Original Research Article

Overexpression of Epidermal Growth Factor Receptor (EGFR) in Esophageal Squamous Cell Carcinoma and its Correlation with Clinicopathological Characteristics in Central Uganda.

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ABSTRACT

Aims: The aims of the current study were to determine the prevalence of epidermal growth factor (EGFR) receptor in patients diagnosed with esophageal squamous cell carcinoma (ESCC) as well as assessing the correlation of overexpression of EGFR with age, gender and tumor grades of the cases.

Study design: This was a cross-sectional analytical study

Place and duration of study: The study was conducted in the pathology laboratory in the department of pathology, Makerere College of Health Sciences, Kampala-Uganda for five months.

Methodology: A sample of 127 archival tissue blocks from patients with ESCC diagnosed between 2010 and 2012 were retrieved from the tissue repository and used to assess overexpression of EGFR using monoclonal mouse Anti-human wild type EGFR antibody. For association between age and overexpression of EGFR, Kruskal- Wallis H test was used and for tumor grade and sex and EGFR, Chi-Square test was performed using SPSS version 16.0. $P < .05$ was considered statistically significant.

Results: The age range of the patients with ESCC in this study was 35-99 years with mean of 59.55 years. The peak age of the cases was 55-64 years. Males and females were 68.5% and 31.5% respectively**.** Moderately differentiated tumors dominated by comprising 59.9%. The prevalence of overexpression of EGFR was 61.4%. The highest overexpression of EGFR was seen in cases with grade 2 compared to grade 1 and 3 but not statistically significant ($P = .255$). Overexpression of EGFR was relatively higher in cases with age ≥ 50 years, but the difference was not statistically significant ($P = .931$). Males expressed relatively higher EGFR than females, however, the difference was not statistically significant ($P = .944$).

Conclusions: Majority of patients with ESCC in Uganda have moderately differentiated tumor and a significant number of them tend to show overexpression of EGFR antigen.

Keywords: Overexpression, Epidermal Growth Factor Receptor, Esophageal squamous cell carcinoma,

Abbreviations: ESCC- Esophageal squamous cell carcinoma, EC- Esophageal carcinoma, FFPE- Formalin fixed paraffin embedded, IHC-Immunohistochemistry, MakCHS- Makerere College of Health Sciences

Introduction

Esophageal squamous cell carcinoma (ESCC) is the malignant tumor in the squamous epithelium that lines the normal esophagus (1) . Esophageal carcinoma (EC) is the eighth most common cancer and the sixth leading cause of cancer related deaths worldwide (2). Of all the cases of EC, 80% mortality occurs within developing countries (3). Wabinga et al (4) studied the trend of ECs in Uganda and it was found that the reason for the rise in the incidence were similar to known associated factors such as smoking, alcoholism, family history, gender and many others. This was similar to what Watanabe et al (5) reported in Japan among the patients with EC and in particular those who had ESCC. The reasons for the rise in the incidence are not very clear apart from the known associated factors such as smoking, alcoholism, family history, gender and many others. Torre and the associates (6) reported that the mean worldwide age-standardized incidence rates (ASRs) for esophageal cancer (EC) were 9.0 and 3.1 per 100,000 in males and females respectively. The ASR for EC in Uganda was reported to be 36.7 and 24.8 per 100, 000 in males and females respectively between 2004- 2008 as reported in by Cheng et al (7).

ESCC develops through accumulation of somatic mutations and epigenetic changes in oncogenes, tumor suppressor genes and cell adhesion molecules (8). Squamous dysplasia and carcinoma in situ of esophagus has been recognized with increased frequency in regions with high incidence of invasive EC (9). The human EGFR is a member of the integral membrane proteins of tyrosine kinase which plays a key role in epithelial cellular growth, proliferation and differentiation (10). EGFR gene protein has been found to be mutated and/or amplified in most malignancies including EC especially ESCC (11). Overexpression of epidermal growth factor receptor (EGFR) has been found to occur in ESCC in 40 – 80% in several studies worldwide

(10). Studies have also shown that increased EGFR overexpression in ESCC has been associated with decreased patient survival, however; this finding has not been consistent in all studies (12). Further studies have also shown an association of EGFR with grade of tumor, lymph node status and poor patient prognosis (13, 14).

The aims of this study were to determine the prevalence of EGFR overexpression in patients with ESCC and also to correlate overexpression of EGFR protein with clinicopathological characteristics including age, gender and tumor grade.

Material and Methods

Patients and tissue samples. In this analytical cross sectional analytical laboratory based study, a total of 127 tissue samples from patients with Esophageal squamous cell carcinoma (ESCC) were obtained from pathology department at Makerere College of Health Sciences (MakCHS) in the central part of Uganda. The tissue samples were of ESCC patients diagnosed histologically from January 2010 to December 2012 and archived in the department. All the tissue blocks were retrieved and selection of the cases was done conveniently The tissue samples were retrieved by a technician who was assigned. Each available case which met the inclusion criteria was included in the study.

The present study was approved by the Ethics Review Committee of the school of biomedical science.

Haematoxylin and Eosin staining. The selected FFPE tissue blocks were first cooled at 4^{0}C temperature on the embedding station for 30 minutes and then sectioned serially at 4.0 microns thickness using a microtome. Serial sections were floated on warm water at 55 ^{O}C in the water bath. For Haematoxylin and Eosin (H & E) staining, the tissue sections were placed on the frosted end of the slide and placed in the oven at 60 ^{0}C for 30 minutes before being stained. Then

they were stained with H & E stains using the protocol which was introduced by Fischer and the associates (15).

Immunohistochemistry (IHC) staining. The 4.0 microns cut sections were placed on the charged slides. The sections were first de-waxed in xylene solution and rehydrated in decreasing concentration of ethanol, subjected to antigen retrieval in 10 mM citrate buffer using microwave irradiation and treated with 3% hydrogen peroxide for blocking endogenous peroxidase. The sections were later incubated with a ready to use primary rabbit monoclonal EGFR antibody at 4˚C overnight. The next day, the slides were stained with a visualizing reagent, 3,3'-diaminobenzidine as a chromogen. In every step, phosphate buffer solution (PBS) was used. Placenta tissue was used as positive control while ESCC tissue devoid of EGFR antibody was used as negative control. The sections were then counterstained with hematoxylin and submitted for examination. The IHC stained slides were then evaluated by expert pathologists. EGFR immunoreactivity was considered positive when tumor cells stained golden brown with varying color intensities. Positive results were reported with regard to intensity of staining and percentage of tumor cells that expressed the receptor. Intensity of staining was scored as 0 (negative), 1+ (weak positive), 2+ (equivocal positive) and 3+ (strong positive). Overexpression of EGFR was considered for score 2+ and 3+ and for those with score 0 and 1+ staining was regarded as negative for EGFR immunoreactivity. Scoring for positivity was considered in over 10% tumor cells (11, 16) .

Statistical analysis. Chi-square test was used to assess the association of tumor grade and gender with overexpression of EGFR whereas Kruskal-Wallis H test was used to assess the association between patients' age and overexpression of EGFR using SPSS 16. 0 version (SPSS, IBM Stat

Inc, USA). $P < 0.05$ in a two-tailed test was considered to indicate statistically significant differences.

Results

A total of 127 esophageal squamous cell carcinoma (ESCC) specimens were studied. The mean age at presentation was 59.65 years (Range: 35 - 99 years, SD = 11.80). Males and females were (68.5%, 87/127) and (31.5%, 40/127) respectively with ratio of 2. 2: 1. Figure 1 shows the age groups of the subjects in this study. Majority of the cases with ESCC (29.1%, 37/127) were in the age group of 55 – 64 years followed by (25.1%, 32/127) in the age group of 65 – 74 years. The rest of the age groups accounted for less than 10% cases in each group.

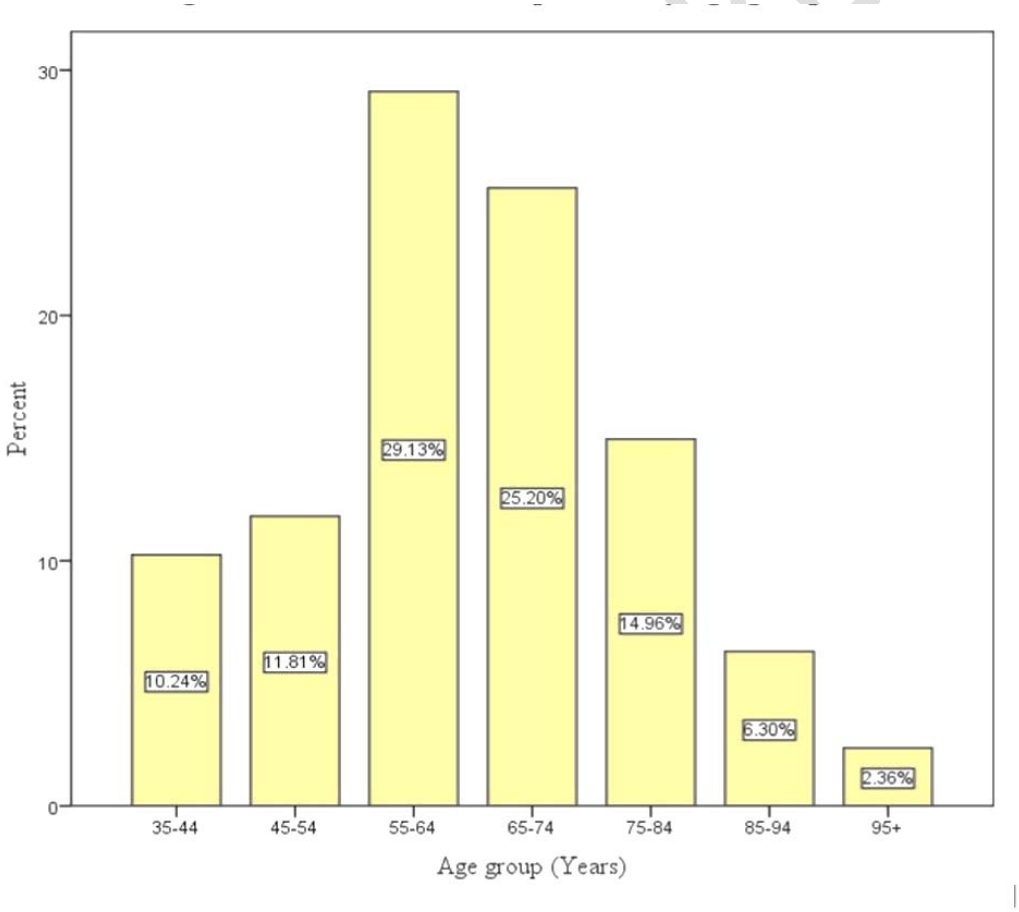

Figure 1: Distribution of the cases with esophageal squamous cell carcinoma by age group.

Figure 2 indicates the distribution of the cases with ESCC by tumor grades. Most of the cases, (59.9%, 76/127) had moderately differentiated ESCC (grade 2) followed by (29.1%, 37/127) cases which had well differentiated ESCC (grade 1) and the remaining (11.0%, 14/127) of ESCC were poorly differentiated (grade 3).

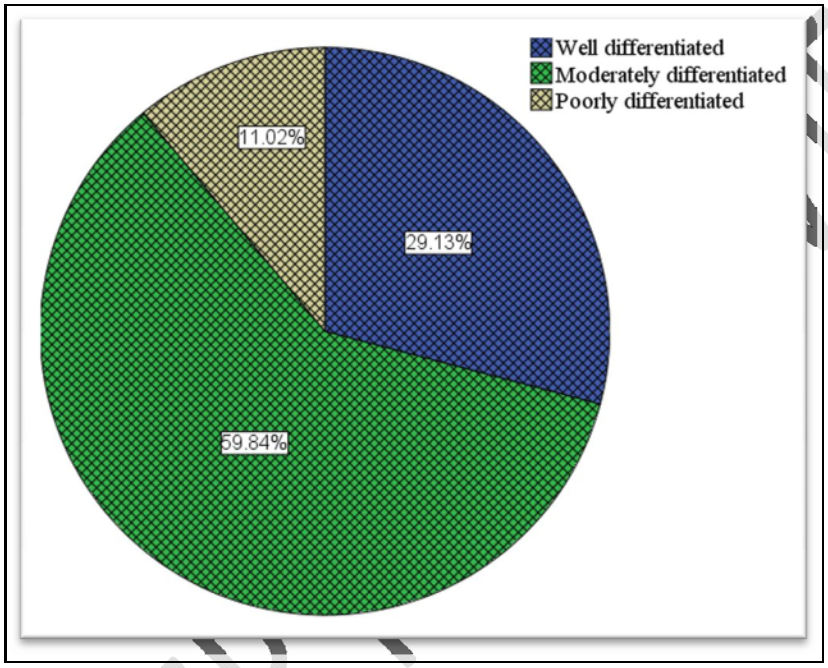

Figure 2: Frequency distribution of the cases of esophageal squamous cell carcinoma by tumor grading

Table 1 represents the ESCC tumor grades among the cases by gender. It was found that, males had more high grade ESCC (14.5%, 11/87) compared to (7.5%, 3/40) among females.

Table 1: Grades of esophageal squamous cell carcinoma according to gender

Tumor grade	Gender			
	Male		Female	
	N	%	N	%
Grade 1	27	21.3	10	7.9
Grade 2	49	38.6	27	21.3
Grade 3	11	8.9	3	2.4
Total	87	68.6	40	31.4

Table 2 below shows expression of EGFR in esophageal squamous cell carcinoma in the cases. The prevalence of EGFR expression in this study was 61.4% and was not associated with age of the patients (P = .931), gender (P = .944) and tumor grade (P = .255). Positive immunoreactivity was found in (61.4%, 78/127) of all the cases and the remaining, (38.6%, 49/127) were negative for EGFR immunoreactivity.

Table 2: EGFR expression among the cases with esophageal squamous cell carcinoma in the study.

EGFR immunoreactivity score	Frequency (N)	Percentage (%)
3+	48	40.2
2+	30	26.0
1+	26	18.1
0	23	15.7
Total	127	100.0

Strong staining of the cases was well seen in well differentiated tumors. The staining pattern was focal (Figure 3), in the sense that some tumor cells were not stained by the antibody. In other

cases of well differentiated tumors, the staining of the tumor cells was well defined to the tumor cells and it was diffuse in nature (Figure 4). Only stromal and reactive inflammatory cells were left unstained. Weak staining was found more in moderately differentiated (Figure 5) and poorly tumors. Staining was more of background than being defined to the tumor cells as it was for well differentiated cases.

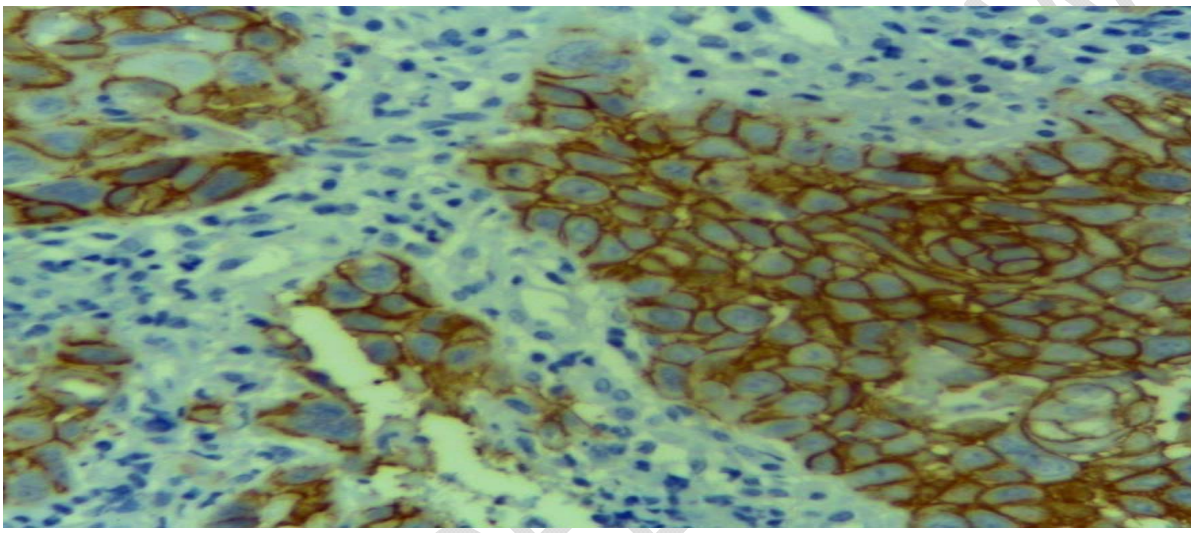

Figure 3: IHC staining showing focal strong staining (3+) of the tumor cells (x400 Original magnification)

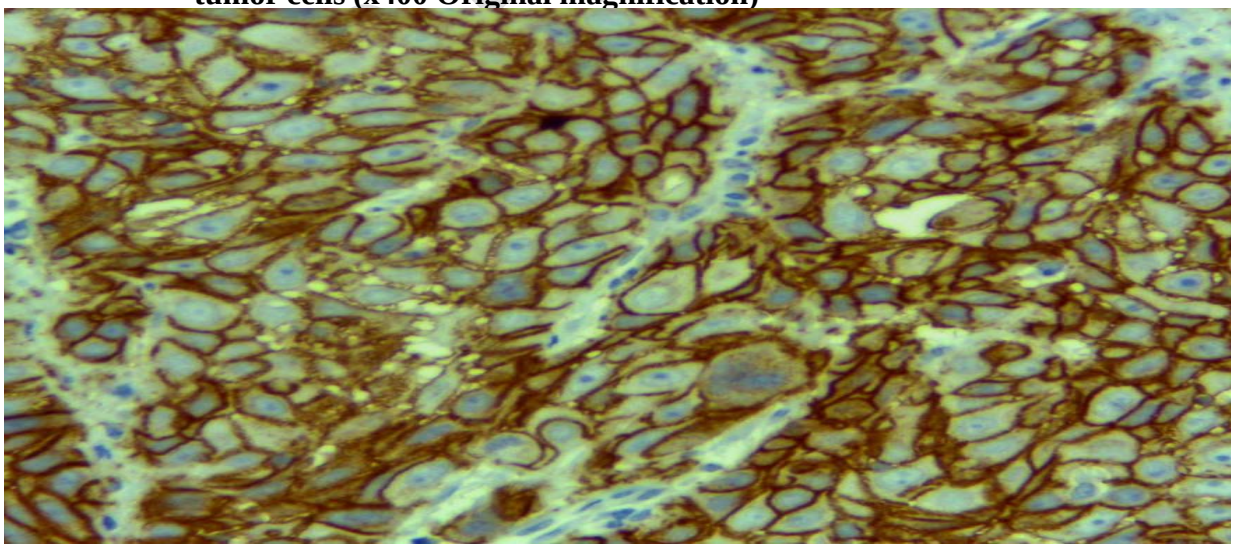

Figure 4: **IHC staining showing focal strong staining (3+) of the tumor cells (x400 Original magnification)**

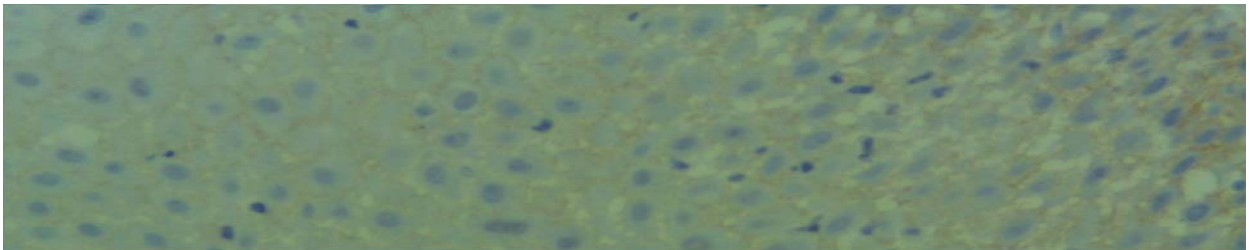

Figure 5: **IHC staining showing weak staining (1+) of the tumor cells (x200 Original magnification)**

Table 3 below shows association with EGFR expression and gender, age group and tumor grade. When the expression of EGFR was compared between males and females, we found that 42.5% of ESCC among males were expressing EGFR compared to 18.9%. The difference was not statistically significant (Chi-square = 0.115, *P* = .944). The expression of EGFR antigen for patients with age 50+ years was higher than those with age below 50 years (48.0% vs 13.8%) but the difference was not statistically significant (Kruskal-Wallis H test = 0.0075, *P* = 931). Moderately differentiated ESCC tumors showed higher expression of EGFR compared to those which were well differentiated and poorly differentiated (35.4% vs 20.5% and 5.5%) but the difference was not statistically significant (Chi-square = 5.327, *P* = .255).

Table 3: Association between EGFR expression and gender, age group and tumor grade of the cases with esophageal squamous cell carcinoma.

		EGFR immunoreactivity				
		Positive		Negative		
Variable		N	%	N	%	P-value
Gender	Male	54	42.5	33	26.0	.944
	Female	24	18.9	16	12.6	
Age group (years)	35-49	17	13.4	11	8.7	.931
	50 -99	61	48.0	38	29.9	
Tumor grade	Grade 1	26	20.5	11	8.7	.255
	Grade 2	45	35.4	31	24.4	
	Grade 3	7	5.5	7	5.5	

Discussion

The current study determined the prevalence of overexpression of EGFR by means of IHC and at the same time the study correlated the level of expression of the gene protein with age, gender and tumor grades; which are the clinicopathological characteristics of the subjects. The mean age of 59.65 years of the cases at presentation in this study was slightly higher than the 55.5 years mean age that was reported by Alema et al (17) among patients with ESCC from Lacor hospital in the northern part of Uganda. The mean ages of the patients with EC in the reports of Ocama and the colleagues from the central part of Uganda (18) and Anvari et al (11) in Iran were almost in keeping with the mean age of the patients in this study. Lin et al (19) in China reported a similar mean age of patients with EC. In all these studies, ESCC was the predominating histopathological type. This implies that EC develops at advanced age. Even when the peak age at presentation of the cases in this study was compared with other studies from other places globally, it was found that there was no significant variation.

For example, a number of studies (18-21) reported a peak age of 53-61 years, almost similar to the peak age of 50-59 years of the cases in this study. The observation that ESCC is more common among males compared to females was in agreement with findings from other studies (17, 18). However, one study reported that the incidence of ESCC in areas where it is prevalent, both males and females are affected equally (19). Parkin et al (20) and Siewert et al (21) at different times they reported that, ESCC is more prevalent among males compared to females and also males tend to have poor prognosis. Likewise, Eroglu et al (22) also reported that the incidence of ESCC between males and females was equal. There is no clearly known reason why EC is more common among males compared to females. However, the difference in predilection of ESCC among males as compared to females is not statistically significant (P = .504) as it was once reported by Kachala et al (23). The tendency of males to indulge in use of most of the stipulated risk factors conveys a message that, it could be the reason why more males are affected compared to females. Meves et al (24) reported that, the risk of alcohol consumption for developing ESCC increases in a linear fashion and smoking increases the risk of developing ESCC by nine-fold as compared to non-smokers (hazard ratio 9.3; 95% CI: 4.0–21.3). Arnal et al (25) reported that most of the risk factors for ESCC which were assessed in their study such as alcoholism and smoking were found more in males compared to females. Wang and associates (26) reported that males carry a high proportion of the risk factors for ESCC compared to females and this is evidenced by the increase of incidence of ESCC in areas where such risk factors have been found to increase among females.

ESCC is a cancer of individuals aged 50+ years regardless of the race. When 50 years was taken as the cut-off for low age for the cases in this study, the finding was that, most of the patients were aged 50+ years compared to the ones who had less than 50 years, although the difference

was not statistically significant (P = .931). This finding is consistent with the previous study which was conducted in Uganda which also showed that the highest age at diagnosis was 50+ years (18).

The degree of ESCC differentiation in this study was similar to what was found by Chen et al (27) in which moderately differentiated cases were the majority (55.6%), followed by well differentiated (32.3%) and the least were poorly differentiated tumors (12.1%). In another study which was done in Japan by Tustumi et al (28) among patients with ESCC, it was found that moderately differentiated cases were the dominating cases constituting 73.7% similar to what we found for the cases in the present study. Despite the fact that in most of studies found in the literature indicate that majority of the patients are either in grade 1 or grade 2; the overall survival of the patients is generally poor due to the fact that tumor differentiation does not reflect the tumor stage and course of the tumor in terms of advancing.

Overexpression or amplification of EGFR has been associated with aggressive biological behaviors of ESCC such as tumor stage, tumor differentiation, lymph node involvement and involvement of surgical margins. For example, in the study of Lin et al (19), it was found that advanced tumor stage and poorly differentiated ESCC were associated with overexpression of EGFR. Li and the colleagues, also reported that positive EGFR overexpression was significantly associated with lymph node metastasis and distant metastasis ($P < .05$) (29). This was similar to what was found in the study of Guo et al (P = .019) (30) in which advanced pathologic stage and lymph node metastasis were associated with high overexpression of EGRF. It has also been reported in the literature that patients who express EGFR have better prognosis than that don't express (11, 19, 28). The prevalence of 61.4% for EGFR overexpression in this study was close to the 64.7% that was reported by Abedi-Ardekani and the associates (16) but lower than 70.0%

which was found by Anvir et al (11). A lower prevalence of 53.6% for EGFR overexpression was reported by Lin et al (19). In all these studies, the scoring method for EGFR overexpression was similar to the method that was used in the current study. The difference in prevalence might have been due to inherent technical staining errors of IHC in their study and also sample size differences. In the study by Wang et al (31) in which the scoring system was different from the one used in this study; positive immunoreactive was regarded for score 3+ only and all cases with score 0, 1+ and 2+ were considered negative.

When degree of differentiation was compared for the cases studied, it was found that, the highest overexpression of EGFR protein (35.4%) was found in cases with moderately differentiated ESCC followed by well differentiated (20.5%) but the difference was not statistically significant (P = .255). The lack of statistical significance in ESCC tumor grades for overexpression of EGFR in this series was in keeping with the communications of Wang et al (31), however, in their study more overexpression of EGFR, (26.0%) was found in poorly differentiated cases. The difference could have been due to the difference in the scoring method between the two series. In the communication of Gao et al (32) and that of Lin and the colleagues (19) both reported that there was no correlation between ESCC tumor differentiation and overexpression of EGFR with P-value of (.882 and .853) respectively. The importance of grade of tumor in EC and ESCC particularly, is controversial. Some studies have shown a more favorable prognosis for well to moderately differentiated tumors than poorly differentiated tumors while other studies highlight the opposite (33). Although gender carries a prognostic role in patients with ESCC, where males have a poor prognosis compared to females, the difference between overexpression of EGFR between males and females in this study was not statistically significant (P = .944) similar to

what was reported by different studies (13, 26, 29) in which the *P*-values were (.120, .850, .410) respectively.

The whole concept of determining the frequency of overexpression of EGFR and then correlating with the clinicopathological characteristics of patients with ESCC is to investigate the indicators for eligibility criteria for the emerging clinical therapies targeting EGFR (34). Studies done on the prognostic role of overexpression of EGFR in patients with ESCC have found that, ESCC tumor with high expression of EGFR protein have poor prognosis compared to those with low expression 19(3535). Therefore, characterization of patients with ESCC for clinical therapies targeting EGFR, would help to improve the prognosis of patients with ESCC.

Conclusions

The age range of the patients with ESCC in this study was 35-99 years. The overexpression of EGFR in patients with ESCC in Uganda was relatively high and majority of the cases showing high overexpression of the EGFR, were of moderately differentiated tumor grade. The findings of this study indicate that majority of the patients with ESCC can benefit from targeted therapy.

Consent

Not applicable

Ethical approval

The permission to conduct this research was sought from the institution review board of the school of biomedical medicine and it was given reference number SBS 062 together with a waiver for using the tissue blocks.

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