

LAB 3

STATUS OF THE TUMOR SUPPRESSOR PROTEINS RB, P53 AND P16^{INK4A} IN HUMAN TUMOR CELL LINES.

Introduction

Genetic alterations occurring in tumor cells involve mutations leading to the oncogene activation and inactivation of the tumor suppressors. The loss of tumor suppressor function can occur by for instance, deletion of the entire gene, missense or nonsense mutation or methylation of the gene, preventing its expression. In order to determine the most effective therapy for a given tumor, it will be more and more important to characterize the status of tumor suppressor genes in the tumor.

The p53 protein is one of the most important tumor suppressor. p53 is a transcriptional factor that can block cell cycle progression in the G1 phase or trigger apoptosis in cells that have suffered genomic injury. Both these responses guard against the replication and amplification of genetic damage thus helping to conserve genetic stability. Mutations in the p53 gene occur at high frequency in human tumors (around 50%). The great majority of p53 mutations are missense mutations that give rise to mutant p53 proteins lacking wild type activity. Missense mutations in p53 gene often lead to the increased mutant p53 protein stability, resulting in its accumulation in tumor cells.

In contrast to the p53 gene, the Rb gene is usually inactivated by nonsense mutations. Inactivation of pRb through nonsense mutation or deletion frequently results in expression of truncated protein in tumor cells. The Rb gene encodes nuclear phosphoprotein (pRb) that is involved in regulation of the cell cycle. Hypophosphorylated pRb blocks the cell cycle in G1 phase. In the absence of Rb, cells fail to halt cell cycle in response to signals which will normally

stop cellular proliferation. The activity of pRb is regulated through phosphorylation by complexes between cyclins and cyclin-dependent kinases (CDK's).

The p16^{INK4A} (henceforth denoted p16) encodes a protein that functions as an inhibitor of CDK4 and CDK6. Binding of p16 to CDK4/6 blocks their complexing with D type cyclins, thus preventing pRb phosphorylation and S-phase entry. Homozygous deletion, point mutation, or methylation of p16 gene results in abolishment of p16 gene expression. These alterations have been found in different types of human tumors.

Aim:

The aim of this lab is to determine the status of tumor suppressor proteins pRb, p53 and p16 in different human tumor cell lines, using three different approaches, namely immunofluorescence staining, Western blot analysis and Polymerase Chain Reaction (PCR).

You will analyze the status of tumor suppressor genes in 3 cell lines of different origin. For the immunostaining, cells have been fixed on a glass slides. Adherent human osteosarcoma Saos-2 cells were grown directly on a cover slips, whereas suspension cells (BL2 Burkitt lymphoma cell line and SW480 colon carcinoma cell line) have been spun down on a glass slide using a special centrifuge (cytospin). Cells were fixed with 4% formaldehyde in PBS for 10 min., permeabilized in 0.2% Triton X-100 in PBS for 2 min. and washed in PBS.

For the detection of proteins by Western blotting you will get cellular lysates.

For PCR analysis genomic DNA prepared from cells will be provided.

Experimental procedures

Immunostaining

1. Wash the slides with fixed cells 3 x10 min.
2. Probe with primary antibody for 1 hour in a wet chamber at room temperature. Antibody should be diluted 1:200 in Blocking buffer. Use 30 µl per glass slide or 50 µl per cover slip.

3. Wash 3X10 min with PBS.
4. Probe with rabbit anti-mouse or goat anti-rabbit antibodies (depending on primary antibody used) conjugated with FITC, diluted in 1:50 in PBS containing 2 µg/ml Hoechst 33342, for 0,5 hour. (30 µl per slide, 50 µl per cover slip)
5. Wash with PBS 3x10 min. Wipe off excess of buffer around the sample, but do not let the sample dry!
6. Mount with mounting solution.

Chemicals:

Buffers:

PBS: phosphate-buffered saline

Blocking buffer: PBS, containing 2% BSA (bovine serum albumin, Sigma)

Mounting solution: Fluorescent medium (DAKO)

Hoechst 33342

Antibodies:

mouse anti-p53 DO1, human-specific (Oncogene Research Products)

mouse anti-Rb 245 (PharMingen)

mouse anti-p16 (Oncogene Research Products)

rabbit anti-mouse FITC conjugate (DAKO)

Western Blot analysis

1. Assemble the apparatus, prepare the resolving gel (6% for Rb, 8% for p53, 12% for p16).
Note!!! Acrylamide is toxic. Avoid skin contact. Always use gloves! Let the gel polymerize for 40 min.
2. Prepare the stacking gel. Polymerization 40 min.
3. Remove the comb, wash the wells with distilled water, assemble the electrophoresis unit, put the running Tris-glycine buffer in.
4. Load your samples, run the gel at 200 v for 30 min.

5. Meanwhile, cut the nitrocellulose membrane and 2 pieces of Whatman paper, gel size. Soak them together with 2 sponges in Transfer buffer for 5 min.
6. Assemble transfer sandwich in a following order: sponge - Whatman paper - gel - membrane - Whatman paper - sponge on a transfer unit. Check for air bubbles before starting transfer. Transfer the proteins at 200 mA for 60 min or at 15 mA overnight.
7. After transfer, wash your membrane in PBS for 2 min., that block with 5% milk in PBS for 0,5 hour
8. Probe with primary antibody. Seal the membrane in a plastic bag, put 5 ml of primary antibody diluted 1:500 in 5% milk in PBS. Incubate for 1 hour on a shaking platform.
9. Wash in PBS 5x5 min.
10. Probe with secondary antibody, anti-mouse or anti-rabbit HRP conjugate, diluted 1:5000 in 5% milk in PBS. Incubate 0.5 hour on a shaking platform.
11. Wash in PBS 5x5 min.
12. Develop with ECL solution (1 ml of solution 1 + 1 ml of solution 2) for 2 min. Don't forget to change the tip!
13. Cover your membrane with plastic, expose to X-ray film for 10 min.
14. Develop the film.

Chemicals

Buffers:

2x Laemmli sample buffer: 4% SDS; 20% glycerol; 120 mM Tris, pH 6.8;
0, 0.1% bromphenol blue; 100 mM DTT

Tris-glycine buffer: 25 mM Tris; 250 mM glycine; 0.1% SDS

Transfer buffer: 25 mM Tris; 190 mM glycine; 20% methanol

PBS

5% milk in PBS

ECL solution 1 and 2 (Amersham)

Resolving gel,

6%: 2.6 ml H₂O; 1.0 ml 30% acrylamide mix (29:1); 1.3 ml of 1.5 M Tris, pH 8.8; 0.05 ml 10% SDS; 0.05 ml 10% ammonium persulfate; 0.003 ml TEMED

8%: 2.3 ml H₂O; 1.3 ml 30% acrylamide mix (29:1); 1.3 ml of 1.5 M Tris, pH 8.8; 0.05 ml 10% SDS; 0.05 ml 10% ammonium persulfate; 0.003 ml TEMED

12%: 1.6 ml H₂O; 2.0 ml 30% acrylamide mix (29:1); 1.3 ml of 1.5 M Tris, pH 8.8; 0.05 ml 10% SD;, 0.05 ml 10% ammonium persulfate; 0.003 ml TEMED

Stacking gel 5%: 1.4 ml H₂O; 0.33 ml 30% acrylamide mix (29:1); 0.25 ml of 1 M Tris, pH 6.8; 0.02 ml 10% SDS; 0.02 ml 10% ammonium persulfate; 0.002 ml TEMED

Antibodies:

mouse anti p53 antibody DO1 (Oncogene Research Products)

mouse anti-Rb antibody 245 (PharMingen)

mouse anti-p16 antibody (Oncogene Research Products)

goat anti-mouse HRP (horseraddish peroxidase) conjugate

PCR

In the clinic, several PCR-based assays are currently in use to screen for genetic changes in human malignancies. The PCR primers used in this assay are designed to specifically detect alterations (if any) in the p53, p16^{INK4A} and pRb genes in the cell lines provided. All PCR reactions will be made in doublets. For each sample you will run a control amplification of the GAPDH (exon 8) gene to check for DNA integrity.

All solutions should be kept on ice during the whole preparation, except for the Taq polymerase that should be kept in the -20°C cooler block at all times.

Always use a new pipette tip when changing solutions.

1. Mark the PCR tubes in an appropriate way. Place on ice.
2. In two different Eppendorf tube, prepare separate master mixes for the gene to be analysed and GAPDH as described (Table 1). Taq polymerase should not be added to the master mix until everything is ready to run the PCR.

Table 1. Master mixes*

Solutions	Amount/reaction (µl)	Total amount (n+1)
Sterile ddH ₂ O	35.8	
10X Taq PCR buffer	5	
DMSO (100%)	3	
dNTPs (2.5 mM)	3	
F-primer (10mM)	1	
R-primer (10mM)	1	
Total volume	48.8	
Taq polymerase (5u/µl)	0.2	

n= total number of reactions.

*Prepare two master mixes:

- Master mix no.1 should contain the analysed gene specific F- and R-primers.
- Master mix no.2 should contain the GAPDH F- and R-primers

3. Put 100 ng of genomic DNA from each sample into the chilled PCR tubes. You should also include a positive (human placenta DNA) and a negative (water) control. Make doublets of all samples and controls.

4. Make sure that the PCR machine is correctly programmed and ready for PCR.

The following program will be used:

Denaturation: 94°C, 2 min, 30sec.

Annealing: 58°C, 20 sec.

Extension: 72°C, 1 min.

Final extension: 72°C, 3 min.

Cool at 4°C.

5. When everything is ready to run the PCR, put the appropriate volume of Taq polymerase into the master mix. Mix thoroughly since Taq polymerase is stored in 50% glycerol and will fall to the bottom of the tube if not mixed.

6. Add 49 µl of the master mix (including Taq polymerase) to each PCR tube. Transfer the tubes directly from the ice to the pre-heated (94°C) PCR machine.

7. Start the PCR program.

8. Prepare an 1.5% agarose gel (in 1XTAE buffer). Make sufficient number of wells for all samples plus one for the size marker on each gel.

9. When the PCR is finished, store samples at -20°C until the agarose gel is ready to be run.

10. Load samples on the agarose gel. Load approximately 10 µl of each reaction mixed with 2µl of a 6X Loading buffer in each well. Do not forget to load the size marker (3µl) in one of the lanes!.

11. Run the gel (100v). When finished, take a photo of the gel.

12. Analyse the results.