CHAPTER III

RESEARCH METHODOLOGY

3.1. Research Question

Is there any association between bcl-2 expression and tumor recurrence in node negative cervical cancer patients who underwent radical hysterectomy?

3.2. Research Objectives

To determine whether negative node cervical cancer patients who underwent radical hysterectomy with tumor recurrence had different proportion of bcl-2 expression compared to those without tumor recurrence.

3.2.1 Primary Objective

To compare the proportion of positive bcl-2 expression in node negative cervical cancer patients, who underwent radical hysterectomy between those with tumor recurrence and those without tumor recurrence.

3.2.2 Secondary Objectives

2.2.1 To compare the score of bcl-2 positive cells (+1, +2, +3) in node negative cervical cancer patients, who underwent radical hysterectomy between those with and without tumor recurrence.

2.2.2 To determine whether bcl-2 expression is an independent factor associated with tumor recurrence.

2.2.3 To examine the association of bcl-2 expression with other prognostic factors for recurrent cervical cancer such as: depth of invasion, lymph-vascular space invasion, and tumor grade.

2.2.4 To determine the association between bcl-2 expression and time to recurrence.

2.2.5 To determine the association between bcl-2 expression and site of recurrence.

3.3. Hypothesis

3.3.1 Research Hypothesis

The proportion of bcl-2 expression in node negative cervical cancer patients, who underwent radical hysterectomy in those with tumor recurrence, is different from the proportion in those without tumor recurrence.

3.3.2 Statistical Hypothesis

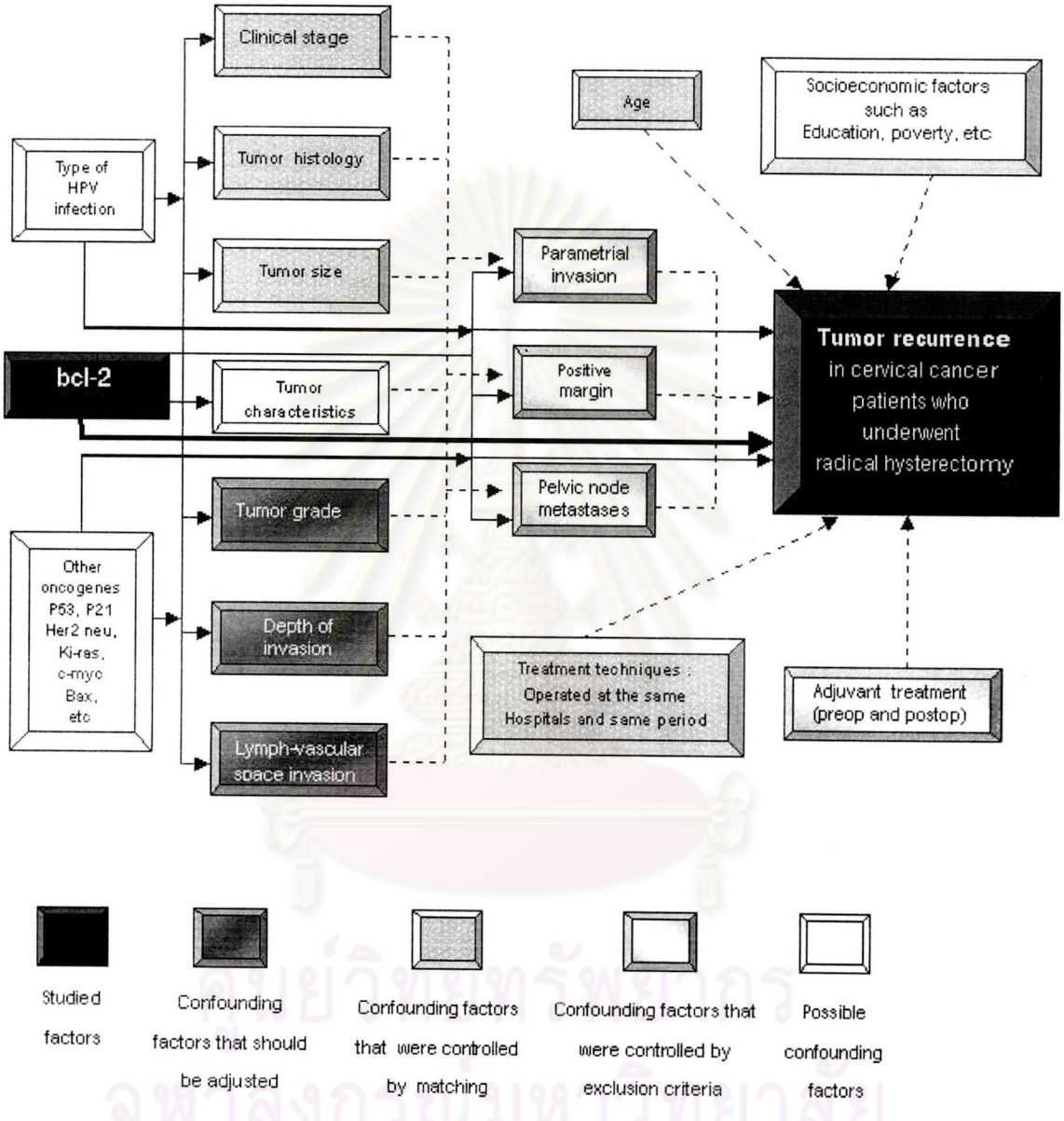
Null and Alternative Hypothesis

H_0 : $\pi_1 = \pi_2$

H_a : $\pi_1 \neq \pi_2$

where π_1, π_2 = population proportion of bcl-2 expression in node negative cervical cancer patients, who underwent radical hysterectomy and developed or did not develop tumor recurrence respectively.

3.4. Conceptual Framework



3.5. Key Word : bcl-2, cervical cancer, radical hysterectomy, recurrence, prognosis.

3.6. Operational Definitions

Scoring of Bcl-2 Expression

Bcl-2 was scored in 4 categories, depending on the proportion of stained tumor cells relative to the overall number of tumor cells, excluding those in areas of necrosis. The tumor cells that were counted as stained tumor cells should have the same intensity of bcl-2 staining as internal positive control (lymphocyte)

Negative	- $\leq$ 5% of tumor cells
Positive +1	- > 5-10% of tumor cells
Positive +2	- > 10-30% of tumor cells
Positive +3	- > 30% of tumor cells

FIGO Staging

: Patients will be restaged according to FIGO staging system 2000[74] by using the clinical data at the time of diagnosis.

Lymph-vascular Space Invasion (LVSI)

: The presence of tumor cells in spaces lined by flattened endothelial cells either at the main tumor bed or away from the tumor.

Depth of Invasion

: The maximal depth of tumor invasion from basement membrane in cases of squamous cell carcinoma, or maximal tumor thickness from basement membrane in cases of adenocarcinoma. Depth of invasion will be categorized into $\leq$1/2 vs. >1/2 of cervical thickness.

Tumor Recurrence

Presence of tumor recurrence was detected by physical examination or imaging techniques. Evidence of recurrence was confirmed by biopsy if possible. (The standard surveillance program after radical hysterectomy 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.)

Time to recurrence was calculated from the date of surgery to the date of first clinical presentation of recurrence.

Site of recurrence will be defined as locoregional recurrence or distant recurrence. The recurrent tumor limited in the pelvis was defined as locoregional recurrence while the tumor found outside the pelvis was defined as distant recurrence.

3.7. Research Design

Matched case-control study.

3.8. Research Methodology

3.8.1 Target Population

Cervical cancer patients who underwent radical hysterectomy with pelvic lymphadenectomy as their primary treatment and had negative pelvic node.

3.8.2 Study Population

Cervical cancer patients , who underwent radical hysterectomy with pelvic lymphadenectomy as their primary treatment at Bangkok Metropolitan Administration (BMA) Medical College and Vajira Hospital or Maharaj Nakorn Chiangmai Hospital between January 1992 and June 1998, and had negative pelvic node.

3.8.3 Definition of Cases

Patients who had tumor recurrence within 5 years after surgery.

3.8.4 Definition of Controls

Patients who had no recurrence after the follow up period of at least 5 years. Controls should be operated in the same hospital as cases, within 10 years of age

different from cases, had the tumor size within 1 cm. different from cases, had the same stage (Ib or IIa) and tumor histology (squamous or non-squamous).

3.8.5 Inclusion Criteria

1. Patients with stage Ib or IIa.
2. Patients underwent radical hysterectomy with pelvic nodes dissection as a primary treatment.
3. Patients who had pathological negative pelvic nodes.

3.8.6 Exclusion Criteria [Reasons for Exclusion]

1. Patients who had other cancers (two primary cancers). [In these cases, sometimes we will not know exactly which cancer the recurrence comes from.]
2. Patients with rare histologic subtypes such as neuroendocrine carcinoma, lymphoma. [For rare histologic subtypes, we will be unable to find the controls that suit all the criteria.]
3. Patients received any form of preoperative and postoperative adjuvant treatment. [We want to study only cases that did not receive adjuvant treatment to find the cases that needs adjuvant postoperative treatment to prevent tumor recurrence.]
4. Patients who had parametrial or surgical margin involvement by hematoxylin-eosin staining (H&E) either from previous pathological report or at this review. [Parametrial invasion and surgical margin involvement are significant prognostic factors for tumor recurrence, and it is quite clear now that these groups of patients should receive adjuvant postoperative treatment.[19]]
5. Patients who had lymph node metastasis at this review. [Lymph node metastasis is the most significant prognostic factors for tumor recurrence, and it is quite clear now that they should receive adjuvant postoperative treatment.[19]]
6. Patients whose paraffin blocks of the tumor are not available. [In these cases, we have no tissue to perform the immunohistochemical staining in order to evaluate the expression of bcl-2.]
7. Patients who died from other causes or loss to follow up before 5 years.

3.8.7 Sample Size Estimation

Sample size for matched case-control study [75]

m = number of discordant pairs

$$m = \frac{[Z_\alpha/2 + Z_\beta\sqrt{P(1-P)}]^2}{(P-1/2)^2}$$

where

Ψ = hypothesized odds ratio

R = estimated odds ratio

P = $\Psi/(1+\Psi)$ = R/(1+R)

M = total number of pairs required on average to yield m discordant pairs

M = $m/(p_0q_1 + p_1q_0)$

p_0 = estimated exposure rate among control

p_1 = $p_0R / [1+p_0(R-1)]$

q = 1-p ($q_0 = 1-p_0$, $q_1 = 1-p_1$)

In this study, we assigned

$Z_\alpha = 1.96$ (two-tailed test of 5% significant level)

$Z_\beta = 0.84$ (power of 80%)

Hypothesized odds ratio = 0.3

P_0 = 0.65 (bcl-2 expression in cervical cancer is around 61-68%[25,41,42,44]

Hence : p1 = (0.65 x 0.3) / [1-(0.65 X 0.7) = 0.36

P = 0.3/1.3 = 0.23

$$m = \frac{[1.96/2 +0.84\sqrt{0.23(0.77)}]^2}{(-0.27)^2} = \frac{[0.98 +0.84(0.42)]^2}{(-0.27)^2}$$

$$= \frac{[1.33]^2}{(-0.27)^2} = 24.3 = 25$$

M = 25 / [(0.65x0.64) + (0.36x0.35)] = 46.1 = 47

The ratio of control : case = 2:1

M' = (c+1)M/2c = (3x47)/4 = 35.2 = 36

According to the calculation, 36 cases and 72 controls are needed. However, three other factors (lymph-vascular space invasion, depth of invasion, tumor grade), that cannot be controlled, might be the confounding factors in determining the association between tumor recurrence and bcl-2 expression. Thus, conditional multiple logistic regression will be used to adjust these 3 factors. Based on the rule of thumb for multiple logistic regression, for altogether 4 independent variables, which affect the recurrent rate, we need about 40 cases of recurrence (10 outcomes of recurrence for each independent variable)[76]

The number of patients undergoing radical hysterectomy and pelvic lymphadenectomy for cervical cancer patients stage Ib-IIa are about 50 - 60 cases per year in Maharaj Nakorn Chiangmai Hospital and 10 - 20 cases per year in BMA Medical College and Vajira Hospital. Since recurrence occurred about 11-18% after radical hysterectomy[9-10,77] and 24-42% of recurrent patients had positive pelvic nodes.[7,9,10,77] This would result in 3-11 cases of tumor recurrence with negative nodes in each year. Of these, some patients may have to be excluded because of unavailable paraffin blocks.

In this study, we plan to recruit all patients in six and a half-year period, January 1992 to June 1998. (Both hospitals keep the paraffin blocks for only 10 years and they have already discarded the blocks before 1992. We need the follow up time of at least 5 years, so we cannot recruit the cases operated after June 1998.) We hope that in this period, we could recruit 40 cases and 80 controls that fulfilled all the criteria.

3.8.8 Methods

1. Research proposal was approved by the ethics committee and the directors of both hospitals before starting.
2. Medical records and follow up information of all cervical cancer patients who underwent radical hysterectomy with pelvic node dissection in the study period were reviewed. All patients that were lost to follow up before 5 years were contacted via telephone or letter to determine tumor recurrence.
3. All recurrent cancer patients operated in the study period were defined as cases.

4. Two controls who were operated on the nearest date were chosen for each case according to the eligibility criteria.
5. Clinical data of cases and controls were recorded.
6. Three-micrometer single sections were prepared from primary cervical cancer tissue from cases and controls and immunohistochemical stained with anti bcl-2 antibody. A single block that contained mostly tumor tissue was chosen, in order to simulate the routine immunohistochemical staining.
7. Intratumor lymphocytes were used as positive control.
8. The negative control consisted of sections that were treated with the same technique with the primary antibody omitted.
9. Two pathologists without the knowledge of clinical information independently reviewed all pathological slides and the bcl-2 immunohistochemical staining.
10. The intraobserver and interobserver reliability of the two pathologists were assessed for the evaluation of the pathology and bcl-2 immunohistochemical staining slides.
11. The disagreement on the interpretation of the pathology and immunohistochemical staining were reported and solved by consensus.

3.8.9 Immunohistochemical Staining

For the immunohistochemical study, 3-micrometer thick sections were cut from paraffin blocks, which contained representative histology of cervical cancer. Paraffin sections were dewaxed with xylene and treated with 95% alcohol. Sections were then treated with 3%H_2O_2 in phosphate buffer saline (PBS) to block endogenous peroxidase activity. For antigen retrieval, they were immersed in 10 mM/L citrate buffer (pH 6.0), and microwaved at 750 W power for 10 minutes. After the buffer had cooled, sections were treated with anti-human bcl-2 oncoprotein mouse monoclonal antibody clone 124 (Dako code No. M0887) diluted 1:50 for 60 minutes at room temperature. Sections were treated for another 10 minutes with the biotinylated link antibody (Dako LSAB code no. K0675 bottle 1). After being rinsed in phosphate-buffered saline (PBS), the sections were coated with streptavidin-HRP (Dako LSAB code no. K0675 bottle 2) for 10

minutes. The reaction product was developed with diaminobenzidine solution for 10 minutes. Sections were then counterstained with Harry hematoxylin, dehydrated through 95% alcohol and absolute alcohol, and were mounted.

3.9. Data Collection

The clinical variables were abstracted from the tumor registry records of gynecologic oncology unit and medical records of both hospitals. The pathologic variables were obtained from the result of reviewing the slides, while the main outcome of interested were obtained from the result of immunohistochemical staining.

3.10. Statistical Analyses

Stata 7.0 and SPSS 11.0 were used for the analysis. The associations of tumor recurrence and bcl-2 expression, together with other pathological characteristics were analyzed by conditional simple logistic regression. Conditional multiple logistic regression was used to determine the effect of each independent factor on tumor recurrence. After adjusting for other independent factors, crude and adjusted odds ratios (ORs) with their 95% confidence intervals were calculated. The association between bcl-2 and other pathological factors were evaluated by chi-square test for univariable analysis, and by multiple logistic regression for multivariable analysis. Mann-Whitney U test and Fisher's exact tests were used to determine the association between bcl-2 expression with time to recurrence and site of recurrence respectively. All associations were calculated using two-sided test and a p-value of 0.05 or less was considered as statistical significance.

3.11. Ethical Considerations

Since this study is a case-control study, the patients did not receive any intervention. Only the medical records, pathological slides were reviewed, and paraffin blocks were recut for immunohistochemical staining. Moreover, the research was start after approval from the directors of both hospitals. All the patients' information will be kept confidentially, and the results will be presented without patients' identification.