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# ORIGINAL ARTICLE

# HER3 overexpression and hypomethylation in colorectal adenocarcinoma

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*Abstract*

*Introduction:* Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cancer in Malaysia. Despite advanced therapies, many cases of recurrence and resistance have been reported. Aberrant DNA methylation of HER3 has been implicated in carcinogenesis of CRC mainly through the regulation of gene expression. Hence, the objective of this study was to determine the status of HER3 DNA methylation and its effects on gene expression in CRC. *Materials and methods:* Fifty-nine of archival formalin-fixed, paraffin-embedded CRC cases with the adjacent normal colon tissues were retrieved. Manual micro-dissection was performed prior to RNA and DNA extraction. HER3 gene expression and DNA methylation status was evaluated by qPCR and methylation-specific PCR (MSP) techniques respectively. *Results:* Upregulation of HER3 mRNA was found in CRC tissue compared to its adjacent normal colon tissue (8.04-fold). Of 59 CRC samples, 8.5% were methylated and 91.5% were unmethylated (hypomethylation). In the adjacent normal colon tissues, methylated and unmethylated tissue were observed in 6.8%and 93.2% respectively. DNA methylation of HER3 showed a significant association with tumour differentiation and tumour location. *Conclusion:* This study showed upregulation and hypomethylation of the HER3 gene in CRC cases. Epigenetic alterations were also found in the adjacent normal colon tissues. Thus, upregulation and hypomethylation of HER3 may play a key role in carcinogenesis of CRC. Hypomethylation of CpG islands might be associated with early steps during carcinogenesis. The findings of this biomarker serve a powerful approach to improve the current diagnostic and therapeutic measures.

*Keywords:* Colorectal cancer, HER3, DNA methylation

## INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related mortality.[1,2] In Malaysia, CRC is the second most common malignancy.[3] Despite many advanced cancer therapies for CRC, there are considerable proportions of cancer patients who respond poorly to therapy and the number of resistance cases increases annually. The current biomarkers available are not very specific and not suitable for population-wide screening purposes. Hence, identification of reliable tumour biomarkers for early detection and therapeutic targets is critically important to reduce the incidence and mortality rate of CRC.

CRC arises as a consequence of accumulation of genetic and/or epigenetic alterations in colonic epithelial cells during neoplastic transformation.[4] These alterations contribute to abnormal expression of genes during carcinogenesis.

Epigenetics are changes that occur on chromosomes without altering its DNA sequence, leading to a heritable and stable phenotype.[5] The most common epigenetic alteration in human tumour is DNA methylation in specific gene promoters. DNA methylation is required for many physiological processes during normal development. However, aberrant DNA

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methylation can cause several pathologic changes including carcinogenesis through regulation on gene expression.[6]

The ErbB family also known as Human Epidermal Growth factor Related (HER) family consists of EGFR (ErbB1 or HER1), ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4).[7] HER receptors play important roles in cell growth, differentiation, proliferation, apoptosis, invasion, survival, and migration.[8,9] HER3 activation is dependent on binding to and/or heterodimerisation with other HER receptors. Aberrant expression of this family receptor is mostly due to overexpression, that can also be observed in other types of human tumours that occur via abnormal signalling of HER pathway.[8,10,11] Alterations due to methylation in the promoter of the tyrosine kinase receptors have been linked to tumour development and progression.[12] The association between aberrant expression and epigenetic alteration of this gene has not been clearly demonstrated. Hence, this study was aimed at determining the status of DNA methylation and its effects on HER3 gene expression in CRC.

## MATERIALS AND METHODS

*Primers*

HER3 primer was designed and synthesised by GeneCopieoa Inc (USA) with accession number NM_001005915 for quantitative Polymerase Chain Reaction (qPCR) method. The primer sequences for Beta actin (β-actin) as the reference gene was 5'-TCACCGAGCG CGGCT-3' (forward) and 5'-TAATGTCACGC ACGATTTCCC-3' (reverse). Methylation Specific PCR (MSP) primers for the methylated and unmethylated HER3, were 5'- ATTTTTAGGTAGGTAAGTGGCGCGA - 3' (forward) and 5'- TCCCAAATAATCCTA ACAAACCGAA-3'(reverse)and 5'- ATTTTTAGGTAGGTAAGTGGTGTGA - 3' (forward) and 5'- TCCCAAATAATCCTAAC AAACCAAA – 3' (reverse), respectively.

*Primary human tissue samples*

Ethics approvals were obtained from National Medical Research Registry (NMRR), Ministry of Health (MOH) and Ethics Committee for Research Involving Human Subjects (Universiti Putra Malaysia). Fifty-nine archival formalin-fixed, paraffin-embedded (FFPE) CRC cases with their adjacent normal colon tissues were retrieved from the Department of Pathology, Hospital Serdang. Manual micro-dissection of selected area of interest (tumour tissue and normal tissue) was performed.

*Quantitative Polymerase Chain Reaction (qPCR)*

Total RNA was extracted from the FFPE tissues using RNeasy FFPE Kit (Qiagen, Hilden, Germany). The purity and quality of RNA were measured using Nanodrop. RNA was then reversed transcribed to cDNA using QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). The qPCR was carried out using QuantiFast SYBR Green Kit (Qiagen, Hilden, Germany). 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. The delta-delta Ct method was used in the analysis of qPCR result.

*Methylation Specific Polymerase Chain Reaction (MSP)*

DNA was extracted from FFPE samples using EpiTect Plus Bisulfite Kit (Qiagen, Hilden, Germany). The extracted DNA was bisulfite converted using EpitectBisulfite 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 53.1°C for unmethylated) for 30s and an extension at 94°C for 30s. Gel electrophoresis of MSP products was conducted on 2% agarose gel and visualised under UV illumination. A sample was classified as methylated if only the methylation amplification was seen. If both methylated and unmethylated amplification were observed the sample was considered as partially methylated. Whereas when unmethylated amplification appeared alone or neither amplification products were found, the samples were labelled as unmethylated.[13] For statistical analysis, the methylation status was classified into methylated (M) and unmethylated (U) group.[14] The methylated group is composed of methylated and partially methylated samples.

*Data processing and statistical analysis*

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 HER3 with demographic and clinicopathological characteristics were analysed using Chi-square test.

## RESULT

Of the 59 CRC obtained the majority of patients (49.2%) were between 50 to 69 years old with average age of 63 years old and range 42 to 85 years old (Table 1). There were slightly more males (54.2%) than females (45.8%). Majority were Chinese 24 (49.2%), followed by Malays, 29 (40.7%), Indians 5 (8.5%) and others 1 (1.7%).

Most of the CRC cases were grade 2 (moderate) (81.4%) and located at the distal part of the colon (78%). A total of 47.5% cases showed lymph node metastasis. Comorbidity in CRC cases included diabetes (30.5%) and hypertension (44.1%). About 8.5% were smokers whereas only 6 CRC patients (10.2%) had family history of CRC. Refer to Table 2.

*Gene expression and MSP of HER3*

Relative quantities of HER3 mRNA in CRC FFPE tissues were expressed as N-fold difference in relation to adjacent normal tissues and normalised to the β-actin as a reference gene. HER3 were found to be upregulated in CRC with 8.04-fold (Fig. 1) compared to adjacent normal tissues. HER3 was methylated in 8.5% of CRC tissues (Fig. 2). The remaining 91.5% were unmethylated. The normal adjacent tissues showed similar pattern where 6.8% and 93.2% were methylated and unmethylated, respectively.

**TABLE 1: Demographic distribution of CRC cases**

| Parameters | n (%) |
|---|---|
| **Age (years)** | |
| < 50 | 10 (16.9) |
| 50-69 | 29 (49.2) |
| >70 | 20 (33.9) |
| **Gender** | |
| Male | 32 (54.2) |
| Female | 27 (45.8) |
| **Ethnicity** | |
| Chinese | 24 (40.7) |
| Malay | 29 (49.2) |
| Indian | 5 (8.5) |
| Others | 1 (1.7) |

However, none of the samples showing fully methylated result. Gel electrophoresis analyses of MSP products for FFPE samples (Fig. 3) displayed partially methylated (a) and unmethylated (b) result.

DNA methylation of HER3 showed significant association with tumour differentiation ($p$ = 0.035, $x^2$ = 6.709) and tumour location ($p$ = 0.007, $x^2$= 7.316) as shown in Table 3 while other parameters showed no association (Table 3). About 44 of patients with unmethylated HER3 were moderately differentiated whereas, 45 of tumours with unmethylated HER3 were located at the distal part of the colon. Nevertheless, DNA methylation of both tumour and normal samples were not significantly associated with HER3 mRNA expression (p>0.05) (Table 4).

**TABLE 2: Clinicopathologic distributions of CRC cases**

| Parameters | n (%) |
|---|---|
| **Tumour grading** | |
| Well | 10 (16.9) |
| Moderate | 48 (81.4) |
| Poor | 1 (1.7) |
| **Dukes' staging** | |
| A | 3 (5.1) |
| B1 | 11 (18.6) |
| B2 | 15 (25.4) |
| C1 | 8 (13.6) |
| C2 | 11 (18.6) |
| D | 11 (18.6) |
| **Tumour location** | |
| Proximal | 13 (22) |
| Distal | 46 (78) |
| **Lymph node metastasis** | |
| Yes | 28 (47.5) |
| No | 31 (52.5) |
| **Diabetes mellitus** | |
| Yes | 18 (30.5) |
| No | 41 (69.5) |
| **Hypertension** | |
| Yes | 26 (44.1) |
| No | 33 (69.5) |
| **Smoking** | |
| Yes | 5 (8.5) |
| No | 54 (91.5) |
| **Family history** | |
| Yes | 6 (10.2) |
| No | 53 (89.8) |

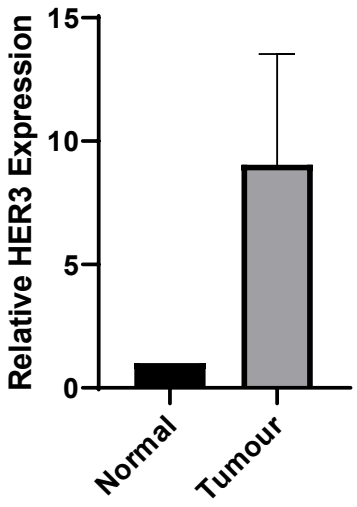


FIG. 1: Normalised expression ratio of HER3 using β-actin as reference gene. HER3 was upregulated in CRC samples as compared to adjacent normal samples.

## DISCUSSION

Colorectal cancer (CRC) is associated with genetic and epigenetic alterations. Recent studies have proved that profound genetic and epigenetic occur in cancer cells during the onset of tumourigenesis and tumour progression.[15,16] The most challenging issue in this study was to deal with high degree of RNA and DNA degradation of the extracted FFPE tissues. There are several procedures to increase the quality and yield of extracted nucleic acids. Lysis process was used to remove the DNA-protein cross links whereas the deparaffinisation process will increase the DNA yield.[17]

*HER3 overexpression*

In this study, overexpression of HER3 was found in CRC compared to adjacent normal samples indicating HER3 overexpression may play a key role in carcinogenesis of CRC. Previous studies have reported mRNA expression of HER3 in CRC[18–21] as well as in other types of cancer such as gastric cancer[22] and lung cancer.[23] HER3 expression impairs molecular signalling leading to excessive cell proliferation due to resistance of the cancer cells to growth inhibitory signals.[24] Moreover, activation of PI3K and AKT pathway signalling by HER3 results in carcinogenesis via biological processes such as translation, anti-apoptosis, cell control and survival.[25] Previous studies also highlighted the role of HER3 in carcinogenesis and resistance to treatment.[21] Zhang *et al*. (2018)[26] proved that cetuximab was able to improve the antitumour activity of AZD6244 by attenuating the activation of HER3/AKT pathway in HT-29 cells and in nude mice. Another study demonstrated that knockdown of HER3 leads to reduction of cell proliferation as well as induces apoptosis, and blocks migration of colon cancer cells.

*Hypomethylation of HER3*

In this study, more than two-third of the CRC samples were unmethylated with only 8.5% methylation were identified. DNA hypomethylation is a global feature of tumours. Previous studies have shown that hypomethylation of DNA was associated with the onset of tumour.[27] Hypomethylation was also found to play a significant, and complementary role in cancer progression.[28,29] Both of these cancer initiation and progression are due to promotion of

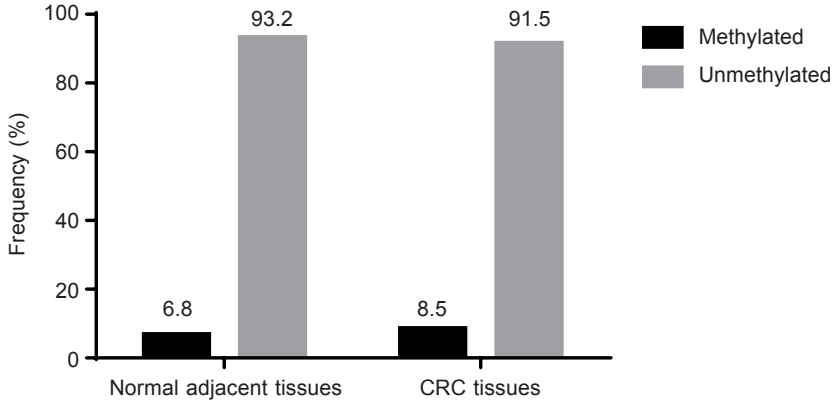


FIG. 2: Frequency of methylation and unmethylation of HER3 for CRC and normal adjacent FFPE tissues.

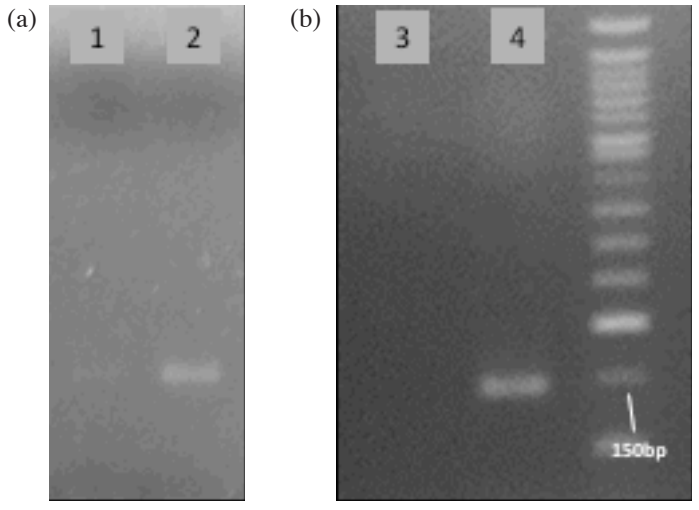


FIG. 3: Representative gel electrophoresis of amplicons from MSP using HER3 methylated primers (lane 1 and 3) and unmethylated primers (lane 2 and 4). Gel (a) showing sample with partially methylated result whereas gel (b) showing unmethylated result.

abnormal genes' activation and genomic instability by DNA hypomethylation.[30,31] Moreover, DNA hypomethylation also contribute to the carcinogenesis by enhancing recombination[32,33], activation of endogenous retroviral elements[34,35] and alteration of chromatin.[36] Hypomethylation and upregulation of HER3 were also found in papillary thyroid carcinoma.[37]

Aberrant DNA methylation pattern of HER3 showed significant association with tumour differentiation and location. In a previous study, HER3 overexpression has been discovered to be associated with low-grade tumour and distal colon location.[38] CRC originating from different parts of colon might have vital molecular differences that lead to different clinical outcome. Patients with right-sided CRC commonly show worse clinical outcome compared to the left-sided CRC.[39–41] Meta-analysis conducted by Yan and his colleague suggested that the overexpression of HER3 have a tendency to occur at left colon and in moderately or well differentiated tumours.[21]

Interestingly, in our study, the corresponding pattern of hypomethylation was found in the normal colon adjacent to the tumour. Our results were consistent with a study by Cheng *et al*.(2010)[42] which also showed hypomethylation of HOXA10 in normal and ovarian cancer. This might suggest the role of epigenetic alterations in carcinogenesis. This phenomenon is known as field defect. A field defect is a field of premalignant tissues in which a new cancer is likely to arise even though it appears to be histologically normal under the microscope. Cells within the field defect in our samples showing increased frequency of epigenetic alteration. This finding is supported by the previous studies.[43–46] Thus, hypomethylation may occur at the early step in carcinogenesis.[27] Hence, investigating molecular alterations in normal tissues adjacent to cancer is mandatory to understand cancer aetiology and to enable early diagnosis and identify molecular targets for chemoprevention.

On the other hand, our results showed that there is no relationship between DNA methylation and HER3 mRNA overexpression. However, DNA hypomethylation facilitates the aberrant expression of oncogenes that leads to transformation and/or tumour progression.[29] This is due to DNA hypomethylation of genes causes genomic instability and activates many genes in signalling pathway.

In conclusion, this study shows aberrant regulation and methylation of HER3, thus suggest the important role of this gene in tumourigenesis of CRC. Epigenetic alterations specifically aberrant DNA methylation are involved in cancer development and progression in addition to genetic mutation. Hence, HER3 has a potential as prognostic factor and therapeutic targets for CRC.

This study provides further information on the roles of HER3 in CRC carcinogenesis. The

**TABLE 3: Association between HER3 expression and DNA methylation status with demographic and clinicopathologic parameters**

| Parameters | HER3 regulation | | *p*-value | Methylation status | | *p*-value |
|---|---|---|---|---|---|---|
| | Up | Down | | Methylated | Unmethylated | |
| **Age (years)** | | | | | | |
| < 50 | 4 | 6 | 0.932 | 0 | 10 | 0.249 |
| 50-69 | 13 | 16 | | 2 | 27 | |
| >70 | 8 | 12 | | 3 | 17 | |
| **Gender** | | | | | | |
| Male | 12 | 20 | 0.410 | 4 | 28 | 0.460 |
| Female | 13 | 14 | | 1 | 26 | |
| **Ethnicity** | | | | | | |
| Malay | 12 | 12 | 0.590 | 1 | 23 | 0.447 |
| Chinese | 11 | 18 | | 4 | 25 | |
| Indian | 2 | 3 | | 0 | 5 | |
| Others | 0 | 1 | | 0 | 1 | |
| **Tumour grading** | | | | | | |
| Well | 5 | 5 | 0.510 | 0 | 10 | **0.035*** |
| Moderate | 20 | 28 | | 4 | 44 | |
| Poor | 0 | 1 | | 1 | 0 | |
| **Dukes' staging** | | | | | | |
| A | 1 | 2 | 0.592 | 0 | 3 | 0.588 |
| B1 | 6 | 5 | | 1 | 10 | |
| B2 | 5 | 10 | | 1 | 14 | |
| C1 | 5 | 3 | | 1 | 7 | |
| C2 | 5 | 6 | | 0 | 11 | |
| D | 3 | 8 | | 2 | 9 | |
| **Tumour location** | | | | | | |
| Proximal | 4 | 9 | 0.338 | 4 | 9 | **0.007*** |
| Distal | 21 | 25 | | 1 | 45 | |
| **Lymph node metastasis** | | | | | | |
| Yes | 12 | 16 | 0.943 | 3 | 25 | 0.905 |
| No | 13 | 18 | | 2 | 29 | |
| **Diabetes mellitus** | | | | | | |
| Yes | 8 | 10 | 1 | 1 | 17 | 0.979 |
| No | 17 | 24 | | 4 | 37 | |
| **Hypertension** | | | | | | |
| Yes | 10 | 16 | 0.589 | 2 | 24 | 0.848 |
| No | 15 | 18 | | 3 | 30 | |
| **Smoking** | | | | | | |
| Yes | 3 | 2 | 0.408 | 0 | 5 | 0.336 |
| No | 22 | 32 | | 5 | 49 | |
| **Family history** | | | | | | |
| Yes | 3 | 3 | 0.691 | 0 | 6 | 0.990 |
| No | 22 | 31 | | 5 | 48 | |

**TABLE 4: Association between HER3 expression and DNA methylation of CRC FFPE samples**

| | HER3 | | Total | *p*-value |
|---|---|---|---|---|
| | Methylated | Unmethylated | | |
| **Gene expression** | | | | |
| Upregulated | 1 | 24 | 25 | 0.558 |
| Downregulated | 4 | 30 | 34 | |

findings of this biomarker serve a powerful approach to improve the current diagnostic measures and to optimize therapeutic decision-making through development of potential novel biomarkers. Hopefully, the number of CRC morbidity and mortality can be reduced with earlier diagnosis of CRC. Hence, further investigation on DNA hypomethylation is required. More study should be conducted focusing on biological alterations in the normal mucosa around neoplastic lesions.

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*Authors'contribution:* R.O: Study plan and conception, laboratory work, data collection, interpretation and manuscript writing. N.M: Supervision, Review and Editing. N.M.A.Z: Data collection. H.F.S: Review and Editing. K.W.N: Sample interpretation. M.O: Review and Editing. All authors have read and approved the final manuscript.

*Conflict of interest:* The authors declare no conflict of interest.

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