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Overexpression of Wildtype *Periostin* and *Transforming Growth Factor Beta I* Genes in Colorectal Carcinoma: A Preliminary Study

Chia Sze Wooi* **and Edmund Sim Ui Hang**
Immunology Laboratory, Department of Molecular Biology,
Faculty of Resource Science and Technology, Universiti Malaysia Sarawak,
94300 Kota Samarahan, Sarawak, Malaysia
**E-mail: szwchia@gmail.com*

ABSTRACT

The majority of deaths from colorectal carcinoma (CRC) occur due to metastasis during the late stage of tumourigenesis. Recently, *periostin*, i.e. a gene encoding a protein which is initially found in osteoblasts, has been reported to be associated with the late-stage tumourigenesis in colon and a variety of human cancers. The researchers investigated the expression of *periostin* mRNA in normal and tumour biopsy specimens using the RT-PCR analysis to elucidate the role of *periostin* in human colorectal carcinoma. The results showed that there was a significantly (P<0.05) higher expression of the *periostin* mRNA in the biopsy specimens obtained from the tumour tissues, as compared to the normal tissues. Nevertheless, sequence analysis revealed no mutation in the full length of the *periostin* gene. As the over-expression of *periostin* in human colorectal carcinoma did not appear to be due to the mutation in the *periostin* gene, the involvement of other collaborative factors was therefore deduced. Consistent with this finding, the researchers focussed on studying the *transforming growth factor (TGF)* β_1 which has been reported to be associated with the increasing in the expression of *periostin*. The analysis (RT-PCR) in this study revealed that *TGF-*β_1 gene was also highly expressed in tumour biopsy specimens (P<0.05). This gene mutation is also absent. These data validated that both *periostin* and *TGF-*β_1 work together to control colorectal organogenesis.

Keywords: Metastasis, colorectal carcinoma, *periostin*, tumourigenesis, RT-PCR, *TGF-*β_1

INTRODUCTION

Colorectal carcinoma (CRC), which is also known as the cancer of the colon and rectum, was recorded as the most commonly diagnosed cancer in men, and the third most common cancer in women (Malaysia National Cancer Registry, 2003). Typically, major fatalities from colorectal cancer are due to the dissemination of the primary tumours, which lead to formation of metastases which are resistant to conventional chemotherapy (Fidler, 1990). This event, known as metastasis, is the hallmark of malignant cancers. Usually, it occurs during the late stage of tumourigenesis to the liver (Galandiuk *et al.,* 1992). For metastasis to take place, the tumour cells must undergo a series of interrelated steps which involve numerous complex molecular interactions (Fidler, 1990; Scanlon and Murthy, 1991).

In the process of identifying genes associated with tumour metastasis, *periostin*, a gene encoding a protein with similarity to the fasciclin family (Takeshita *et al.,* 1993), has been shown to promote tumour metastasis and angiogenesis

*Corresponding Author

of the colon cancer (Bao *et al.,* 2004). Formally known as the osteoblast-specific factor 2 (OSF-2), *periostin* is found to be over-expressed in several human tumours including ovarian carcinoma (Gillan *et al.,* 2002), colon cancer (Bao *et al.,* 2004; Sim *et al.,* 2006), breast cancer (Shao *et al.,* 2004), nasopharyngeal carcinoma (NPC) (Chang *et al.,* 2005), oral cancer (Siriwardena *et al.,* 2006), head and neck squamous cell carcinoma (HNSCC) (Kudo *et al.,* 2006) and papillary thyroid carcinomas (Puppin *et al.,* 2008). *Periostin* activates the serine/threonine kinase (Akt/PKB) signaling pathway, which is known to increase cellular and endothelial cell survival, by promoting angiogenesis (Bao *et al.,* 2004). An exposure of colorectal cancer cells to anti-*periostin* antibodies activated apoptosis and potentiates the effects of 5-fluorouracil chemotherapy (Tai *et al.,* 2005). Therefore, further studies are suggested to target this protein as a therapeutic option in colorectal cancers.

Although several *periostin* regulating genes have been reported, including bone morphogenetic protein-2 (BMP-2) (Lindner *et al.,* 2005), the bHLH transcription factor (TWIST) (Oshima *et al.,* 2002) and fibroblast growth factor 2 (FGF2) (Li *et al.,* 2004), transforming growth factor beta (*TGF-β*) has been identified as one of the possible candidates for the regulating factor which is responsible for the over-expression of *periostin* in the colorectal cancer in this study, since *periostin* contains similar structure to *β*ig-h3, a molecule induced by *TGF-β*. This gene plays an important role in controlling proliferation, differentiation, and is involved in many important cellular functions (Derynck *et al.,* 2001; Xie *et al.,* 2003). Three highly homologous isoforms of the *TGF-β* (*TGF-β_1, TGF-β_2, and TGF-β_3*) have been reported in mammals (Friedman *et al.,* 1995). Mutations have been reported in transforming growth factor beta receptor two (*TGF-βRII*) gene (Takenoshita *et al.,* 1997).

To date, there has been no report written on the mutation analysis of the *TGF-β_1* gene in colon cancer. The presence of *TGF-β* has been reported to increase the expression of *periostin* in primary osteoblast cell (Horiuchi *et al.,* 1999). In addition, *TGF-β* has also been shown to be involved in tumour progression by modulating angiogenesis in colorectal cancers (Xiong *et al.,* 2002). There has been no report on the expression or mutation analysis of the human *periostin* gene in colorectal cancer. Hence, this study aimed to look at the association between the over-expression with the mutations which might be present within the full length sequences of human *periostin,* as well as in the *TGF-β1* gene.

MATERIALS AND METHODS

Total RNA Specimens

The total RNA from the colorectal biopsy tissues (labelled in numeric number) were provided by the Institute for Medical Research (IMR), Kuala Lumpur (courtesy of Pauline Balraj). The samples from the IMR were initially provided by Hospital Universiti Kebangsaan Malaysia (courtesy of Prof. Dr. A. Rahman A. Jamal) to the IMR, where the total RNA extraction using trizol method was subsequently carried out. The research group was provided with these samples (total RNAs only) as the group is a part of the national team in the multi-institutional research programme approved by the National Biotechnology Directorate (a division within the Ministry of Science, Technology and Environment), and coordinated by the IMR. Two sets of commercially available total RNA, designated as CN and CT, were purchased from BD Biosciences, Inc., USA and Chemicon, Inc., USA. The total RNA was quantitated using a spectrophotometer (Biochrom, England).

Primer Design

The upstream and downstream synthetic oligonucleotide primers were constructed using Primer 3.0 free-ware (*http://frodo.wi.mit.edu/*). The primers for the genes of interest were *periostin* (5'-AATCATCCATGGGAACCA GA-3' and 5'-TATTCACAGGTGCCAGC AAA-3'), *TGF-β_1* (5'-CCCGTCGGTCGCTAG

CTC-3' and 5'- CGTGTACTGGCCGTTACC TT-3') and *GAPDH* (5'-TGCACCACCAA CTGCTTAGC-3' and 5'-GGCATGGACTG TGGTCATGAG-3').

Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) Analysis

The first-strand cDNA was prepared using Moloney Murine Leukemia Virus (MMLV) RT enzyme and a range of reagents from Promega, USA. The PCR was performed using PTC-200 Peltier Thermal Cycler, with an initial 2 minutes of denaturation at 95˚C, followed by 35 cycles of 30 seconds of denaturation at 95˚C, 45 seconds of annealing at 58ºC, 1 minute of elongation at 72˚C, and ended with 5 minutes of final elongation at 72˚C. The total 25µl PCR reaction volume contained 1µl of first-strand cDNA template, 1X PCR buffer, 1.5mM $MgCl_2$, 0.2mM each of dNTP, 1µM of upstream and downstream *periostin* or *TGFβ_1* primers and 1 unit of Go*Taq* DNA polymerase (Promega, USA).

Cloning of Partial cDNA Fragments and Sequencing

Purified PCR fragments were cloned into Promega pGEM®-T Easy Vector System II and transformed into chemically competent *E. coli* JM109. Positive colonies with insert of interest were screened using T7 and SP6 promoter primers. The positive clones were selected for the downstream plasmid DNA isolation, using Promega Wizard® *Plus* Minipreps DNA Purification Systems and the procedures were carried out according to the manufacturer's instructions. After that, the isolated plasmids were sent to FirstBase Sdn. Bhd. for sequence acquisition.

Statistical Analysis

The RT-PCR images were captured using the Alpha DigiDoc™ Imaging System and the quantification of the bands intensity (ng) was performed using the AlphaEase®FC Stand Alone software. The statistical values were analysed using the SPSS Paired Sample t-Test to check for significant differences between the normal and tumour samples (in this case, *P*-value < 0.05 is considered as significant).

RESULTS AND DISCUSSION

Expression Analysis of Periostin *and* TGF-β_1 *mRNA*

Quantitative RT-PCR yielded the PCR product with band size of 602 bp from the eighteen samples. Over-expression of *periostin* was observed in all nine tumour samples. In this study, a significant difference ($P<0.05$) was found in the level of mRNA expression of *periostin* between the local or commercial normal and tumour samples. Nevertheless, the *periostin* expression level was not compared among the tumour and metastatic tumour since the samples used in this study were mainly of Dukes'B stage. Overall, although the sample size was rather small, the consistency of the preliminary result with the findings of Bao *et al.* (2004) and Sim *et al.* (2006) further supported the expression behaviours of *periostin* in colorectal carcinoma. For the analysis of *TGF-β_1*, a product band of 869bp was amplified from all the normal and tumour samples. The expression of *TGF-β_1* gene was found to be significantly higher in all cases of colorectal tumour samples ($P<0.05$). This is consistent with the other findings (Friedman *et al.,* 1995; Xiong *et al.,* 2002), in which *TGF-β_1* demonstrated high levels of expression in the tumour samples from colorectal carcinoma.

In this study, *GAPDH* was used as the house-keeping gene to show that all the samples tested had equimolar starting concentrations. The values of both genes (*periostin* and *TGF-β_1*) were normalised with the *GAPDH* values before it was continued with the statistical analyses (Table.1). The representative RT-PCR results for *periostin* and *TGF-β_1* are shown in *Figs. 1* and *2*, whereas *Fig. 3* shows a gel photo of the *GAPDH* expression.

TABLE 1
Distribution of the samples according to their origin and the RT-PCR signal of *periostin* and *TGF-β_1*

No.	Samples		RT-PCR value (ng) after normalisation with GAPDH	
	Local biopsy	Commercially available	*Periostin*	*TGF-β_1*
1		CN1	1.25	5.40
2		CT1	1.55	6.80
3		CN2	0.24	0.05
4		CT2	0.63	3.50
5	61N		0.30	1.66
6	61T		0.54	2.10
7	63N		0.31	0.00
8	63T		0.54	1.57
9	67N		0.45	3.00
10	67T		0.57	3.10
11	69N		0.41	1.92
12	69T		0.57	3.10
13	43N		0.13	0.07
14	43T		0.57	0.10
15	44N		0.07	1.58
16	44T		0.46	1.70
17	53N		0.13	0.02
18	53T		0.46	3.10

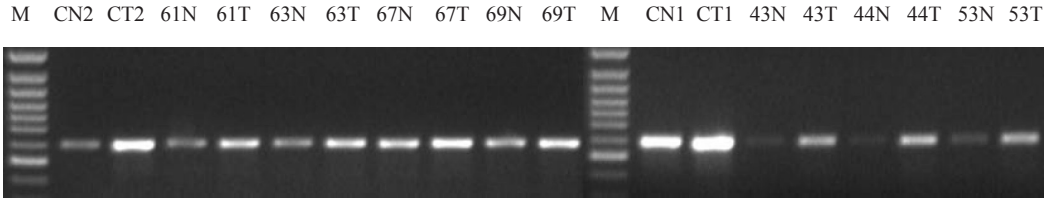

Fig. 1: Agarose gel (1.5% w/v) electrophoresis analysis of the PCR amplified periostin *gene with lane M, 100bp DNA ladder (Fermentas), lane 2-11 and lane 13-20 represent amplicon (602bp) from nine normal (N) and tumour (T) tissues. Note that all samples were paired normal and tumour tissues, except for the CN/CT*

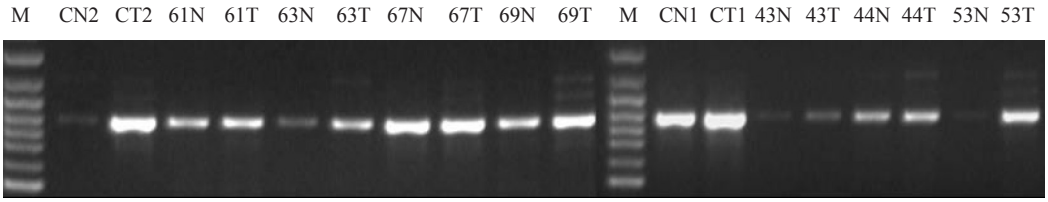

Fig. 2: Agarose gel (1.5% w/v) electrophoresis analysis of the PCR amplified TGF-β_1 *gene with lane M, 100bp DNA ladder (Fermentas), lane 2-11 and lane 13-20 represent amplicon (869bp) from nine normal (N) and tumour (T) tissues. Note that all the samples were paired normal and tumour tissues, except for the CN/CT*

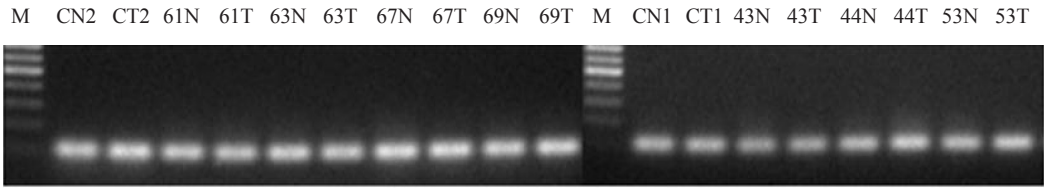

Fig. 3: Agarose gel (1.5% w/v) electrophoresis analysis of the housekeeping gene GAPDH with lane M, 100bp DNA ladder (Fermentas), lane 2-11 and lane 13-20 represent amplicon (87bp) from nine pairs of normal (N) and tumour (T) tissues

Mutational Analysis of Periostin *and* TGF-β_1 *Gene*

The full length of the *periostin* gene, including all nine tumours and five normal samples (CN1, CN2, 44N, 67N, 69N) were sequenced. The sequence analysis of all the nine tumour samples showed no mutation throughout the 2,511 bp open reading frame; however, for samples 53T, 61T and 69T, there is a 16bp nucleotide repeat inserted after the termination codon in the 3' untranslated region (*Fig. 4*). After that, 69T was compared to the corresponding paired 69N and found that the repetition fragment was not present in the normal. Nevertheless, the

TCTCAG*TGA*AAATCCAAAAACCAGAAAAAAATGTTTATACAACCCTAAGTCAATAACCTG
ACCTTAGAAAATTGTGAGAGCCAAGTTGACTTCAGGAACTGAAACATCAGCACAAAGAAG
CAATCATCAAATAATTCTGAACACAAATTTAATATTTTTTTTTCTGAATGAGAAACATGAG
GGAAATTGTGGAGTTAGCCTCCTGTGG**AGTTAGCCTCCTGTGG**TAAAGGAATTGAAG
AAAATATAACACCTTACACCCTTTTTCATCTTGACATTAAAAGTTCTGGCTAACTTTGGAA
TCCATTAGAGAAAAATCCTTGTCACCAGATTCATTACAATTCAAATCGAAGAGTTGTGA
ACTGTTATCCCATTGAAAAGACCGAGCCTTGTATGTATGTTATGGATACATAAAATGCAC
GCAAGCCATTATCTCTCCATGGGAAGCTAAGTTATAAAAATAGGTGCTTGGTGTACAAAA
CTTTTTATATCAAAAGGCTTTGCACATTTCTATATGAGTGGGTTTACTGGTAAATTAT
GTTATTTTTTACAACTAATTTTGTACTCTCAGAATGTTTGTCATATGCTTCTTGCAATGC
ATATTTTTTAATCTCAAACGTTTCAATAAAACCATTTTTCAGATATAAAGAGAATTACTTC
AAATTGAGTAATTCAGAAAAACTCAAGATTTAAGTTAAAAAGTGGTTTGGACTTGGGAA

Fig. 4: Nucleotide 2,514 – 3,213 of human periostin nucleotide sequence. The stop codon, TGA (nucleotide 2,520-2,522), is italicized and highlighted. The putative 16bp sequence is underlined and the repeated sequence is highlighted and denoted in bold

researchers were unable to sequence the other paired normal (53N and 61N), and since the samples provided from the IMR were limited in quantity. Due to the fact that the number of the samples used in this study was rather small, future studies with more samples are required to ascertain the findings of this study. As for the *TGF-β_1*, the sequence analysis of all nine tumours and two normal samples (CN2 and 67N) showed no mutation throughout the gene. Together, these data suggested that the over-expression of both *periostin* and *TGF-β_1* was not due to the mutation of the gene itself.

CONCLUSIONS

The expression of *periostin* and *TGF-β_1* mRNA was detected at a significantly higher level in the tumour biopsy specimens suggesting that both genes might play a role in the carcinogenesis of colorectal cancer. However, no mutation was detected in the coding region of *periostin* and *TGF-β_1* genes, suggesting that their over-expression might be due to the effect of other cis-trans elements. Future studies, consisting of more samples, are therefore required to ascertain the findings of this study.

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