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Quantitative analysis of the expression of *p53* gene in colorectal carcinoma by using real-time PCR

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Abstract. Colorectal carcinoma ranks third among ten leading causes of cancer in Malaysia. The colorectal carcinoma tumourigenesis involves the inactivation of tumour suppressor genes, and activation of proto-oncogenes. The *p53* is one of the tumour suppressor genes that is involved in the colorectal carcinogenesis. The *p53* gene is located on human chromosome 17p13.1 and comprises of 11 exons. Deficiencies in the *p53* gene can cause the cancerous cells to spread to distant organs such as liver, lungs, lymph nodes, spine and bone. The most common *p53* abnormalities that can lead to the metastasis of colorectal tumours are mutation and deregulation of the gene. In this study, nine colorectal carcinoma samples were used to establish a simple and sensitive strategy in the study on *in vivo p53* expression by using real-time LightCycler SYBR Green I technology.

INTRODUCTION

In Malaysia, colorectal carcinoma is the third leading cause of cancer in the population (Lim & Halimah, 2003). Colorectal carcinoma develops through a cascade of histopathological and genetic defects, which is known as adenoma to carcinoma sequence (Kinzler & Vogelstein, 1996). The *p53* genetic aberration is involved in the late stage of colorectal tumourigenesis (Vogelstein & Kinzler, 1996). This tumour suppressor gene has been mapped to human chromosome 17p13.1 and it plays an important role in cellular growth control (McBride *et al.*, 1986). Under stress condition, the wild type *p53* binds to DNA, stimulates transcription of several genes and mediates cell cycle arrest in the G1 phase for DNA repair or initiates apoptosis when the damaged DNA cannot be successfully repaired (Kumudini *et al.*, 2002). If the *p53* gene is mutated, the damaged DNA remains unrepaired and mutations become fixed in the dividing cells. Subsequently, malignant transformation of the cancerous cells occurs and starts to accumulate the mutated *p53* gene (Kumudini *et al.*, 2002).

Consequently, mutations and deregulation of the *p53* gene are commonly found in cancers (Wunderlich *et al.*, 2000). More than 75% of colorectal carcinomas are due to mutation in the *p53* gene (Erhan *et al.*, 2002), while over-expression of the gene is associated with advanced malignancies (Auvinen *et al.*, 1994). Recent preliminary reports revealed that over-expression of *p53* are an indicator of poor prognosis and survival in the patient (Remvikos *et al.*, 1992; Sun *et al.*, 1992; Zhao *et al.*, 2005). However, other reports correlated low expression of the *p53* gene and adverse outcome (Kumudini *et al.*, 2002). Whether *p53* expression level is associated with patient survival and stages of the diseases in colorectal tumour is still uncertain and remains to be scrutinized.

More studies are needed to solve these controversies.

A method to accurately and efficiently detect *p53* gene expression is urgently needed because of the prognostic importance of this marker. Therefore, in this study, we have developed a strategy by using real-time PCR to compare level of *p53* mRNA transcripts in colorectal carcinomas with the normal mucosal tissues.

MATERIALS AND METHODS

Tissue samples

This study was based on total RNA extracted from colorectal carcinomas of nine patients. Normal mucosal tissues were taken from the distant resection margin from the same patients. Among the nine paired samples, eight total RNA samples (C039, C044, C046, C050, C051, C053, C067 and C069) were provided by Dr Edmund Sim Ui Hang, Universiti Malaysia Sarawak (UNIMAS). One fresh tumour and its corresponding normal tissue were obtained from a patient who underwent surgical resection at the Damansara Specialist Centre (6N and 6T). One normal control total RNA (C045N) was taken from the Molecular Pathology Unit, Institute for Medical Research (IMR). Details of the patients are given in Table 1.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from frozen tissue using RNA Pure Kit (Roche Diagnostics GmbH Mannheim, Germany). The cDNA was reverse transcribed from 150 ng of total RNA by using 1st Strand cDNA Synthesis Kit with random hexamers (Roche Diagnostics GmbH Mannheim, Germany).

Relative gene expression by quantitative real-time PCR

To quantify the amount of mRNA in tumour and the corresponding normal mucosa, we performed real-time PCR (LightCycler 1.5) by using DNA Master SYBR Green 1 (Roche Diagnostics GmbH Mannheim, Germany). A PCR reaction mixture of 20 µl containing 5 mM of $MgCl_2$, 2 µl of SYBR Green 1 mix, 2-4 µl of cDNA and a pair of primers. Two pairs of primers were separately used: one pair to amplify the p53 gene, the other pair for the endogenous control gene, *GAPDH*. The forward and reverse primers for *p53* and *GAPDH* genes were designed according to Ohtani *et al*. (2003) and Bong (2005), respectively. The oligonucleotides used were as follow: *p53*: 5'-AGA GTC TAT AGG CCC ACC CC-3' and 5'-GCT CGA CGC TAG GAT CTG AC-3', *GAPDH*: 5'-CAT GGG GAA GGT GAA GGT CGG A-3' and 5'-TTG GCT CCC CCC TGC AAA TGA G-3'.

Real-time PCR was carried out on LightCycler instrument (Roche Diagnostics) in capillary glass tube. The amplification program consisted of 1 cycle of 95°C with a 10 min hold, followed by 40 cycles of 95°C with a 10 s hold, annealing temperature at 64°C (*p53*) or 58°C (*GAPDH*) with a 5 s hold, and 72°C with a 10 s hold. Additional step was added for fluorescence data acquisition at an elevated temperature for *p53* amplification, which is 89°C with a 1 s hold. This is followed by melting curve analysis, which ran for 1 cycle at 95°C with a 0 s hold, 64°C (*p53*) or 58°C (*GAPDH*) with a 15 s hold, and 95°C with a 0 s hold at the step acquisition mode. A negative control was included in each run to access specificity of primers and possible contamination.

Standard curves were generated using diluted purified *p53* and *GAPDH* PCR products synthesized from normal colon designated as 045N. The crossing points (Ct) were determined as the PCR cycle at which increased reporter fluorescence above the baseline could be detected. The gene expression could be obtained via the logarithmic plot of fluorescence signal above the background noise. Then, the gene expression or concentration of *p53* gene was normalized with *GAPDH*. The relative quantification of *p53* gene was calculated using the following formula:

$$\text{Ratio} = \frac{a / b}{c / d}$$

where a: measured expression of *p53* gene in tumour sample
b: measured expression of the housekeeping gene, *GAPDH* in tumour sample
c: measured expression of *p53* gene in normal sample
d: measured expression of the housekeeping gene, *GAPDH* in normal sample

The PCR efficiencies for the amplification of the *p53* and *GAPDH* genes were calculated using the following formula:

$$\text{PCR efficiency} = 10^{-1/\text{slope}}$$

RESULTS

All colorectal samples tested using real-time SYBR Green I technology showed no primer-dimers in the melting curve analysis of *GAPDH* (Figure 1). Primer-dimers were observed in the *p53* amplification. Therefore, an additional step was performed for fluorescence data acquisition at an elevated temperature of 89°C to eliminate the primer-dimers formation. The melting curve for *p53* gene before and after elevation temperature are shown in Figures 2(a) and (b).

Standard curves for the *p53* and housekeeping gene, *GAPDH* were successfully obtained (Figures 3 and 4). The PCR amplification efficiencies for *p53* and *GAPDH* genes were 2.0 and 1.8, respectively (Figures 3 and 4). Linear regression for analysis of standard curves showed high correlation: r = - 0.99 and r = -1.00 for *p53* and *GAPDH*, respectively. The number of target genes was extrapolated from a standard curve equation and the relative quantification of colorectal carcinomas examined were calculated and summarized in Table 1.

DISCUSSION

Our results revealed expression of *p53* mRNA in all samples analysed. Among 9 samples examined in our study, 2 samples (C044 and 6T/6N) revealed up-regulation of *p53* transcripts by 1.8-fold and 1.4-fold, respectively. The C044 and 6T/6N were from 2 patients with different sex, age, tumour location and differentiation. In

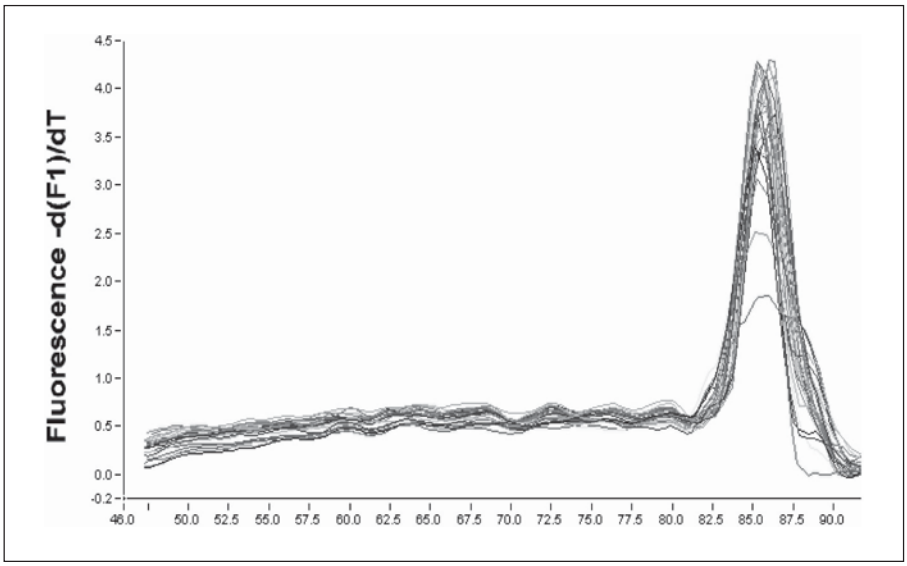

Figure 1. The melting curves of *GAPDH* gene for all samples tested. One specific peak was observed and no primer-dimers were formed during the amplification.

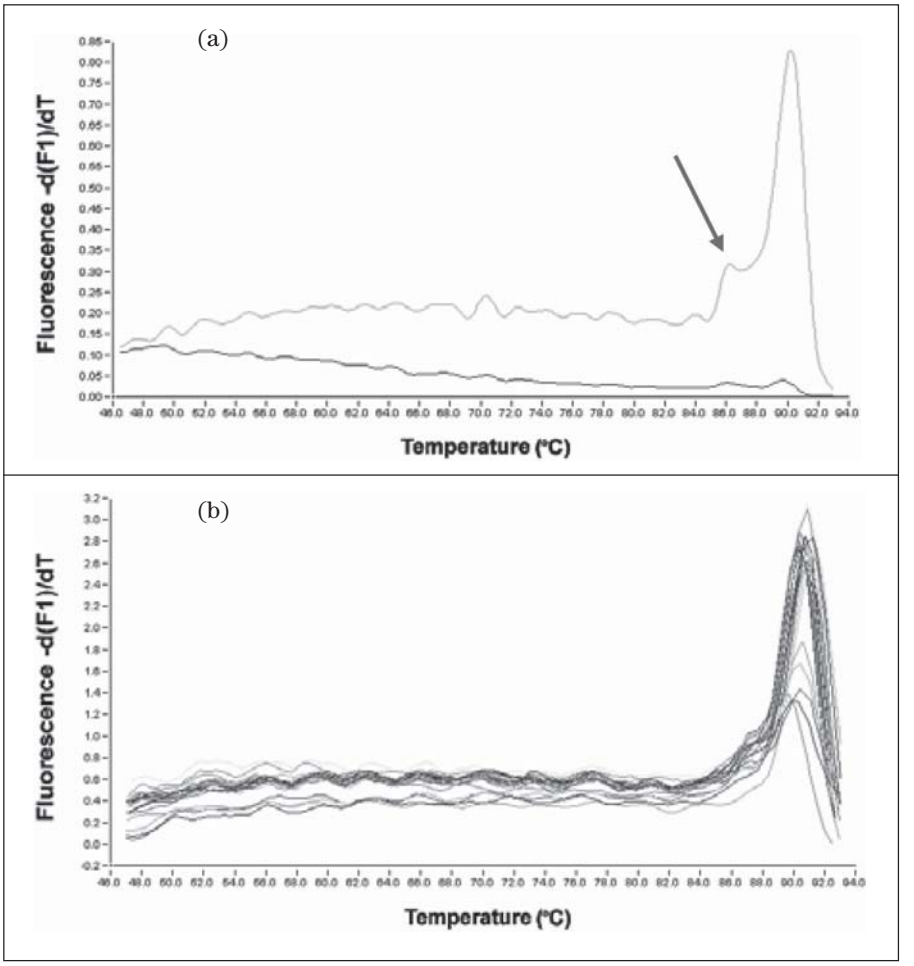

Figure 2. (a) Melting curve analysis of *p53* gene before elevation of temperature generated primer-dimers after 30 cycles of amplification. The arrow shows the unspecific amplification. (b) The *p53* amplification with elevated temperature at 89°C generated a specific peak in the melting curve.

other aspect, 6T/6N was diagnosed as Dukes' C adenocarcinoma with a lymph node invasion while Dukes' stage for C044 is unknown. Although tumour stage for C044 is unknown but we predicted it as late Dukes' B tumour since the cancerous cells has invaded the serosa layer of the colon. Based on our findings, we revealed that over-expression of *p53* mRNA in colorectal carcinoma is not significantly related with patients' sex, age, tumour location and differentiation. It is most likely associated with tumour stages, mostly in higher or advanced tumours. Our finding is consistent with Garrity *et al.* (2004), in which *p53* over-expression is more frequently detected in Dukes' C rather than Dukes' B tumours. However, further study using larger sample size with different tumour stages is needed to validate our findings.

Although our results need further investigation, we have established an approach to quantify *p53* expression by using LightCycler SYBR Green I technology. We measured *p53* expression at an elevated temperature of 89°C to eliminate fluorescence signal that was caused by primer-dimers. The real-time PCR analysis showed a sensitivity of *p53* detection of up to 4 X 10^{-4} ng of cDNA in normal control. Unlike the *p53* ampli-

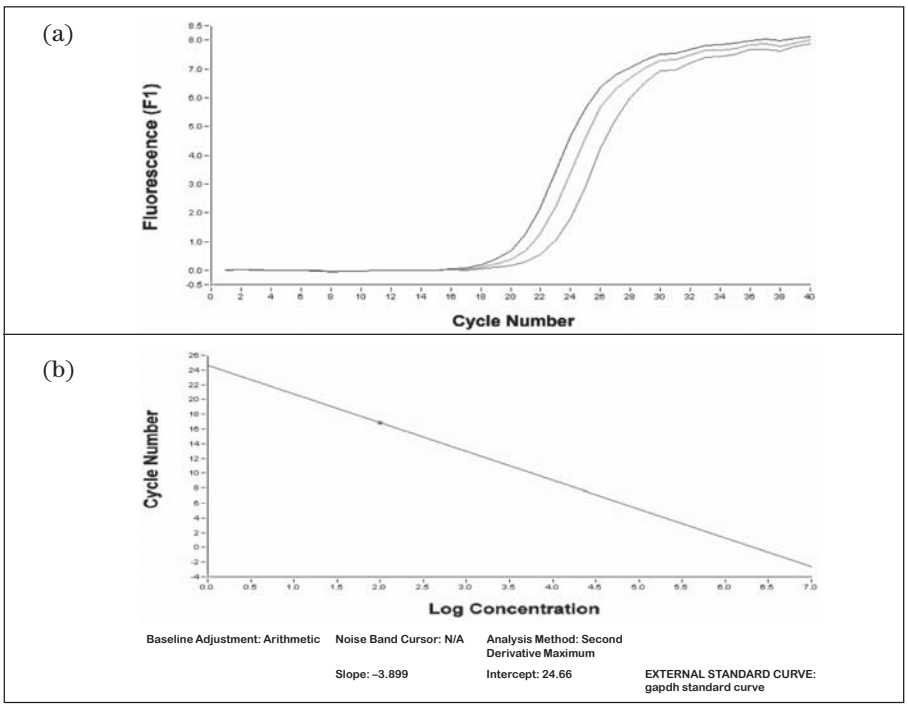

Figure 3. (a) The standard curve for *GAPDH* gene was successfully obtained by using serial dilutions purified target PCR products. (b) The standard curve was imported to create the scatter plot of the log concentration vs cycle number with r = -1.00.

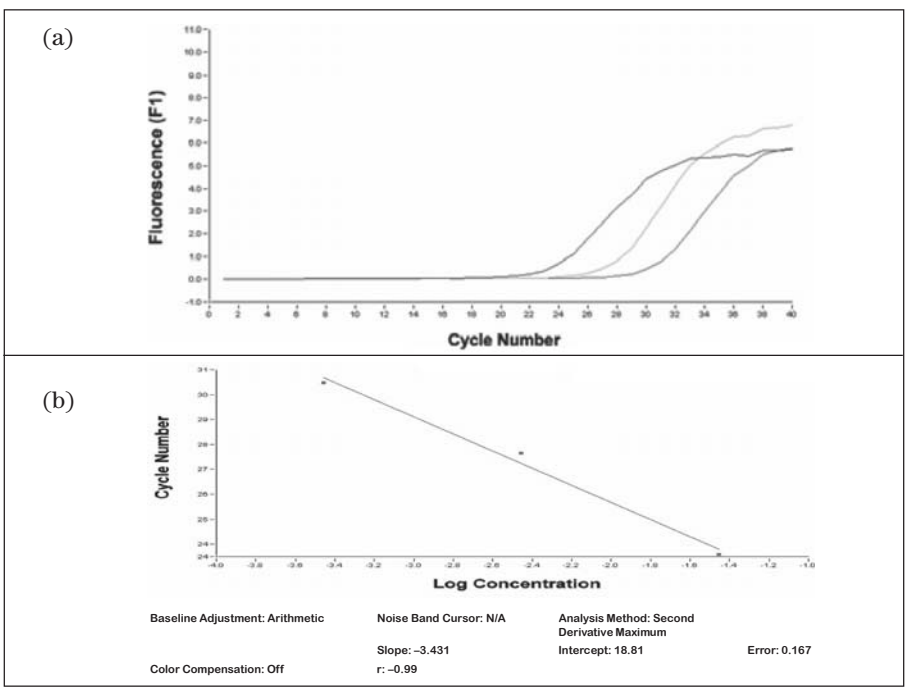

Figure 4. (a) The standard curve for *p53* gene was successfully obtained by using serial dilutions purified target PCR products. The standard curve contained 3.5 x 10^{-2} ng, 3.5 x 10^{-3} ng and 3.5 x 10^{-4} ng of cDNA. (b) The log concentration was plotted against the crossing points (cycle number) to create a *p53* standard curve with r = -0.99.

Table 1. Expression of *p53* in nine histopathologically confirmed colorectal patients. Ratio in positive indicates higher expression while negative indicates lower expression in tumour

Sample No.	Age	Gender	Race	Diagnosis	Ratio *p53* mRNA: tumour/normal
C039	67	M	M	Colon: microglandular adenocarcinoma, Dukes' B.	-0.2
C044	73	F	C	Rectosigmoid: well-differentiated adenocarcinoma with serosal margin invasion.	+1.8
C046	66	M	M	Rectosigmoid colon: moderate well-differentiated adenocarcinoma, Dukes' B.	-2.2
C050	66	F	C	Sigmoid & ascending colon: well-differentiated adenocarcinoma, Dukes' B.	-2.0
C051	61	F	C	Sigmoid colon & ileum: well-differentiated adenocarcinoma, Dukes' B.	-1.6
C053	53	F	M	Rectosigmoid colon: moderate-differentiated adenocarcinoma, Dukes' B.	-1.9
C067	NA	F	C	Colon: well-differentiated adenocarcinoma with deep margin involvement, Dukes' B. Tubulovillous adenoma with an invasive adenocarcinoma within its core.	-2.1
C069	NA	F	C	Colon (hepatic flexure): poorly-differentiated adenocarcinoma, Dukes' B2.	-1.5
6T/6N	71	M	M	Rectosigmoid: moderate-differentiated adenocarcinoma with involvement of a lymph node. Dukes' C	+1.4

fication, no elevated temperature step is needed to amplify the *GAPDH* gene. Apart from that, the PCR efficiencies and r-values for *p53* (PCR efficiency = 2.0; r = -1.00) and *GAPDH* (PCR efficiency = 1.8; r = -0.99) are acceptable in our study (Grunenwald *et al*., 2005).

By using real-time PCR approach, the risk of contamination is reduced, as it requires no post-PCR sample preparation. In addition, the detection of PCR products occurs during the log phase of amplification instead of the plateau phase, which gives a more accurate quantification of target concentration. Our study shows that real-time PCR is a simple, reliable and sensitive assay for quantifying *p53* expression at the mRNA level with excellent turnaround time (less than 1 hour). This protocol is suitable for use in cancer diagnosis. Future study using hybridisation probes instead of SYBR Green I reaction will enable more specific and optimum amplification.

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