Detection of EGFR mRNA in Colorectal Carcinoma

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Colorectal carcinoma is a common malignant disease worldwide, including Myanmar. Epidermal Growth Factor Receptor (EGFR) has role in colorectal carcinogenesis and its overexpression is considered to be related with prognosis and disease management. This study was to find the association between EGFR mRNA (one of the biomarkers) and pathological features among colorectal carcinoma in Myanmar population. The aim was to detect circulating EGFR mRNA by nested RT-PCR method in histologically confirmed colorectal carcinoma. Total 84 cases of colonoscopic biopsy proven colorectal carcinoma cases were recruited and collected 2 ml of blood before operation and only 68 cases were eligible for the study. The commonest site of tumor occurrence was rectum 61.8% (42/68) and the most common tumor type was adenocarcinoma 86% (59/68). The most frequent histological grade was moderately differentiated type 41/68 (60.3%), followed by well differentiate type 21/68(30.9%), and 6/68 (8.8%) of poorly differentiated type. According to Astler-Coller staging, 45/68(66.2%) were in stage B2 followed by 8/68(11.8%) in stage D, 7/68(10.3%) in stage B1, 5/68(7.4%) in stage C2 and 3/68(4.4%) in stage C1, respectively. EGFR mRNA was detected in 19/68(27.9%) of colorectal carcinoma which were 23.8% (5/21), 26.9% (11/41) and 50% (3/6) of well, moderate and poorly differentiated adenocarcinoma, respectively. EGFR mRNA was detected in 15.4% (8/52), 62.5% (5/8) and 75% (6/8) of Astler-Coller stage B, stage C and stage D, respectively. The findings pointed out that EGFR mRNA was frequently detected in high grade tumor, as compare to low grade, although significant association was not observed. However, EGFR mRNA was frequently detected in advanced stage of tumor (stage C and D), which was statistically significant with the 'p' value of 0.001. But the association between sites of tumors, histological types and EGFR mRNA was not observed. Since EGFR mRNA was more detected in high grade and advanced stage tumor and it indicates metastatic potential. EGFR mRNA assay might represent a suitable marker for detection of circulating tumor cells in colorectal carcinoma patients for staging and prediction of cancer progression and metastasis.

Keywords: EGFR mRNA, Colorectal carcinoma, Nested PCR

INTRODUCTION

Colorectal carcinoma (CRC) is one of the leading causes of mortality and morbidity in the world.[1] In United States, there are more than 130,000 new cases and nearly 50,000 deaths from CRC each year.[2] According to admission data of North Okkalapa General and Teaching Hospital (NOGTH), there were 186 cases (6.1 percent of total cancer patients), 351

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cases (14.2 percent of total cancer patients), and 382 cases (9.7 percent of total cancer patients) of CRC in 2014, 2015, and 2016, respectively. Epidermal growth factor receptor (EGFR) is known as HER-1 or erb-B1.[3] Binding of EGF to EGFR produces a biological signal to cell that promote tumor growth, cell invasion, metastasis, repair and angiogenesis, which are essential for ongoing survival of tumor.[4]

One of the concerns of research is the identification of molecular markers that could predict the biological behavior of individual tumors and guide treatment strategies. Over last decades, great interest has been focused on EGFR signaling network because of its role in tumor development and aggressiveness. While EGFR mutations events are rare in CRC, aberrant activation of the EGFR signaling network frequently occurs through the alterations of downstream elements in the signaling cascade.[5] Aberrant activation of the EGFR signaling network can also result from the over expression of ErbB family members.[6]

RNA identification is superior than others because it detects mainly on viable tumor cell and only viable cells can produce mRNA and then extracellular RNA is rapidly degraded.[7] Circulating EGFR messenger RNA (EGFR mRNA) is one of the biomarkers and detected by PCR methods for identification of early metastasis, targeted therapy and prognosis assessment.

A study by Ho Pun Cheung, *et al* (2010) stated that EGFR mRNA may be useful to predict the distant metastasis in locally advanced cancer.[6] In this study, circulating EGFR mRNA was detected by nested RT-PCR method and found out the association between EGFR mRNA and pathological features.

MATERIALS AND METHODS

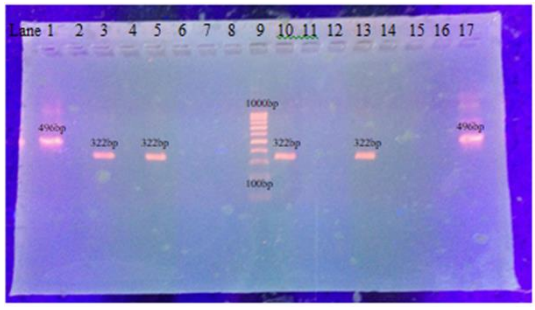
This study was a cross-sectional descriptive study and carried out for two years (October, 2016 to October, 2018) at NOGTH, University of Medicine 2, Yangon and DMR. Patients with colonoscopic biopsy confirmed cases before operation were included in this study and cases which have been treated with chemotherapy and radiotherapy were excluded. Also other cancers such as breast cancer, lungs cancers, etc, were excluded by history taking. Sample size calculation was as follow:

$$N = \frac{Z\alpha/2\ p\ (1-p)}{d^2}$$ [8]

N= Minimum required sample
Zα=Statistics for alpha error 1.96 for α = 0.05
P=Proportion of positive of EGFR in colorectal carcinoma is 80% [9]
d^2 =Margin of error (precision) in this study, d^2 is set at 0.10
Required sample=62
Dropout rate 10%=6

Minimum (68) cases were needed for study. Total 84 patients with colonoscopic biopsy confirmed CRC were recruited. After getting informed consent, 2 ml of venous blood was collected and stored at -20˚C and waited for biopsy results. Only 68 patients were operated and histologically confirmed as CRC. After that stored blood samples were processed for detection of EGFR mRNA by nested RT-PCR method at Pathology Research Division, DMR. Resected organs (colon and lymph nodes) sent to the histopathology section of Department of Pathology, NOGTH were examined for tumors sites, histological diagnosis, grading and staging (Astler-Coller staging), and all the data from getting each patient were recorded in pro-forma.

Ribopure RNA extraction kit and Thermo Scientific cDNA synthesis kit were used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was used as internal control. For EGFR mRNA, total (20 µL) reaction solution contained 10 µL PCR master mix, 3 µL DW, 1 µL (20 pmol) of each primer, and 5 µL cDNA. First PCR reaction had 30 cycles consisting of 5 cycles of 30s at 94˚C, 45s at 60˚C, and 45s at 72˚C and 25 cycles of 30s at 94˚C, 45 s at 55˚C, and 45s at 72˚C. After the first round, 1 µl of first PCR amplification product was added to the second PCR solution. The second PCR reaction had 35 cycles consisting of 5 cycles of 30s at 94˚C, 45s at 60 ˚C, and 45s at 72˚C and 30 cycles of 30s at 94˚C, 45 s at 55˚C, and 45s



Lane 9 - 100 bp ladder
Lane 8 - Negative control
Lane 10 - 322 bp distinct DNA band of case 2 (used as positive control)
Lane 3, 5 & 13 - EGFR positive cases (case- 23, 25, 30) (322bp)
Lane 2, 4, 6, 7, 11, 12, 14, 15 & 16 - EGFR negative cases (case- 22, 24, 26, 27, 28, 29, 31, 32 & 33)
Lane 1 &17 - GAPDH (case 24 & 31) (496 bp)

Fig. 1. Amplified PCR product of cases (22 to 33)

at 72˚C cycle. Final product had 322 base pair (Fig.1). Positive cases were confirmed by genetic sequencing.

Data entry and data analysis

Data entry and data analysis were 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 grading (well, moderate, poorly) and staging (Astler-Coller staging) was summarized as frequency tables. The association between circulating EGFR mRNA, histological types, grading and staging was tested using Chi-square test. Statistical analysis 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 of University of Medicine 2, Yangon. The participants eligible to selection criteria were explained in detail about the study by 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 written informed consents were obtained. The rules of privacy and confidentiality were strictly maintained. Only coded system was used and research information was kept by a password-protected file in the computer.

RESULTS

Total 84 cases of CRC were recruited and only 68 cases were eligible and studied. Sixteen cases were drop out. All cases were recruited from NOGTH. Mean age of patients with CRC was 55.72±13.9 years. Youngest patient with CRC was 22 years and oldest patient was 94 years old. The commonest age group was 51-60 years and represented for 33.80% (23/68), followed by 25% (17/68) in 61-70 years, 13.20% (9/68) in 41-50 years, 10.20% (7/68) in 31-40 and 71-80 years, 6% (4/68) in ≤30 years, and 1.5% (1/68) in >81years age group. Ratio of male to female was 1:1.2 with 30 (44.1%) cases were males and 38 (55.9%) cases were females.

Rectum was the commonest site of tumor with 61.8 % (42/68). Second commonest site was ascending colon with 23.5% (16/68) followed by transverse colon 8.8% (6/68), descending colon 2.9% (2/68) and sigmoid colon 2.9% (2/68). More than two third of cases were adenocarcinoma type with 86.8% (59/68) followed by mucinous adeno-carcinoma 7.4% (5/68), signet ring cell type 4.4% (3/68) and squamous cell carcinoma 1.5% (1/68). More than half of the cases were in moderately differentiated, 60.3% (41/68) followed by well differentiated, 30.9% (21/68) and poorly differentiated, 8.8% (6/68). Two third of cases, 66.2% (45/68) were in stage B2 and the rest were 11.8% (8/68) in stage D, 10.3% (7/68) in stage B1, 7.4% (5/68) in stage C2 and 4.4% (3/68) in stage C1. There was no stage A.

In total 68 cases of CRC, only 27.9% (19/68) were EGFR mRNA positive cases and 72.1% (49/68) cases showed negative EGFR mRNA. Among 16 cases of tumor from ascending colon, only 25% (4/16) gave positive EGFR mRNA. In 42 cases of rectum, 31% (13/42) were EGFR mRNA

positive. Half of the cases from descending colon and sigmoid colon gave positive EGFR mRNA. There was no significant association between EGFR mRNA and site of tumors.

In total 59 cases of adenocarcinoma type of colorectal carcinoma, only 27.1% (16/59) showed EGFR mRNA positive and in mucinous adenocarcinoma type, only 20% (1/5) gave EGFR mRNA positive. For signet ring cell carcinoma, 66.7% (2/3) showed EGFR mRNA positive. However, the significant association was not observed between different histological types of colorectal carcinoma and EGFR mRNA (Table 1).

Table 1. Association between EGFR and histology types

Histological types	EGFR		Total N(%)
	Positive N(%)	Negative N(%)	
Adenocarcinoma	16 (27.1)	43 (72.9)	59 (100)
Mucinous adenocarcinoma	1 (20)	4 (80)	5 (100)
Signet ring cell carcinoma	2 (66.7)	1 (33.3)	3 (100)
Squamous cell carcinoma	0 (0)	1 (100)	1 (100)

N=Number

Among 21 cases of well differentiated tumor, only 23.8% (5/21) showed EGFR mRNA positive. In 40 cases of moderately differentiated tumor, 26.9% (11/41) gave EGFR mRNA positive and in poorly differentiated group, 50% (3/6) of the cases gave EGFR mRNA positive. Although significant association was not observed between EGFR mRNA and different histological grading, EGFR mRNA was frequently detected in high grade tumors, i.e. poorly differentiated tumors in compare with low grade tumors (Table 2).

Out of total 52 cases of stage B (including B1 and B2), 15.4% (8/52) gave EGFR mRNA positive. For stage C (including C1 and C2), 62.5% (5/8) showed EGFR mRNA positive and in stage D, 75% (6/8) gave EGFR mRNA positive.

Table 2. Association between EGFR and ` histology grades

Histological grades	EGFR		Total
	Positive N (%)	Negative N (%)	
Well	5 (23.8)	16 (76.2)	21
Moderate	11 (26.9)	30 (73.1)	41
Poorly	3 (50)	3 (50)	6

Table 3. Association between EGFR and Astler-Coller staging

Stages	EGFR				Total
	Positive		Negative		
	Number	(%)	Number	(%)	
A	-		-		-
B	B1- 1 B2- 7	8 (15.4)	B1-6 B2-38	44 (84.5)	52
C	C1- 2 C2- 3	5 (62.5)	C1- 1 C2- 2	3 (37.5)	8
D	6 (75)		2 (25)		8

P value=0.001

The *significant* association between EGFR mRNA expression and Astler-Coller staging was observed (p=0.001). EGFR mRNA was frequently detected in advanced stage of tumor, ie., stage C and stage D (Table 3).

DISCUSSION

This study evaluated EGFR mRNA by nested RT-PCR in peripheral blood of 68 primary CRC patients and detected EGFR mRNA in 27.9% (19/68) of total cases. The findings were within the range of findings from others. Spaulding and Spaudling (2002) reported that expression of EGFR had varying figures ranged from 25-82%.[10] Clark *and co-authors* (2003) detected EGFR mRNA in 12.5% of peripheral blood of colon carcinomas.[11] Tsouma, *et al* (2010) observed 19.3% of EGFR transcripts in peripheral blood of total 88 patients with primary CRC.[12] Teama and Agwa (2010) detected EGFR in 41.7% (15/36) of colon cancer.[13] Nannini, *et al* (2009) stated that EGFR mRNA was observed in 62% of CRC patients.[14]

This study used nested RT-PCR method to detect EGFR mRNA in CRC whereas others used qRT-PCR and multiple RT-PCR methods. Since different detection systems have their unique sensitivity, the discrepancy in findings may be one of the consequences.

In this study, EGFR mRNA was frequently detected in high grade tumors in compare with low grade. However, statistically significant association was not observed between tumor location, histological grades, histological types and EGFR mRNA which were in agreement with study of Nannini, *et al* (2009) who observed no significant association between tumors location, histological grades and EGFR mRNA.[14]

According to Astler-Coller staging, there was no case of stage A in current study. EGFR mRNA was detected in 15.4% in Stage B (B1 and B2), 62.5% in stage C (C1 and C2), and 75% in Stage D. Based on current findings, EGFR mRNA were frequently occurred in more advanced stage of cancer and showed statistically significance association with p value of 0.001. Current study pointed that EGFR mRNA was detected more in advanced stage compared to early stage of CRC.

Similarly, De Luca, *et al* (2000) observed EGFR mRNA in 73%, 55% and 29% of Stage D,C and B, respectively.[15] Also, Giacomelli, *et al* (2003) reported that EGFR was expressed in 100% of Stage III/IV and 9% of Stage I/II and showed significant association.[16] Teama and Agwa (2010) observed significant association of EGFR mRNA and Astler-Coller staging in CRC with 81.8% detected in stage IV and 11.1% detected in Stage II.[13] Tsouma, *et al* (2010) reported that EGFR mRNA was detected in 35.5%, 14.8% and 7.7% of Stage D, Stage C, Stage B, respectively with $p < 0.001$, and suggested that EGFR as possible marker for advanced disease.[12]

Current study used high sensitive nested RT-PCR to detect EGFR mRNA in peripheral blood and had also found EGFR mRNA in early stage of disease. Similarly, De Luca, *et al* (2000) observed EGFR mRNA in CRC patients of early resectable stage and clinical follow-up of these patients had been started to assess whether patients at high risk of relapse might be recognized by using nested RT-PCR EGFR mRNA assay.[15] Differently, a study by Leitzel, *et al* (1998) failed to demonstrate EGFR transcripts in localized cancer patients.[17]

In this study, EGFR mRNA was detected in 15.4% of Stage B and 62.5% of Stage C, although they had no distant metastasis yet, tumor cells were going around the circulation and they will metastasize soon. Findings from Giacomelli, *et al* (2003) showed patients with persistence of high EGFR levels after surgery had high risk of relapse and suggested a clinical use of pre-operative EGFR RT-PCR assay in the prediction of tumor recurrence.[16] Indeed, if EGFR expression can identify patients with high risk of developing distant metastasis and additional treatment might be considered.

In conclusion, identification of high risk patients with distance metastasis represents a major challenge in management of cancer. Quality of surgery (evaluated by circum-ferential resection margin), degree of bowel wall invasion by primary tumor, and lymph nodes status are currently the most reliable indicators for metastasis risk assessment.[18] However, these parameters are not sufficient, as patients with tumors belonging to the same TN category may have different outcomes. So, additional biological markers of aggressive disease are needed for appropriate treatment strategies. Therefore, current findings suggested that EGFR assay can be useful for prediction of high risk patients and targeted therapy may be effective to prevent the metastasis in these high risk patients in the future.

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