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Expression of *Periostin* and Identification of *Periostin*-Isoforms in Colorectal Carcinoma

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ABSTRACT *Periostin*, a gene that is initially associated with bone and tooth development, is linked to tumourigenesis of colorectal carcinoma (CRC). Increasing evidence via functional study revealed this protein as an oncogenic promoter or, in certain circumstances, as a tumour suppressor. Therefore, in order to verify the role of *periostin* in CRC, we performed gene expression study using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis in local and commercially available colorectal cancer samples. Expression of *periostin* mRNA was detected at a significantly higher level in the tumour specimens ($P < 0.05$), suggesting this gene as a putative oncogene. Further studies using PCR and gene sequencing assays revealed the presence of ten *periostin* isoforms that harbour deletion at specific C-terminus region, possibly caused by alternative splicing events. Three isoforms were identical to published data of *periostin* (GenBank), while the remaining variants that have not been reported may provide new information on the genetics of colon cancer.

ABSTRAK *Periostin*, sejenis gen yang terlibat dalam perkembangan tulang dan gigi, telah dikaitkan dengan ketumbuhan kanser (tumourigenesis) kolorektal. Bukti-bukti melalui kajian tentang fungsinya menunjukkan bahawa protein ini memainkan peranan sebagai pemangkin oncogen, atau, sebaliknya sebagai penghalang ketumbuhan tumor. Oleh itu, untuk memastikan peranan gen *periostin* dalam kanser kolorektal, analisis pengekspresan telah dijalankan dengan menggunakan teknik separa-kuantitatif RT-PCR pada sampel biopsi tempatan dan komersial. Kadar pengekspresan gen *periostin* didapati lebih tinggi dalam spesimen tumor ($P < 0.05$), ini menunjukkan gen ini berkemungkinan berperanan sebagai oncogene. Kajian seterusnya menggunakan teknik PCR dan analisis jujukan gen menunjukkan kehadiran sepuluh isofom gen *periostin* yang mengalami kehilangan bahagian C-terminal tertentu, mungkin disebabkan acara alternative splicing. Tiga isofom adalah sama dengan data gen *periostin* yang telah diterbitkan dalam GenBank, manakala isofom-isofom lain yang belum dikenal pasti akan membekalkan informasi baru dari segi genetik dalam kanser kolorektal.

(CRC, tumourigenesis, *periostin*, RT-PCR, C-terminal)

INTRODUCTION

Colorectal carcinoma (CRC) is a cancer of the colorectal system that usually arises from benign adenomatous polyps which can occur by hereditary or sporadic (non-inherited) mean. In Malaysia, the National Cancer Registry (2003) rated CRC as the commonest cancer among men (14.2%) and third most common cancer in women (10.1%), with the highest incidence rate in the Chinese population compared to other ethnic groups. Studies have identified several critical genetic alterations that somehow lead to predisposition of colorectal cancer, among which are mutation in *APC*, *K-Ras* and *p53* genes [1].

The development of metastatic tumours consists of a series of interrelated steps including proliferation of neoplastic cells, initiation of angiogenesis, penetration into circulation, detachment of cell aggregates, survival in the circulation, cell arrest in the distant capillary beds, extravasation and proliferation within the organ parenchyma [2]. Since metastasis is responsible for most cancers death, attempt to identify genes involves in the metastatic process offers a promising approach for cancer therapy.

Recent findings revealed *periostin* is closely related to the late stage tumourigenesis in colon cancer [3]. *Periostin* was initially isolated from mouse osteoblastic cell line, MC3T3-E1, as osteoblast specific-factor 2 [4]. It has a typical N-terminus signaling peptide sequence and four repeated domains (RDs) which shares homology with the insect axon guidance protein FAS-1[5]. *Periostin* was initially found to be expressed in osteoblast cell as a cell adhesive molecule and is involved in osteoblast recruitment, attachment, and spreading [4]. Recent studies revealed its expression in developing teeth, embryo and adult heart, vascular remodeling and a variety of cancer cells [6, 7, 8, 9, 10, 3].

Differential expression pattern of *periostin* is discovered in a variety of human cancers. Mechanistically, *periostin*-mediated tumour metastasis is associated with the ability to enhance cellular survival under stress conditions by activating the Akt/PKB signaling pathway [3]. It acts as an angiogenic factor that triggered over-expression of vascular endothelial growth factor receptor Flk-1/KDR through an integrin $\alpha_v\beta_3$-focal adhesion kinase-mediated signaling pathway. Hence, epithelial cell tumours have the potential to promote the proliferation, migration, and vessel formation activities [11]. These reports suggest that increased *periostin* expression could promote metastasis. The role of *periostin* in the development of human cancers has been a controversial issue since the expression of *periostin* was detected to be over-expressed in ovarian epithelial cells [10], colon cancer tissues [3], breast cancer tissues [11], non-small cell lung cancers [12], soft tissue liposarcomas [13], nasopharyngeal carcinoma [14], oral cancer [15], head and neck cancer [16] and gastric cancer [17], but is found to be down-regulated in bladder cancer [18], and lung cancers [19]. In a previous study by us [20], *periostin* was discovered to exhibit remarkably high expression in the tumour sample via microarray approach. To further establish the profile of *periostin* activity in colorectal cancer, we studied the expression of *periostin* mRNA in a bigger sample size using semi-quantitative RT-PCR.

In addition, we studied the isoforms of *periostin* since isoforms of *periostin* have been isolated from periodontal ligament (PDL), periosteum, heart valves, ovarian cancer and bronchial asthma [5, 4, 21, 22, 10, 23], but never before reported in CRC. The alternatively spliced isoforms consist of deletion or addition of one or more exonic region at the C-terminus. This event gives rise to multiple isoforms, which largely undiscovered. To identify possible *periostin* isoform in CRC, we performed RT-PCR assay and sequencing analysis in normal and tumour colorectal samples. Since little is known about the *periostin* isoforms and its functions in human colorectal cancer, these findings will provide important information of *periostin*'s involvement in the development of colorectal carcinoma.

MATERIALS AND METHODS

Tissues and Total RNA

Local CRC biopsy samples from 10 patients with stage II and III adenocarcinoma, including 7 female and 3 male patients were used in this study. These paired biopsies designated in numeric form were initially provided by Hospital Universiti Kebangsaan Malaysia (courtesy of Prof. Dr. A. Rahman A. Jamal) to the Institute for Medical Research (IMR), where total RNA extraction using trizol method was subsequently carried out. Our group was provided with these samples (total RNAs only) as it is 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 IMR. In this study, two sets of total RNAs in used were purchased from BD Biosciences (Clontech, USA) and Chemicon® (International, Inc., USA) and were designated as CN2, CT2 and CN3, CT3. The normal local biopsies were paired (adjacent) to the tumour tissues except for commercial samples. The total RNAs from local biopsies samples were isolated using Trizol (Invitrogen, USA) method. Frozen tissue was cut into small pieces and the tissue was homogenized using polytron homogenizer with one microliter of Trizol reagent. After incubation at RT for 10 minutes, 200 µl of chloroform were added and the mixture was allowed to stand at RT for 3 minutes, and then centrifuged at 10,000 rpm for 15 minutes at 4°C. The colourless layer (top layer) was transferred into a new tube, followed by addition of 0.5 ml isopropanol to precipitate the RNA. Following incubation at RT for 10 minutes, and centrifugation at 10,000 rpm for 10 minutes at 4°C, the pellet was initially washed with 75% ethanol (1 ml ethanol/ 1ml Trizol ratio) and further washed with 1 ml of absolute ethanol.

The air-dried pellet was dissolved in 70 µl of elution buffer.

Semi-quantitative RT-PCR assay

Total RNA (1µg) from commercially available sources and local biopsies samples were reverse transcribed using Moloney Murine Leukemia Virus (MMLV) RT enzyme (Promega, USA) in the presence of 0.5 µg $Oligo(dt)_{15}$ primer (Promega, USA), 1 X MMLV buffer, 1 u/µl RNasin® Plus RNase Inhibitor, 0.2 mM dNTPs and ultra-pure water to a final reaction volume of 20 µl. The reaction mixture was incubated at 42°C for 1 hour, heated to 70°C for 15 minutes and chilled on ice for subsequent amplification. One to two microlitres of cDNA was used as template in PCR amplification with Go*Taq* DNA polymerase (Promega, USA). Because of the limited amount of total RNA available, *β-actin* primer was included together with *periostin* primer in a duplex PCR manner to determine the sample to sample variations in total RNA amounts. *β-actin* primer was used because it represents classical control for quantitative RT-PCR experiments. Both primer pairs were optimized to ensure the specific amplification of the PCR products and the absence of any primer or artifacts. The PCR reaction was carried out using PTC-200 Peltier Thermal Cycler (MJ Research) 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 57°C, 1 minute of elongation at 72°C; and ending with 5 minutes of final elongation at 72°C. Amplified PCR products were assessed on 2% agarose gels in 1XTAE via electrophoresis. The details of *periostin* and *β-actin* primers used in duplex PCR (OSF and ACTB), and primers for amplifying *periostin* isoforms (OSF-T1, OSF-T2 and OSF-T3) are shown in Table 1.

Quantitative analysis and statistical methods

The RT-PCR images were captured using Alpha DigiDoc™ Imaging System and quantification of the bands intensity (ng) was performed using AlphaEase®FC Stand Alone software. The intensities of each spot, quantified after background correction, were normalized with internal control for initial variations in sample concentration. For both quantitative and statistical analysis, the values for *periostin* and *β-actin* from duplex PCR were averaged from 4 replicates. Statistical values were analyzed using SPSS Paired Sample T-Test to check for significant differences between normal and tumour samples (P-value < 0.05).

Cloning and sequence analysis

The PCR products from ten sets of normal and tumour samples (43, 44, 53, 56, 61, 63, 67, 69, C2 and C3) were excised and purified, prior to cloning into pGEM®-T easy vector (Promega, USA). Plasmids with insert of interest were purified using Wizard® *Plus* SV Minipreps (Promega, USA) and sent to First Base Laboratory Sdn. Bhd. for sequence procurement.

RESULTS

Periostin is differentially expressed in tumour colorectal carcinomas

To determine if the *periostin* is over-expressed in tumour colorectal tissues, we examined the total RNA of normal and tumour colorectal tissues via RT-PCR assay using specific primer designed from mRNA region. The PCR product (594bp) that was procured from 24 samples (Figure 1) showed consistent expression of *periostin* in the normal tissues and up-regulated expression in the tumour counterpart. *β-actin* (an estimated product size of 234bp) was used as the control in this study. Quantitative analysis (in fold change) of all tumour samples showed *periostin* to exhibit higher expression relative to paired normal. *Periostin* expression higher than 1 fold was frequently observed in the tumour samples (58.33%, 7 out of 12 pairs). We only observed 33.33% (4 out of 12 pairs) that showed a normal tumour ratio of higher than 2 fold, and 8.33% (1 out of 12 pairs) that was higher than 5 fold. Out of the 12 CRC cases, there were 10 and 2 cases of stage II and III, respectively. We were unable to compare the expression (fold change) between both stages since the sample size of stage III was small. Therefore, we compared all the 12 pairs of *periostin* tumour expression relative to its paired normal, and the expressions were statistically significant (P =0.009).

Table 1. List of primers used in PCR experiments

PRIMER NAME	SEQUENCE (5' → 3')	NUCLEOTIDE POSITION	SIZE (bp)	Ta (°C)
OSF (F)	TCAGCACAAAGAAGCAATCA	2,620 – 3,213	594	58
OSF (R)	TTCCCAAGTCCAAACCACTT			
OSF-T1 (F)	CTTCAAAGAAATCCCCGTGA	1,991 – 2,404	304, 243, 153	57
OSF-T1 (R)	AAATGACCATCACCACCTTCA			
OSF-T2 (F)	AACCAAAGTTGTGGAACCAA	2,033 – 2,461	429, 339, 237, 149	57
OSF-T2 (R)	AACTTCCTCACGGGTGTGTC			
OSF-T3 (F)	GGAAATGATCAACTGCTGGAA	1,917 - 2,461	467, 376, 292, 202	57
OSF-T3 (R)	AACTTCCTCACGGGTGTGTC			
ACTB (F)	GGACTTCGAGCAAGAGATGG	736 - 969	234	58
ACTB (R)	AGCACTGTGTTGGCGTACAG			

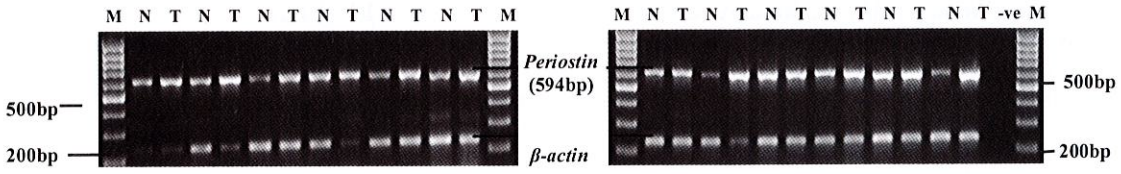


Figure 1(a). Relative expression of *periostin* and *β-actin* between normal (N) and tumour (T) samples on 2.0% (w/v) agarose gel. Product size for *periostin* was sequenced and confirmed as 594bp.

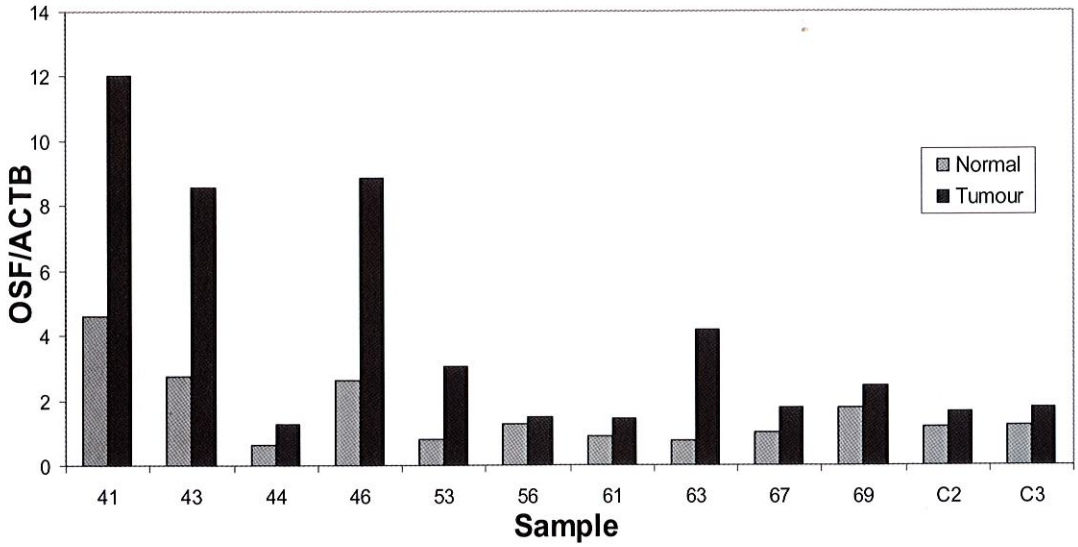
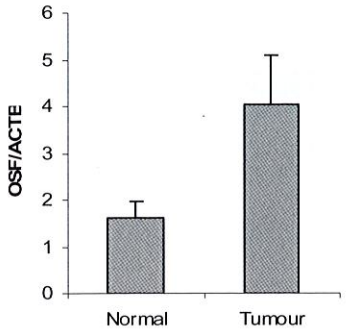


Figure 1(b). Bar chart showing the mean values of normalized data of *periostin/β-actin* mRNA ratios in 12 CRC paired samples. All samples were paired normal and tumour tissues except for commercial C2 and C3. For each sample, measurements were done in 4 replicates.



	MEAN	N	STD. ERROR MEAN
Normal	1.63	12	0.34
Tumour	4.05	12	3.67

Figure 1(c). Mean expression between the normal and tumour CRC samples studied. Error bars represent standard deviations (SD). The average OSF/ACTB ± SD value is 0.009 ± 2.6. The table beside the graph shows statistical analysis of the data.

Generation and analysis of ten periostin isoforms

To establish a comprehensive profile for *periostin* isoform dominantly expressed in colorectal tissue, we continue to analyze the cDNA by PCR using three sets of primers on 20 samples (8 paired local biopsies and 2 set of commercially available samples). The paired primers (OSF-T1, OSF-T2 and OSF-T3), were designed to flank C-terminal region that are different between *periostin* and its isoforms (region between Exon 16 and Exon 22). Multiple segments of 3' end of the *periostin* cDNA were generated by PCR from both the normal and its counterpart, and subsequently cloned. Sequence analysis showed that there were ten isoforms of *periostin* generated by deletion of one or more exons. As shown in Table 2, most of the deleted exon comprises Exons 17, 18, 19 and 21. We aligned *periostin* and its putative isoforms (Figure 2) and found that all forms contain a N-terminus signal peptide, a cysteine-rich domain, four internal homologous repeats, and a C-terminal hydrophilic domain.

Among the ten isoforms identified, Isoform 3 is identical to GenBank data of *periostin* with accession no: BC106710, BC106709 and D13665, whereas Isoform 4 and 5 are identical to the data with Accession: AY140646 and AY918092. Single amino acid changes were detected in six isoforms (Figure 2). In *periostin* Isoform 1 and 4, the amino acid glycine (G) at 697 amino acid (aa) position is replaced with arginine (R). For both Isoform 3 and 5, glutamine (E) at 727 aa is replaced with lysine (K), and for Isoform 6 and 7, glycine (G) residue at 757 aa is replaced with serine (S). Although deletion or insertion involved the entire exon, however, the number of nucleotide residues in some exon is not multiple of three, therefore leading to a frame-shift in translation in certain isoforms.

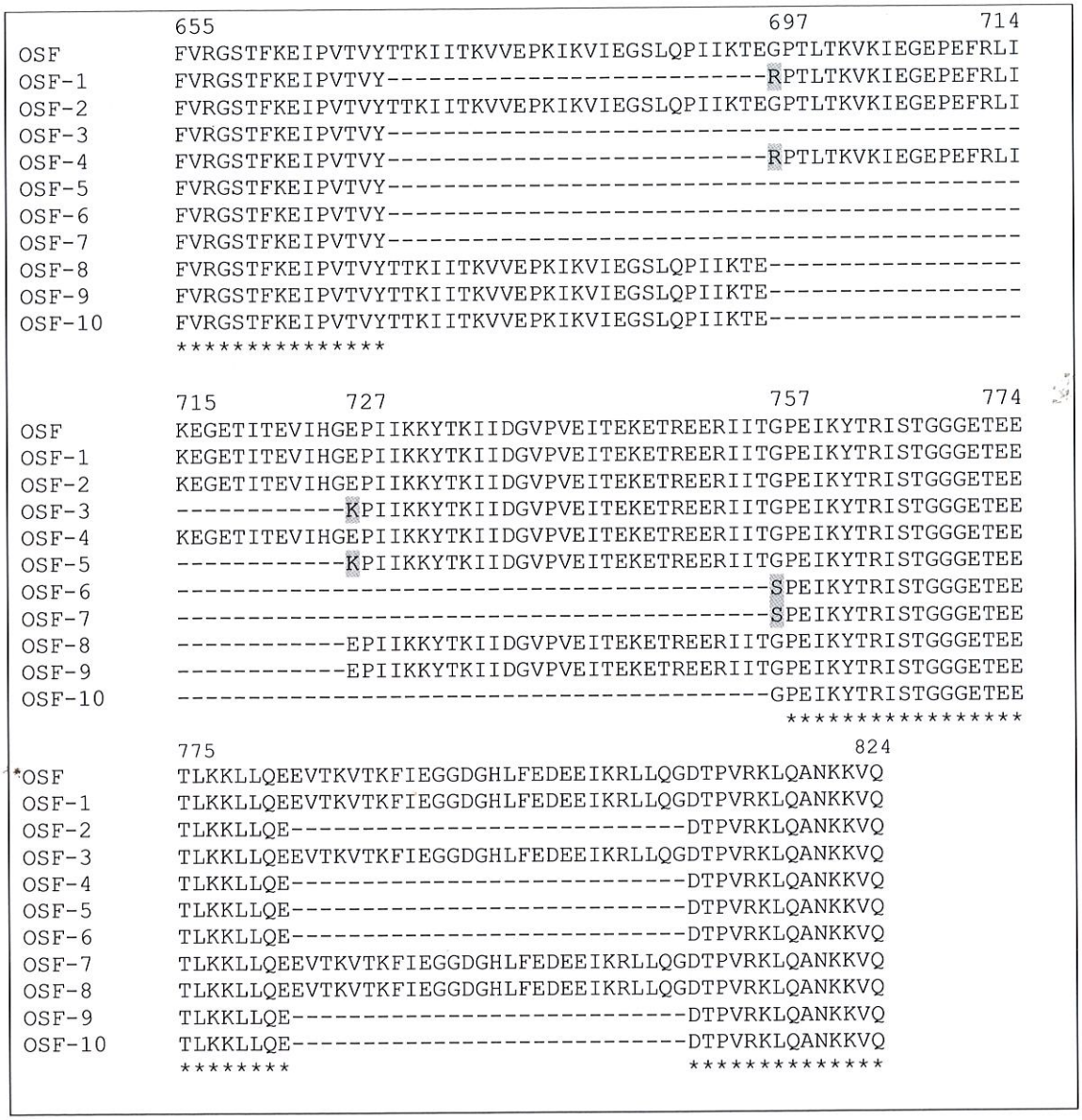


Figure 2. Alignment of periostin protein (OSF) with the proposed *periostin* isoforms (OSF-1 to OSF-10) in the region spanning from Exon 16 (655 aa) to Exon 22 (824 aa), with the region containing most of the differences between 670 and 810 aa. *Periostin* (OSF) is designated by the accession number NM_006475.

Table 2. Exon arrangement of *periostin* (NM_006475) and *periostin* isoforms

ISOFORMS	EXON	SIZE (bp)
Periostin	E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, E17, E18, E19, E20, E21, E22, E23	3,213
1	E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, Bbb, E18, E19, E20, E21, E22, E23	3,132
2	E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, E17, E18, E19, E20, Bbb, E22, E23	3,129
3	E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, Bbb, E18, E19, E20, E21, E22, E23	3,042
4	E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, Bbb, E18, E19, E20, Bbb, E22, E23	3,048
5	E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, Bbb, E18, E19, E20, Bbb, E22, E23	2,958
6	E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, Bbb, E18, E19, E20, Bbb, E22, E23	2,868
7	E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, Bbb, E18, E19, E20, E21, E22, E23	2,952
8	E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, E17, E18, E19, E20, E21, E22, E23	3,123
9	E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, E17, E18, E19, E20, Bbb, E22, E23	3,039
10	E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, E17, E18, E19, E20, Bbb, E22, E23	2,949

Bbb Deleted exon(s) region

DISCUSSIONS

To date, altered *periostin* expression pattern is mostly reported to be associated with late stage tumourigenesis (particularly metastasis) in several human cancers [10, 3, 11, 16]. There are also studies that showed down-regulated expressions of *periostin* in certain cancers and a variety cancer cell lines [19, 18]. Hence, the actual role of *periostin* in human cancers remains unclear. Here, we reported the over-expression of *periostin* in all (100%) colorectal carcinoma tissues studied. Our finding, which tally with the finding of Bao *et al.* (2004) in colon cancer, suggests that over-expression of *periostin* correlates with tumour development in CRC. Previous studies [3, 11, 15] also showed that *periostin* is involved in invasion, angiogenesis and metastasis in cancer cells. However, we are unable to compare the expression pattern with the tumour invasiveness since our samples are mainly of primary tumour tissues (stage II).

We found that *periostin* was expressed in several isoforms that differ in its 3' region in both normal and tumour samples. *Periostin* C-terminus which is believed to undergo splicing events eventually gives rise to different splice variants. Further analysis in this segment by sequence analysis showed that the spliced regions can be further classified according to Exon 17, 18, 19 and 21 (in this study), or into six different cassettes [4]. Horiuchi *et al* (1999) reported that deletion or insertion of any of the fragment will not lead to a frame-shift in translation since the number of nucleotides residues of the fragment is a multiple of three, however, we discovered that for certain isoform where the numbers of nucleotide residues do not qualify for a complete reading frame, frame-shift in translation occurs. Consequently, different amino acid was generated. Alteration of single amino acid was observed in Isoform 1, 3, 4, 5, 6 and 7 (Figure 2). The significance of this alteration in *periostin* isoforms remains to be elucidated.

The differences between the *periostin* isoforms may have significant functional consequences. Yoshioka *et al.* (2002) and Kim *et al.* (2005) have shown that the C-terminus of *periostin* is required for the tumour suppressor activity of this protein. Mutant with deleted C-terminal region showed weaker suppressor function of cell invasiveness than the mutant with only the C-terminal region [18]. However, Yoshioka *et al* (2002) revealed the suppressive function of C-

terminal region in cases whereby the N-terminus is truncated. *Periostin* mutant lacking the signal peptide (N-terminus) are not secreted into the surrounding and remain in cells. Interestingly, we found a putative nuclear localization signal (NLS) at 815 aa – 831 aa in the C-terminal end using motif scan. This was also reported by Litvin *et al* (2004) in mouse *periostin*. Theoretically, a NLS directs transportation of peptide to the nucleus. This was supported by detecting periostin protein with deletion of N-terminal region in both cytoplasm and nucleus by fluorescence microscopy [19]. In other words, the secretory form of periostin protein may confer an advantage by altering the microenvironment of cells allowing the induction of metastasis or angiogenesis. Thus, the N-terminal region containing the typical signal peptide sequence may also play a role in the expression of cell invasiveness. In our study, although NLS was detected in all *periostin* isoforms, N-terminal region was present in all ten isoforms including the full length *periostin*, suggesting *periostin* is in their secretory form in CRC. This further strengthened the notion that *periostin* could be a putative oncogene in tumourigenesis of CRC. We found that most of the isoforms identified lack either one or more exons in the C-terminus. Since C-terminus of *periostin* harbour the suppressive function, *periostin* isoforms with spliced C-terminal may lose its suppressor activity and become oncogenic. This suggests that *periostin* isoforms with differentially spliced C-terminus may be expressed differentially during development of late stage CRC.

Our study introduced for the novel notion that *periostin* isoforms have a role in CRC. However, much has yet to be done to examine the expression and the possible function(s) of *periostin* isoforms. Two areas of investigation are crucial for future work. Firstly, to study the expression of the identified ten *periostin* isoforms at the protein level using Western blot approach. Secondly, to test the role of each *periostin* isoforms in CRC, using *in vitro* technique for more comprehensive functional and gene-gene interaction studies.

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