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# Immunohistochemical Biomarkers of Survival in Patients With Adenocarcinoma of the Uterine Cervix Receiving Chemoradiotherapy

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**Abstract.** *Background/Aim: To determine the prognostic effects of immunohistochemical biomarkers for predicting chemoradiotherapy (CRT)-based treatment outcomes in patients with adenocarcinoma of the uterine cervix. Materials and Methods: This study included 42 patients receiving definitive CRT. According to the International Federation of Gynecology and Obstetrics staging system, 13, 21, and 8 patients were classified as having stage IB2, II, and III disease, respectively. Baseline immunohistochemical biomarkers, including those for hypoxia, cell proliferation, cell adhesion, immunogenicity, and evasion of apoptosis, were analyzed using tissue microarrays from biopsy specimens. Results: Myeloid cell leukemia-1 (MCL1) overexpression and the presence of pelvic lymph node metastasis were two prognostic factors for inferior cancer-specific survival. A higher H-score for c-MYC proto-oncogene, bHLH transcription factor (c-MYC) was associated with lower pelvic relapse-free survival. Conclusion: For patients with adenocarcinoma of the uterine cervix requiring definitive CRT, treatment outcomes can be stratified by the immunohistochemical biomarkers MCL1 and c-MYC for cancer death and local failure, respectively.*

Adenocarcinoma (AC) of the uterine cervix constitutes approximately 10-20% of all uterine cervical carcinomas with a trend toward a rising incidence (1-3). Possible reasons for this increase include obesity, nulliparity, and human papillomavirus-18 infection (4, 5). AC can be localized deep in the endocervical canal and easily be missed with the usual sampling in screening programs. Some studies indicated that AC and squamous cell carcinoma (SCC) behave differently in epidemiology (2-7), and have differential genomic expression (8, 9). In addition, they have diverse prognostic factors and patterns of failure after similar treatments (4, 6, 10-13). Currently, most treatment knowledge of cervical AC comes from studies where the majority of patients had SCC. Therefore, molecular profiling of novel treatment strategies specifically for AC is imperative.

Given that chemoradiotherapy (CRT) has been the standard of care for patients with locally advanced cervical cancer worldwide, radioresistance or treatment failure is a clinically relevant problem. Patients with cervical AC primarily treated with radiotherapy have inferior outcomes compared with those with SCC (4, 12-14). As advances in molecular profiling have allowed for the identification of biomarkers of many biological characteristics in tumor cells, biomarkers in standard treatment are of interest for their potential role in the design of personalized therapeutic strategies targeting individual tumors. In cervical cancer, several biomarkers for RT-based treatment have been validated by patient survival or recurrence data (15, 16). These biomarkers fall into categories according to biological function including hypoxia, cell proliferation, cell adhesion, immunogenicity, and evasion of apoptosis (15). There is a great need to identify biomarkers since there are few studies on CRT-based prognostic factors specifically for cervical AC. Hence, this study was conducted to investigate the impact of pretreatment immunohistochemical (IHC) markers and clinical parameters on CRT-based treatment in these patients.

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*Key Words:* Cervical adenocarcinoma, chemoradiotherapy, immunohistochemistry, prognosis, myeloid cell leukemia-1.

## Materials and Methods

*Study population*. This retrospective study included 42 patients newly diagnosed with AC of the uterine cervix between July 2009 and December 2015. All patients had undergone $^{18}$F-fluorodeoxyglucose positron-emission tomography/computed tomography (PET/CT) for staging and had received allocated external-beam radiotherapy and intracavitary brachytherapy. Concurrent chemotherapy consisted of weekly administration of 40 mg/m$^2$ of cisplatin. The eligibility criteria included patients with stage IB2 to IIIB disease in accordance with staging system of the International Federation of Gynecology and Obstetrics (FIGO) (17). Accordingly, 13, 21, and eight patients were classified as having stage IB2, II, and III disease, respectively. The median age of our patients was 55 years. Because PET/CT has high sensitivity and specificity in detecting the nodal status in cervical cancer, the diagnosis of pelvic lymph node (PLN) metastasis was based on PET/CT. Patients with para-aortic lymph node metastasis were excluded. In addition, we excluded patients who were histologically diagnosed with adenosquamous carcinoma. This study was approved by the local Institutional Review Board (CMUH107-REC3-008). Patient characteristics are listed in Table I.

Table I. *Patient characteristics (n=42).*

| Variable | Value |
|---|---|
| Age, years | |
| Median (range) | 55 (33-77) |
| FIGO stage | |
| IB2 | 13 (31%) |
| IIA-IIB | 21 (50%) |
| IIIA-IIIB | 8 (19%) |
| Maximum tumor dimension, cm | |
| Mean±SD (range) | 5.7±1.1 (3.9-8.6) |
| Pelvic lymph node metastasis | |
| Negative | 24 (57%) |
| Positive | 18 (43%) |
| Pretreatment hemoglobulin, g/dI | |
| Mean±SD (range) | 10.3±3.0 (3.5-14.3) |
| Carcinoembryonic antigen, ng/dI | |
| Mean±SD (range) | 36.2±22.9 (0.4-528.8) |
| External beam radiotherapy, Gy | |
| Whole pelvis, Gy | |
| Median (range) | 45 (39.6-54) |
| Bilateral parametrial boost, central shielding, Gy | |
| Median (range) | 54 (50.4-57.6) |
| Pelvic lymph node boost, Gy | |
| Median (range) | 64 (60-66) |
| Brachytherapy | |
| 2-Dimensional* | |
| Number of patients | 14 |
| Cumulative EQD2 of point A, $Gy_{10}$ | |
| Mean±SD | 84.3±7.3 |
| 3-Dimensional brachytherapy# | |
| Number of patients | 28 |
| Cumulative EQD2 of D90, $Gy_{10}$ | |
| Mean±SD | 88.1±10.3 |

FIGO: International Federation of Gynecology and Obstetrics; EQD2: equivalent dose in 2 Gy; HR-CTV: high-risk clinical target volume. *6 Gy to point A per session for 5 courses. #HR-CTV >6.5 Gy per session for 5 courses.

*Immunohistochemistry*. IHC biomarkers, namely endogenous hypoxic [glucose transporter 1 (GLUT1), carbonic anhydrase IX (CAIX), and hypoxia-inducible factor 1-alpha (HIF1α)], angiogenesis or metastasis ([vascular endothelial growth factor (VEGF), c-MET]), cell proliferation [epidermal growth factor receptor (EGFR), c-MYC, insulin-like growth factor 1 receptor (IGF1R)], cell to cell adhesion (E-cadherin, vimentin), evasion to apoptosis [B-cell lymphoma 2 (BCL2), BCL2-associated protein X (BAX), myeloid cell leukemia 1 (MCL1)], and immunogenic or inflammatory biomarkers [programmed cell death protein ligand 1 (PD-L1), tumor necrosis factor-α (TNFα), calretinin, galectin-9, and chemokine ligand 5 (CCL5)] were analyzed using tissue microarrays from incisional biopsy specimens before treatment. Each tumor was represented by one tissue core on a tissue microarray. Furthermore, 4-μm-thick paraffin sections were deparaffinized and microwaved according to standard procedures before being processed for IHC staining. The antibodies for staining of biomarkers are detailed as follows: polyclonal rabbit antihuman Glut1 (1:200; GTX15309; GeneTex; Santa Barbara, CA, USA), liquid mouse monoclonal CAIX (1:200; clone TH22; Novocastra; Santa Barbara, CA, USA), monoclonal mouse antihuman VEGF (1:800; NB100-664; Novus Biologicals; Santa Barbara, CA, USA), HIF-1α rabbit polyclonal (1:200; ab10625; Abcam; Santa Barbara, CA, USA), rabbit monoclonal EGFR (1:100; clone EP22; Zeta; Santa Barbara, CA, USA), liquid mouse monoclonal Bcl-2 (1:100; clone bcl-2/100/D5; Novocastra), monoclonal rabbit anti-Met (c-Met) antibodies (1:200; EP1454Y; Abcam), monoclonal mouse IgG human c-MYC antibody (1:100; clone #9E10; bio-techne, Devens, MA, USA), and monoclonal mouse IgG human MCL1 antibody (1:50; clone #4B7; GeneTex), monoclonal rabbit IgG anti-human Bax antibody (clone #E63; Santa Barbara, CA, USA), polyclonal goat IgG anti-human/mouse IGF-1R antibody (catalog #391-GR; bio-techne), monoclonal rabbit IgG anti-human Vimentin antibody (clone #SP20; Fremont, CA, USA), monoclonal rabbit IgG anti-human E-cadherin antibody (clone #EP700Y; Fremont, CA, USA), polyclonal rabbit IgG anti-human/mouse TNF-α antibody (catalog #GTX110520; GeneTex), polyclonal rabbit IgG anti-human/mouse calretinin antibody (catalog #GTX111627; GeneTex), polyclonal rabbit IgG anti-human galectin-9 antibody (catalog #GTX127352; GeneTex), polyclonal rabbit IgG anti-human/mouse CCL5 antibody (NBP1-19769; bio-techne).

The staining slides were scored by two pathologists blinded to the clinical outcome. Except for PD-L1, IHC results of the aforementioned biomarkers were scored by a semiquantitative approach used to assign an H-score to tumor samples (18). The H-score takes into consideration the staining intensity in conjunction with the percentage of cells staining positively. Staining intensity was graded as 0, 1, 2, and 3 corresponding to negative, mild, moderate, and strong staining, respectively. The percentage of positively stained tumor cells was estimated by the observers. The total number of neoplastic cells in the field and the number of neoplastic cells stained at each intensity were counted. The following formula was applied: H-score=[% of cells stained at intensity category 1 (neoplastic cells with mild staining) ×1] + [% of cells stained at intensity category 2 (neoplastic cells with moderate staining) ×2] + [% of cells stained at intensity category 3 (neoplastic cells with strong staining) ×3]. Accordingly, the H-scores were calculated, ranging from 0 to 300, with 300 being equal to 100% of tumor cells stained strongly (3+).

Tumor biomarker PD-L1 was evaluated through IHC staining using the DAKO clone 22C3 pharmDx kit (DAKO, Carpinteria, CA, USA). PD-L1 expression was scored according to the combined positive score, which is the number of PD-L1 -staining cells (tumor cells, lymphocytes, macrophages) at any intensity divided by the total number of viable tumor cells, multiplied by 100 (19).

*Treatment*. The treatment was described previously (20, 21). All patients were treated with intensity-modulated RT. The total dose applied to the pelvis was 45 Gy, administered in 25 fractions over a 5-week period. Following pelvic irradiation, the bilateral parametrium was boosted from 50.4 to 54 Gy.

After adequate tumor regression, high-dose-rate intracavitary brachytherapy was performed once or twice a week using an $^{192}$Ir remote afterloading technique concurrently with pelvic irradiation or parametrial boosting. Before January 2013, the standard prescribed dose for each session of brachytherapy was 6.0 Gy to Point A, with five sessions. After January 2013, 28 patients were treated with three-dimensional image-based brachytherapy according to the recommendations of the Groupe Européen de Curiethérapie and the guidelines specified by the European Society for Radiotherapy and Oncology (22). The details of the cumulative dose are summarized in Table I.

Chemotherapy consisted of weekly 40 mg/m$^2$ doses of cisplatin, administered intravenously to a total dose of 60 mg.

*Follow-up*. After completion of RT, patients were regularly followed-up every 2 months for the first year, and every 3 to 4 months thereafter. Besides a routine pelvic examination, the serum level of the tumor marker carcinoembryonic antigen was examined during each follow-up. Additionally, a radiographic examination was performed every 6 months. Patients exhibiting symptoms of central-pelvic recurrence underwent a salvage hysterectomy or pelvic exenteration, if feasible. Patients with distant metastasis were treated with systemic chemotherapy.

*Statistical analysis*. To examine correlations between the aforementioned parameters and tumor recurrence, receiver operating characteristic (ROC) curves were constructed to evaluate the optimal predictive performance among the various IHC and clinical parameters, such as maximum tumor dimension and pretreatment hemoglobulin (Hb) (23). In addition, binary logistic regression analysis was performed to determine the independent factors among all IHC biomarkers for predicting clinical outcomes. The quantitative differences between H-scores of the biomarkers and clinical parameters were examined using the Mann–Whitney *U*-test. The outcome endpoints were cancer-specific survival (CSS), distant metastasis-free survival (DMFS), and pelvic relapse-free survival (PRFS), all of which were calculated using the Kaplan–Meier method. The log-rank test and Cox regression analysis were performed to examine the effects of explanatory variables on these endpoints. The stage, age, PLN status, maximum tumor dimension, Hb level, and predictive IHC markers were included for multivariate analysis. Patient survival was measured from the date of initiation of RT to the last follow-up. Two-tailed tests were used, and values $p<0.05$ was considered statistically significant. All calculations were performed using SPSS, Version 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

## Results

*Treatment outcomes*. After a median follow-up duration of 48 months (range=7-120), 29 patients were alive and 13 patients had died of cancer progression. Twenty-three patients had no evidence of disease progression. Eight out of the 19 patients with disease progression had infield recurrence, five had distant metastasis, and six had both. None of the 14 patients with infield recurrence experienced sole relapse of PLN. In summary, 14 patients had local residual or recurrent tumors at primary sites, whereas 11 patients experienced distant metastasis. Seven patients underwent salvage operation for residual or recurrent primary tumor, whereas 10 received systemic chemotherapy after the diagnosis of distant metastasis.

*Predictive ability of IHC biomarkers*. All aforementioned IHC biomarkers were retrieved. Table II lists the biomarkers and the area under the ROC curve. The c-MYC H-score most accurately predicted the presence of local residual or recurrent tumors (AUC=0.71, $p=0.03$). Logistic regression analysis showed the c-MYC H-score had the highest predictive value in the cohort [odds ratio (OR)=1.30, 95% confidence interval (CI)=1.001-1.061; $p=0.045$]. Based on Youden's index, we found that an optimal cut-off for the c-MYC H-score was 27 (AUC=0.68, $p=0.06$).

The ROC curves showed that the MCL1 H-score was the sole biomarker that predicted death from cancer (AUC=0.69, $p=0.06$). In logistic regression analysis, however, the MCL1 H-score failed to attain statistical significance probably due to restricted events ($p=0.058$). When using an optimal cut-off of 115 for the MCL1 H-score, the AUC for death from cancer was 0.69 ($p=0.055$).

None of the other IHC biomarkers, including those for hypoxia, cell adhesion, or immunogenicity biomarkers, appeared to be prognostic for the three study endpoints for this cohort. Therefore, the MCL1 and c-MYC H-scores, combined with age, stage, maximum tumor dimension, pretreatment Hb, PLN status, and brachytherapy schemes (2D *versus* 3D) were selected for multivariate Cox regression model for survival analyses.

*Prognostic factors for CSS, PRFS, and DMFS*. As summarized in Table III, Cox regression analysis indicated that the MCL1 H-score (hazard ratio (HR)=12.82, 95% CI=1.53-107.38; $p=0.019$) and presence of PLN metastasis (HR=4.49, CI=1.21-16.68; $p=0.025$) were two factors prognostic for CSS. As depicted in Figure 1, the 4-year CSS of patients with and without PLN disease was 52% and 85% ($p=0.02$), and among patients who had tumors with a MCL1 H-score >115 and ≤115, it was 40% and 84%, respectively ($p=0.004$). The trend remained statistically significant when dichotomizing the patients with a cutoff using the median

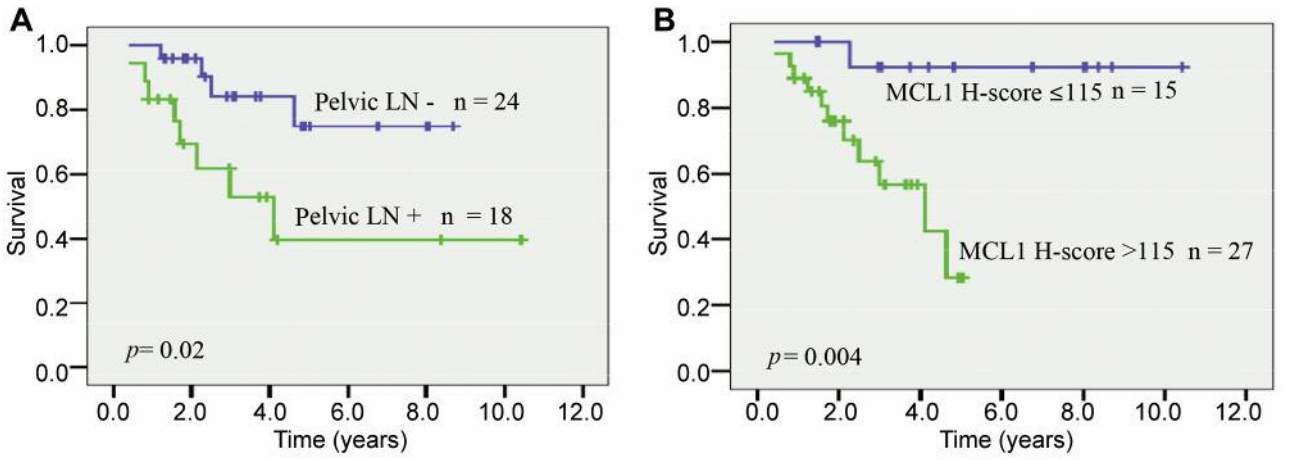


Figure 1. *Cancer-specific survival in patients who had tumors with and without pelvic lymph node (LN) metastasis (A), and according to myeloid cell leukemia-1 (MCL1) H-score (>115 vs. ≤115) (B).*

MCL1 H-score of 130. The 4-year CSS of patients in high and low MCL1 expression groups was 60% and 79%, respectively ($p$=0.048).

Cox regression analysis disclosed that the c-MYC H-score was the sole predictor of poor PRFS (HR=1.03, CI=1.01-1.05; $p$=0.011). Using the optimal cut-off of 27 for the c-MYC score, the 4-year PRFS of patients with tumors with high and low c-MYC H-scores was 52% and 77%, respectively ($p$=0.048, Figure 2).

None of the IHC biomarkers were prognostic for DMFS; the major determinants of poor DMFS were PLN disease (HR=5.41, CI=1.47-19.86; $p$=0.011) and FIGO stage III disease (HR=3.38, CI=1.17-9.79; $p$=0.02). In multivariate analysis, age, tumor size, pretreatment Hb, and brachytherapy schemes were not identified as independent prognostic factors for the aforementioned endpoints.

*Quantitative differences between MCL1 or c-MYC H-scores and clinical parameters*. Using the Mann–Whitney *U*-test, an association analysis was carried out to investigate the quantitative difference between MCL1 and c-MYC H-scores according to dichotomized clinical parameters including stage, PLN status, tumor dimension (median value of 5.6 cm), and pretreatment Hb (median value of 10 gm/dI). As shown in Figure 3A, patients presenting with pretreatment Hb <10 g/dl had tumors with a significantly higher mean MCL1 H-score (26.6 *vs.* 17.7, $p$=0.021). In addition, a lower Hb was associated with tumors having a higher mean vimentin to E-cadherin ratio (0.15 *vs.* 0.05, $p$=0.037; Figure 3B). MCL1 and c-MYC H-scores were not related to stage, PLN status, or maximum tumor dimension. None of the clinical parameters or IHC biomarkers, including GLUT1 and HIF1α, were associated with c-MYC H-score.

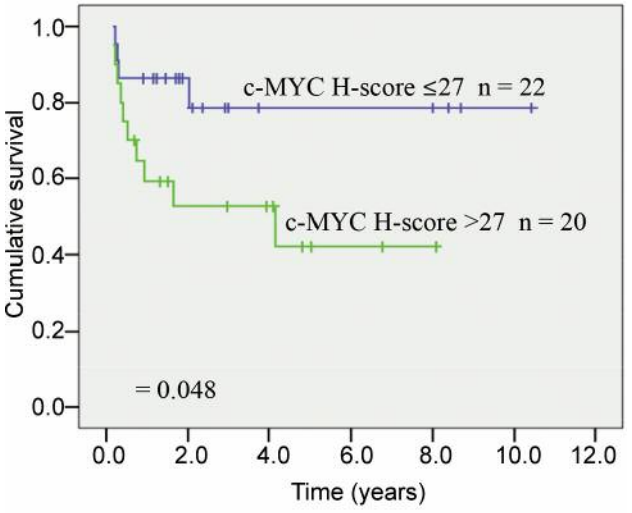


Figure 2. *Pelvic relapse-free survival in patients according to tumor MYC proto-oncogene, bHLH transcription factor (c-MYC) H-score (>27 vs. ≤27).*

*Impact of combined overexpression of MCL1 and c-MYC on survival*. Because a recent study disclosed that co-amplification of *c-MYC* and *MCL1* increases cancer stem cells in chemotherapy-resistant triple-negative breast cancer (24), the impact of combined MCL1 and c-MYC overexpression on survival was analyzed. Using the optimal values for both biomarkers, 18 tumors were identified as having co-overexpression. As shown in Figure 4, the 4-year CSS of patients with tumors with and without co-overexpression was 54% and 83% ($p$=0.026). The 4-year PRFS of patients with tumors with and without co-overexpression was 59% and 73% ($p$=0.15), whereas the 4-year DMFS for the two groups was

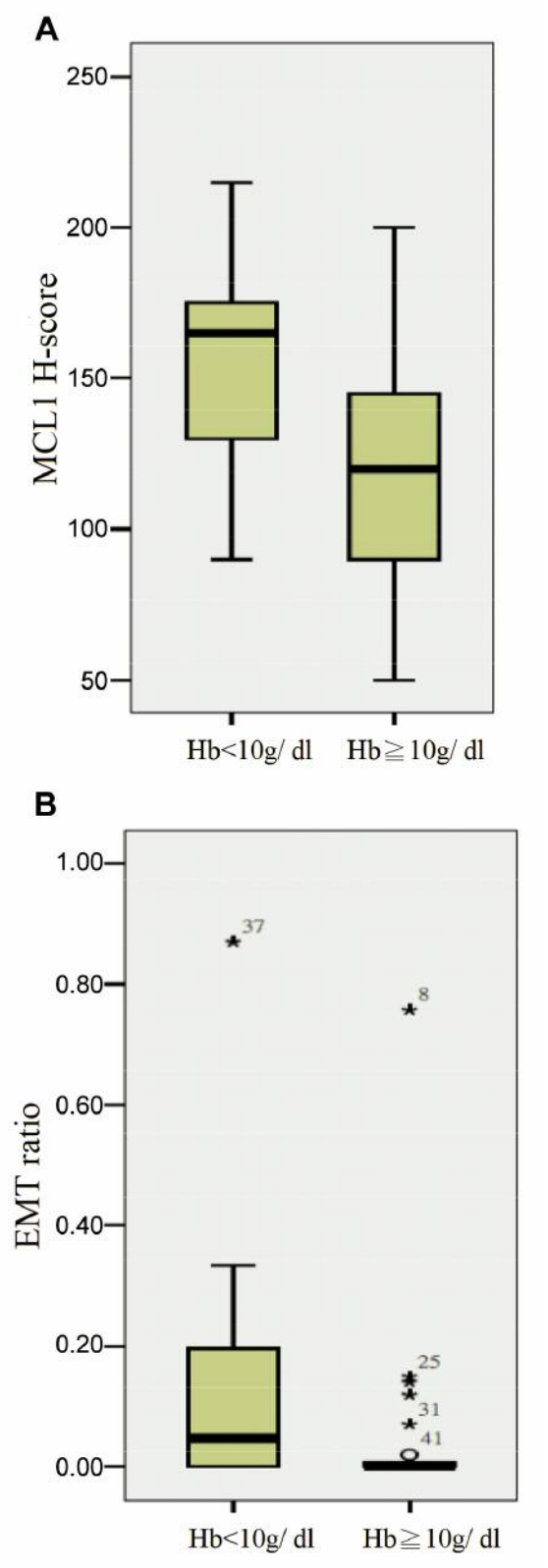


Figure 3. *Myeloid cell leukemia-1 (MCL1) H-score (A), and vimentin-to-E-cadherin (EMT) ratio according to pretreatment hemoglobulin (B) (p=0.021 and p=0.037, respectively).*

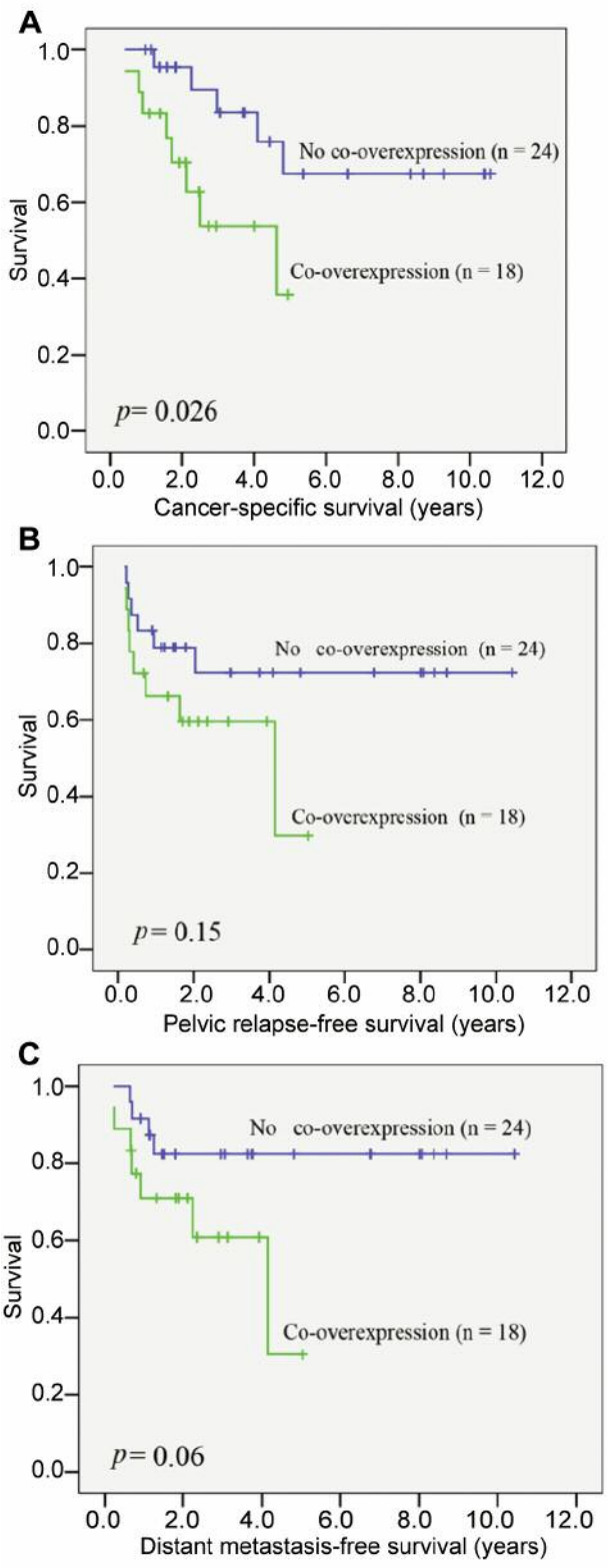


Figure 4. *Cancer-specific (A), pelvic relapse-free (B) and distant metastasis-free (C) survival curves of patients with tumors with and without co-overexpression of myeloid cell leukemia-1 (MCL1) and MYC proto-oncogene, bHLH transcription factor (c-MYC).*

Table II. *Predictive immunohistochemical and clinical parameters and the area under the receiver operating characteristic curve (AUC).*

| | Cancer death | | Local failure | | Distant metastasis | |
|---|---|---|---|---|---|---|
| Variable | AUC | *p*-Value | AUC | *p*-Value | AUC | *p*-Value |
| MCL1 H-score | 0.69±0.09 | 0.06 | 0.61±0.09 | 0.25 | 0.60±0.10 | 0.32 |
| c- MYC H-score | 0.59±0.10 | 0.33 | 0.71±0.08 | 0.03 | 0.49±0.11 | 0.95 |
| EGFR H-score | 0.38±0.10 | 0.19 | 0.53±0.07 | 0.77 | 0.34±0.10 | 0.74 |
| PD-L1 combined positive score | 0.44±0.10 | 0.51 | 0.64±0.09 | 0.15 | 0.44±0.11 | 0.54 |
| TNFα H-score | 0.42±0.10 | 0.43 | 0.38±0.09 | 0.22 | 0.41±0.09 | 0.39 |
| Calretinin H-score | 0.43±0.10 | 0.48 | 0.52±0.09 | 0.81 | 0.39±0.10 | 0.29 |
| Maximum tumor dimension | 0.60±0.10 | 0.30 | 0.50±0.10 | 0.97 | 0.62±0.10 | 0.26 |
| Pretreatment hemoglobulin | 0.49±0.10 | 0.88 | 0.41±0.10 | 0.34 | 0.45±0.11 | 0.62 |
| Pretreatment serum CEA | 0.56±0.10 | 0.55 | 0.63±0.09 | 0.18 | 0.59±0.09 | 0.38 |

CEA: Carcinoembryonic antigen; EGFR: epidermal growth factor receptor; MCL1: myeloid cell leukemia-1; c-MYC: MYC proto-oncogene, bHLH transcription factor; PD-L1: programmed cell death protein ligand 1; TNFα: tumor necrosis factor-α.

Table III. *Multivariate analyses using Cox regression analysis for cancer-specific (CSS), pelvic relapse-free (PRFS), and distant metastasis-free (DMFS) survival.*

| | CSS | | | | PRFS | | | | DMFS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Univariate | | Multivariate | | Univariate | | Multivariate | | Univariate | | Multivariate | |
| Variable | *p*-Value | HR | 95% CI | *p*-Value | *p*-Value | HR | 95% CI | *p*-Value | *p*-Value | HR | 95% CI | *p*-Value |
| Clinical parameters | | | | | | | | | | | | |
| FIGO stage | | | | | | | | | | | | |
| IIIA-IIIB *vs*. IB-IIB | 0.06 | | | 0.29 | 0.78 | | | | 0.04 | | | 0.20 |
| IIB-IIIB *vs*. IB | 0.011 | 2.72 | 0.96-7.68 | 0.06 | 0.12 | | | | 0.019 | 3.38 | 1.17-9.79 | **0.025** |
| Pelvic lymph nodes (+ *vs*. −) | 0.005 | 4.49 | 1.21-16.68 | **0.025** | 0.83 | | | | 0.02 | 5.41 | 1.47-19.86 | **0.011** |
| Age (continuous) | 0.49 | | | | 0.18 | | | | 0.54 | | | |
| Maximum tumor dimension (continuous) | 0.12 | | | | 0.60 | | | | 0.18 | | | |
| Pretreatment hemoglobulin (continuous) | 0.39 | | | | 0.10 | | | | 0.78 | | | |
| Brachytherapy 3D *vs*. 2D | 0.88 | | | | 0.85 | | | | 0.89 | | | |
| Immunohistochemical biomarkers | | | | | | | | | | | | |
| MCL1 H-score (continuous) | 0.036 | 12.82 | 1.53-107.38 | **0.019** | 0.17 | | | | 0.30 | | | |
| MYC H-score (continuous) | 0.026 | 1.03 | 0.99-1.05 | 0.06 | 0.008 | 1.03 | 1.01-1.05 | **0.011** | 0.49 | | | |

HR: Hazard ratio; CI: confidence interval; FIGO: International Federation of Gynecology and Obstetrics. Cox regression model with stepwise procedure was adopted to identify the prognostic factors. Statistically significant results in multivariate analysis are shown in bold.

60% and 83% ($p$=0.06) (Figure 4). However, there was no statistically significant trend of quantitative differences between co-overexpression and the other IHC biomarkers, including hypoxia or biomarkers of epithelial-mesenchymal transition (EMT). In addition, no association was found between co-overexpression and clinical parameters.

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## Discussion

An understanding of cancer phenotypes from genomic expression, IHC profiling, or imaging studies allows oncologists to use individualized therapy. Given that there are significant differential genomic expression changes between AC and SCC of the uterine cervix, the two tumor types behave differently (4, 8, 9), and some studies have indicated that patients with cervical AC experience inferior CRT-based treatment outcomes (4, 12-14), there is a need to explore the biological mechanisms or tumor microenvironment specifically for patients with AC. To date, no comprehensive IHC studies for this specific tumor type are available for clinical practice. Our work here was a pilot study to compare wide-ranging quantitative IHC biomarkers in predicting the outcomes of patients with locally advanced cervical AC receiving definitive CRT. The assessment of

various biomarkers revealed that MCL1 and c-MYC overexpression played roles in inferior CSS and PRFS, respectively. In addition, co-overexpression of the two markers was associated with lower CSS. Our findings revealed that certain biological characteristics of the tumors might supplement well-known clinical prognostic factors in predicting CRT-based treatment outcomes. Before initiating a novel therapeutic strategy for cervical AC, validation studies are required to confirm the findings.

In a recent review of the majority of studies on biomarkers for cervical SCC, the authors suggested that the most promising targets are apoptosis proteins, ΔNp73 and BCL2; and hypoxia-related proteins galectin-1 and HIF1α, which are associated with radioresistance or poorer prognosis (15). Unlike the results of published results on cervical SCC, none of the studied IHC biomarkers representing hypoxia, cell adhesion, or immunogenicity appeared to be prognostic for the study endpoints. We identified MCL1, an anti-apoptotic member of the BCL2 family of apoptosis-regulating proteins, as an independent factor for cancer death. MCL1 overexpression has been reported in some hematological cancer and solid tumors (25). MCL1 blocks the progression of apoptosis by binding the pro-apoptotic proteins BCL2 homologous antagonist killer (BAK) and BAX, which are capable of forming pores in the mitochondrial membrane, allowing the release of cytochrome c into the cytoplasm (26, 27). Although our data showed the MCL1 H-score did not correlate with the pretreatment Hb by Spearman's correlation test (coefficient=–0.25, $p$=0.12), an Hb level of less than 10 g/dl was associated with higher MCL1 expression. Alternatively using the median cut-off of MCL1, the pretreatment Hb value was also inversely correlated with MCL1 expression (11.2 g/dI in the low expression group *vs*. 9.3 g/dl in the high expression group, $p$=0.04). Tumor hypoxia may directly contribute to the radioresistance of cancer. Some articles suggested the impact of pretreatment Hb or blood transfusion on CRT outcome in patients with cervical cancer (23, 28). In anemic patients, tumor oxygenation is compromised due to a reduced oxygen transport capacity of the blood. Accordingly, a direct association between hypoxia and anemia appears likely (28). Given none of the other IHC parameters, including the hypoxia markers, was significantly related to MCL1 expression, it would be interesting to know whether overexpression of MCL1 was the chicken or the egg for tumor hypoxia, or the engine of proliferation. Because MCL1 is not a biomarker for tumor hypoxia, the interplay between MCL1 and other survival pathways merits further studies.

The *c-MYC* oncogene is overexpressed in the majority of human cancers and contributes to the cause of at least 40% of tumors (29). *c-MYC* drives the metabolic changes which are important to support the increased need for nucleic acids, proteins and lipids necessary for rapid cellular proliferation (30). However, *c-MYC* expression is tightly regulated, and its level of expression is influenced at the transcriptional level by a number of transcriptional regulatory motifs (31). Several studies have shown that overexpression of *c-MYC* contributed to cancer radioresistance (32-36). c-MYC is involved in the repair of DNA double-strand breaks through the regulation of non-homologous end joining and homologous recombination repair (32). In addition, c-MYC promotes radioresistance through transcriptional activation of *CHEK1* and *CHEK2* checkpoint kinases through direct binding to the *CHK1* and *CHK2* promoters in a stem cell-like population of nasopharyngeal cancer cells (33). Furthermore, a metabolism-associated or radioresistance-related pathway, such as HIF1α, has been reported to have dramatic effects on c-MYC function (37). As our study revealed that HIF1α and other IHC markers were not statistically associated with c-MYC H-scores, this implied that the mechanism for inferior tumor control by c-MYC overexpression is not directly linked to the Warburg effect (30). Therefore, c-MYC expression confers a molecular mechanism of radioresistance which might be complex and needs to be investigated further.

On the other hand, because MCL1 has a protective role in delaying apoptosis induced by *c-MYC* overexpression (38), and c-MYC and MCL1 enhance the stem cell-like potential of breast tumors *via* the hypoxia pathway (24), further clinical investigations are required to confirm the clinical impact of co-overexpression for other cancer types. To our knowledge, we are the first to show a cooperative role of c-MYC and MCL1 in predicting CSS for patients with cervical AC. Furthermore, co-overexpression had a marginal impact on DMFS. Although quantitative differences between co-overexpresssion and hypoxia or stemness pathways were not found in our data as previously described (24), this novel discovery warrants additional molecular investigations.

EMT plays an important role in tumor invasion, metastasis, and prognosis, including cervical cancer (9, 39). Two hallmark EMT proteins, E-cadherin and vimentin, are tightly controlled during EMT through multiple signal transduction pathways. In this study, we applied the continuous values of the H-score rather than grading of the immunoreactive score to quantify the staining intensity of E-cadherin or vimentin. Therefore, the role of EMT has been examined when stratifying the CRT outcome. Despite the results showing that the three EMT parameters were not associated with the outcome, the finding that a lower Hb level was associated with tumors having a higher mean vimentin to E-cadherin ratio might provide clinical insight. Some *in vivo* and *in vitro* studies of tumor cells of different origins indicated that hypoxia marker HIF1α may be directly involved in the down-regulation of E-cadherin through up-regulation of the EMT-inducing transcription factors *SNAIL* and *TWIST* (40, 41).

The findings of this study should be interpreted cautiously because they represent a retrospective study at a single institution. External validation studies using an independent data set and a large sample size are necessary to confirm these findings. Furthermore, the precise molecular pathway that MCL1 or c-MYC overexpression confers to poor CRT-based outcomes could not be clarified through association studies between IHC biomarkers and clinical parameters. Additional molecular studies are recommended to elucidate the underlying biological mechanisms, as well as the interplay with other survival pathways. Finally, the association between DNA sequencing or transcriptomes and the protein product should be investigated to outline a comprehensive biological mechanism of radioresistance or distant metastasis for these patients. For example, whether the co-amplification of *MCL1* and *c-MYC* genes causes the overexpression of the two IHC markers should be verified. Nevertheless, the strengths of this study include the uniform treatment strategies, and wide-ranging analyses of the IHC biomarkers. Our findings provide an indication that future studies can clarify the mechanisms related to failure of CRT. In addition, this study took the initial step to enable the tailoring of CRT to specific biological characteristics of patients with cervical AC. Future studies should include information on next-generation sequencing and enroll patients prospectively. Oncologists might then be able to then assess the feasibility of personalized therapy for high-risk patients, such as salvage surgery, dose escalation schemes, and a novel combination therapy.

## Conclusion

For patients with AC of the uterine cervix requiring definitive CRT, CSS can be stratified by the IHC biomarker MCL1, and the presence of PLN metastasis. The c-MYC H-score most accurately predicted the presence of local residual or recurrent tumors after CRT. In addition, co-overexpression of MCL1 and c-MYC led to more cancer deaths. External validation studies are required to verify our findings.

## Conflicts of Interest

All Authors declare no conflicts of interest in regard to this study.

## Authors' Contributions

YC Lin, RY Chen, and SW Chen were responsible for the study design. All Authors collected the data. YC Lin, RY Chen, and SW Chen performed statistical analyses, interpreted data, and drafted the article. All Authors provided some intellectual content. SW Chen and YC Lin approved the version to be submitted. All Authors read and approved the final article.

## Ethical Approval

This study was approved by a local Institutional Review Board [CMUH 107-REC3-008].

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