

Association between cyclo-oxygenase-2 overexpression and missense *p53* mutations in gastric cancer

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Summary Wild-type *p53* competitively binds to the promoter region of COX-2 in vitro and inhibits its transcription. We examined the association between *p53* mutation and COX-2 expression in gastric cancer. COX-2 over-expression was seen in 19 (48.7%) cases. These tumours had more lymph-node metastasis ($P = 0.048$) and tended to have a poorer survival ($P = 0.07$). Missense mutations of *p53* were detected in 20 (51.3%) patients and had a significantly stronger COX-2 expression than tumours without *p53* mutation ($P = 0.016$). Our results suggest a link between *p53* mutation and COX-2 overexpression in gastric cancer. © 2001 Cancer Research Campaign <http://www.bjcancer.com>

Keywords: cyclo-oxygenase-2; *p53* gene; mutation; gastric cancer

Epidemiological studies have reported a 40–50% reduction in risk of developing colorectal cancer among chronic non-steroidal anti-inflammatory drug (NSAID) users (Thun et al, 1991; Giovannucci et al, 1994). Subsequent studies had implicated the cyclo-oxygenase-2 (COX-2) enzyme, an inducible form of prostaglandin synthase responsible for the conversion of arachidonic acid into prostaglandins, as the link between these observations (Dubois et al, 1998; Taketo, 1998). Notably, COX-2 is not just a marker of inflammation but is actively involved in the carcinogenesis process. Overexpression of COX-2 in colorectal cancer has been associated with angiogenesis (Tsujii et al, 1998), lymphatic invasion, metastasis (Tsujii et al, 1997) and poor prognosis (Sheehan et al, 1999). In this context, COX-2 overexpression is also frequently detected in gastric tumours (Ristimaki et al, 1997; Murata et al, 1999) as well as in premalignant gastric lesions (Sung et al, 2000). Similar to colonic cancer, gastric tumours with COX-2 over-expression appeared to have more frequent lymphatic invasion (Murata et al, 1999).

Nonetheless, the mechanism leading to COX-2 overexpression in tumour remains elusive. A recent in-vitro study suggested that wild-type *p53*, a major tumour suppressor gene that is involved in the control of cell cycle progression, DNA integrity and cell survival, inhibits the binding of TATA-binding proteins (TBP) to the promoter region of *Cox-2* gene (Subbaramaiah et al, 1999). Thus, levels of prostaglandin E_2 (PGE_2) were 10-time lower in cells with wild-type *p53* than in those with mutated *p53*, suggesting the potential interaction of *p53* and COX-2 in cancer cells. While both *p53* mutation (Imazeki et al, 1992; Renault et al, 1993; Uchino et al, 1993) and COX-2 over-expression was frequently reported in gastric cancers, their interactions had not been properly evaluated. This study examined the correlation between *p53* mutation and COX-2 expression in gastric cancer.

MATERIALS AND METHODS

Patients

Patients with adenocarcinoma of stomach who had undergone gastrectomy in the Prince of Wales Hospital of Hong Kong were examined. A total of 39 patients were included (male:female = 21:18; median age of 69 years, ranges 29–80 years). 32 (82.1%) cases had tumours located in the distal stomach and there were 7 (17.9%) cases of proximal cancer. 22 (56.4%) of these tumours were intestinal type whilst the rest were classified as diffuse type according to Lauren classification. Early gastric cancer as defined by lesions confined to the gastric mucosa and submucosa was seen in 4 cases. All patients were regularly followed up after surgery (median 23 months, range 3–149 months). Survival was measured from the time of surgery till death.

COX-2 immunohistochemistry

Archive pathological specimens were retrieved. 5- μ m thick formalin-fixed and paraffin-embedded gastrectomy sections was retrieved. Sections were deparaffinized and endogenous peroxidase activity was blocked with 3% H_2O_2 in Tris-buffered saline (TBS). Non-specific binding was blocked with 5% rabbit serum (DAKO, Glostrup, Denmark) in TBS, and the tissues were incubated with antibody against COX-2 (1:100, Santa Cruz, Santa Cruz, CA) in TBS containing 2% rabbit serum and 1% bovine serum albumin. This was followed by sequential incubation with biotinylated rabbit anti-goat immunoglobulins (1:400, DAKO) and avidin-biotin peroxidase complex (DAKO) respectively. Colour was developed in DAB solution (Sigma, St Louis, MO) and counterstained with Mayer's haematoxylin. Negative control was performed by incubating samples without the primary antibody.

COX-2 expression was scored semi-quantitatively according to the percentage of positively stained tumour cells: grade 0 = no expression, 1 = <10%, 2 = 10–30%, 3 = 30–60% and 4 = >60% expression. A minimum of 10 high power view was used to assess COX-2 expression level in tumour cells (Fig. 1). Positive staining

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in stromal tissues and inflammatory cells was not counted. Two independent investigators (KFT and TLL) who were blinded to the *p53* mutation statuses performed the assessment. The mean expression level (in percentage) was used in subsequent analysis. In discordant cases (when inter-observer differences were greater than 30%), the two investigators would review the slides again. This immunostaining result had been validated by in-situ hybridization by using anti-sense COX-2 RNA probe (Sung et al, 2000). Over-expression of COX-2 was defined as grade 2 or above expression.

p53 mutation analysis

The formalin-fixed, paraffin-embedded tissues were retrieved and cut into 7 μm sections. Area containing cancer was carefully microdissected. DNA extraction was performed by using High Pure PCR Template Preparation Kit (Roche, GmbH, Germany) as described by the manufacturer. Mutations in *p53* were determined by PCR-based single strand conformational polymorphism (SSCP) of exons 5–8, where most mutations were detected. The primers used for amplification were previously reported (Hse et al, 1991; Okamoto et al, 1991). The PCR reaction mixtures contained 1 \times PCR Buffer, 2.5 mM MgCl_2 , 0.1 mM of each dNTPs, 0.25 μM of each primer, 0.625 U Taq polymerase (Gibco BRL, Rockville, MD), and 1 μl of DNA template in a 25 μl reaction volume. Nested PCR was performed and 0.3 μl ($\sim 3 \mu\text{Ci}$) of ($\alpha\text{-}^{32}\text{P}$)dCTP (NEN, MA) was added into the second amplification. The MKN-45 human gastric cancer cell line (Riken Cell Bank, Tokyo, Japan), that has wild-type *p53* (Matozaki et al, 1992), was included as normal control.

For SSCP, 5 μl of the nested PCR products were mixed with 45 μl of loading dye (95% formamide, 0.05% xylene cyanol and 0.05% bromophenol blue). The mixture was heated to 95°C for 10 minutes and then put in ice immediately. A 4 μl aliquot was loaded into 8% non-denaturing polyacrylamide gel with 5% glycerol. Electrophoresis of the gel was carried out at room temperature for 18 hours. The gel was dried and exposed to X-ray film at -80°C with intensifying screen for 1 to 2 days. The presence of an abnormal band shift when compared to wild-type control was noted. Amplifications were repeated for samples showing band shifts to ensure that consistent result were obtained. The shifted bands were excised from the polyacrylamide gel and eluted by Milli-Q water. Direct DNA sequencing was performed by using ABI Prism 310 Genetic Analyzer according to standard protocol (Perkin Elmer, Branchburg, NJ). Both forward and reverse primers were used for sequencing and all positive samples were repeatedly tested.

Statistics

All statistical calculations were performed by SPSS for Windows software (version 9.0). Fisher exact test was used for categorical data and Mann-Whitney U test was used in the comparison of COX-2 expression between patients with and without *p53* mutation. Survival data was summarized by Kaplan-Meier curve and compared by log-rank test. A *P* value (two-tailed) of less than 0.05 was considered statistically significant.

RESULTS

COX-2 expression

30 (74.4%) cases of gastric tumours showed COX-2 expression (grade 1 and above) while COX-2 overexpression (grade 2 and above) was detected in 19 (48.7%) cases. Tumours with COX-2

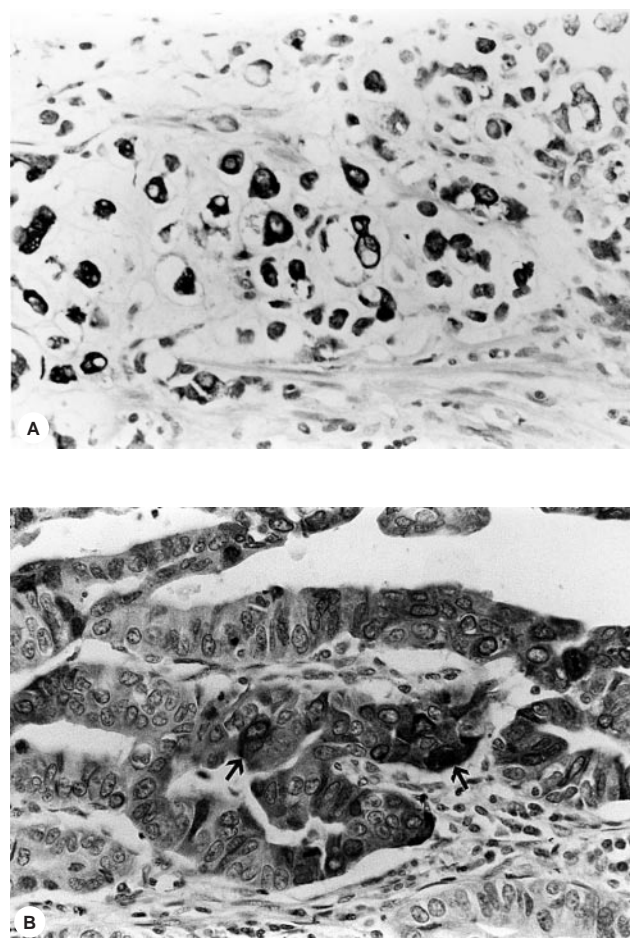


Figure 1 (A) A representative sample of diffuse type gastric cancer that showed discohesive sheets of tumour cells ($\times 400$). Majority of cancer cells ($>60\%$, grade 4) exhibited strong cytoplasmic staining for COX-2 (dark colour). (B) Intestinal type gastric cancer showing malignant glandular pattern. More than 10% (grade 2) of tumour cells demonstrated COX-2 expression (dark colour staining). Arrows indicated some of the positively stained cells ($\times 400$)

over-expression had more lymph-node metastasis (78.9% versus 45%, $P = 0.048$). However, there was no significant difference in demographic data, histological types and tumour locations between tumours with and without COX-2 over-expression (Table 1). There was a trend towards better prognosis for tumours without COX-2 overexpression (median survival 68.8 vs 28.8 months; log rank test, $P = 0.07$; Figure 2).

p53 mutation

p53 mutation was detected in 20 tumours (51.3%). All mutations were missense mutations leading to amino acid substitutions (Table 2). Mutations in exon 5 were the most commonly detected (60%), followed by mutations in exon 7 (25%). 18 (90%) of these mutations were G:C \rightarrow A:T transition. Gastric tumours with missense *p53* mutations had a significantly higher level of COX-2 expression than tumours with wild-type *p53* (median scores: 3 versus 1, $P = 0.016$, Figure 3). There was no correlation between *p53* mutation and clinicopathological features of tumours including age of patients, types of tumor, presence of lymph node metastasis and survival (Table 1).

Table 1 Association of COX-2 expression and p53 mutation with clinicopathological features of gastric cancer

	COX-2 expression level			p53 mutation		
	2-4 (n = 19)	0-1 (n = 20)	P	Present (n = 20)	Absent (n = 19)	P
Median age	58	63	NS ^a	58	63	NS
Male:female	10:9	11:9	NS	9:11	12:7	NS
Histological type						
Intestinal	10	12	NS	11	11	NS
Diffuse	9	8		9	8	
Tumour location						
Distal	16	16	NS	15	17	NS
Proximal	3	4		5	2	
Early cancer	1	3	NS	2	2	NS
Lymphatic involvement	15	9	0.048	15	9	NS

^aNS = not significant.

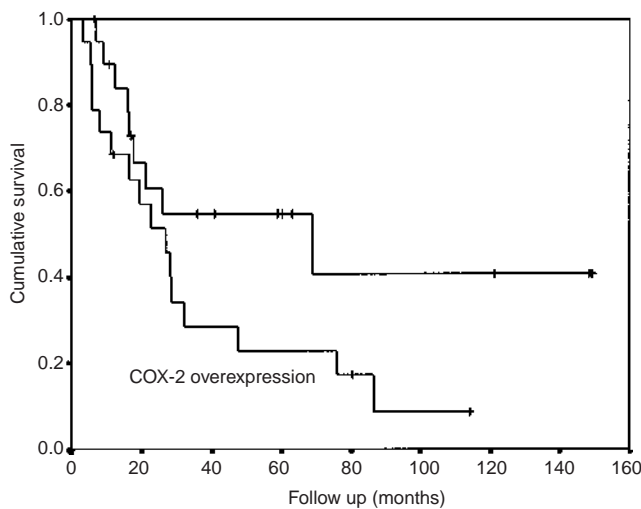


Figure 2 Kaplan-Meier curve of gastric cancer with and without COX-2 overexpression. The upper line represented tumours without COX-2 overexpression (grade 0 or 1) whilst the lower line indicated cancers with COX-2 overexpression (median survival 28.8 months versus 68.8 months; $P = 0.07$, log rank test)

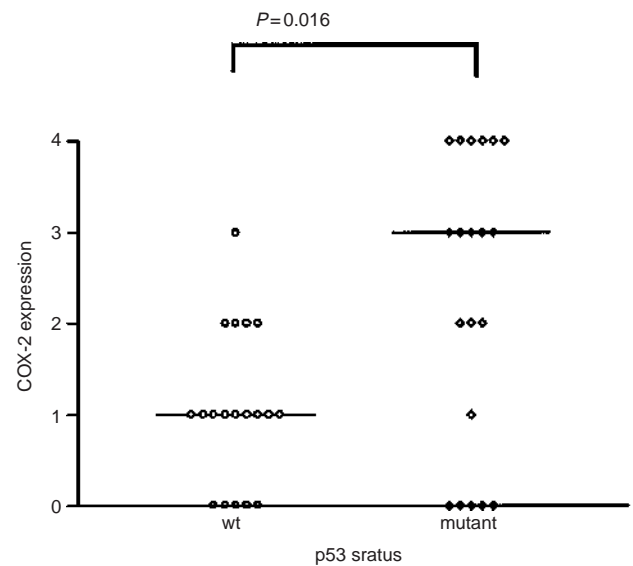


Figure 3 The scattergram of COX-2 expression in p53 mutated tumours (mutant) versus tumours without wild-type p53 (wt). The horizontal line represents the median value of COX-2 expression

DISCUSSION

The role of wild-type p53 protein is multiple and expression of high level of p53 results in cell cycle arrest and apoptosis. Furthermore, wild-type p53 could serve as a transcriptional activator of genes containing the p53-binding sites as well as inhibiting gene transcription (Ko and Prives, 1996). Thus, mutations in the core domain of p53 may alter its binding ability and affect its role in transcriptional regulation. One of the ways by which p53 inhibits gene expression is via interaction with the TATA-binding proteins (TBP) and thereby interfering with the assembly of a functional transcription initiation complex (Seto et al, 1992; Mack et al, 1993). Accordingly, a recent in-vitro study demonstrated that wild-type p53 inhibits the formation of the complex between TBP and human Cox-2 promoters in a cell-free system (Subbaramaiah et al, 1999). Intuitively, p53 mutation would result in loss of inhibitory effect on COX-2 expression in human cancer.

We showed that tumours with p53 missense mutation, which leads to amino acid substitution, had a higher level of COX-2 expression when compared to tumours without p53 mutation. To our knowledge, this is the first report of this correlation in human cancer. In contrast to other tumour-related genes such as the oncogenes of the ras family, mutations in 'hotspot' codons accounted for only about 20% of all p53 mutations reported so far. As in this study, mutation in codons 175 and 248 were detected in 3 cases only (14.3%) and diverse mutation patterns were observed for the remaining samples. Notably, most of these mutations were G:C → A:T transition as previously reported (Imazeki et al, 1992; Renault et al, 1993). This transition has been linked to exposure to nitrous oxide (Nguyen et al, 1992). On the other hand, with the diverse mutation patterns, it is not difficult to anticipate the heterogeneous COX-2 expression level among tumours with different p53 mutations. It would be interesting to study the in-vitro promoter activity and responses by using reporter assays or DNA-binding assays with various forms of p53 variants.

Table 2 Summary of p53 mutation in gastric cancer cases

Case no	Site	Type	COX-2 expression	p53 mutation	Exon	Codon	Mutation	Amino acid
3	A	Diffuse	0	No				
9	A	Diffuse	1	Yes	5	140	ACC>ATC	Thr>Ile
10	A	Intestinal	1	No				
11	A	Intestinal	4	Yes	5	175	CGC>CAC	Arg>His
18	B	Intestinal	0	No				
24	B	Intestinal	1	No				
26	A	Intestinal	2	Yes	5	129	GCC>GTC	Ala>Val
28	A	Intestinal	1	No				
31	A	Intestinal	1	No				
33	A	Intestinal	2	No				
36	A	Intestinal	3	Yes	5	146	TGG>CGG	Trp>Arg
44	C	Intestinal	0	No				
47	A	Intestinal	2	No				
50	C	Intestinal	0	Yes	5	154	GGC>AGC	Gly>Ser
53	B	Diffuse	4	Yes	7	248	CGG>CAG	Arg>Gln
55	B	Diffuse	4	Yes	5	181	CGC>TGC	Arg>Cys
57	A	Diffuse	2	No				
59	B	Diffuse	2	Yes	5	155	ACC>ATC	Thr>Ile
62	B	Diffuse	4	Yes	7	250	CCC>CTC	Pro>Leu
70	A	Intestinal	0	Yes	5	146	TGG>CGG	Trp>Arg
72	C	Diffuse	4	Yes	5	175	CGC>CAC	Arg>His
77	C	Diffuse	1	No				
78	A	Diffuse	0	Yes	5	170	ACG>ATG	Thr>Met
80	C	Intestinal	0	Yes	8	262	GGT>AGT	Gly>Ser
90	C	Intestinal	3	Yes	5	171	GAG>AAG	Glu>Lys
93	A	Intestinal	3	Yes	6	192	CAG>CAC	Gln>His
99	A	Diffuse	3	No				
101	B	Diffuse	0	Yes	8	267	CGG>CAG	Arg>Gln
102	B	Intestinal	1	No				
104	B	Intestinal	1	No				
106	A	Diffuse	4	Yes	5	177	CCC>CTC	Pro>Leu
108	A	Intestinal	3	Yes	6	193	CAT>AAT	His>Asn
110	A	Diffuse	0	No				
112	C	Intestinal	2	Yes	7	230	ACC>GCC	Thr>Ala
115	A	Diffuse	1	No				
116	A	Intestinal	3	Yes	7	245	GGC>AGC	Gly>Ser
117	A	Intestinal	0	No				
120	A	Diffuse	1	No				
121	A	Diffuse	2	No				

A = antrum, B = body, C = cardia.

In this study, COX-2 expression was also detected in gastric tumour with wild-type p53. This finding is not unexpected since other factors are also involved in regulation of COX-2 expression. For example, *H. pylori*-associated gastritis has been associated with upregulation of COX-2 (Fu, 1999). This is turn may be triggered by pro-inflammatory factors such as cytokines, tumour necrosis factor- α , and nuclear factor- κ B (Dubois et al, 1998). Recently, the *APC* gene was also found to play a role in the translational regulation of COX-2 in colorectal cancer cell line (Hsi, 1999). Given the complexity of regulation of COX-2 expression, wild-type p53 is probably just one of the many factors responsible for the inhibition of COX-2 transcription in normal tissues.

A modest increase in lymphatic involvement was detected among tumours with COX-2 overexpression. In this regard, it is not surprising to observe a trend towards lower survival in these tumours when compared to tumours without COX-2 overexpression. Similar findings had been reported in colorectal as well as in gastric cancer (Murata et al, 1999; Sheehan et al, 1999). Taken together, these results suggested the prognostic significance of COX-2 in gastrointestinal tumours and thus, COX-2 is probably playing an active role in tumorigenesis and is not just an epiphenomenon.

In conclusion, we have demonstrated that gastric tumours with missense p53 mutations were associated with higher level of COX-2 expression, suggesting the potential role of wild-type p53 in the regulation of COX-2 expression. Studies that look into the regulation of COX-2 expression in cancer may offer a new insight into gastric carcinogenesis and plausibly, chemoprevention pathways.

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