

Original Research Article

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

ABSTRACT

Aims: the aims of the current study were to determine the prevalence of epidermal growth factor (EGFR) receptor in patients diagnosed with oesophageal squamous cell carcinoma as well as assessing the correlation of overexpression of EGFR with age, gender and tumour 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 between January 2013 and May 2013.

Methodology: A sample of 127 archival tissue blocks from patients with OSCC 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 (Dako-Denmark) antibody. For association between age and overexpression of EGFR, Kruskal- Wallis H test was used and for tumour grade and sex and EGFR, Chi-Square test was performed using SPSS version 16.0. P value less than .05 was considered statistically significant.

Results: The prevalence of overexpression of EGFR in this study was 61.4%. Moderately differentiated tumours dominated by comprising 59.9%. The highest overexpression of EGFR was seen in cases with grade 2 compared to grade 1 and 3 but the difference was 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 OSCC in Uganda have moderately differentiated tumour and a significant number of them tend to show overexpression of EGFR antigen.

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

Abbreviations: OSCC- Oesophageal squamous cell carcinoma, OC- Oesophageal carcinoma, FFPE- Formalin fixed paraffin embedded, IHC-Immunohistochemistry, MakCHS- Makerere College of Health Sciences

Introduction

Oesophageal squamous cell carcinoma (OSCC) is the malignant tumour in the squamous epithelium that lines the normal oesophagus [1]. Oesophageal carcinoma (OC) is the eighth most common cancer and the sixth leading cause of cancer related deaths worldwide [2]. Of all the cases of OC, 80% mortality occurs within developing countries [3]. Wabinga et al [4] in 2000 reported that, the trend of OCs in Uganda. 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 in 2013 reported that the mean worldwide age-

standardized incidence rates (ASRs) for oesophageal cancer (OC) were 9.0 in males and 3.1 per 100,000 in females [5]. The ASR for OC in Uganda was reported to be 36.7 for males and 24.8 for females per 100, 000 between 2004- 2008 as reported in 2015 by Cheng et al [6].

OSCC develops through accumulation of somatic mutations and epigenetic changes in oncogenes, tumour suppressor genes and cell adhesion molecules [7]. Squamous dysplasia and carcinoma in situ of Oesophagus has been recognized with increased frequency in regions with high incidence of invasive OC [8]. 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 [9]. EGFR gene protein has been found to be mutated and/or amplified in most malignancies including OC especially OSCC [10]. Overexpression of epidermal growth factor receptor (EGFR) has been found to occur in OSCC in 40 – 80% in several studies worldwide [11]. Studies have also shown that increased EGFR overexpression in OSCC 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 tumour, lymph node status and poor patient prognosis [13].

The purpose of this study was to determine the prevalence of EGFR overexpression among OSCC patients and also correlate with clinicopathological characteristics in Uganda.

Material and Methods

This was a cross sectional analytical laboratory based study involving 127 tissue blocks of cases diagnosed with OSCC from 2010 to 2012. The study was carried out in the pathology laboratory at the department of Pathology of the College of Health Sciences, Makerere University. We, two authors and one technician, retrieved all the tissue blocks. Selection of the cases was done

conveniently. Every available case, meeting the inclusion criteria was included in the study. The selected FFPE tissue blocks were serially sectioned at 4.0 microns thickness using a microtome. Serial sections were floated on warm water at 55°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°C for 30 minutes before being stained. Then they were stained with H & E stains. And they were submitted to the two independent senior pathologists who were blinded of the clinical symptoms and stage of the cases. Diagnosis and grading of the cases were made.

Comment [ANR1]: What is the inclusion criteria?

Comment [ANR2]: Senior is obsolete words

Comment [ANR3]: How do you grade the cases? At least provide reference or standard protocol.

For Immunohistochemistry (IHC) staining, the 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 (DAKO) as a chromogen. In every step, phosphate buffer solution (PBS) was used. The sections were then counterstained with hematoxylin and viewed under the light microscope (Leica MD500, Tokyo, Japan). Placenta tissue was used as positive control while OSCC tissue devoid of EGFR antibody was used as negative control. The IHC stained slides were then submitted to the two senior independent pathologists in a blinded manner for being reported.

Comment [ANR4]: Not consistent with previous paragraph

Comment [ANR5]: Unclear words, for being reported to whom?

EGFR immunoreactivity was considered positive when tumour cells stained golden brown with varying colour intensities. Positive results were reported with regard to intensity of staining and percentage of tumour 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 were regarded as negative for EGFR immunoreactivity. Scoring for positivity was considered in over 10% tumour cells [6, 10, 14].

For association between categorical variables; Chi-square test was performed whereas association between continuous and categorical variables; Kruskal-Wallis H test was performed using SPSS 16.0 version (SPSS, IBM Stat Inc, USA). For statistical significance in difference of the compared variables, a P-value < .05 was applied.

Results

A total of 127 OSCC 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) with ratio of 2.2: 1 respectively. Figure 1 shows the age groups of the subjects in this study. Majority of the cases with OSCC (29.1%, 37/127) were in the age group of 50 – 59 years followed by (25.1%, 32/127) in the age group of 60 – 69 years. The rest of the age groups accounted for less than 10% cases in each group.

Comment [ANR6]: Not clear, which one is used for testing Chi-square or Kruskal-Wallis H test? The sentence is contradicted with the one in the abstract

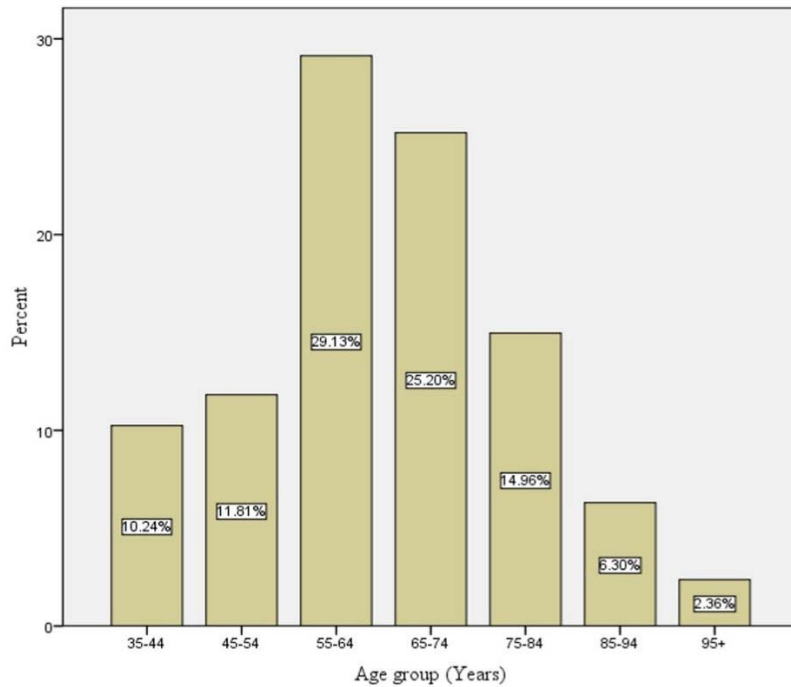


Figure 1 Distribution of the cases with oesophageal squamous cell carcinoma by age group.

Figure 2 indicates the distribution of the cases with OSCC by tumour grades. Most of the cases diagnosed with OSCC in this study, (59.9%, 76/127) were moderately differentiated (grade 2) followed by (29.1%, 37/127) with well differentiated (grade 1) and the remaining 11.0%, 14/127) were poorly differentiated (grade 3).

Comment [ANR7]: Does this means there is a small fraction of patient that did not diagnosed with OSCC?

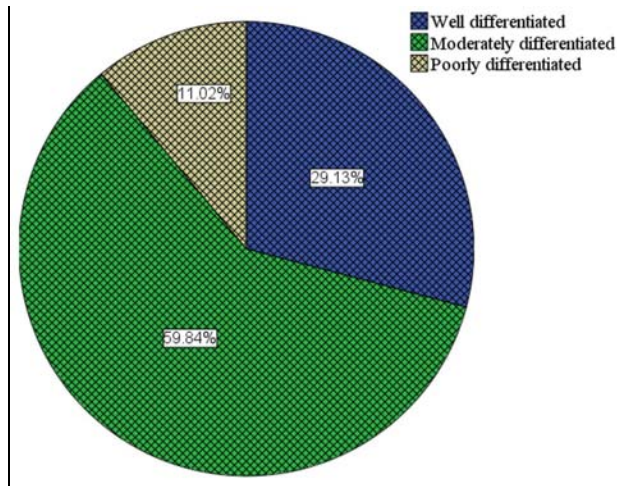


Figure 2 Frequency distribution of the cases of oesophageal squamous cell carcinoma by **tumour differentiation**

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

Table 1 Grades of oesophageal squamous cell carcinoma according to gender

Tumour 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%. Positive immunoreactivity was

Comment [ANR8]: What does tumor differentiation means? What is the criteria of this 3 groups?

Comment [ANR9]: How do you make grade classification? Make it clear in the method section. What is the criteria to classified as Grade 1, 2 and 3

Comment [ANR10]: Is this significantly different?

seen in (61.4%, 78/127; 2+ and 3+ score) and the remaining (38.6%, 49/127; 0 and 1+ score) were negative for EGFR immunoreactivity.

Comment [ANR11]: In what? Missing word

Comment [ANR12]: Do not use () if you want to include the numbers in the sentence.

Table 2 EGFR expression among the cases with oesophageal 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 tumours. The staining pattern was focal (Figure 3), in the sense that some tumour cells were not stained by the antibody.

Comment [ANR13]: Explain what does focal mean and why this is happen. And how this result can affect the result?

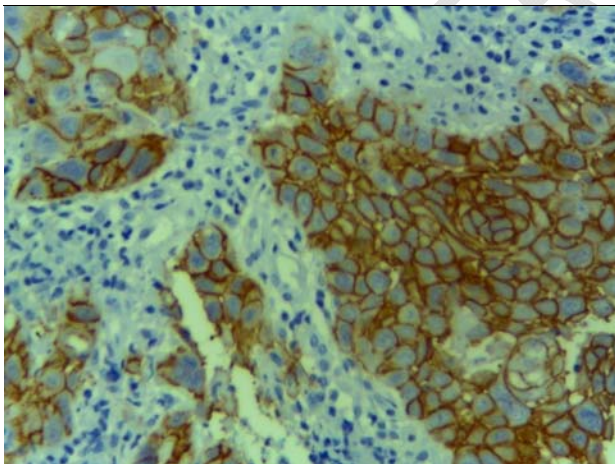


Figure 3 Photomicrograph of IHC staining showing focal EGFR cell membrane staining of immunoreactivity (3+) in a well differentiated OSCC (x400 magnification)

In other cases of well differentiated tumours, the staining of the tumour cells was well defined to the tumour cells and it was diffuse in nature (Figure 4). Only stromal and reactive inflammatory cells were left unstained.

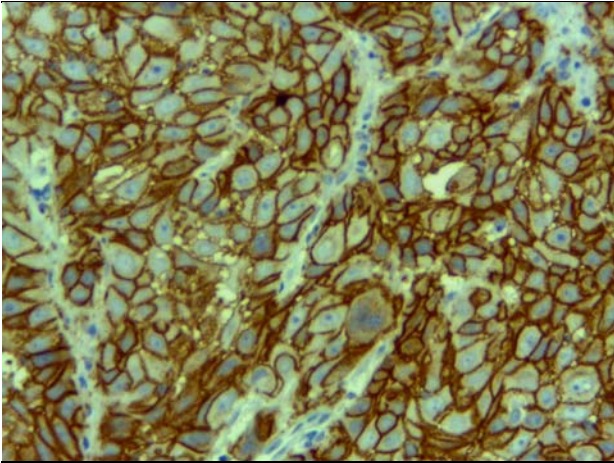


Figure 4 Photomicrograph of IHC staining showing diffuse EGFR cell membrane staining of immunoreactivity (3+) in a well differentiated OSCC (x400 magnification).

Comment [ANR14]: Images in figure 3, 4 and 5 are best to be put side by side as one figure, more easily to be compared

Weak staining was found more in moderately differentiated (Figure 5) and poorly tumours. Staining was more of background than being defined to the tumour cells as it was for well differentiated cases.

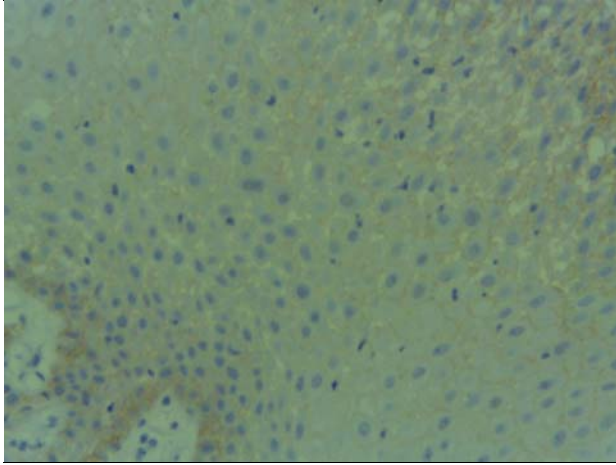


Figure 5 Photomicrograph of IHC staining showing weak EGFR cell membrane staining (+1) in a moderately differentiated OSCC (x400 magnification)

Table 3 below shows association with EGFR expression and gender, age group and tumour grade. When the trend of EGFR expression was associated with gender of the subjects, it was found that the difference was not statistically significant although males had a high proportion of EGFR antigen compared to the females. The expression of EGFR antigen between the two extremes of the age groups for the cases with OSCC studied was almost similar between the two groups and the difference was not statistically significant. Expression of EGFR among the cases with moderately differentiated OSCC cases was highest of all the three grade groups, but the difference between grades was not statistically significant ($P = .255$).

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

		EGFR immunoreactivity				P-value
		Positive		Negative		
Variable		N	%	N	%	
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	
Tumour 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	

Comment [ANR15]: Decipher the statistics method that was used here. Is it Chi square or Kruskal-Wallis H test? And if one of the study use here, where do you use the other statistic test?

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 tumour 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 [15] among patients with OSCC from Lacor hospital in the northern part of Uganda. The mean ages of the patients with OC in the reports of Ocama and the colleagues from the central part of Uganda [16] and Anvari et al [10] in Iran were almost in keeping with the mean age of the patients in this study. Lin et al [17] in China reported a similar mean age of patients with OC in 2017. In all these studies, OSCC was the predominating histopathological type. This implies that OC 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 [1, 2, 16, 17] reported a peak age of 53-61 years which almost similar to the peak age of 50-59 years of the cases in this study. The observation that OSCC is more common among males compared to females was in agreement with a large number of similar findings documented in the English literature [15, 16]. However, studies have shown that the incidence of OSCC in areas where it is prevalent, both males and females are affected equally. There is no clearly known reason why OC is more common among males as compared to females. However, the difference in predilection of OSCC among males as compared to females is not statistically significant ($P = .504$) as it was once reported by Kachala et al [18]. The tendency of males to indulge in use of most of the stipulated risk factors, conveys a message that, it could be why they are more affected compared to females. In the review article of Meves et al [19] reported that, Anderson and the associates found that the risk of alcohol consumption for developing OSCC increases in a linear fashion and smoking increases the risk of developing OSCC by nine-fold as compared to non-smokers (hazard ratio 9.3; 95% CI: 4.0–21.3).

Oesophageal carcinoma, commonly OSCC, 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 below, 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 [20].

The degree of OSCC differentiation in this study was similar to what was found by Chen et al [21] in which moderately differentiated cases were the majority (55.6%), followed by well differentiated (32.3%) and the least was poorly differentiated tumours (12.1%). In another study which was done in Japan by Tustumi et al [22] among patients with OSCC, it was found that

Comment [ANR16]: What do you mean by English literature? English language literature are to wider, you need more specific which reference do you attend to focus on.

Comment [ANR17]: Which studies (more than one study) do you mean here? Does this refer to other studies?

Comment [ANR18]: Unclear sentence structure. Please rewrite this sentence and revise the use of commas, they make the sentence hard to digest.

Comment [ANR19]: You should add references to show that that all the risk factor are occurrence in males compare to females.

Comment [ANR20]: Explain why the current study the result is not statistically significant.

moderately differentiated cases were the dominating cases constituting 73.7% similar to what we found for the cases in the present study, however, 71.9% of the cases in their study were poorly differentiated and 61.5% were well differentiated. Despite 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 tumour differentiation does not reflect the tumour stage and course of the tumour in terms of advancing.

Comment [ANR21]: 73.7 and 71.9%? not clear

Overexpression or amplification of EGFR has been associated with aggressive biological behaviours of ESCC such as tumour stage, tumour differentiation and many others. It has also been reported in the literature that patients who express EGFR have better prognosis than that don't express [10, 17, 23]. The prevalence of 61.4% for EGFR overexpression in this study was close to the 64.7% that was reported by Abedi-Ardekan and the associates [15] but lower than 70.0% which was found by Anvir et al [10]. A lower prevalence of 53.6% for EGFR overexpression was reported by Lin et al [15]. 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 inherent technical and also sample size differences. In the study by Wang et al [24] 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, the prevalence for EGFR overexpression was 49.2%, lower than the one in the current series.

Comment [ANR22]: Explain why differentiation is not reflected the tumour stage.

Comment [ANR23]: ESCC?

Comment [ANR24]: Reference?

Comment [ANR25]: Incomplete words

Comment [ANR26]: Of course if the positive group limit cut smaller, the prevalence will be low, the sentence is not important. You should decipher why the study put 0, 1+, 2+ as negative? Give more argument, strength and weakness of both method.

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 OSCC followed by well differentiated (20.5%) but the difference was not statistically significant ($P = 0.255$). The lack of statistical significance in OSCC tumour grades for overexpression of

EGFR in this series was in keeping with the communications of Wang et al [24], 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 [23] and that of Lin and the colleagues [17], both reported that there was no correlation between OSCC tumour differentiation and overexpression of EGFR with P value of 0.882 and 0.853 respectively. The importance of grade of tumour in OC and OSCC particularly, is controversial. Some studies have shown a more favorable prognosis for well to moderately differentiated tumours than poorly differentiated tumours while other studies highlight the opposite [25].

Although gender carries a prognostic role in patients with OSCC, 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 [24-26] in which the P -values were .120, .850 and .410 respectively.

Activation of EGFR signaling has been implicated in metastasis via modulation of cell adhesion, angiogenesis, invasion and migration [27]. For example, Yoshida et al [28] reported that oesophageal tumour cells that activate EGFR, increase the expression of matrix metalloproteases (MMPs) which are important for the degradation of extracellular matrix (ECM); a process that is necessary for tumour invasion and metastases. Additionally, EGFR induces re-localization of E-cadherin from the lateral adhesion sites to a more uniform distribution over the cell surface which correlates with change in cell morphology and increased invasiveness [29]. On the other hand, EGFR upregulates integrin molecules, leading to cohesion of the transformed cell to the vasculature, hence promoting metastasis [30]. A consequence of unregulated and improper

Comment [ANR27]: Incorrect structure leads to incorrect statement. The cells active EGFR? Then increase MMPs?

receptor activation in induction of signals that promote proliferation, survival, migration and angiogenesis all of which are important in tumour development and progression [28, 30].

Comment [ANR28]: What is the relevance of this paragraph in the discussion part? The literature study should be connected to result study if you want to put them in the discussion part

Conclusions

The overexpression of EGFR in patients with OSCC in Uganda was significant and majority of the cases showing high overexpression of the EGFR, were of moderately differentiated tumour grade.

Comment [ANR29]:

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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