Cervical cancer is the second most common cancer among women world-wide, with about 470,000 newly diagnosed cases and almost 250,000 deaths every year. Furthermore, cervical cancer is the most common cause of death from gynecological malignancy in developing countries.

Typically, type III radical hysterectomy (RH) is prescribed for patients diagnosed with stage IB-IIA cervical cancer who can tolerate an aggressive surgical approach, and want to avoid the long-term adverse effects of radiation therapy. For stage IIB patients, radiation therapy has been commonly prescribed. However, in some countries in Europe and Asia, type III radical hysterectomies are prescribed for patients with stage IB-IIIB cervical cancer. Various clinico-pathological variables evaluated as possible prognostic factors for survival after RH, including depth of invasion, parametrial margin involvement, pelvic lymph node metastasis, number of lymph node metastases, histological type and pre-treatment squamous cell carcinoma (SQCC)-antigen level, are significant factors for clinical outcome.

In addition to these clinico-pathologic variables, increasing attention has focused on various new approaches to molecular and genetic changes in cervical cancer. Molecular and genetic alterations are important in the pathogenesis of cervical cancer, especially in association with human papillomavirus (HPV) infections. One of considerable interest is c-MYC gene status. c-MYC activation, triggered by insertion of HPV DNA sequences into near the c-MYC locus, is now regarded to be an important genetic event in cervical oncogenesis. c-MYC gene maps to chromosomal band 8q24, which is reportedly a common site for integration of HPV DNA. Gene amplification is an important mechanism for protein overexpression and oncogene activation in tumor cells. c-MYC gene amplification is well documented in cervical cancer. Amplification of the c-MYC gene is also addressed in breast cancer, bladder cancer, prostate cancer and lung cancer. Although strong prognostic impact of c-MYC gene amplification on histologic grade, disease stage and patients survival have been well documented in certain types of tumors, data on prognostic significance of both gain and amplification of c-MYC is lacking in cervical cancer.

Therefore, in this study, we assessed c-MYC gene status in human cervical cancer samples using fluorescence in situ hybridization (FISH) to analyze the relation between c-MYC gene amplification and clinico-pathologic factors. The prognostic value...
of c-MYC was evaluated in conjunction with immunohistochemical analysis for Ki67, p53 and bcl2, reportedly c-MYC-related markers.\textsuperscript{17}

**MATERIALS AND METHODS**

**Patients**

We evaluated archival paraffin-embedded tumor tissues from 144 patients from 1997 to 2004, with primary, untreated and histologically confirmed cervical cancer with International Federation of Gynecology and Obstetrics (FIGO) stage IB to IIB. All patients underwent type III RH with pelvic and/or paraaortic lymph node dissection. After radical surgery, selected patients with high risk prognostic factors such as positive surgical margin, lymph node metastasis, large tumor size (>4 cm) or parametrical involvement were referred for postoperative concurrent cisplatin-based chemo-radiation therapy (CCRT). After surgery, patients were examined every three months for 2 years, then every 6 months for the next 3 years, and annually thereafter.

Hematoxylin and eosin (H&E)-stained slides were reviewed in each case to confirm the original diagnosis by two pathologists (TJK and AWL) independently. Tumor specimens were histologically diagnosed as SQCC (n = 108), adenocarcinoma (ADCA; n = 23) and adenosquamous carcinoma (ADSC; n = 13).

Disease free survival time was calculated as the time that recurrence was first suspected. Cancer specific survival was defined as the interval between the histological confirmation of disease and cancer related death or last observation taken. The data were censored at the last follow-up period for living patients. Study design, data collection and analysis followed the principles of the Declaration of Helsinki. This study was approved by the Institutional Review Board (IRB) of The Catholic University of Korea (IRB number KC10SIS0475).

**Tissue microarray**

Twelve tissue microarrays were constructed from archival formalin-fixed and paraffin-embedded cervical cancer tissue blocks and paired normal cervix tissue using a manual tissue arrayer (Quick-Ray Manual Tissue Microarrayer, Unitma, Seoul, Korea). Review of H&E stained slides led to selection of the representative tumor area to be studied. Then, a single core was obtained from the corresponding area of tissue block (donor block) using a 3.0 mm diameter punch and transferred to the recipient paraffin block. Each of the twelve recipient blocks consisted of 13 tumor cores, 13 paired normal cervix cores and three different control cores. The control cores consisted of a normal tonsil tissue, a normal lung tissue and a normal colonic mucosa.

**Immunohistochemistry**

Four-micrometer sections of the paraffin-embedded tissue arrays were submitted for immunohistochemistry. The tissue arrays were processed in an automatic immunohistochemistry staining machine using the standard protocols (Lab Vision Autostainer, Lab Vision, Fremont, CA, USA) with Dako ChemMate EnVision system (Dako, Carpenteria, CA, USA). The following antibodies were used: bcl2 (1:50, Invitrogen, Carlsbad, CA, USA), p53 (1:50, Dako) and Ki67 (1:50, Dako). Immunohistochemistry of bcl2 and p53 were considered to be positive when more than 10% of the cytoplasm and nuclei showed positive staining, respectively. Ki67 expression was scored semiquantitatively based on the positive nuclear staining fraction of the tumor cells (score 0, no staining; score 1+, 1-10%; score 2+, 11-25%; score 3+, 26-50%; score 4+, 51-100%). For purposes of statistical analysis, scores of 0 to 2 were considered as a low Ki67 labeling index (LI) and scores of 3 and 4 were considered as high Ki67 LI.

**FISH**

Five-micrometer thick sections were cut from the paraffin-embedded tissue array, attached to slides, subjected to deparaffinization and hydration process and then probed using a directly-labeled centromere-specific probe for chromosome 8 (CEP8) and a region-specific probe for c-MYC (Vysis LSI MYC [8q24] Spectrum Probe, Abbott/Vysis, Abbott Park, IL, USA). Experiments were performed according to the manufacturers’ guidelines. First, the samples were treated with diluted wash buffer for 2 minutes. They were treated with pre-treatment solution at 95-99°C for 10 minutes and washed with wash buffer twice for 3 minutes each. Next, samples were treated with pepsin reagent for 10 minutes, washed twice with wash buffer for 3 minutes and dehydrated. Subsequently, the samples were treated with the c-MYC/CEP8 probe mixture at 80°C for 10 minutes, incubated in a 37°C humidified hybridization chamber for 16 hours, and treated with stringent wash buffer twice for 3 minutes each. Next, the samples were dehydrated, and slides were treated with fluorescent mounting solution containing 4’-6-diamidino-2-phenylindole (DAPI) and imaged. c-MYC gene sta-
tus was examined independently by two pathologists based on previous study: the number of dots for CEP8 and c-MYC was counted at least in 60 nuclei per sample to verify copy number estimation. The amplification of c-MYC gene was defined as c-MYC/CEP8 ratio ≥ 2.0. Ratios of > 1.0 and < 2.0 represented gain of c-MYC gene, and a ratio of ≤ 1.0 as normal.

Statistical analyses

Statistical analyses were performed using SPSS ver. 12 (SPSS Inc., Chicago, IL, USA). The relationship between c-MYC status and clinicopathologic parameters and the association between c-MYC status and immunohistochemical markers were evaluated by a chi-square test or Fisher’s exact test. Disease free survival and cancer specific survival were analyzed using the Kaplan-Meier method. Differences in survival curves were assessed using the Log-rank test. Multivariate Cox proportional hazards regression analysis was performed to obtain a model for prognostic factors using backward selection strategies. Statistical significance was set at p < 0.05.

RESULTS

The median age at diagnosis was 49 years (range, 25 to 72 years). Of the 144 patients, lymph node metastases were observed in 38 (26.4%) cases. FIGO classifications were as follows: 88 (61.1%) were stage IB, 15 (10.4%) were stage IIA and 41 (28.5%) were stage IIB. RH alone was performed in 88 (61.1%) cases. Fifty six (38.9%) cases received RH plus concurrent chemoradiotherapy (CCRT). The mean duration of follow-up was 54 months (range, 1 to 147 months) (Table 1).

Table 1. Distribution of c-MYC status and clinicopathologic parameters in cervical cancer

<table>
<thead>
<tr>
<th></th>
<th>n (%)</th>
<th>≥Gain</th>
<th>Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>p-value</td>
</tr>
<tr>
<td>Mean age (range, yr)</td>
<td>49 (25-71)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>75 (52.1)</td>
<td>12</td>
<td>63</td>
</tr>
<tr>
<td>≥50</td>
<td>69 (47.9)</td>
<td>14</td>
<td>55</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SQCC</td>
<td>108 (75.0)</td>
<td>14</td>
<td>94</td>
</tr>
<tr>
<td>ADCA</td>
<td>23 (16.0)</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>ADSC</td>
<td>13 (9.0)</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤4</td>
<td>109 (75.7)</td>
<td>20</td>
<td>89</td>
</tr>
<tr>
<td>&gt;4</td>
<td>35 (24.3)</td>
<td>6</td>
<td>29</td>
</tr>
<tr>
<td>Parametrial invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>100 (69.4)</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>Present</td>
<td>44 (30.6)</td>
<td>6</td>
<td>38</td>
</tr>
<tr>
<td>Positive surgical margin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>133 (92.4)</td>
<td>25</td>
<td>108</td>
</tr>
<tr>
<td>Present</td>
<td>11 (7.6)</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>106 (73.6)</td>
<td>21</td>
<td>85</td>
</tr>
<tr>
<td>Present</td>
<td>38 (26.4)</td>
<td>5</td>
<td>33</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IB</td>
<td>88 (61.1)</td>
<td>17</td>
<td>71</td>
</tr>
<tr>
<td>IIA</td>
<td>15 (10.4)</td>
<td>3</td>
<td>12</td>
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<tr>
<td>IIB</td>
<td>41 (28.5)</td>
<td>6</td>
<td>35</td>
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<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RH only</td>
<td>88 (61.1)</td>
<td>17</td>
<td>71</td>
</tr>
<tr>
<td>RH+CCRT</td>
<td>56 (38.9)</td>
<td>9</td>
<td>47</td>
</tr>
<tr>
<td>Median follow-up duration (range, mo)</td>
<td>54 (1-147)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In bold, p < 0.05.
*Evaluated by Kaplan-Meier method; †Evaluated by Fisher’s exact test; ‡SQCC vs ADCA and ADSC: cases ≥ gain: p = 0.006, amplification: p = 0.711; SQCC vs ADCA: cases ≥ gain: p = 0.003, amplification: p = 0.658; SQCC and ADSC vs ADCA: cases ≥ gain: p = 0.004, amplification: p = 0.661; §Stage I vs stage II. SQCC, squamous cell carcinoma; ADCA, adenocarcinoma; ADSC, adenosquamous carcinoma; FIGO, International Federation of Gynecology and Obstetrics; RH, radical hysterectomy; CCRT, postoperative concurrent chemo-radiation therapy.
Results of c-MYC FISH

c-MYC gene alterations were found in 118 of 144 cervical cancers by FISH (Fig. 1). c-MYC copy number per nucleus was ranged from 0.09 to 8.13 (mean, 2.55). The number of CEP8 signals per nucleus was ranged from 0.24 to 4.19 (mean, 1.93). Ratio of c-MYC/CEP8 ranged from 0.1 to 13.8 (mean, 1.40). One hundred eight out of 144 cervical cancers (75.0%) showed gain of the c-MYC gene. Ten of 144 cervical cancers (6.9%) showed amplification of the c-MYC gene. c-MYC copy numbers per nucleus with normal, gain and amplification were ranged from 0.09 to 2.68 (mean, 1.57; standard deviation, 0.47), from 1.45 to 6.38 (mean, 2.55; standard deviation, 0.86) and from 2.90 to 8.13 (mean, 4.84; standard deviation, 1.98), respectively (Fig. 2).

Association with the clinicopathologic parameters and immunohistochemical markers

c-MYC gene alteration was not associated with clinicopathologic parameters except tumor histology. It correlated with SQ-CC histology (94/108, 87.0%) better than ADCA (14/23, 60.9%) or ADSC (10/13, 76.9%) (p = 0.006). On the other hand, c-MYC amplification showed no statistically different correlation according to histological types: seven (6.8%) of 101 SQCC, two (8.7%) of 23 ADCA and one (7.7%) of 13 ADSC (p = 0.925) (Table 1). Other clinicopathologic variables, including age (< 50 years vs ≥ 50 years), tumor size (≤ 4 cm vs > 4 cm), parametrial invasion, positive surgical margin, lymph node metastasis, and stage (stage I vs II) did not correlate with c-MYC gene alteration.

Table 2. Association between c-MYC status and immunohistochemical markers in cervical cancer

<table>
<thead>
<tr>
<th>c-MYC status</th>
<th>Absent (%)</th>
<th>Present (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>Present</td>
<td>0.013</td>
<td>1.000</td>
</tr>
<tr>
<td>Amplification</td>
<td>Absent</td>
<td>102 (70.8)</td>
<td>8 (5.6)</td>
</tr>
</tbody>
</table>

In bold, p < 0.05.

*a* c-MYC gain or amplification vs no c-MYC alteration; *b* c-MYC amplification vs c-MYC gain or no c-MYC alteration; *c* evaluated by chi-square test; *d* evaluated by Fisher’s exact test.

LI, labeling index.
In immunohistochemical analysis, cases with gain or amplification showed significant association with low Ki67 LI ($p = 0.013$) but showed no association with p53 and bcl2 expressions. On the other hand, cases with c-MYC amplification showed significant association with absence of bcl2 expression ($p = 0.048$) but not with Ki67 LI and p53 (Table 2).

**Survival analysis**

At the time of analysis, the number of disease recurrence and cancer specific death was 21 and 21, respectively. On univariate analysis, the conventional prognostic parameters, including tumor size, parametrial invasion, positive surgical margin, lymph node metastasis, CCRT and stage reached significance for disease free and cancer specific survival. But, even though p53 ($p = 0.287$) and bcl2 ($p = 0.135$) showed trends for worse cancer specific survivals, Ki67 LI and expression of p53 and bcl2, assessed by immunohistochemistry, had no statistically significant impact on disease free and cancer specific survival. c-MYC gain (vs normal) did not show statistically significant association with progression free and cancer specific survival ($p = 0.783$ and $p = 0.869$, respectively). However, c-MYC amplification-positive group had a significantly worse disease free and cancer specific survival ($p = 0.007$ and $p = 0.020$, respectively).

**Fig. 3.** Kaplan-Meier survival curves for (A) disease free survival and (B) cancer specific survival stratified by c-MYC amplification.
survival than did the c-MYC amplification-negative group (p = 0.007 and p = 0.020, respectively) (Table 3, Fig. 3). A multivariate analysis using the Cox proportional hazard model was performed and included tumor size, parametrial invasion, positive surgical margin, lymph node metastasis, postoperative CCRT, stage and c-MYC amplification. Four variables, parametral invasion (p < 0.015, only in disease free survival), positive surgical margin (p = 0.036 and p = 0.009, disease free survival and cancer specific survival, respectively), stage (p = 0.010; only in cancer specific survival) and c-MYC amplification (p = 0.028 and p = 0.025; disease free survival and cancer specific survival, respectively) were independent prognostic factors for cervical cancer patients (Table 3).

**DISCUSSION**

Implementation of cervical cancer screening programs has greatly reduced disease incidence and mortality. However, cervical cancer is still the second most common malignancy affecting women worldwide, leading to approximately 250,000 deaths each year.

Activation of c-MYC, c-Ha-RAS and fibroblast growth factor receptor 3 have all been observed in cervical cancer. Of these, there is a close relationship between c-MYC and HPV DNA integration. Preferential integration of specific types of HPVs near the c-MYC locus in cervical carcinoma and an association between c-MYC activation and integration of HPV DNA at the c-MYC locus are well documented, which implies that secondary chromosomal aberrations that occur during HPV DNA integration are important to carcinogenesis.

The developmental and oncogenic roles of c-MYC are well established. c-MYC acts as a key regulator of major cell functions such as proliferation, apoptosis, differentiation and DNA metabolism. These findings are not surprising in view of the multifunctional nature of oncogene, with pivotal roles in proliferation, differentiation and cell death. Presently, cases with c-MYC gene gain showed lower Ki67 LI compared to those with normal c-MYC gene, which indirectly supports the view that the role of c-MYC gene copy alteration is on apoptosis rather than proliferation. In general, c-MYC gene alteration is associated with high Ki67 index, but a few reports showed an inverse association of c-MYC gene alteration with Ki67 index.

Interestingly, a carcinogenic role of c-MYC was demonstrated by its suppression in a pancreas beta cell model that suppression of c-MYC induced apoptosis by coexpression of Bcl-xl and would trigger rapid progression to invasive tumors. On the other hand, our study revealed a significant association between c-MYC amplification and absence of bcl2 expression in cervical cancer, and also revealed that c-MYC amplification was significantly associated with shorter disease free and cancer specific survival, independent of tumor stage at diagnosis. This finding was not expected and the reason may be that c-MYC amplified tumor is more resistant to chemo-radiation induced DNA damage probably due to low proliferative activity.

Previously, the status of c-MYC oncogene was evaluated using fluorescent quantitative polymerase chain reaction in 20 SQCC samples, which showed amplification in 67% patients with SQCC, which defined gene amplification as c-MYC copy numbers greater than the mean value +2 standard deviation of patient with normal histology. Another report on 84 cervical cancer samples using interphase FISH suggested that c-MYC was amplified in up to 25% of cases. In our study, c-MYC gain was found in 81.9% of cases but c-MYC amplification was found only in 6.9% of cases, which could be explained by our different signal scoring method, in contrast to previous reports. In the former study with FISH method, amplification was defined as 3 or more copies per nucleus in more than 50% of counted cells. In contrast, we examined the ratio c-MYC per CEP8 and defined amplification as cases with c-MYC/CEP8 ratio ≥ 2.0.

Interestingly, although the number of positive samples in our study was small, c-MYC amplifications correlated strongly with shorter disease free survival and cancer specific survival. On the contrary, c-MYC gain did not show significant correlation with disease recurrence and cancer related death. The guideline for c-MYC amplification used in our study well reflected biologically aggressive tumor characteristics. The other independent poor prognostic indicators in this study were positive surgical margin for both disease recurrence and cancer related death, parametrial invasion for disease recurrence, and stage for cancer related death.

Previous reports suggest that there are significant differences in gene expression between SQCC and ADCA of the uterine cervix. ADCA of uterine cervix is more likely to be locally advanced when diagnosed, less responsive to radiation therapy, and, when matched stage for stage, more likely to result in death. Our study revealed different distribution of c-MYC gene status according to histologic types. Predilection of c-MYC alteration to SQCC indirectly reinforces the different genetic background between SQCC and ADCA. However, in our study, the histologic types showed no differences in amplification of c-MYC and there were no differences in disease free and cancer specific
survival within tumor types, which could be due to the unbalance of the number of histological types and due to small number of c-MYC amplification cases.

In this study, two treatment groups were included: RH only and RH plus CCRT. However, adjuvant CCRT did not gain statistically significant benefits on disease free survival or cancer specific survival in multivariate analysis, which may due to relatively limited number of our cases than previous large series study.\(^{50}\)

There are some limitations to the generalization of these results. First, we cannot exclude selection bias because all the enrolled patients were surgically resected. Second, the number of c-MYC amplification is small. Third, we included heterogenous histologic types. Fourth, this is a retrospective study.

Despite these limitations, this is the first study to examine both c-MYC gain and amplification in large number of cervical cancer patients and to reveal the association of c-MYC amplification with disease recurrence and cancer specific survival.

In conclusion, c-MYC gene has gained new interest as a biomarker for detection of HPV associated genomic instability in cervical epithelial cells and a potentially important prognostic marker. As a consequence, presence of c-MYC amplification could be an important key to study the biology of cervical cancer and can represent an important and useful tool to facilitate the development of new tailored therapeutic approaches for cervical cancer patient after radical surgery. This study objectively demonstrated the prognostic significance of c-MYC gene status using FISH. c-MYC amplification, not gain, was a negative independent prognostic factor for disease free and cancer specific survival in cervical cancer. So we recommend more aggressive adjuvant therapy on cases with c-MYC amplification and more conservative therapy on cases with c-MYC gain or no c-MYC alterations.

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