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Prognostic Significance of Amplification of the *c-MYC* Gene in Surgically Treated Stage IB–IIB Cervical Cancer

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Background: Mutations of *c-MYC* have been described in cervical cancer. However, association between *c-MYC* gene status and its prognostic significance have not been clarified. **Methods:** Tissue microarray sections from 144 patients with stage IB-IIB cervical cancer treated by radical hysterectomy were analyzed by fluorescence *in situ* hybridization using a region-specific probe for *c-MYC* and a centromere-specific probe for chromosome 8. **Results:** Seventy five percent (108/144) of *c-MYC* gain and 6.9% (10/144) of *c-MYC* gene amplification were observed. *c-MYC* gene alteration was more frequently observed in squamous cell carcinoma than adenocarcinoma or adenosquamous carcinoma and were associated with low Ki67 labeling index ($p=0.013$). *c-MYC* amplification was not associated with clinicopathologic parameters except absence of bcl2 expression ($p=0.048$). Survival analysis revealed that patients with *c-MYC* amplification were significantly associated with higher risk of disease recurrence ($p=0.007$) and cancer related death ($p=0.020$). However, *c-MYC* gain was not associated with unfavorable outcome. Multivariate analysis proved *c-MYC* amplification as independent prognostic factors of shorter disease free survival and cancer-related death ($p=0.028$ and $p=0.025$, respectively). **Conclusions:** *c-MYC* amplification, not gain, is an independent prognostic marker for shorter disease free and cancer specific survival in cervical cancer treated by radical hysterectomy.

Key Words: Uterine cervical neoplasms; *In situ* hybridization, fluorescence; MYC; Hysterectomy; Prognosis

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.[1] Furthermore, cervical cancer is the most common cause of death from gynecological malignancy in developing countries.[2]

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.[3] 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-IIB cervical cancer.[4] 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.[5]

In addition to these clinico-pathologic variables, increasing attention has focused on various new approaches to molecular and genetic changes in cervical cancer.[6] Molecular and genetic alterations are important in the pathogenesis of cervical cancer, especially in association with human papillomavirus (HPV) infections.[7] 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,[8] *c-MYC* gene maps to chromosomal band 8q24, which is reportedly a common site for integration of HPV DNA.[9] Gene amplification is an important mechanism for protein overexpression and oncogene activation in tumor cells.[10] *c-MYC* amplification is well documented in cervical cancer.[11] Amplification of the *c-MYC* gene is also addressed in breast cancer,[12] bladder cancer,[13] prostate cancer,[14] and lung cancer.[15] Although strong prognostic impact o f *c-MYC* gene amplification on histologic grade, disease stage and patients survival have been well documented in certain types of tumors,[16] 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 clinicopathologic factors. The prognostic value

of *c-MYC* was evaluated in conjunction with immunohistochemical analysis for Ki67, p53 and bcl2, reportedly *c-MYC*-related markers.[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 parametrial 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 patient. 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 KC10SISI0475).

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, Carpinteria, 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 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 semiquantatively 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-diamidine-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 immunohistochemial 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

	n (%)	*c-MYC* status					
		≥Gain			Amplification		
		Absent	Present	p-value	Absent	Present	p-value
Mean age (range, yr)	49 (25-71)						
<50	75 (52.1)	12	63	0.504[a]	70	5	0.891[a]
≥50	69 (47.9)	14	55		64	5	
Histology							
SQCC	108 (75.0)	14	94	**0.006**[a,c]	101	7	0.925[b,c]
ADCA	23 (16.0)	9	14		21	2	
ADSC	13 (9.0)	3	10		12	1	
Tumor size (cm)							
≤4	109 (75.7)	20	89	0.872[a]	103	6	0.257[b]
>4	35 (24.3)	6	29		31	4	
Parametrial invasion							
Absent	100 (69.4)	20	80	0.360[a]	96	4	0.068[b]
Present	44 (30.6)	6	38		38	6	
Positive surgical margin							
Absent	133 (92.4)	25	108	0.689[a]	123	10	1.000[b]
Present	11 (7.6)	1	10		11	0	
Lymph node metastasis							
Absent	106 (73.6)	21	85	0.465[a]	100	6	0.456[b]
Present	38 (26.4)	5	33		34	4	
FIGO stage							
IB	88 (61.1)	17	71	0.621[a,d]	83	5	0.455[a,d]
IIA	15 (10.4)	3	12		15	0	
IIB	41 (28.5)	6	35		36	5	
Treatment							
RH only	88 (61.1)	17	71	0.621[a]	83	5	0.455[a]
RH+CCRT	56 (38.9)	9	47		51	5	
Median follow-up duration (range, mo)	54 (1-147)						

In bold, $p<0.05$.

[a]Evaluated by Kaplan-Meier method; [b]Evaluated by Fisher's exact test; [c]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; [d]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).

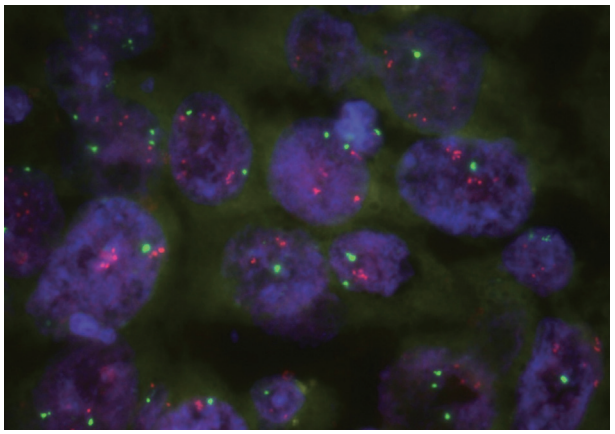

Fig. 1. Example of cervical cancer with *c-MYC* amplification: dual-color fluorescence *in situ* hybridization detected the copy numbers of *c-MYC* range from four to eight copies per cells (red color) and one to two copies of chromosome 8 centromere (green color).

Association with the clinicopathologic parameters and immunohistochemical markers

c-MYC gene alteration was not associated with clinicopathologic parameters except tumor histology. It correlated with SQCC 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 altera-

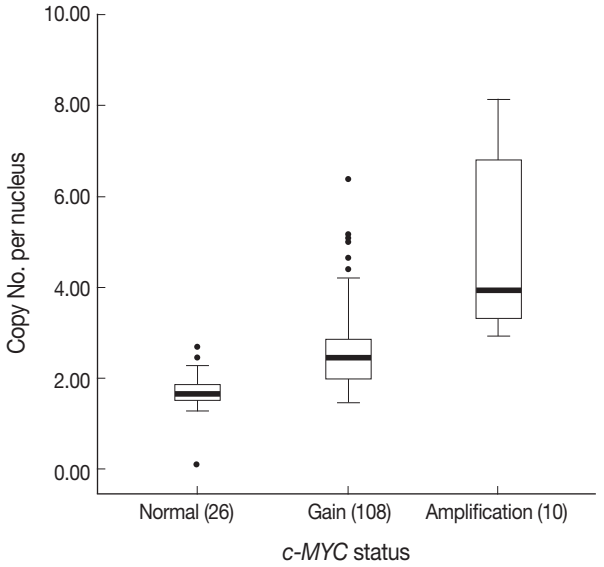

Fig. 2. The ranges of *c-MYC* copy number per nucleus in cervical cancer with normal, gain and amplification.

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

		n (%)	*c-MYC* status					
			Gain or amplification[a]			Amplification[b]		
			Absent (%)	Present (%)	p-value[c]	Absent (%)	Present (%)	p-value[d]
Ki67 LI	Low	110 (76.4)	15 (10.4)	95 (66.0)	**0.013**	102 (70.8)	8 (5.6)	1.000
	High	34 (23.6)	11 (7.6)	23 (16.0)		32 (22.2)	2 (1.4)	
p53	Negative	88 (61.1)	18 (12.5)	70 (48.6)	0.348	81 (56.3)	7 (4.9)	0.741
	Positive	56 (38.9)	8 (5.6)	48 (33.3)		53 (36.8)	3 (2.1)	
bcl2	Negative	69 (48.6)	11 (7.6)	58 (40.3)	0.527	61 (42.4)	8(5.6)	**0.048**
	Positive	75 (51.4)	15 (10.4)	60 (81.9)		73 (50.7)	2 (1.4)	

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.

Table 3. Univariate and multivariate analysis of disease free survival and cancer specific survival of patient with cervical cancer

	Disease free survival				Cancer specific survival			
	Univariate	Multivariate			Univariate	Multivariate		
	p-value	HR	95% CI	p-value	p-value	HR	95% CI	p-value
Age (≥50 yr)	0.665				0.835			
Histology (ADCA)	0.696				0.397			
Tumor size (>4 cm)	**0.008**	1.814	0.733-4.493	0.198	**0.003**	1.877	0.692-5.094	0.216
Parametrial invasion	**<0.001**	3.184	1.253-8.096	**0.015**	**0.001**	1.035	0.243-4.408	0.963
Positive margin	**0.002**	3.544	1.084-11.583	**0.036**	**<0.001**	4.463	1.463-13.613	**0.009**
LN metastasis	**0.002**	2.089	0.808-5.398	0.128	**0.008**	1.558	0.581-4.180	0.379
CCRT	**0.002**	1.002	0.200-5.016	0.998	**0.002**	0.930	0.207-4.185	0.925
Higher stage (II)	**0.001**	1.426	0.307-6.628	0.651	**<0.001**	4.081	1.410-11.814	**0.010**
High Ki67 LI	0.496				0.287			
p53 (+)	0.461				0.135			
bcl2 (+)	0.848				0.911			
c-MYC gain (vs nomal)	0.783				0.869			
c-MYC amplification (vs gain plus normal)	**0.007**	3.655	1.154-11.576	**0.028**	**0.020**	3.782	1.186-12.060	**0.025**

In bold, $p<0.05$.
HR, hazard ratio; CI, confidence interval; ADCA, adenocarcinoma; LN, Lymph node; CCRT, postoperative concurrent chemo-radiation therapy; LI, labeling index.

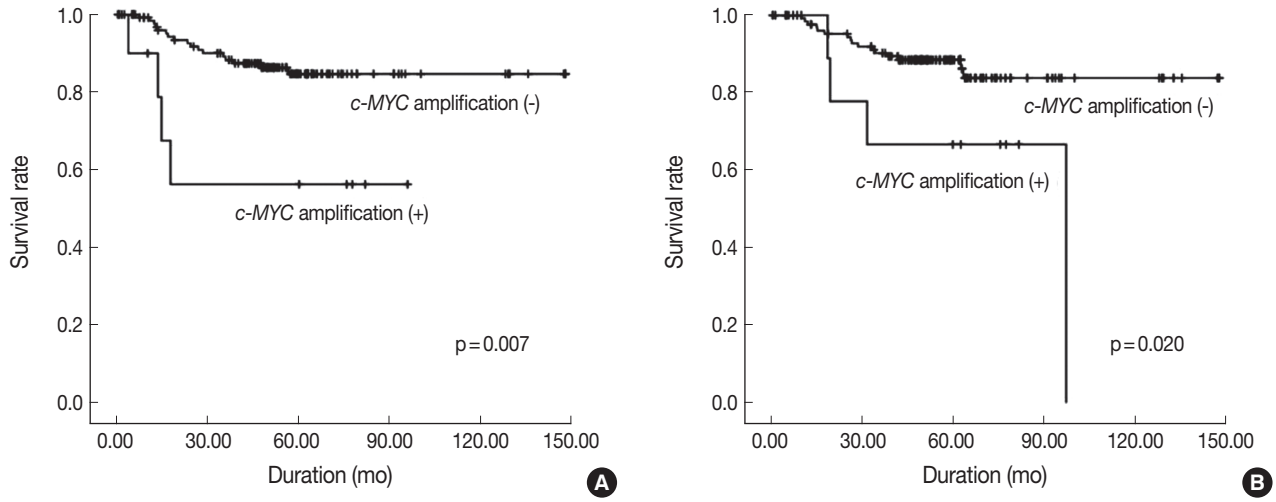

Fig. 3. Kaplan-Meier survival curves for (A) disease free survival and (B) cancer specific survival stratified by *c-MYC* amplification.

tions (Table 1).

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 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, parametrial 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.[18] However, cervical cancer is still the second most common malignancy affecting women worldwide, leading to approximately 250,000 deaths each year.[19]

Activation of *c-MYC*,[20] c-Ha-RAS[21] and fibroblast growth factor receptor 3[22] 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[23] and an association between c-MYC activation and integration of HPV DNA at the *c-MYC* locus[8] are well documented, which implies that secondary chromosomal aberrations that occur during HPV DNA integration are important to carcinogenesis.[24]

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.[25] 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.[26] 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-x(L) and would trigger rapid progression to invasive tumors.[27] 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.[28] Another report on 84 cervical cancer samples using interphase FISH suggested that *c-MYC* was amplified in up to 25% of cases.[20] 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.[29] 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.[30]

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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