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Short Communication

RNA profiling of cyclooxygenases 1 and 2 in colorectal cancer

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Cyclooxygenases (particularily Cox-2) are involved in carcinogenesis and metastatic cancer progression. The expression profiles of the cyclooxygenases and the roles they play in established tumours of similar stage remains unclear. We report that Cox-1 and Cox-2 expression is highly variable in Dukes' C tumours, and changes in Cox-1 expression may be of importance.
British Journal of Cancer (2004) **91,** 1015 – 1018. doi:10.1038/sj.bjc.6602119   www.bjcancer.com
Published online 24 August 2004
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Colorectal cancer is the third most common cancer in the Western world and despite advances in surgery, adjuvant therapies and screening, little impact on the mortality rates has been seen (Jemal *et al*, 2003). A greater understanding of the molecular mechanisms underlying carcinogenesis and progression is leading to novel treatment strategies.

Cyclooxygenases (Cox) are responsible for the metabolism of arachidonic acid into prostaglandins (Church *et al*, 2003). Two isoforms exist, termed Cox-1 and Cox-2 (Vane, 1971; Xie *et al*, 1991). Increased expression of Cox-2 has been implicated in carcinogenesis and metastatic progression in many forms of human cancer (Church *et al*, 2003). For example, increased expression of Cox-2 protein has been shown to correlate with tumour invasiveness and metastasis (Chen *et al*, 2001). In addition, 100% of metastatic lesions had positive immunohistochemical staining for Cox-2 *vs* 72% of primary tumours (Zhang and Sun, 2002). Cox-2 inhibitors are now being evaluated as adjuncts to chemotherapy for colon cancer (Blanke, 2002; Blanke and Masferrer, 2003).

Initial evidence with regard to the expression of Cox-1 suggested a minimal role in colonic neoplasia, with several studies demonstrating minimal expression of Cox-1 with little variability in polyps and established tumours (Eberhart *et al*, 1994; Sano *et al*, 1995). More recent evidence suggests that Cox-1 expression and activity may have a role to play in the carcinogenic process (Takeda *et al*, 2003). For example, reduced polyp formation was seen in MIN mice lacking a functional Cox-1 gene (Chulada *et al*, 2000) and Cox-1 expression may promote carcinogenesis in lung and gynaecological tissues, both synergistically with and independently of Cox-2 (Hasturk *et al*, 2002; Sales *et al*, 2002; Gupta *et al*, 2003). We used real time PCR to investigate Cyclooxygenase 1 and 2 expression profiles in invasive colonic tumours. Our aims were to define cyclooxygenase expression patterns in established colorectal tumours compared to adjacent normal mucosa and correlate this with clinicopathological variables and patient outcome.

MATERIALS AND METHODS

Patients

In total, 51 stage III (Dukes' C) colorectal cancer patients had tumour and adjacent normal bowel mucosa samples collected at the time of surgical resection by The Siteman Cancer Center Tissue Procurement Core. The median age of these patients was 68 (range 39 – 96 years). All samples were snap frozen and stored at −80°C until used for RNA extraction. In total, 29 patients (56.9%) were male. Approval for this study, including the genomic analysis of the tissue samples, was obtained from the Washington University in St Louis Human Studies Committee. All patients gave informed consent. Clinical data were collected prospectively and used to compare expression with tumour differentiation, anatomic location (either left or right colon), survival, recurrence (both metastatic and local recurrence), patient gender and age.

RNA extraction and real time PCR for cyclooxygenases

The TRIzol RNA isolation kit (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction from the paired tumour and normal mucosa. Areas of high cellularity on light microscopy (median 86%, range 65 – 95%) were chosen from each tissue sample. RNA was quantified and assessed for purity by measurement of OD260 and OD280 using a UV fiberoptic spectrophotometer (Nanodrop Technologies, Rockland, DE, USA) and was qualitatively assessed by measurement of relative 28S and 18S ribosomal band intensities using a Bioanalyzer and RNA NanoChip capillary gel electrophoresis assay (Agilent Technologies, Palo Alto, CA, USA). RNAs were reverse-transcribed into cDNA samples using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Primers and probes for the Real Time PCR for Cox-1 and Cox-2 RNA were designed using the Primer Express Software (ABI, Foster City, CA, USA) (Table 1). The probe and primer sets were synthesised by

Table 1 RT–PCR probes for Cox-1, Cox-2 and the reference gene APP. The fluorophores FAM, JOE, TAMRA were used, as indicated

Gene	Primer	Primer or probe sequence
Cox-1	Probe	5′-FAM/CCTTCCATCCGGTGGCCTATTCCA/36-TAMRA
	Forward primer	5′-CTGCCCTCCTCAAGACTTTAGCTT
	Reverse primer	5′-TCCAACTGATTTAAGCAAAAGAGGAAT
Cox-2	Probe	5′-FAM/AATCAAGCCTGGCTAGCATGCTG/36-TAMRA
	Forward primer	5′-TGAAGCCAATTCAGTAGGTGCAT
	Reverse primer	5′-ATCGCTAAAAGAAAAGAAAAGGA
APP reference gene	Probe	5′-JOE/AGTTCAGCCTGGACGATCTCCAGCC/TAMRA
	Forward primer	5′-CTCATGCCATCTTTGACCGA
	Reverse primer	5′-GGGCATCAACAGGCTCAACT

Integrated DNA Technologies (Coralvile, IA, USA). The relative RNA quantitation was assessed by Taqman real time PCR using an ABI PRISM 7700 analyser (Applied Biosystems, Foster City, CA, USA). All real time PCR assays were performed in triplicate.

Measurement of relative RNA expression levels

The relative expression levels were calculated using the modified comparative CT method (Pfaffl, 2001). The PCR efficiencies were calculated from standard curves using the formula $E = 10[-1/\text{slope}]$ where E is the efficiency and slope is the slope of the standard curve. Standard curves for the reference and cyclooxygenase genes constituted separate experiments using pooled colorectal cancer RNA samples (data not shown). The APP gene was used as the internal reference. The relative expression level of the RNA for each Cox gene was normalised to the APP gene and to one of all of the 102 tissue samples. The calibrator sample chosen was that which had the maximum C_T value, that is, the lowest expression level. The normalised relative expression levels for each gene was calculated using the following formula (Pfaffl, 2001):

$$\frac{E_{\text{target}}^{\Delta C_T \text{ target (calibrator−sample)}}}{E_{\text{reference}}^{\Delta C_T \text{ reference (calibrator−sample)}}}$$

where E_{target} is the real-time PCR frequency of the target gene transcript and $E_{\text{reference}}$ is the real time PCR efficiency of the reference gene transcript.

Statistics

Statistical analyses were performed using GraphPad InStat version 3.05 for GraphPad Software (San Diego, CA USA). Wilcoxon matched pairs test and Spearman's Rank Correlation coefficient were used to evaluate the differences seen in expression levels of the Cox enzymes between the samples. Kruskal–Wallis Test (Nonparametric ANOVA) and Mann–Whitney U test was used to compare cyclooxygenase expression and clinical and pathological variables. Kaplan–Meier analyses were carried out when comparing survival times. The P-values of < 0.05 were considered to be significant.

RESULTS

Substantial variation in the expression of Cyclooxygenase 2 mRNA was observed in normal mucosa (33-fold) and tumour tissues (51-fold). Variable Cox-1 expression was also seen in normal mucosa (68-fold) and tumour (40-fold). Cox-2 was significantly upregulated in the tumour samples compared to paired mucosal tissues (median tumour : normal ratio = 1.54, range 0.20–8.96, $P = 0.012$, Figure 1B). In contrast, tumour Cox-1 expression was significantly lower than normal mucosal samples (median tumour : normal ratio = 0.48, range 0.01–2.85, $P < 0.0001$, Figure 1A). The expression levels of each enzyme in normal mucosa also correlated to the expression seen in paired malignant mucosa (Cox-1, $r_s = 0.63$, $P < 0.0001$; Cox-2, $r_s = 0.33$, $P = 0.008$).

Cyclooxygenase-2 expression in tumour tissues did not correlate with disease recurrence ($P = 0.16$), tumour differentiation ($P = 0.26$), gender ($P = 0.2$), age > 70 ($P = 0.06$), or site of tumour ($P = 0.84$). Cox-1 expression similarly did not show any significantly different expression in tumour or normal mucosa in relation to these clinicopathological variables.

The relationships between Cox-1 and Cox-2 were also examined (Figure 3A and B). In normal tissue a linear relationship could be
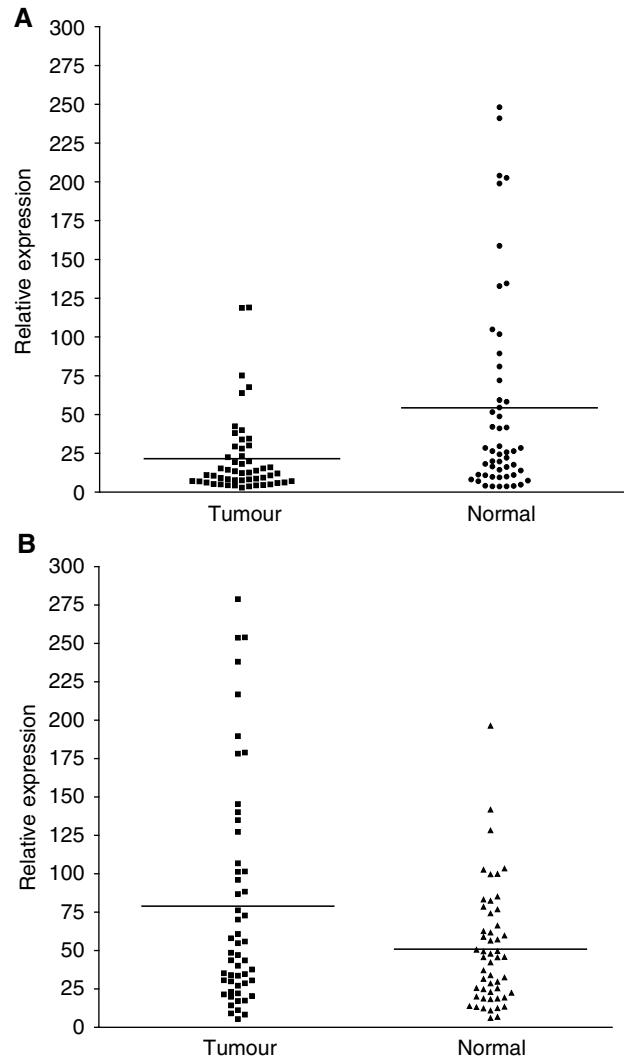
Figure 1 Comparison of variations in expression of Cox 1 (**A**) and Cox 2 (**B**) paired normal mucosa and tumour tissue samples. Horizontal lines represent the mean for each population.
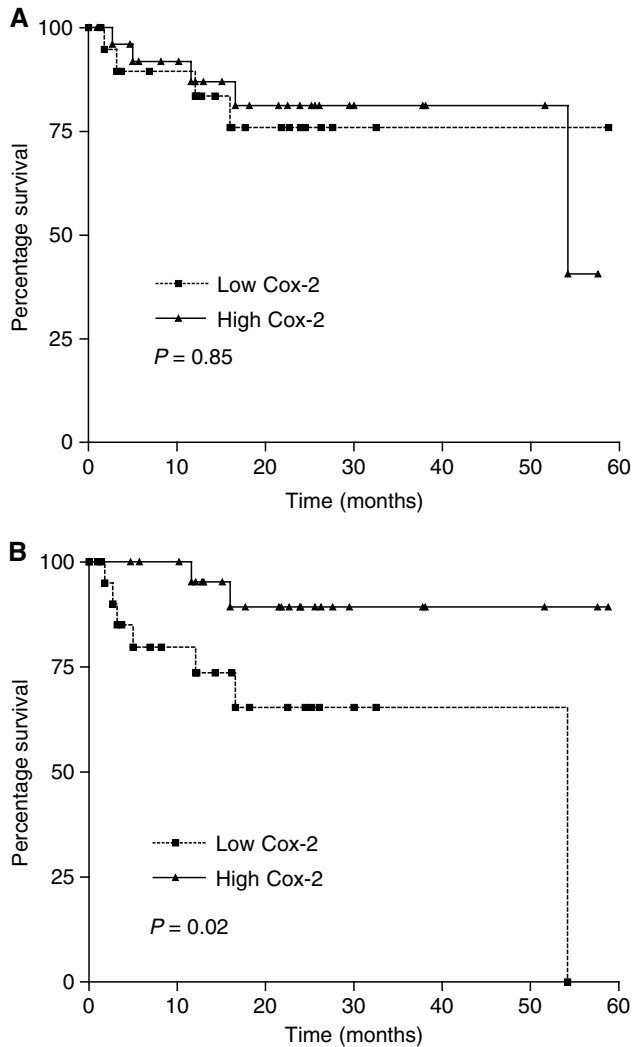
Figure 2 Relationship between patient survival and Cox-2 expression in tumour tissues (**A**) and normal mucosa (**B**).

Figure 3 Relationship between Cox-1 and Cox-2 expression in normal mucosa (**A**) and tumour (**B**).

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seen between Cox-1 and Cox-2 expression (Figure 3A, $r^2 = 0.32$). However this relationship was clearly not maintained in the tumour tissues (Figure 3B, $r^2 = 0.003$) with an increased expression of Cox-2 protein relative to Cox-1 expression.

Patients were divided into high or low expression groups using the median expression values for each cyclooxygenase gene. No significant differences in cancer-specific survival were seen using Cox-1 expression in normal ($P = 0.26$) or malignant tissues ($P = 0.36$). Cox-2 expression in the tumour did not correlate with survival ($P = 0.85$, Figure 2A) but patients expressing high levels of Cox-2 in the normal mucosa appeared to have a survival advantage ($P = 0.02$; Figure 2B).

DISCUSSION

Cox-1 and Cox-2 expression seen in normal and malignant mucosa showed wide variation, even in the context of patients with the same clinical disease stage. The validity of such variation could be confirmed with immunohistochemistry but the recovery of tissue slides for inclusion in this pilot study was not possible. The previously reported immunohistochemical studies have also shown large differences in the staining intensity, and the numbers
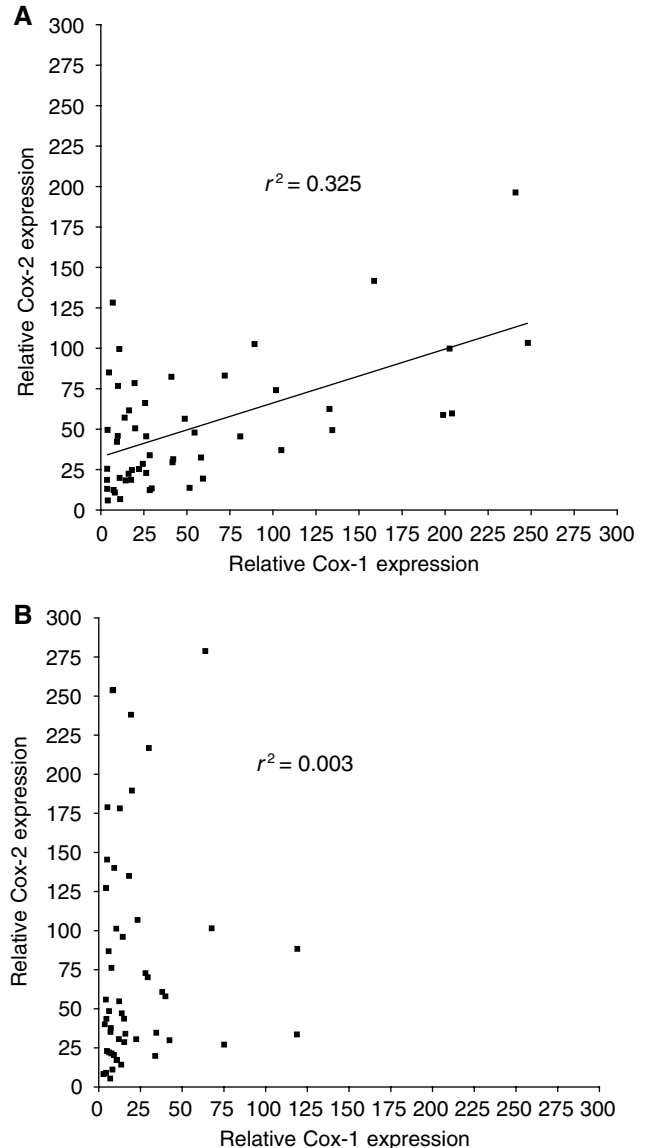
of cells expressing the Cox-2 protein (Hao *et al*, 1999; Masunaga *et al*, 2000; Cianchi *et al*, 2001). Our RNA expression data highlight such previously observed variability. These and other studies have been able to show that such elevated expression of Cox-2 correlated with clinicopathological variables. However, thresholds for positivity in these studies were low, including cells weakly stained, and sections with less than 10% of epithelial cell population deemed to be positive (Cianchi *et al*, 2001; Zhang and Sun, 2002). In addition, these studies utilised samples obtained across various disease stages. The increased expression of cyclooxygenase-2 mRNA in tumour in this study is consistent with these previous studies (Church *et al*, 2003).

A direct molecular basis for the upregulation of Cox-2 in polyps and cancer is still poorly defined. However, one mechanism may be the clonal expansion of tumour cells that express Cox-2. Such increased expression seems to increase tumour angiogenesis and decrease cellular apoptosis, leading to improved overall cellular viability compared to tumours not aberrantly expressing this protein (Church *et al*, 2003).

We were not able to show differences in cancer-specific survival or disease recurrence in patients expressing high levels of Cox-2 in tumour. This may reflect the fact that our samples are from a well-defined stage of disease progression, that is, Dukes' C tumours. Previously, it has been suggested that Cox-2 expression is associated with poorer outcomes; however, these studies compared expression across clinical disease stages and were not able to demonstrate any predictive potential independent of Dukes' stage (Sheehan *et al*, 1999; Masunaga *et al*, 2000). Patients with a high level of Cox-2 expression in the normal mucosa did seem to have survival advantage. The reasons for this observation are not easily explained and conflicts with some previous studies that examined the expression of the cyclooxygenases in the malignant tumour (Church *et al*, 2003).

The expression levels of Cox-1 also demonstrated considerable variation in RNA expression in normal and malignant tissues. This contrasts with the previously accepted opinion that Cox-1 exists as a house keeping gene, which is not subject to variable expression (Sano *et al*, 1995). More recent evidence suggests that Cox-1 is inducible and can be upregulated in malignant tissues (Sales *et al*, 2002; Gupta *et al*, 2003). We have shown that Cox-1 seemed to be downregulated in colorectal tumour specimens. Indeed, a synergistic relationship of the cyclooxygenases in the early stages of carcinogenesis has been suggested, with Cox-1 having a role initially followed by a rise in Cox-2 expression as the malignant process continues (Takeda *et al*, 2003). Our data confirm an altered regulation of Cox-1 expression between normal and malignant tissues, consistent with such suggestions. It has also been suggested that the increases of Cox-2 expression and the tissue-specific prostaglandin E Synthetase often seen in malignant tissue may be dependent on the expression of Cox-1, at least initially (Takeda *et al*, 2003). There is emerging evidence that Cox-1 may have a role to play in carcinogenesis in other solid tumours such as ovarian (Gupta *et al*, 2003) and skin cancer (Tiano *et al*, 2002). This may mean that the nonspecific cyclooxygenase inhibitors, such as sulindac and aspirin, may be more important agents in the prevention of colonic polyps, if compared to the Cox-2 specific inhibitors, such as celecoxib and rofecoxib, which are currently being studied in this context. However, the reduction in Cox-1 expression in more advanced disease supports the view that as additions to adjuvant therapy regimes specific Cox-2 inhibitors should be more effective.

ACKNOWLEDGEMENTS

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