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ORIGINAL ARTICLE

Upregulation and Hypomethylation of EGFR in Formalin-fixed Paraffin Embedded (FFPE) Tissues of Colon Adenocarcinoma

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ABSTRACT

Introduction: Colorectal cancer (CRC) is the third most common cancer worldwide. Even though many cancer therapies have been developed, considerable proportions of patients respond poorly to therapy and the number of resistance cases increases. CRC emerges as a result of genetic and/or epigenetic modifications of epidermal growth factor receptor (EGFR) in colonic epithelial cells during tumourigenesis. Determination of DNA methylation status of EGFR is very crucial to further understand the role of this gene in carcinogenesis. However, the applicability of formalin-fixed paraffin embedded (FFPE) tissues in molecular studies is still limited due to high degradation of the nucleic acids. Hence, this study aimed to determine the gene expression and DNA methylation status of EGFR in FFPE CRC samples. **Methods:** Fifty-nine of archival FFPE CRC cases with the adjacent normal colon tissues were retrieved. Manual micro-dissection was performed prior to RNA and DNA extraction. EGFR expression and DNA methylation status was evaluated by qPCR and methylation specific PCR (MSP) techniques respectively. **Results:** EGFR was overexpressed in 54.2% (p-value=0.021) of CRC cases. Hypomethylation of EGFR was discovered in 81.4% and 79.7% of FFPE CRC tissues and normal adjacent tissues respectively. No significant association was found between DNA methylation and mRNA levels of EGFR. **Conclusion:** Determination of gene expression and DNA methylation in FFPE tissues were successfully carried out. The overexpression and hypomethylation of EGFR strongly suggest its important role in CRC tumourigenesis. Hypomethylation in normal tissue adjacent to the tumours indicates this epigenetic change occurs at the early step in carcinogenesis.

Keywords: EGFR, Colon adenocarcinoma, Formalin-fixed paraffin embedded (FFPE), DNA methylation

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INTRODUCTION

Colorectal cancer (CRC) is the second most fatal type of cancer and is the third most common cancer worldwide (1,2). In Malaysia, CRC incidence has increased over the years (3). To this day, surgical resection is the only curative approach for CRC, however the number of recurrences and metastases cases has increased year by year. Moreover, it is estimated that the cost for CRC management in Malaysia for new cases alone is RM108 million per year (4).

CRC is associated with genetic and epigenetic modifications. Recent studies have shown that profound genetic and epigenetic changes occur in cancer cells during the onset of tumorigenesis and tumour progression (5,6). Therefore, potential molecular biomarkers are extremely needed to improve diagnosis, prognosis and therapeutic intervention of CRC personalized therapy (7,8).

According to Datta et al. (2008) (9), methylation of tyrosine kinase receptors have been linked to cancer development. In addition, epigenetic modifications in tyrosine kinase receptors might be one of the factors causing acquired resistance to conventional cancer therapy (10).

Epidermal growth factor receptor (EGFR) is a member of human epidermal growth factor receptor (HER) family. HER family (EGFR, HER2, HER3 and HER4) serves as potential therapeutic target or prognostic factor for various types of cancers particularly ovarian, breast and lung cancers (11). Overexpression of HER family has been associated with oncogenic transformation

(12). In addition, alterations due to methylation in the promoter of the tyrosine kinase receptors have been associated with cancer progression (9). The EGFR gene is on chromosome 7p12-13 and its protein consists of 170 kDa protein (13). EGFR comprised of ligand binding domain, transmembrane domain and tyrosine kinase domain (14). EGFR activation will lead to cellular growth. Excessive production of EGFR is normally due to receptor overexpression, autocrine signalling or mutation. Cell proliferation due to EGFR pathway activation will ultimately lead to apoptosis resistance in cancer cells (15). Overexpression of EGFR has been detected in breast, oesophagus, bladder and lung cancer (10,11,16).

Most studies related to gene signature and gene expression involved the usage of fresh frozen tissue samples which are more reliable for molecular investigation. However, frozen samples are not routinely available in clinical practice compared to FFPE tissues. FFPE is an invaluable repository of molecular information for genetic and epigenetic studies. Since the FFPE tissues were routinely prepared for histopathologic diagnosis, it serves as the most available clinical samples for research and is often accompanied with sufficient follow-up data and broad range of tissue types. However, the applicability of this tissue type in molecular studies is still limited due to the effect of the fixation process causing high degradation and fragmentation of nucleic acids. Therefore, the fragments of nucleic acids contained in FFPE tissues are smaller which are usually less than 300 bp (17). There is also cross-linking of DNA and proteins during tissue preparation which increases the rate of degradation. In addition, the long storage of the tissue blocks can further compromise the quality of FFPE-derived nucleic acid (18).

Despite these limitations, FFPE provides many advantages which include easy handling, low cost and suitable for large scale application (19). Moreover, FFPE is the best options for retrospective analyses in investigating high sample numbers in parallel (20). Hence, FFPE tissues serve as one of the essential choices in molecular research to obtain the genetic and epigenetic information of certain diseases.

Presently, two monoclonal antibodies; Cetuximab and Panitumumab are the examples of EGFR targeted therapy used to treat CRC. These antibodies will bind to the extracellular domain of EGFR to inhibit EGFR activation (21). Immunohistochemistry staining (IHC) was commonly used to determine the EGFR expression and to select the patients who could benefit from this treatment especially in FFPE samples. However, the reliability of this technique for this purpose is debatable (22). Previous studies have proved that aberrant DNA methylation of EGFR has been implicated in carcinogenesis of CRC mainly through the regulation of gene expression. Hence, gene expression and DNA methylation seems to be more efficient to assess the EGFR expression even in FFPE tissues. Therefore, the present study was designed to determine the gene expression and DNA methylation status of EGFR in CRC FFPE tissues of patients in Hospital Serdang using quantitative polymerase chain reaction (qPCR) and methylation specific (MSP) respectively.

MATERIALS AND METHODS

FFPE samples

Fifty-nine FFPE samples were retrieved from the Department of Pathology, Hospital Serdang. Ethics approvals were obtained from National Medical Research Registry (NMRR), Ministry of Health (MOH) (Reference number: NMRR-12-1403-13767) and Ethics Committee for Research Involving Human Subjects (Universiti Putra Malaysia) (Reference number: FPSK_Mei(13)02). Approximately, four sections of 5 μm of samples were sectioned from each block using microtome. For tumour and normal tissues within the same blocks, the selected areas were micro-dissected manually. In this process, the tumour and normal tissues of the unstained sections on slides were marked accordingly. Then, the respective tissues were scraped off the slides using a sterile scalpel prior to RNA and DNA extraction.

Gene expression

For EGFR primer, Quantitect Primer Assays from Qiagen, Germany were used. The primer sequences for Beta actin (β-actin) as the reference gene was 5′-TCACCGAGCGCGGCT-3′ (forward) and 5′-TAATGTCACGCACGATTTCCC-3′ (reverse). RNA extraction was carried out using RNeasy FFPE Kit from Qiagen, Germany. The quantity and quality of extracted RNA was determined using NanoDrop 1000 Spectrophotometer V3.7 (Thermo Fisher Scientific, USA). The purity and concentration of extracted RNA were between 1.8 - 2.1 and ≥ 50 ng/μl respectively. Reverse transcription process was carried out using Quantitect Reverse Transcription Kit (Qiagen, Germany) and followed by quantitative polymerase chain reaction (qPCR) using QuantiFast SYBR Green Kit from Qiagen. The amplification conditions consisted of a PCR initial heat activation at 95°C for 5 minutes, denaturation at 95°C for 10 seconds and the last step was the combination of annealing and extension at 60°C for 2 minutes. The last 2 steps were repeated for 40 cycles. The amplification was conducted using the Eppendorf Mastercycler ep realplex real-time PCR system.

DNA methylation

Determination of DNA methylation status was carried out using methylation specific PCR (MSP) technique. The sequences of EGFR primer set were adapted from previous study (23). MSP primers for the methylated and unmethylated EGFR, were 5′-GGTTGGGTTTGTAAGTTCGC - 3′ (forward) and 5′- ATAAACAACGATAACCCCCG -3′ (reverse) and

5′- GGTTGGGTTTGTAAGTTTGT - 3′ (forward) and 5′- ATAAACAACAATAACCCCCA – 3′ (reverse), respectively. DNA was extracted from FFPE samples using EpiTect Plus Bisulfite Kit (Qiagen, Hilden, Germany). The extracted DNA was bisulfite converted using Epitect Bisulfite Conversion Kit (Qiagen, Hilden, Germany). MSP was performed using Epitect MSP Kit (Qiagen, Hilden, Germany). The amplification condition involved initial activation step (95°C for 10 minutes), denaturing (94°C for 15 seconds), annealing (56°C for methylated and 51.5°C for unmethylated) for 30s and an extension at 94°C for 30 s. Gel electrophoresis of MSP products was conducted on 2% agarose gel and visualized under UV illumination.

Analysis

The delta-delta Ct method was used in the analysis of qPCR results. Statistical analysis was carried out using GraphPad Prism 6.0 and Statistical Packages for Social Sciences (SPSS) software version 20.0. The variables and parameters were analysed using descriptive analysis. The association between the expressions and DNA methylation status of EGFR with demographic and clinicopathological characteristics were analyzed using Chi-square test. The p-value of less than 0.05 was considered significant.

Ethical clearance

This study was approved by National Medical Research Registry (NMRR), Ministry of Health (MOH) (Reference number: NMRR-12-1403-13767) and Ethics Committee for Research Involving Human Subjects (Universiti Putra Malaysia) (Reference number: FPSK_Mei(13)02).

RESULTS

Analysis of qPCR result revealed that EGFR was significantly upregulated in 54.2% (N=32) compared to adjacent normal samples using beta actin as reference gene as shown in Fig. 1 (p-value = 0.021). The mean fold change for EGFR expression was 18.22.

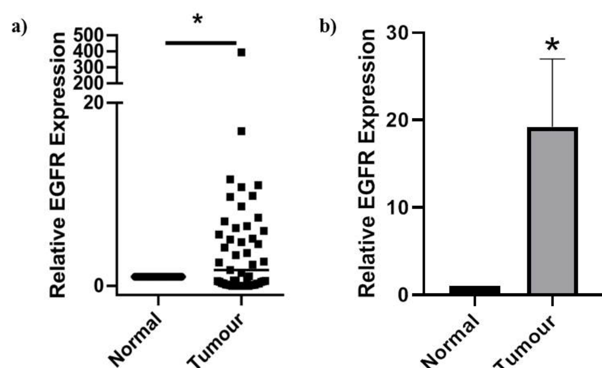

Fig 1: Relative expression of EGFR using β-actin as reference gene in CRC cases was shown in (a) and (b). (a) represents the distribution of 59 CRC and 59 normal samples according to the relative EGFR expression. EGFR was significantly up-regulated in CRC samples (54.2%) as compared to adjacent normal samples. (b) represents the average of fold change for the samples which was 18.22. (p<0.05).

The CRC and normal adjacent FFPE tissues were subjected to MSP for determination of DNA methylation status. A sample was classified as methylated if only the methylation amplification was seen. If both methylated and unmethylated amplifications were observed the sample was considered as partially methylated. When unmethylated amplification appeared alone or neither amplification products were found, the samples were labelled as unmethylated (23). For statistical analysis, the methylation status was classified into methylated (M) group (for methylated and partially methylated samples) and unmethylated (U) group (24).

EGFR was found to be methylated in 18.6% of CRC samples and unmethylated in 81.4%. Same trend was seen in normal adjacent tissues where EGFR were methylated and unmethylated in 20.3% and 79.7% of normal samples respectively (Fig. 2).

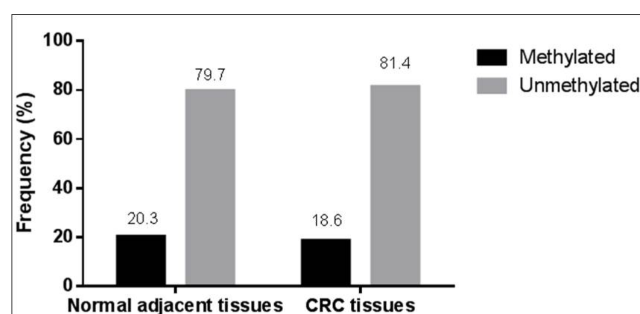

Fig 2: Frequency of methylated and unmethylated EGFR in CRC and normal adjacent FFPE tissues. EGFR was methylated in 18.6% of CRC samples and unmethylated in 81.4% of CRC samples. EGFR was methylated in 20.3% and unmethylated in 79.7% of normal adjacent tissues.

Fig. 3 showing the representative gel electrophoresis of amplicons from MSP using EGFR methylated primers (lane 1, 3 and 5) and unmethylated primers (lane 2, 4 and 6). In gel (a), a single band was seen only in lane with methylated primer indicating hypermethylation of EGFR in that sample. Two bands in gel (b) showing EGFR were partially methylated. A band was detected in gel (c) only at lane with unmethylated primer (lane 6) indicating unmethylation or hypomethylation of EGFR for that particular sample.

The association between EGFR expressions with DNA Methylation was determined using chi-square tests.

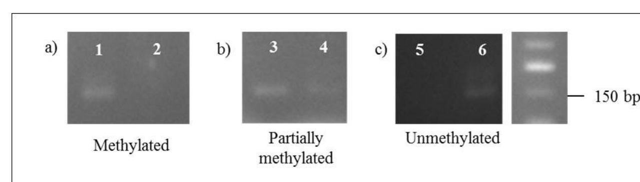

Fig 3: Representative gel electrophoresis of amplicons from MSP using EGFR methylated primers (lane 1, 3 and 5) and unmethylated primers (lane 2, 4 and 6). In gel (a), a single band was seen only in lane 1 indicating hypermethylation of EGFR. Two bands in gel (b) (lane 3 and 4) showing EGFR were partially methylated. A band in gel (c) (lane 6) indicates unmethylation of EGFR for that particular sample.

There was no apparent significant association between EGFR expression and DNA methylation of CRC FFPE samples identified (Table I). There were also no significant association of regulation and DNA methylation of EGFR with demographic and clinicopathological parameters of CRC FFPE samples (Table II).

Table I: Association between EGFR expression and DNA methylation of CRC FFPE samples

Gene expression	EGFR		Total	p-value
	Methylated	Unmethylated		
Upregulated	8	24	32	0.172
Downregulated	3	24	27	

DISCUSSION

Determination of gene expression and DNA methylation in FFPE tissues were successfully carried out. The most challenging issue in this study was to deal with the high degree of RNA and DNA degradation of the extracted FFPE tissues. There were several procedures performed to increase the quality and yield of extracted nucleic acids. For example, lysis process was used to remove the DNA-protein cross links (25) whereas the deparaffinization process will increase the DNA yield (26).

DNA methylation analysis using FFPE tissues was

Table II: Association between EGFR expression and DNA methylation status with demographic and clinicopathologic parameters

Parameters	EGFR regulation		*p*-value	EGFR Methylation status		*p*-value
	Up	Down		Methylated	Unmethylated	
Age (years)						
< 50	7	3	0.420	2	8	0.964
50-69	16	13		5	24	
>70	9	11		4	16	
Gender						
Male	16	16	0.543	6	26	0.982
Female	16	11		5	22	
Ethnicity						
Malay	14	10	0.297	4	20	0.905
Chinese	16	13		6	23	
Indian	2	3		1	4	
Others	0	1		0	1	
Tumour grading						
Well	3	7	0.139	2	8	0.809
Moderate	28	20		9	39	
Poor	1	0		0	1	
Dukes' staging						
A	1	2	0.584	0	3	0.652
B1	4	7		1	10	
B2	8	7		4	11	
C1	5	3		1	7	
C2	8	3		2	9	
D	6	5		3	8	
Tumour location						
Proximal	10	3	0.63	3	10	0.951
Distal	22	24		8	38	
Lymph node metastasis						
Yes	18	10	0.141	6	22	0.602
No	14	17		5	26	
Diabetes mellitus						
Yes	9	9	0.665	4	14	0.917
No	23	18		7	34	
Hypertension						
Yes	14	12	0.957	5	21	0.918
No	18	15		6	27	
Smoking						
Yes	3	2	1	2	6	0.496
No	29	25		9	45	
Family history						
Yes	3	3	1	0	6	0.494
No	29	24		11	42	

successfully carried out in many of previous studies (23,27) while other studies showed the reproducible result and good correlation between fresh tissues and FFPE tissues (28,29). These results are in line with a finding by Ludgate et al. (2017) (30) , which also successfully extracted DNA from FFPE tissues for DNA methylation downstream application. Zhang et al. (2017) has proved that FFPE material from CRC as a valuable source of materials for DNA methylation studies. (31). According to this study, FFPE specimens provide great value for high throughput genomic studies.

In this study, the mean fold change for EGFR expression was 18.22 indicating the overexpression of EGFR in CRC FFPE tissues compared to adjacent normal samples. This result suggests the vital role of EGFR in carcinogenesis of CRC. Our finding was supported by Motalleb et al. (2014) (32) that has discovered the EGFR gene was overexpressed in 80% of FFPE CRC tissues. An earlier study also showed that the EGFR gene was found in 95.6% of FFPE CRC metastatic tissues (33). High mRNA levels of EGFR was also found in FFPE samples of locally advanced rectal cancer (LARC) patients developing metastases (34).

Upregulation of EGFR was found to be associated with proliferation, differentiation and migration of cancer cells (35,36). EGFR executes these functions by promoting RAS/MAPK and PI3K/Akt signalling cascade (16). Several previous studies proved the correlation of EGFR overexpression with pathological severity. High expression of EGFR indicates the tumour is more severe. There was positive correlation between overexpression of EGFR with poor prognosis and aggressive tumour behaviour of CRC (37). The possibility of recurrence and death increased in rectal cancer cases with high levels of EGFR expression (34). This is consistent with what has been found in a study by Cui et al. (2012) in ovarian cancer (38). According to this study, overexpression of EGFR was higher in poorly differentiated and metastatic ovarian cancer. The expression of EGFR also was higher in advanced Dukes' stage of CRC or gastric cancer (39) and in poorly differentiated of non-small cell lung cancer (NSCLC) with high degree of invasion (40).

Aberrant DNA methylation can be either hypomethylation or hypermethylation and is usually associated with upregulation and downregulation of specific genes which leads to tumourigenesis. In this study, EGFR was hypomethylated in CRC FFPE tissues signifying the role of EGFR in pathogenesis of CRC. Hypomethylation is an early event in CRC (41) since high levels of hypomethylation were found in pre malignant lesions and subsequently in tumour progression of CRC (42,43). Our outcome is in accordance with a finding discovered by Demurtas et al. (2017) (44) which has discovered the hypomethylation of EGFR in 50% of CRC cases. Nonetheless, our data is contradictory with previous finding which revealed that almost 63% of CRC cases demonstrated 10% to 50% of EGFR methylation and 2% of cases with more than 50% of EGFR methylation using pyrosequencing (45).

The corresponding pattern of EGFR hypomethylation was found in the normal colon adjacent to the tumour. This finding was in parallel with a study by Sugai et al. (2017) (46) which also showed aberrant DNA methylation of SFRP1 and SFRP2 in both normal and CRC tissues. Aberration of DNA methylation in the normal colon adjacent to the tumour may indicate worse prognosis after removal of the tumours. This is due to field cancerization where the premalignant tissues appear normal histologically, but displayed genetic and epigenetic aberration. In addition, our results are in line with the fact that hypomethylation occurs at the early step in carcinogenesis (47). More study should be conducted focusing on biological alterations in the normal mucosa around a neoplastic lesion to help us to understand the mechanism by which the tumours interact with its microenvironment.

Our results have shown that there is no significant association between DNA methylation and overexpression of EGFR. This result is in line with previous studies by Chiadini et al. (2015) (45) and Scartozzi et al. (2011) (48) which have reported no correlation between methylation and expression of EGFR in CRC. On the contrary, Liu et al. (2017) (49) have found significant association of hypermethylated-low expressed EGFR in CRC. No association was also found between expression and DNA methylation status of EGFR with demographic and clinicopathological characteristics of CRC patients involved in this study. However, a study conducted by Teama and Agwa (2010) discovered significant association between EGFR expression with the gender and stage of CRC patients (50). Scartozzi et al. (2011) on the other hand found no significant association between aberrant DNA methylation of EGFR with any patients characteristics (48).

In our study, no significant association was found might be due to the low number of samples used. Larger sample size will increase the statistical power and the possibility to find significant relationships between variables are higher. In addition, more reliable association will be obtained in greater sample size. Apart from that, this study involves samples with high heterogeneity, such as age, ethnicity, gender and stage. Low sample size with high heterogeneity will display low correlation between two measurements.

CONCLUSION

In conclusion, qPCR and MSP serve as valuable tools for determination of gene expression even in FFPE samples. This study has also demonstrated that FFPE serves as valuable materials for genetic and epigenetic studies. Hypomethylation and overexpression of EGFR may play

a significant role in CRC even though our findings found no significant relationship between DNA methylation and mRNA expression. Hypomethylation is associated with aberrant gene activation that leads to increase in gene transcription and activation of oncogenes. Further investigation on prognostic value of EGFR hypomethylation is required.

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