# Down-regulation of cyclooxygenase-2 expression but up-regulation of cyclooxygenase-1 in renal carcinomas of the Eker (*TSC2* gene mutant) rat model

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(Received August 2, 2002/Revised October 29, 2002/Accepted November 6, 2002)

**Although it is generally accepted that cyclooxygenase (Cox)-2 is overexpressed in carcinomas, few studies have examined Cox-2 expression in renal carcinoma (RC). The Eker rat RC is an example of a Mendelian dominantly inherited carcinoma, and in the present study, expression of Cox-2 in the Eker rat RC was examined. Reverse transcription polymerase chain reaction indicated constitutive expression of Cox-2 mRNA in the normal control kidney and non-tumor part of Eker rat kidney. Unexpectedly, expression of Cox-2 mRNA was down-regulated in four Eker RCs from two Eker rats, and in the cell line Lk9ds. Immunohistochemical analysis failed to reveal Cox-2 protein staining in Eker RCs. As a control to Cox-2 expression, Cox-1 expression was examined. Interestingly, in contrast to the down-regulated Cox-2 expression, Cox-1 mRNA expression was induced in these four Eker RCs and cell lines. Cox-2 and Cox-1 expression were further examined in six additional Eker RCs. In total, Cox-2 mRNA expression was down-regulated in eight out of ten Eker RCs and cell lines, while Cox-1 mRNA expression was up-regulated in nine out of ten Eker RCs and cell lines. (Cancer Sci 2003; 94: 22–25)**

Cyclooxygenase (Cox) catalyzes the formation of prostaglandins (PGs) from arachidonic acid, and there are two distinct Cox enzymes, Cox-1 and Cox-2. It is generally thought that Cox-1 is a constitutively expressed housekeeping gene responsible for various functions, including cytoprotection of the stomach, vasodilation in the kidneys, and production of thromboxane by platelets.[1, 2] Cox-2, the inducible isoform of Cox, is responsible for the rapid production of PGs in response to various inflammatory stimuli.[1, 3, 4] Furthermore, Cox-2 is associated with carcinogenesis,[5–7] and overexpression of Cox-2 contributes to the development of tumors in several tissues.[8–10] However, the expression pattern of Cox in the kidney is different from those of other organs. In the normal kidney, Cox-1 is only weakly expressed, but Cox-2 is constitutively expressed.[11] Furthermore, it is not known whether Cox-2 is also overexpressed or not in renal carcinomas (RCs). There have only been a few studies on Cox-2 expression in RCs. In a study of Cox-2 expression in three canine RCs, Cox-2 was expressed at high levels in two of them.[12] The Eker rat RC is an example of a Mendelian dominantly inherited carcinoma in an experimental model.[13, 14] A rat homologue of the human tuberous sclerosis gene mutation has been revealed to be the predisposing gene in the Eker rat,[15, 16] and the Eker rat is a suitable animal for elucidating the mechanism of hereditary RCs. Therefore, in the present study, we examined whether Cox-2 is overexpressed or not in RCs of Eker rats.

## Materials and Methods

**Eker rat and its cell line.** In the first set of experiments, two female Eker rats (Eker rats No. 1 and No. 2), which spontaneously developed RCs, were used at 24–25 months of age. In Eker rat No. 1, one RC and one adjacent non-tumor tissue area in the right kidney and one RC in the left kidney were sampled. In Eker rat No. 2, two RCs in the left kidney were sampled. Kidney tissue samples from an intact Fischer 344 rat were used as normal control samples.

In the additional experiment, four Eker RCs from three Eker rats (Eker rats No. 3, No. 4 and No. 5) and two other RC samples from Eker rat No.1 were used.

All animals were housed and treated in accordance with institutional guidelines. The animals were anesthetized with ether before sacrifice. On sacrifice, tissues were removed from each animal, and then immediately frozen in liquid nitrogen and stored at −80°C for RNA analysis. Lk9dL and Lk9dR are cell lines derived from Eker RCs in the right and left kidneys.[17]

**Reverse transcription polymerase chain reaction (RT-PCR).** RNA was isolated with TRIZOL (Gibco BRL, Rockville, MD), washed with 75% ethanol, air-dried, and dissolved in diethyl pyrocarbonate-$H_2O$. Aliquots were used to synthesize cDNA, and the remainder was stored at −80°C. RNA samples of 3 $\mu$g (7 $\mu$l of total RNA) were heated to 65°C for 3 min and then cooled on ice. After the addition of 10 m*M* dithiothreitol (DTT), 1× first-strand buffer (Gibco BRL), 0.5 m*M* each of deoxynucleotide triphosphates, 20 pmol of $C_6$ random primer and 200 units of Superscript II (Gibco BRL), RT reactions were performed in a final volume of 20 $\mu$l at 37°C for 1 h. RT-PCR analysis was performed as previously described.[18] Four microliters of each reaction mixture was used for RT-PCR in a 20 $\mu$l mixture containing 0.75 m*M* $MgCl_2$, 0.2 m*M* (each) deoxynucleotide triphosphates, 1× PCR buffer (Toyobo, Tokyo), 0.05 units of *Taq* polymerase (Toyobo) and, 3 $\mu$*M* each of forward and reverse primers. cDNA was amplified by means of an appropriate number of cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1.5 min. The gene-specific primers for Cox-2, the inducible form of nitric oxide synthase (iNOS), and glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) were as follows: Cox-2 (Genbank Acc#S67722), (sense) 5′-GGAGAGAAGGAAATGGCTGCA-3′, (antisense) 5′-ATCTAGTCTGGAGTGGGAGG-3′; Cox-1 (Genbank Acc#U03388), (sense) 5′-TTTGCACAACACTTC-ACCCACCAG-3′, (antisense) 5′-AAACACCTCCTGGCC-CACAGCCAT-3′; iNOS (Genbank Acc#L09126), (sense) 5′-TGGGAATGGAGACTGTCCCAG-3′, (antisense) 5′-GGGATC-TGAATGTGATGTTTG-3′; GAPDH (Genbank Acc#X02231), (sense) 5′-ATGGTGAAGGTCGGTGTGAACG-3′, (antisense) 5′-GTTGTCATGGATGACCTTGGCC-3′.

**Immunohistochemical analysis.** For immunohistochemical analysis of Cox-2, a female Eker rat, which had developed RCs, was used at 15 months of age. RCs from the Eker rat were fixed with 10% phosphate-buffered formalin, embedded in paraffin, and then sectioned at 5 $\mu$m thickness. A section was treated with 3% $H_2O_2$ for 10 min to inactivate endogenous peroxidase,

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and then incubated with 10% goat non-immune serum (Vector Laboratories, Inc., Burlihgame, CA) at 37°C for 20 min to block nonspecific binding. The specimen was then incubated with anti-rat Cox-2 antibodies (IBL, Gunma) overnight at 4°C, and then with the secondary antibodies (biotinylated goat anti-rabbit IgG) (Vector Laboratories, Inc.), followed by incubation with avidin-biotin-peroxidase and staining with the diaminobenzidine substrate.

## Results

**Expression of Cox-2 in Eker RCs and a cell line.** Expression of Cox-2 mRNA was examined in four RCs derived from two Eker rats No. 1 and No. 2, and cell line Lk9d. RT-PCR analysis indicated that Cox-2 mRNA was constitutively expressed in a normal control kidney (Fig. 1A) and in the non-tumor part of a kidney of an Eker rat with RCs (Fig. 1A). However, Cox-2 mRNA expression was down-regulated in both Eker RCs (Fig. 1A) and cell line Lk9ds (Fig. 1B). Immunohistochemical analysis of Cox-2 was performed in Eker RC using antibodies against Cox-2. Immunohistochemistry failed to reveal staining of Cox-2 protein in the tumor part of an Eker RC (Fig. 2).

As a control, Cox-1 mRNA expression was examined in the same samples as used in Fig. 1. In normal kidneys, Cox-1 mRNA was only weakly expressed. However, expression of Cox-1 mRNA was up-regulated in both Eker RCs (Fig. 3A) and cell line Lk9ds (Fig. 3B). Expression of Cox-1 was also observed in the non-tumor part of a kidney of an Eker rat.

Expression of iNOS, which is another inducible enzyme, was examined. In a normal control kidney, iNOS mRNA was not expressed (Fig. 4A). In Eker RCs, iNOS mRNA was expressed in two of four RCs (Fig. 4A), but it was not expressed in the non-tumor part of an Eker RC (Fig. 4A). Furthermore, iNOS mRNA was expressed in cell line Lk9ds (Fig. 4B). Although iNOS mRNA was expressed in Eker RCs, its expression was not consistent.

**Cox-2 expression in additional Eker RCs.** Expression of Cox-2 was further examined in additional six Eker RCs. In RCs from Eker rat No. 3 and No. 4, Cox-2 mRNA expression was not down-regulated (Fig. 5). Intensity of Cox-2 mRNA expression in these two Eker RCs was almost the same as in the normal kidney. In two RC samples from Eker rat No. 5, expression of Cox-2 mRNA was down-regulated (Fig. 5). In two more RC samples from Eker rat No. 1, Cox-2 mRNA expression was down-regulated. Expression of Cox-1 mRNA was up-regulated in RC samples from Eker rat No. 3, No. 5 and No. 1 (Fig. 5). In the RC from Eker rat No. 4, Cox-1 mRNA expression was slightly induced.

## Discussion

It is generally accepted that overexpression of Cox-2 is common in carcinogenesis.[5–7] The role of overexpressed Cox-2 in the development of human colorectal cancers has been well studied.[7, 8, 19] The involvement of Cox-2 expression in tumor development has also been reported in several types of tumors, including prostate cancers,[20] cervical carcinomas,[21] and urinary bladder carcinomas.[22] Furthermore, direct genetic evidence that Cox-2 plays a key role in tumorigenesis in APC delta 716 knockout mice has been obtained.[23] Although Cox-2 expression is repressed in most tissues in intact animals, Cox-2

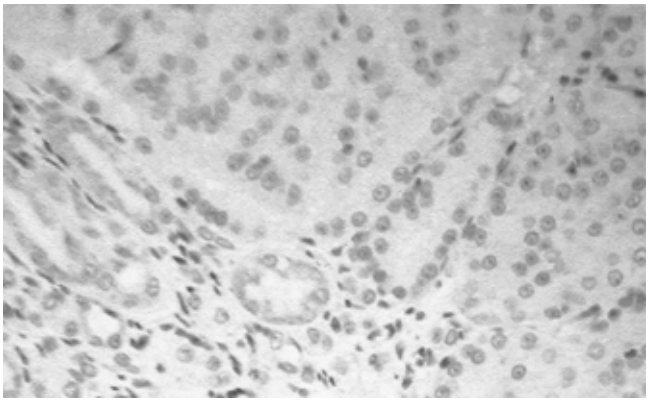

**Fig. 2.** Immunohistochemical analysis of Cox-2 in spontaneously induced Eker RCs. Immunohistochemical analysis was performed on spontaneously induced Eker RCs using antibodies against Cox-2 (×20).

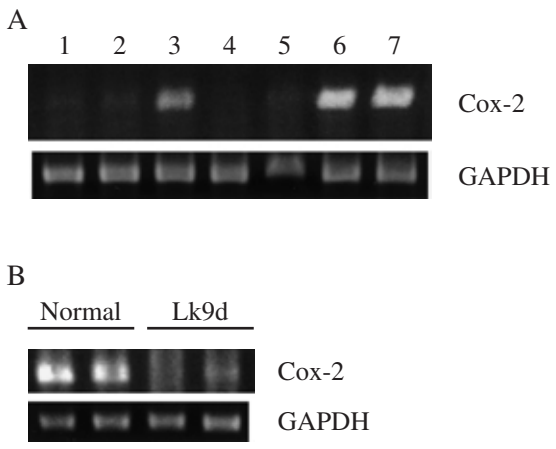


**Fig. 1.** Cox-2 mRNA expression in spontaneously induced Eker RCs and Lk9d cells. RNA samples from spontaneously induced Eker RCs and Lk9d cells were subjected to PCR analysis with *Cox-2* gene-specific primers for 26 cycles of amplification. A. From left: 1st, RC from Eker rat No. 1 right kidney; 2nd, RC from Eker rat No. 1 right kidney; 3rd, adjacent non-tumor tissue from Eker rat No. 1 right kidney; 4th and 5th, RC from Eker rat No. 2 left kidney; 6th and 7th, normal right and left kidney from normal control Fischer 344 rat. B. From left: 1st and 2nd, normal kidney; 3rd, Lk9dL; 4th, Lk9dR.

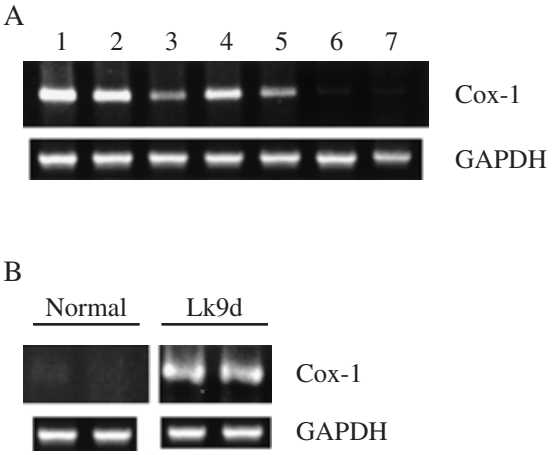


**Fig. 3.** Cox-1 mRNA expression in spontaneously induced Eker RCs and Lk9d cells. Expression of Cox-1 mRNA was measured in the same RNA samples as used in Fig. 1. RNA samples were subjected to PCR analysis with *Cox-1* gene-specific primers for 26 cycles amplification. A. From left: 1st and 2nd, RC from Eker rat No. 1 right kidney; 3rd, adjacent non-tumor tissue from Eker rat No. 1 right kidney; 4th and 5th, RC from Eker rat No. 2 left kidney; 6th and 7th, normal right and left kidney from normal control Fischer 344 rat. B. From left: 1st and 2nd, normal kidney; 3rd, Lk9dL; 4th, Lk9dR.

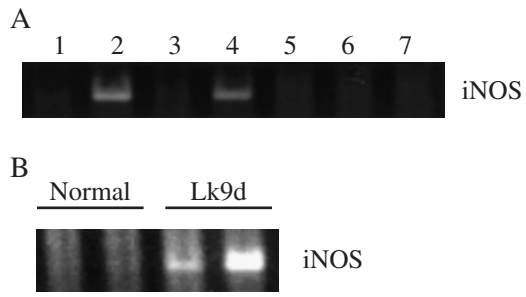


**Fig. 4.** iNOS mRNA expression in spontaneously induced Eker RCs and Lk9d cells. The same RNA samples as used in Fig. 1 were subjected to PCR analysis with *iNOS* gene-specific primers for 30 cycles of amplification. A. From left: 1st and 2nd, RC from Eker rat No. 1 right kidney; 3rd, adjacent non-tumor tissue from Eker rat No. 1 right kidney; 4th and 5th, RC from Eker rat No. 2 left kidney; 6th and 7th, normal right and left kidney from normal control Fischer 344 rat. B. From left: 1st and 2nd, normal kidney; 3rd, Lk9dL; 4th, Lk9dR.

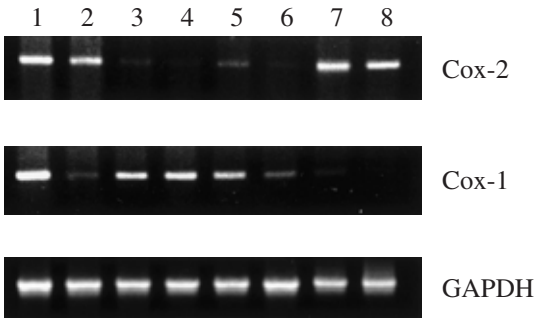


**Fig. 5.** Cox-2 and Cox-1 mRNA expression in spontaneously induced Eker RCs. RNA samples were subjected to PCR analysis with *Cox-2* or *Cox-1* gene-specific primers for 26 cycles amplification. From left: 1st, RC from Eker rat No. 3; 2nd, RC from Eker rat No. 4; 3rd and 4th, RCs from Eker rat No. 5; 5th and 6th, RCs from Eker rat No. 1; 7th and 8th, normal control Fischer 344 rat kidney.

mRNA is constitutively expressed in the normal kidney.[11] In the present study, Cox-2 mRNA was expressed in the normal kidney. Interestingly, Cox-2 mRNA expression was down-regulated in spontaneously induced Eker rat RCs. Immunohistochemical analysis failed to stain the Cox-2 protein in the tumor part of an Eker RC, supporting the results of RT-PCR. Since the major origin of Eker RC is proximal tubules,[13] Cox-2 expression in the proximal region seemed to be decreased in the Eker RCs. Moreover, we are examining more tumor samples to elucidate whether Cox-2 expression is differently regulated in the different stages of tumor development.

As a control to Cox-2, Cox-1 mRNA expression was measured. In contrast to the down-regulated Cox-2 expression, Cox-1 mRNA expression was up-regulated in these Eker RC samples. Thus, there might be some linkage between Cox-1 and Cox-2 expression in Eker RCs.

Cox-2 is known to be inducible, and iNOS is also induced as part of the inflammatory response. In some cancers, such as human gastric cancers[24, 25] and human ovarian cancers,[26] iNOS plays a role in the development of carcinomas by being coexpressed with Cox-2. In the present study, however, expression of iNOS mRNA in Eker RCs did not correlate with the expression of Cox-2 mRNA, indicating that iNOS expression is regulated differently from that of Cox-2 in Eker RCs.

Expression of Cox-2 was further examined in additional Eker RCs, and was down-regulated in eight out of ten Eker RCs and in cell lines. In contrast, Cox-1 expression was up-regulated in nine out of ten Eker RC samples. From these results we conclude that Cox-2 expression was down-regulated and Cox-1 was up-regulated in Eker RCs. It was reported that in addition to Cox-2, Cox-1 contributes to intestinal tumorigenesis in *Apc* gene knockout mice.[27] As one possibility to explain down-regulation of Cox-2 expression and up-regulation of Cox-1 expression in Eker RCs, it is probable that Cox-1, but not Cox-2, might be involved in the development of Eker RCs.

Accumulating data indicate that the overexpression of Cox-2 is common during carcinogenesis, and Cox-2 expression is up-regulated in case of some canine RCs,[12] but there are only a few reports regarding reduced expression of Cox-2 in carcinomas. In this work, down-regulation of Cox-2 and up-regulation of Cox-1 was seen in certain Eker RCs. The Eker rat is a suitable animal for elucidating the mechanism of hereditary RCs, and a study on Cox expression in Eker RCs may lead to further understanding of the role of Cox in the development of carcinomas.

We wish to thank Mr. H. Mitani and Y. Hirayama for helping us to prepare Eker RC samples and cell lines, and S. Ebata for immunohistochemical staining.

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