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Immunohistochemical Expression (IHC) and Fluorescence In Situ Hybridization (FISH) of EGFR in Colorectal Carcinoma

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Adenocarcinoma of the colon is the most common malignancy of the gastrointestinal tract and is a major cause of morbidity and mortality worldwide. Epidermal growth factor receptor (EGFR) is also known as HER-1 or erb-B1. The beginning of EGF to EGFR produces a biological signal to the cell that initiates several functions that promote tumor growth, including cell invasion and metastasis, repair and new blood vessel formation. Thus, EGFR is recognized as an important player in colorectal cancer (CRC) initiation and progression. In this study, total 61 cases of colorectal carcinoma were included and histological grading, immuno-histochemical (IHC) expression and fluorescence in situ hybridization of EGFR were conducted. For the grading, 11/61(18%) cases were well differentiated, 38/61(62%) cases were moderately differentiated and 12/61(20%) were poorly differentiated colorectal carcinoma. EGFR IHC immune expression was positive in 50/61(82%) cases and negative in 11/61(18%) cases. All 11 cases of well differentiated cases gave EGFR IHC positive immunoreaction. Among the 38 cases of moderately differentiated adenocarcinoma, 30 cases showed EGFR IHC positivity and 8 cases gave no reaction. Nine out of 12 cases of poorly differentiated adenocarcinoma showed EGFR IHC positive and 3 cases gave no reaction. Half (10/20, 50%) of the EGFR IHC highly positive cases showed FISH positive and other half cases give FISH negative reaction. Detection of EGFR is mainly for anti-EGFR targeted therapy. Therefore, this study aids in selection of patients for anti-EGFR targeted therapy and helpful in treatment options and disease management. Moreover, EGFR FISH can be tested together with KRAS mutation and can predict the treatment response and the disease outcome.

Keywords: Colorectal carcinoma, Epidermal growth factor receptor, Immunohistochemistry, Fluorescence in situ hybridization

INTRODUCTION

Adenocarcinoma of the colon is the most common malignancy of the gastrointestinal (GI) tract and is a major cause of morbidity and mortality worldwide. Approximately 1.2 million new cases of colorectal adenocarcinoma, and 600,000 associated deaths, occur each year worldwide. Thus, colorectal adenocarcinoma is responsible for nearly 10 percent of all cancer deaths.[1] In Myanmar, according to cancer registry of Yangon General Hospital, there were 334 (5.1 percent) cases of colorectal carcinoma out of 6841 cases in 2014 and 757 (13.7 percent) cases of colorectal carcinoma out of 5509 cases in 2012.[2] There have been many studies concerning colorectal carcinoma in Myanmar. In 2012, there was a study on correlation between serum vascular endothelial growth factor and tumor-node-metastasis staging of colorectal adenocarcinoma.[3] Significance of EA, CA 19-9 and BETA HCG serum tumor-markers in colorectal carcinoma was also studied.[4]

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Moreover, histopathological spectrum of colonoscopic biopsy was done in a study done by Myint Myint San.[5] By using the immunohistochemical method, there was a study on the significance of Ki-67 antigen, carcinoembryonic antigen and vascular endothelial growth factor receptor-1 immunoexpression in colorectal carcinoma.[6] However, the study related with EGFR and colon cancer has not been carried out yet.

Epidermal growth factor receptor (EGFR) belongs to HER family of cell surface receptors. EGFR is also known as HER-1 or erb-B1 is a ubiquitous 170-kd membranous-spanning glycolprotein and EGFR gene is located in the short arm of chromosome 7.[7] The epidermal growth factor receptor is composed of three domains: (1) an extracellular domain that recognizes and binds ligands specifically, such as epidermal growth factor (EGF), transforming growth factor (TGF)-α and amphiregulin which bind specifically to EGFR; (2) a hydrophobic transmembrane domain that is involved in interactions between cell surface receptors; and (3) an intracellular domain that serves as a site of tryosine kinase activity.[8]

The beginning of EGF to EGFR produces a biological signal to the cell that initiates several functions that promote tumor growth, including cell invasion and metastasis, repair and new blood vessel formation (angiogenesis), all of which are essential components to the ongoing survival of the tumor.[9] EGFR is recognized as an important player in colorectal cancer (CRC) initiation and progression.[10]

In human colorectal cancer, EGFR is also associated with tumor development and progression. EGFR is overexpressed in up to 82% of colorectal cancers.[11] It has been well documented that overexpression of EGFR in colon cancer may be linked to an advanced stage of the disease[12] or may predict a potential metastatic risk.[13] Based on the importance of the EGFR axis in colorectal cancer, drugs that interfere with various functional domains of the receptor have been developed.[8] EGFR expression in the tissue can be assessed by many methods such as immunohistochemical (IHC) method, and fluorescence in situ hybridzation (FISH) method. Immunohistochemical method is detection of EGFR protein expression in tissue and can be visualized as brown color membrane staining.

Fluorescence in situ hybridization is a form of detection of EGFR gene copy number within the chromosome and it can be seen as color dots and assess the increase or decrease number. Probe signals are enumerated in individual nuclei if they are bright, distinct, and easily assessable against a dark background relatively free of fluorescent particles and haziness. The EGFR gene assessment of fluorescence in situ hybridization is carried out by counting gene specific signals and corresponding control signals in 40 nuclei, at two areas of colorectal carcinoma. Overlapping or damaged nuclei were disregarded. A cell with a normal number of copies of the EGFR gene or chromosome 7 status is characterized by 2 EGFR and 2 CEP7 signals per nucleus.

Many studies indicated that EGFR gene copy number could influence response to anti-EGFR mAbs cetuximab or panitumumab therapy in CRC. In the retrospective analysis conducted by Moroni, *et al*. where EGFR genomic gain was assessed by fluorescence in situ hybridization (FISH), there was a significant association between high EGFR copy numbers and better response to anti-EGFR mAbs cetuximab or panitumumab.[14]

Both IHC and FISH analysis on EGFR can be used for assessment of targeted therapy. However, it was stated that targeted therapy efficacy outcomes is more favorable with FISH analysis than IHC because the analysis of EGFR protein expression did not identify consistent trends related to efficacy outcomes across the range of IHC values.[15]

At the present, the study concerning with FISH method has not been established yet. Therefore, for the development of new method and interest in the colon cancer, both IHC and FISH method were used to detect EGFR expression and find the association. It is hoped that detection of EGFR can predict the severity and prognosis assessment, and useful in targeted therapy of colon cancer patients.

MATERIALS AND METHODS

Study design

It was a cross-sectional descriptive study on all patients who attended the surgical ward of Yangon General Hospital and were histologically diagnosed as colorectal cancer during the period of two years (2016-2017).

Sample size

Minimal required sample is calculated by using following formula.

$$N = \frac{n^2\ 1\text{-}\alpha / 2\ p\ (1\text{-}p)}{\delta^2}$$

Confident interval (1-α) = 95%
N = Minimum required sample
p = EGFR expression in colorectal carcinoma is expected to be 80% [13], 0.805
δ^2 = Margin of error (precisim) in this study δ^2 is set at 0.10
Minimum required sample = 61
Total 61 cases will be needed for the study

This study recruited the tissue sample which were sent to Pathology Department, Yangon General Hospital for routine diagnosis. Only paraffin blocks were collected and the sections were cut into 4 μm thickness. After that, haematoxyline and eosin staining for histological grading, EGFR IHC staining were conducted. For immunohistochemical staining, primary antibody; anti-EGFR (SP9) rabbit monoclonal antibody and secondary antibody; HRP conjugated anti-rabbit IgG were used. Olympus ordinary microscope was used for histology and EGFR IHC scoring.

For FISH, following deparaffinization and dehydration, pretreatment and enzyme digestion were done using proteinase K. Zyto Light SPEC EGFR/CEN7 dual color probe was used to detect EGFR gene pattern by FISH. Probes were applied to target area and denatured at 85°C for 10 minutes before overnight hybridization at 37°C. Post-hybridization washes were done in 0.4% SSC/0.3% NP40 at 73°C for 2 minutes and in 2X SSC/0.1% NP40 for 1 minute. Slides were counterstained with 4', 6'-diamidino-2-phenylindole and were stored at -20°C before evaluation. Axio imager optical sectioning microscope (Carle Zeiss) was used for EGFR FISH gene pattern analysis and image capture.

Working definition of EGFR IHC scoring[16]

- Tumors are deemed EGFR positive when ≥1% of the tumor cells exhibited membranous staining of any intensity.
- EGFR IHC scored 1+ means weak intensity and faint brown membranous staining.
- EGFR IHC scored 2+ means moderate intensity and medium brown membranous staining.
- EGFR IHC scored 3+ means strong intensity, dark brown or black membranous staining producing a thick outline, complete or incomeplete of the neoplastic cells.

EGFR FISH[17]

Normal disomy	=2 gene copies in >90% of cells
Trisomy	=3 gene copies in >10% of cells and ratio gene/ chromosomes 2
Low polysomy	=4 gene copies in >10% but <40% of cells and ratio gene/ chromosomes 2
High polysomy	=4 gene copies in>40% cells and ratio gene/chromosomes 2
Gene amplification	= ratio gene/chromosome >2 or 15 gene copies in10% of cells

Trisomy, low polysomy, high polysomy, and/or gene amplification were considered EGFR-FISH positive.

Normal disomy was considered EGFR-FISH negative.

Statistical analysis

All the data from each patient were recorded in proforma and data analysis was conducted with statistical Package for Social Science (SPSS version 16.0). Simple descriptive analysis for each single variable was done. Categorical variables such as histological grade (grade 1, 2 and 3) and EGFR immunoexpression were summarized as frequency tables. The association of EGFR IHC with age, sex, site of tumour and tumour grading was done by using Statistical Package for Social Science (SPSS version 16.0).

Ethical consideration

This study was done only after getting approval from Ethics Review Committee, DMR. The participants eligible to the selection criteria were explained in detail about the study by the investigator with information sheet. Only after they have fully understood the nature of the study, objectives, methodology, procedures, duration, risks and benefits, they were invited to take part in the study and the written informed consents were obtained. The investigator strictly maintained the rules of privacy and confidentiality. Only coded system was used and research information was kept by a password-protected file in the investigator's personal computer.

RESULTS

In the present study, total 61 cases of colorectacl carcinoma were included. The ages ranged from 18 to 86 years. The mean age was 46 years. Sixty percent of cases (37/61) were in ≤50 years age group and 40% (24/61) were in >50 years age group. There were total 61 cases with 28(46%) male and 33(54%) female in this study. Most of the tumour (47/61, 77%) were located in rectum followed by colon (14/61, 23%).

Among 61 cases of colorectal carcinoma, 62% (38/61) were in moderately differentiated group followed by 20% (12/61) cases in poorly differentiated and 18% (11/61) cases in well differentiated group (Fig. 1).

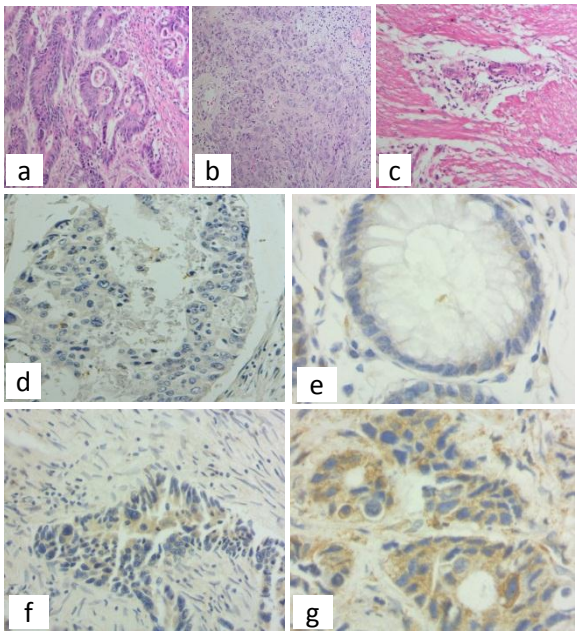

Fig. 1. (a) Well differentiated adenocarcinoma colon (b) Moderately differentiated adenocarcinoma colon (c) Poorly differentiated adenocarcinoma with signet-ring cells (d) EGFR IHC negative (e) EGFR IHC 1+ (f) EGFR IHC 2+ (g) EGFR IHC 3+

Table 1. Distribution of EGFR FISH in colorectal carcinoma

EGFR FISH		Number of cases	Percentage	
Negative	Disomy	10	50%	
Positive	Trisomy	6	30%	50%
	Polysomy	3	15%	
	Amplification	1	5%	
	Total	20	100%	

Disomy only is EGFR FISH negative and other trisomy, polysomy and gene amplification means EGFR FISH positive (Table 1 & Fig. 2).

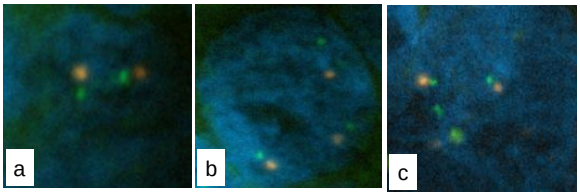

Fig. 2. (a) EGFR FISH disomy: 2 green and 2 red signal (b) EGFR FISH trisomy: 3 green and 3 red signal (c) EGFR FISH gene amplification: >2 EGFR and CEN 7 ratio

Table 2. Association of EGFR immunohistochemical expression with age, gender, sites of tumor and different histological gradings of colorectal carcinoma

	EGFR		Total number	P value
	Positive n (%)	Negative n (%)		
Age				
≤50	30(81)	7(19)	37	0.553
>50	20(83)	4(17)	24	
Gender				
Male	22(79)	6(21)	28	0.380
Female	28(85)	5(15)	33	
Site				
Colon	12(86)	2(14)	14	0.512
Rectum	38(81)	9(19)	47	
Grading				
Well	11(100)	-	11	
Moderately	30(79)	8(21)	38	0.130
Poorly	9(75)	3(25)	12	

EGFR=Epidermal growth factor receptor

Table 2 shows the association of EGFR IHC expression with age, sites of tumor and gradings of colorectal carcinoma. Out of 61 cases of colorectal carcinoma, 82% (50/61 cases) were EGFR IHC positive and only 18% (11/61 cases) were EGFR IHC negative.

DISCUSSION

Adenocarcinoma of the colon is the most common malignancy of the GI tract and is a major cause of morbidity and mortality worldwide. Approximately 1.2 million new cases of colorectal adenocarcinoma, and 600,000 associated deaths, occur each year worldwide. Thus, colorectal adenocarcinoma is responsible for nearly 10 percent of all cancer deaths.[1] The present study evaluated the overall immunohistochemical expression of EGFR in colorectal carcinoma. It gives brown to dark brown color in cytoplasm and membrane staining, which can be observed in tumor cells. In this study, 82% (50/61) cases of colorectal carcinoma gave EGFR IHC positive reaction which is significant frequency compares to others. In a study by Cappuzzo, *et al*. EGFR

IHC expression in colorectal carcinoma was 80.5% (66/85).[18] Similar finding was observed in this study.

For the grading of the colorectal carcinoma cases, 18% were well differentiated, 62% were moderately differentiated and 20% were poorly differentiated colorectal adenocarcinoma. All the 11 cases of well differentiated cases gave EGFR IHC positive immunoreaction. Among the 38 cases of moderately differentiated cases, 30 cases showed EGFR IHC positivity and 8 cases gave no reaction. Nine out of 12 cases of poorly differentiated cases were EGFR IHC positive and only 3 cases showed no reaction.

In a study by Arkom, *et al*. a significant association between EGFR IHC and tumour differentiation was found,[19] although other studies have shown no such correlation.[20] In the present study, the association between EGFR IHC and tumor grades, age, sex, and sites of tumor was not observed wich is similar to some study.[21] The difference in findings may be due to type of study design and sample size. Many studies described that EGFR overexpression can be considered as poor prognostic factor. However, this study cannot show EGFR as a poor prognostic factor. Detection of EGFR by IHC is useful for selection of targeted therapy, since anti-EGFR strategies are offering a new hope for patients with cancer.

Next, total 20 cases of EGFR IHC highly positive cases were selected and observed for EGFR gene pattern by FISH. Fifty percent (10/20) was disomy that means EGFR FISH negative. Another 50% was EGFR FISH positive with 6/20 cases trisomy, 3/20 cases of low polysomy and 1/20 case of gene amplification, respectively.

In the present study, assessment of EGFR gene copy number is for the establishment of FISH methodology and hoped to be useful in targeted therapy in future. EGFR gene copy number analysis, when performed from areas with highest EGFR IHC expression, is a highly promising method for predicting the efficacy of anti-EGFR therapy in locally advanced or metastatic CRC.[21]

Currently, patients with metastatic CRC are screened for KRAS status and only those with KRAS wild type (WT) tumours receive anti-EGFR therapy. This selection is not absolute and about half of the patients with KRAS WT tumours will receive the anti-EGFR monoclonal antibodies unnecessarily. Together with KRAS analysis, EGFR gene copy number identifies the responsive patients more accurately than either test alone. Algars A, *et al.* suggestthat anti-EGFR such as cetuximab and panitumumab should not be offered to KRAS WT patients with EGFR gene copy number <4.0.[22]

In conclusion, EGFR is highly expressed in colorectal carcinoma and targeted therapy with anti-EGFR can be used. Although EGFR expression is not associated with tumour grading, EGFR IHC expression is useful for selection of area for in situ analysis. EGFR gene copy number analysis by FISH is helpful in prediction of respond to treatment. Patients with low EGFR gene copy number are indeed unlikely to respond to treatment and have poor prognosis compared to increased gene copy number. Additional study with larger sample size and comparative study should be conducted.

Competing interests

The authors declare that there is no competing interest.

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