CHAPTER 2

RESEARCH DESIGNS, SCOPE AND METHODS

Subjects

Paraffin tissue blocks from 90 cervical cancer patients who underwent RDH with PLD as a primary treatment in Maharaj Nakhon Chiang Mai Hospital between 1992-1997 were included in this study. These patients were divided to two groups:

1. Cases group

—Thirty patients who have recurrence within 5 years after surgery.

2. Controls group

— Sixty patients who have no recurrence after the follow up period of at least 5 years. Control should be operated in the same hospital as cases and within 10 years of age different from cases. These group have the tumor size within 1 cm. Different from cases, have the same stage and histology type.

Tumor recurrence

Presence of tumor recurrence was primarily detected by physical examination or imaging techniques. Evidence of tumor recurrence was also confirmed by biopsy if possible. The standard surveillance program after RDH included clinical history and physical examination every 3-4 months in the first 2 years, and every 6 months during the third to the fifth year. After 5 years of uncomplicated follow up, the patients were seen annually. Patients were investigated for recurrence from clinical history and physical examination clue.

Tissue staining

Haematoxylin and eosin

To demonstrate general histology of cervical cancer, tissue sections will be stained by the standard protocol for Harris' s haematoxylin and eosin staining. See the appendix for making up the stain and solution.

Procedure

1. Deparaffinized sections and dehydrate in graded alcohol and hydrate to distilled water.
2. Stained in freshly filtered Harris's haematoxylin for 6 minutes.
3. Washed in running tap water for 5 minutes.
4. Differentiated in 1% acid alcohol, 1 to 2 dips
5. Washed briefly in running tap water
6. Placed in ammonia water until sections are bright blue
7. Washed throughly in running tap water for 10 minutes
8. Placed in 80% ethyl alcohol for 1 to 2 minutes
9. Counterstained in eosin-phloxine solution for 2 minutes.
10. Dehydrate and clear through 2 changes each of 95% ethyl alcohol, absolute ethyl alcohol, and xylene, 2 minutes each
11. Mount with mounting medium

Immunohistochemistry

In this study, the method of Avidin-Biotin Complex (ABC) is chosen for immunohistochemical staining. Tissue sections will be processed as briefly described below.

Tissue sections of the tumor will be deparaffinized, dehydrated in graded alcohols ending in distilled water. The pressure cooker will be used for antigen retrieval. Section will be boiled in TE-buffer (10 mM Tris, 1 mM EDTA, pH 9.0) for 7 minutes. After cooling by running tap water for a few minutes, the blockage of endogenous peroxidase will be performed in 3% hydrogenperoxide (H_2O_2) for 30 minutes at room temperature. Then, the sections will be incubated in 3% normal horse serum for 20 minutes to prevent non-specific binding.

Consequently, monoclonal mouse anti-human p53 protein (DAKO) at 1:2000 dilution in 3% normal horse serum will be applied to the sections and leave at room temperature for 1 hour. After washing with phosphate buffer saline (PBS), the sections will be incubated with biotinylated horse anti-mouse immunoglobulin (1:100) for 30 minutes at room temperature. The sections will then be incubated in Avidin-Biotin Complex (ABC kit, Vector Laboratories vectastain®), diluted in PBS at a concentration of 1:300 for 60 minutes at room temperature. After being rinsed three times in PBS, the sections will be reacted in 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 10 minutes. The section will be stopped by washing the sections in distilled water and running tap water for 5 minutes. For counterstaining, sections will be left in haematoxylin for a 1 minute, and then be washed in running tap water to remove access stain. All sections will be dehydrated in 95%, 100% alcohol and cleared in xylene respectively, and mounted with permount. The sections of colorectal cancer will be used as a positive control. The negative control sections will be processed by the same technique with the primary antibody omitted.

Data collection and evaluation

Two gynecologic pathologists without the prior knowledge of clinical information will independently review all pathological slides of cervical cancer and then all the pathological results (histologic type, histologic grade, depth of invasion and presence of lymph vascular space invasion) will be recorded. Positive immunostaining of p53 will be scored in all sections. The clinical variables will be abstracted from the tumor registry records of gynecologic oncology unit of the hospital. The pathologic variables will be obtained from the result of reviewing the slides, while the main outcome of interested will be obtained from the result of immunohistochemical staining.

Definition of clinicopathological variables

FIGO staging

Patients will be restaged according to FIGO staging system 2000 [6] by using the clinical data at the time of diagnosis.

Lymphovascular space invasion (LVSI)

LVSI will be delineated by the presence of tumor cells in luminal space lined by flattened endothelial cells in the presence or absence of lymphocytes either at the main tumor bed or away from the tumor.

Depth of invasion

The maximum depth of tumor invasion from basement membrane in case of squamous cell carcinoma, or maximal tumor thickness from surface of tumor in case of adenocarcinoma.

Scoring of p53 protein expression

p53 expression will be scored based on the proportion of stained tumor cells relative to the overall number of tumor cells, excluding those in tumor area. At least 1,000 cells per section were counted in 25-30 random 100x fields. The p53 immunoreactivity, only a distinct brown nuclear staining was scored as positive. The scores will be given in 4 categories as the following:

Negative(-) = < 5% of tumor cells Positive+2 = 10-30% of tumor cells

Positive+1 = 5-10% of tumor cells Positive+3 = > 30% of tumor cells

Statistical analyses

The SPSS window 12.0 program package was used for statistical analyses, the association among p53 protein expression, tumor recurrence, and other clinicopathologic characteristics will be analyzed by Mantel-Haenzel test. Condition multiple logistic regression will be used to identify the correlation between p53 protein expression and prognostic factors for cervical cancer. Adjusted odd ratio with 95% confidence interval will be calculated and a p-value of 0.05 or less than will be considered as statistical significance.