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Original Research

Immunohistochemical profiling of Bcl-2 and EGFR proteins in cervical carcinoma at a tertiary hospital in Ghana

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Abstract

Background: Using Immunohistochemistry (IHC) to assess the expression of EGFR and Bcl-2, and their correlations with clinicopathological features in a cohort of cervical cancer cases.
Methodology: A retrospective and descriptive study in Komfo Anokye Teaching Hospital (KATH) Kumasi, Ghana. Patients were women diagnosed with cervical cancer at KATH from January 2015 to December 2016. For inclusion, suitable archived formalin fixed paraffin-embedded (FFPE) cases with adequately preserved tissue blocks and available clinicopathological data were selected for Tissue Microarray (TMA), otherwise, the cases were excluded. One hundred and thirty-five out of 230 cervical cases met the inclusion criteria. IHC assesses EGFR and Bcl-2 expressions, correlating with clinicopathological features in cervical cancer cases, enhancing molecular-clinical insights in the Ghanaian context.
Results: The mean age of the cases was 58.9 (SD ± 17.88). Predominantly, 96.3% of the cases were of the squamous cell carcinoma (SCC) subtype. The majority of the cases (49.63%) were grade III. EGFR and Bcl-2 were expressed in 35.2% and 25.7% of the cases, respectively. Neither EGFR nor Bcl-2 showed any significant correlation with age, subtype, and histological grade. Significant inverse correlation was, however, observed between EGFR and Bcl-2 expression ($P<0.001$).
Conclusion: The age stratification shown in this study confirms earlier reports of late age at diagnosis, mostly observed in our setting, and therefore emphasizes the need for effective and population-wide screening programs to ensure early diagnosis. The significant inverse correlation between EGFR and Bcl-2, suggestively leans towards a possible role played by EGFR in downregulating Bcl-2.

Keywords: Cervical Cancer; EGFR; Bcl-2; Clinicopathological Characteristics; Tissue Microarray; Immunohistochemistry.

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Introduction

Cervical cancer is an important gynecological health challenge worldwide. It is the fourth most common malignancy globally, claiming over 250,000 deaths annually. Significant improvements in diagnosis, prevention, and treatment have been achieved particularly in high-income countries due in part to the implementation of population-wide screening programs, the use of Human papillomavirus (HPV) DNA testing, and the administration of HPV vaccines. [1,2]

In contrast, low- and middle-income countries (LMIC) bear a disproportionate burden, accounting for approximately between 40 - 87% of global cervical cancer cases and 30 - 85% of related deaths. [2-4] Sub-Saharan African countries are disproportionately affected with higher incidence and mortality rates of the disease than any other region of the world.[5] Here in Ghana, the disease is the second leading cause of cancer prevalence and mortality [6, 7], thus presenting a major health challenge among Ghanaian women. Higher incidence and mortality rates observed in LMICs are attributable to low access to screening programs, non-implementation of prevention modalities, late stage at diagnosis, and ineffective and inadequate treatment options. [1, 8, 9]

Several risk factors of cervical cancer have been documented with oncogenic HPV accounting for the majority of cases. [9-11] However, the processes of carcinogenesis are mostly associated with increased stimulation of cell division and proliferation, deregulation in growth inhibitors, disturbances in immune surveillance, and alterations in apoptosis. 12] Persistent infection with HPV can lead to malignant cellular modifications ranging from cervical intraepithelial neoplasia (CIN) 1, CIN2, and CIN3, with subsequent progression into invasive cervical cancer. [12] Consequently, there is continual increasing interest in further research into tumor biomarkers or signaling pathways that could improve prognosis and therapeutics. 13, 14 This is very crucial, particularly to LMICs who share the highest proportions of the global cervical cancer-related burden.

The anti-apoptotic B cell lymphoma protein (bcl-2), a 25-kDa protein encoded by the bcl-2 gene, has been documented to facilitate the protection of tumour cells and ensure cell survival. This apoptotic inhibitory ability is dependent on the expression of bcl-2 and its association with bax, another family member protein.[15]

Epidermal growth factor receptor (EGFR), a member of the erbB family of tyrosine kinase receptor proteins, is involved in cell division and proliferation as well as cancer development. EGFR initiates a signaling cascade via ligand-incited dimerization with subsequent activation of tyrosine kinase and myriad downstream effectors. EGFR overexpression has been observed in solid tumours such as breast, head-and-neck, ovarian, and non-small-cell lung cancer [16-20], and such cancers are characterized by aggressiveness, increased proliferation, and high metastasis. [20, 21] The significance of the EGFR signaling cascade in cancer development has made it a promising target for anti-cancer drug design and development. [19-21]

EGFR and Bcl-2 have been studied previously in cervical cancer; however, there are some conflicting results on the prognostic significance of these proteins. Moreover, the expression profiles and the roles played by these key proteins in cervical tumours among women from sub-Saharan Africa remain largely unknown. This study was therefore designed to assess, using immunohistochemistry, the expression profile of Bcl-2 and EGFR proteins in a cohort of cervical cancer patients in Ghana and their correlation with clinicopathological features.

Methodology

Ethical considerations

Ethical approval (CHRPE/AP/314/20) was obtained from the Committee on Human Research, Publications and Ethics, School of Medicine and Dentistry, Kwame Nkrumah University of Science and Technology (KNUST), and the Research and Development Unit, Komfo Anokye Teaching Hospital (KATH) (REG NO: RD/CR18/203) on "Molecular profiling of cervical cancer in Kumasi.

Study design and tissue samples

A retrospective and descriptive study was employed to assess the expression profile of Bcl-2 and EGFR on Formalin-fixed Paraffin-Embedded (FFPE) cervical cancer tissues at the pathology Department of the KATH, from January 1, 2015, to December 31, 2016. The study outlines the generation of cervical tumor microarray of 135 out of 230 cases that were diagnosed within the study period. Patients' data including age, histologic type, and histologic grade were sourced from the hospital records. All consecutive malignant cases seen within the study period were included while cases with missing patient records, missing or damaged tissue blocks, and ambiguous diagnoses were excluded. Haematoxylin and Eosin (H&E) stained slides were prepared from the FFPE tissue blocks and reviewed by an independent pathologist using the Leica DM200 LED microscope (Leica MICROSYSTEMS). This review was conducted in accordance with the ESMO Clinical Practice Guidelines for the diagnosis, treatment, and follow-up of cervical cancer to confirm diagnosis of the disease and Royal College of Pathologists, Britain.[22] Representative tumor sites were mapped for TMA preparation, while clinicopathological information was assessed and noted.

Tissue microarray (TMA) preparation

TMA's were constructed using an automated TMA machine (TMA Master by 3DHISTECH-2016) and a Leica EG1150H Paraffin embedding station. Using a TMA map as a guide, two cylindrical cores (1mm each) were punched out of the marked areas of the donor's block and inserted into pre-drilled holes in the recipient block. The blocks were thereafter placed on a mould warmer section of the Leica EG1150H Paraffin embedding station for a few minutes to ensure the tissue cores were sunk uniformly into the recipient block. Before Immunohistochemical analysis was performed, the slices were incubated overnight at 37 degrees Celsius.

Immunohistochemistry (IHC)

Immunohistochemical staining was carried out according to standard procedures, as previously outlined.[23] Thin sections (3μm) were made from each TMA block and spread onto Super Frosted Plus slides. The slides were deparaffinized in xylene and rehydrated in a decreasing grade of alcohol (absolute, 90, 70), diluted with tris buffered saline (TBS). The slides were then washed in distilled water and incubated in a pressure cooker for antigen retrieval. Background staining and non-specific antibody binding were prevented using hydrogen peroxide and casein solutions, respectively. Immunohistochemical dilutions for Bcl-2 and EGFR were performed according to the manufacturer guidelines as detailed in Table 1. Optimal tissue sections were incubated in the diluted primary antibodies. The sections were then immersed in a secondary antibody conjugated with peroxidase and anti-peroxidase before being developed in diaminobenzidine (DAB) tetrahydrochloride. They were then counterstained in haematoxylin, dehydrated in various percentages of alcohol (70, 95, and absolute), and mounted with DPX mountant.

Table 1 **Antibodies employed for the study**

Antibody	Clone	Dilution	Control	Company
EGFR CST	D38B1	50 CST Diluent	Lung	CST
BCL2 (Human)	124	2500	Tonsil	Dako

Scoring of IHC

For the assessment of the biomarker count, five (5) randomly selected high-power fields were examined on uniformly stained slides. TMA sections were evaluated to determine the presence of positive staining. The tumors were scored by employing slight modifications to the previously described method. [24] 0 represented < 10% positive cells, 1 represented 10-40%, 2 represented 41-70%, and 3 represented ≥ 70%. Positivity of a cell is characterized by a color intensity equivalent to or considerably greater than the control.

Data analysis

Data analyses were performed using Statistical Package for Social Sciences for Windows version 26 (SPSS, Chicago, IL, USA). The relationships between the immunohistochemistry profiles and clinicopathological parameters were assessed using the Chi-squared test of associations. All statistical tests were two-sided and considered significant at $p<0.05$ with a confidence interval set at 95%.

Results

A total of 135 cervical cases met the inclusion criteria and were representative of this study. Table 2 details the descriptive statistics of the cases' demographics and histological characteristics. The ages of the cases ranged from 31-115 years with a mean age of 58.9 years (SD ± 17.88). The modal age was seen in the 50-59 - year group. Squamous cell carcinoma (SCC) was the predominant histological type (96.3%). The non-keratinizing variant of SCC had the highest frequency (53.1%). The majority of the cases were high grades, with grades II and III accounting for 33.3% and 49.6% respectively.

Table 2: Summary of Descriptive Statistics of the Cervical Cancer Cases

Clinicopathological parameters	Number of cases	Percentage (%)
Age groups (years)		
30-39	27	20
40-49	17	12.6
50-59	36	26.7
60-69	19	14.1
70 and older	36	26.7
Total	135	100
Histological type		
SCC	130	96.3
Adenocarcinoma (ADC)	5	3.7
Total	135	100
SCC variant		
Basaloid squamous cell carcinoma (BSCC)	6	4.6
Keratinizing	55	42.3
Non-keratinizing (NK)	69	53.1
Missing	5	-
Total	135	100
Tumour grade		
Grade I	23	17.0
Grade II	45	33.3
Grade III	67	49.6
Total	135	100

*All percentages were calculated on the number of valid cases

Immunohistochemical staining of EGFR and Bcl-2

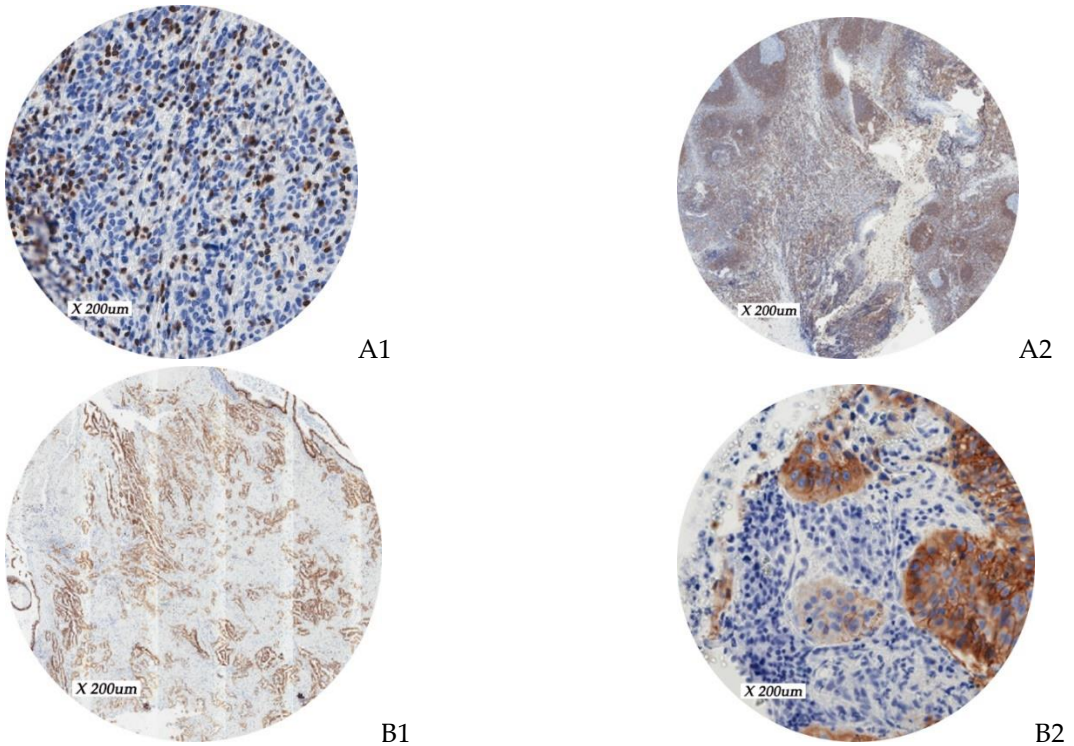
Bcl-2 protein was positively stained in 35.2% of the cases. EGFR expression was observed in 25.7% of the cases as detailed in Table 3. Figure 1 illustrates the photomicrographs of tissue cores of EGFR and Bcl2 depicting their IHC stains.

Table 4 outlines the distribution of EGFR and Bcl-2 expression among the clinicopathological features. Percentages of positive and negative expressions are presented along with p-values. The display of positive and negative expressions for both proteins exploring their interactions is also presented (Table 5).

Table 3 Frequency of EGFR and Bcl-2 Immunohistochemical Staining in Cervical Cancer Cases

Biomarker	Number of cases	Percentage (%)
EGFR		
Positive	43	35.2
Negative	79	64.8
Missing	13	-
Total	135	100.
Bcl-2		
Positive	29	25.7
Negative	84	74.3
Missing	22	-
Total	135	100.0

**Missing cases were unsuitable for immunohistochemistry*



A1 A2

B1 B2

Figure 1. Immunohistochemical Stained Photomicrographs: (A1) tonsil-control sample showing positive Bcl-2 antibody stain (A2) Representative cervical sample demonstrating positive Bcl-2 antibody staining. (B1) lung-control sample showing positive EGFR antibody stain (B2) Representative cervical sample depicting positive EGFR antibody staining. Positive stain is characterized by deep golden brown coloration.

Table 4 Expression profile of EGFR and Bcl2 with clinicopathological features

		EGFR			Bcl-2		
		Positive (%)	**Negative (%)**	**p-value**	**Positive (%)**	**Negative (%)**	**p-value**
Clinicopathological features							
Age groups	30-39	9 (20.9)	18 (22.8)		3 (10.3)	23 (27.4)	
	40-49	0 (0)	10 (12.7)		2 (6.9)	7 (8.3)	
	50-59	14 (32.6)	19 (24.1)	0.15	14 (48.3)	19 (22.6)	0.07
	60-69	7 (16.3)	9 (11.4)		2 (6.9)	11 (13.1)	
	≥70	13 (30.2)	23 (29.1)		8 (27.6)	24 (28.6)	
Histological types	SCC	40 (32.8)	71 (58.2)		26 (23)	76 (67.3)	
	ADC	2 (1.6)	3 (2.5)	0.78	3 (2.7)	2 (1.8)	0.079
Histological Grade	Grade 1	5 (4.1)	12 (9.8)		7 (6.2)	10 (8.8)	
	Grade 2	18 (14.8)	24 (19.7)	0.437	11 (9.7)	26 (23)	0.136
	Grade 3	20 (16.4)	43 (35.2)		11 (9.7)	48 (42.5)	

Table 5 Association and Interaction between Bcl-2 and EGFR in Cervical Cancer

		EGFR			
		Positive (%)	Negative (%)	Chi-squared	p-value
Bcl-2	Positive (%)	21 (18.6)	8 (7.1)	22.029	0.000
	Negative (%)	20 (17.7)	64 (56.6)		

Discussion

In this study, 135 cases with a complete data set and FFPE tissue blocks out of the 230 cases that were seen over the study period were analyzed. The highest number of cases occurred in the sixth decade (50–59-year group). This mirrors the findings in Ghana. [25,,26] A meta-analysis observed a bimodal age distribution in cervical cancer in some regions with the first peak in those studies occurring at younger ages, just after sexual debut, while the other occurred 45 years and above. [11] The age stratification observed in our setting can be attributed to the natural aging of the population and the absence or insufficiency of screening programs among females within these specific age brackets. [27, 28]

The mean age of 58.9 years in our study closely aligns with the findings of a previous study in the same institution. Consistent with several earlier studies in Ghana, this figure has been documented [26], and in other LMICs. [27-32] Our mean age at diagnosis was, however, way above that seen among the high-income countries.[33-36] Lower mean age observed among high-income countries can be attributed to the availability and access to cervical cancer screening programs that facilitate early detection and diagnosis. Squamous cell carcinoma and Adenocarcinoma are known to have a distinctive clinicopathological characteristics and a conflicting prognostic significance. [37, 38] However, both earlier and recent literature have demonstrated the prognostic significance of these histological variants with most studies reporting squamous cell carcinoma to predict better prognosis compared to adenocarcinoma.[39-42]

Although, Squamous cell carcinoma accounted for majority (96.3%) of the cervical cancer cases in our setting and may predict comparatively better prognosis, it does not translate into providing a better overall survival outcome in our setting owing in part to the high proportions of cases that are presented at a later stage, with high histological grade. Unsurprisingly, histological grade III accounted for high proportions of our cases (49.6%) which was followed by grade II (33.2%). This is consistent with findings from previous works conducted in Ghana and Nigeria, where majority of the tumors are of high histological grades. [29, 31, 43]

EGFR protein was expressed in 35.2% of our cervical cases. Prior Immunohistochemical studies of cervical carcinomas have shown a wide range of EGFR expression levels (6-90%). [16,18,44,45] EGFR is overexpressed in many carcinomas including cervical cancer due to its crucial downstream pathways in carcinogenesis, in regulating apoptosis, cell migration, cell growth and angiogenesis.

Our study did not find any significant association between EGFR and the clinicopathological features. Previous studies have shown conflicting results on the prognostic significance of EGFR with some works reporting no association between EGFR and prognostic parameters [21,46-48], while others have shown otherwise. [49-51] These differences may be as a result of variations in methodological protocols including the source and dilutions of the antibodies employed to assess positive Immunohistochemical reactions. [45] As a membrane protein, Bcl-2 expression in cervical cancer is known to inhibit apoptosis and extend the cell's life cycle by preventing Ca^{2+} release in the cell, shutting down cell nuclear transportation, and acting as an antioxidant. [52, 53] Prior studies have reported between 25-77% Bcl-2 expressions in cervical cancers. [54-56] In this study, Bcl-2 was expressed in 25.7% of the cervical cancer cases which is consistent with previous work [56], but way below that observed by others. [57, 58] This disparity could arise as a result of differences in the study populations, methodologies, and the specific characteristics of the cervical cancer cases under investigation in these separate research endeavors. [45]

The expression of Bcl-2 did not show any significant association with age, tumor grade, and histologic subtype, which is in keeping with similar studies [15, 55, 57] however, [59, 60] reported otherwise. A number of studies have documented positive correlation between Bcl-2 expression and a 5-year survival rate in cervical cancer patients, which may be suggestive of Bcl-2 role in controlling apoptosis but failing to control cell proliferation. [61] However, our study could not make inferences based on patients' survival due to unavailability of follow-up data at our center.

We assessed the correlation between Bcl-2 and EGFR and found a significant negative association between the two ($p<0001$). This finding raises the possibility of Bcl-2 downregulation by EGFR, as previously documented. [62, 63] This negative association could imply a regulatory mechanism where the overexpression of EGFR might influence the expression levels of Bcl-2, potentially contributing to altered cellular processes in cervical cancer. Further studies are needed to elucidate the mechanisms and clinical implications of this observed correlation between Bcl-2 and EGFR.

Conclusion

To summarize, no significant association was found between Bcl-2 and EGFR expression with age, histological subtypes, and histological grade, indicating that these markers may not serve as strong prognostic indicators in our context. However, the observed significant inverse correlation between Bcl-2 and EGFR may be suggestive of downregulation of Bcl-2 by EGFR. A larger case series with survival data is required to assess the association of these proteins with clinicopathological features in our setting.

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