagulation, spin samples at 700 x g for 15 min at room temperature and collect serum. Aliquot your samples and store at –20C. Use sample diluted (100 ul) or diluted with sample dilutent (e.g. 1:5).

6. Preparation of working solutions (room temperature 18-25 C).

- Solution 1: Anti-p53-POD (bottle1):

  Reconstitute the lyophilizate in 1 ml of redist. Water for 10 min and mix thoroughly. The reconstituted solution is stable for at least 3 months at 4 C.

- Solution 2: 2a-2f: p53 Standards (bottle 2a-2f):

  Reconstitute the lyophilizates in 500 ul of redist. Water for 10 min and mix thoroughly. Keep the portion required for the test series at 4 C. Aliquot the rest to be used for subsequent standard curves and store this at –20 C.

- Solution 3: Incubation buffer/sample diluent (bottle3):

  Ready-to-use, stable at 4 C.

- Solution 4: Washing buffer, 10x (bottle 4):

  Dilute content of the bottle 1:10 with redist. Water(100ml plus 900 ml). Stable at 4 C.

- Solution 5: TMB substrate solution (bottle 5):

  The substrate solution is ready-to-use. Fill the required quantity in a suitable vial immediate before use. The solution reacts to light if exposed over a longer period. Do not pipette direct from the bottle

into the wells. The solution is stable until the expiry date of the pack if stored at 4 C.

- Solution 6: TMB stop solution (bottle 6):

  Ready-to-use, stable at room temperature.

- Solution 7: Anti-p53-POD, working dilution (1:15):

  Do only prepare the amount of immunoreagent you need for the day. For example, to prepare 10.5 ml of immunoreagent, add 700 ul anti-p53-POD (solution 1) to 9.8 ml of incubation buffer (solution 3) and mix. This amount is suficient for 96 wells.

7. Assay procedure

7.1 General recommendations

Important: All reagents should be equilibrated at room temperature (18-25 C) before starting the test. Only the microtiter plate included must be used for the assay.

Sample and standard solutions must be pipetted very carefully.

Reagents of different lots must not be used in one test series.

7.2 Pipetting scheme for the microtiter plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bl	Bl	P2	P2								
B	Sa	Sa										
C	Sb	Sb										
D	Sc	Sc										
E	Sd	Sd										
F	Se	Se										
G	Sf	Sf										
H	P1	P1									P41	P41

Legend:

Bl = blank (= incubation buffer)

Sa-Sf = standards 2a-2f

P1-41 = sample 1-41

7.3 Working instructions

7.3.1 Pipetting of standards/samples and antibody

Pipette first 100 ul of standards (solutions 2a-2f) or samples (for dilution see under sample preparation, chapter 5.) very carefully into the wells. Then add 100 ul of anti-p53-POD (solution 7) to all wells supposedly containing standard/samples. Cover the microtiter plate tightly with the included adhesive cover foil and incubate for 2 hours at room temperature on a shaker. Astandard curve has to be determined individually for each experiment.

7.3.2 Washing

Remove incubation buffer thoroughtly by tapping off or suction. Rinse wells five times with 300 ul washing buffer (solution 4) and remove washing solution carefully.

7.3.3 Substrate reaction

Pipette 200 ul of substrate solution (solution 5) into the wells. Cover the microtiter plate tightly with the adhesive cover foil included and incubate at room temperature on a shaker at 300 rpm until color developments is sufficient for photometry detection (10-20 min).Protection from light will reduce the background level of the substrate!

7.3.4 Stop reaction

Pipette 50 ul of stop solution (solution 6) into each well. Incubate the plate for approx. 1 min on the shaker at 300 rpm (or mix thoroughly).

7.3.5 Instruments and Measurements

Assay results are quantitated spectrophotometrically at 450 nm using a microtiter plate reader (reference wavelength: 690 nm) against blank. Measure at 370 nm (reference wavelength: 492 nm) against blank. Maximal absorbance reading for standard 2f should be 2.5.

7.3.6 Interpretation

Average the values from the double absorbance readings from standards/samples. The calibration curve is constructed by plotting the average absorbance values of standards (y-axis) versus the standard concentrations (x-axis), or by processing the

data with an appropriate laboratory data system. Sample concentrations can then be determined from the calibration curve. Samples with values exceeding the measurement range should be diluted further and run again. In this case, the additional dilution factor has to be taken into account when calculating the amount.

8.Related products:

Product	Pack size	Cat. No.
Anti p53 protein, mutant	100 ug	1 696 823
Anti p53 protein, pan	200 ug	1 810 928
Anti p53 protein, pan, Biotin labeled	150 ug	1 810 936
Anti p53 protein, pan, POD labeled	50 U	1 810 944

9.Quick reference protocols

9.1 Required solutions (sufficient for 100 tests)

Solution	Content	Used for
1	Anti-p53-OPD (bottle 1):	Solution 7
7	Anti-p53-OPD working dilution: for 10.5 ml of reagent add 700 ul of solution 1 to 9.8 ml of solution 3 and mix thoroughly	Step 1
2a-2f	P53 standards (bottle 2a-2f): dissolve in 500 ul redist. Water	Step 1
3	Incubation buffer/sample diluent (bottle 3): ready-to-use solution	Samples and solution 7
4	Washing buffer (bottle 4): dilute 1:10 with redist. Water	Step 2
5	TMB substrate solution (bottle 5): ready-to-use solution	Step 3
6	Stop reagent (bottle 6): ready-to-use	Step 4b

9.2 Working procedure flow sheet

Steps	Procedure	Volume/well	Time/Temperature
1	Pipette first 100 ul of solution 2a-2f or (un-)diluted sample respectively into MTP wells according to the pipetting scheme Pipette 100 ul of solution 7 in to MTP well	100 ul 100 ul	Incubate for 2 h at RT 300 rpm on a shaker
2	Wash five times with solution 4	5 x 300 ul	5 x 1 min
3	Add 200 ul of solution 5 to MTP wells	200 ul	Approx. 10-20 min at RT 300 rpm on a shaker
4a	Alternative without stop reagent: measure absorption at 370 nm (reference wave length: 492 nm)		
4b	Alternative with stop reagent: add 50 ul of solution 6 to MTP well and subsequently measure absorption at 450 nm (reference wavelength 690 nm)	50 ul	Approx. 1 min at RT 300 rpm on a shaker

MTP = microtiter plate

RT = room temperature

ประวัติผู้ทำวิทยานิพนธ์

นายธีรยุทธ นัมคณิสรณ์ เกิดเมื่อวันที่ 6 สิงหาคม พ.ศ.2510 ที่อำเภอเมือง จังหวัดจันทบุรี สำเร็จการศึกษาระดับปริญญตรี แพทยศาสตร์บัณฑิต จากคณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ในปีการศึกษา 2533 หลังจากนั้นทำงานเป็นแพทย์ประจำ โรงพยาบาลเขาคิชฌกูฏ กิ่งอำเภอเขาคิชฌกูฏ จังหวัดจันทบุรีเป็นเวลา 2 ปี และรักษาการตำแหน่งผู้อำนวยการโรงพยาบาลเขาคิชฌกูฏ กิ่งอำเภอเขาคิชฌกูฏ จังหวัดจันทบุรีเป็นเวลา 1 ปี หลังจากนั้นเข้ารับการฝึกอบรมแพทย์ประจำบ้าน ภาควิชาอายุรศาสตร์ โรงพยาบาลจุฬาลงกรณ์ ระหว่างปี พ.ศ. 2537-2540 และสอบได้วุฒิบัตรผู้มีความรู้ความชำนาญทางวิชาชีพเวชกรรมสาขาอายุรศาสตร์ทั่วไป เมื่อปี พ.ศ.2540 หลังจากนั้นในปี พ.ศ.2540-2541 ได้กลับไปทำงานเป็นแพทย์ประจำอยู่โรงพยาบาลศูนย์พระปกเกล้า จังหวัดจันทบุรี กลุ่มงานอายุรกรรมเป็นเวลา 1 ปี ปัจจุบันอยู่ระหว่างการฝึกอบรมแพทย์ ประจำบ้านต่อยอดสาขาอายุรกรรมมะเร็ง ภาควิชาอายุรศาสตร์ โรงพยาบาลจุฬาลงกรณ์