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Sphingosine-1-phosphate/sphingosine-1-phosphate receptor 2 signaling induces COX-2 expression in Wilms tumor

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Abstract

Purpose—Cyclooxygenase-2 (COX-2) has been reported to be ubiquitously expressed in Wilms tumor, the most common malignant renal tumor in children. However, the regulation mechanism of COX-2 expression remains unexplored.

Materials and Methods—Quantitative real-time PCR and western blot analysis were performed to detect COX-2 mRNA and protein expression in WiT49 cells upon the stimulation by sphingosine-1-phosphate (S1P) as well as $S1P_2$ and COX-2 mRNA expression in 10 fresh frozen Wilms tumor tissues and their matched normal tissues. Overexpression, blockade and downregulation of $S1P_2$ were performed using adenoviral transduction, $S1P_2$ antagonist JTE-013 and siRNA transfection, respectively. The level of prostaglandin E_2 (PGE_2) in WiT49 cells was determined by gas chromatography/mass spectrometry.

Results—S1P induced COX-2 mRNA and protein expression in WiT49 cells in a concentration-dependent manner. Overexpression of $S1P_2$ in WiT49 cells led to a significant increase in COX-2 mRNA and protein expression as well as subsequent PGE_2 synthesis. In addition, pretreatment of those cells overexpressing $S1P_2$ with $S1P_2$ selective antagonist JTE-013 completely blocked S1P-induced COX-2 protein expression. In accordance with these results, silencing of $S1P_2$ in WiT49 cells downregulated S1P-induced COX-2 expression. Further research on 10 Wilms tumor specimens found that $S1P_2$ mRNA was greatly increased in Wilms tumor.

Conclusions—S1P induced COX-2 expression in Wilms tumor, and this effect was mediated by $S1P_2$. This finding extends the biological function of $S1P_2$ and provides the biochemical basis for the development of inhibitors targeting S1P/COX-2 signaling pathway.

Keywords

COX-2; sphingosine 1-phosphate; sphingosine 1-phosphate receptor 2; WiT49; Wilms tumor

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Introduction

Cyclooxygenase (COX) is a key, rate-limiting enzyme that converts arachidonic acid into prostaglandins (PGs). Two isoforms of COX enzymes have been characterized: COX-1, which is constitutively expressed in most mammalian tissues,[1] and COX-2, which is induced by various factors including mitogens, hormones, serum and cytokines.[2] Interestingly, COX-2 has been found to be overexpressed in various human cancers. It plays a crucial role in carcinogenesis through synthesis of PGs which stimulate PGs receptors with subsequent enhancement of cellular proliferation, promotion of angiogenesis, inhibition of apoptosis and suppression of immune responses.[3] With the successful use of celecoxib, the first approved selective COX-2 inhibitor, to reduce the formation of colorectal polyps in patients with familial adenomatous polyposis and inhibit experimentally-induced tumorigenesis in several animal models,[4] COX-2 was proposed to be a potential molecular target for cancer chemoprevention.

Sphingosine-1-phosphate (S1P) is an important bioactive lipid that exerts a wide variety of cellular functions via interaction with five G protein-coupled receptors (named $S1P_{1-5}$).[5] Many studies have shown that S1P and its receptors are involved in diverse processes such as angiogenesis, cardiac development, neuronal survival, immunity and recently carcinogenesis.[6, 7] Interestingly, S1P has been shown to induce COX-2 expression in some cell types.[8–13] In addition, Lee et al. found that COX-2 was ubiquitously expressed in human primary Wilms tumors and the specific COX-2 inhibitor SC-236 suppressed tumor growth and inhibited tumor angiogenesis in an orthotopic xenograft model.[14] These findings drove us to investigate the role of S1P regulation of COX-2 expression in Wilms tumor.

In this study, we aimed to study the regulation of COX-2 by S1P in WiT49 cells, a well-characterized Wilms tumor cell line,[15] and to determine which S1P receptor was responsible for this effect. In addition, we studied the expression of COX-2 and its related S1P receptor in 10 Wilms tumor specimens.

Materials and Methods

Cell culture, siRNA transfection and adenoviral transduction

WiT49 cells were cultured as described previously.[15] Transfection of small interfering RNA (siRNA) oligonucleotide duplexes to block $S1P_2$ expression was done using Oligofectamine reagent (Invitrogen) according to manufacturer's instructions. The sequences of siRNA (Dharmacon) were as follows: UACCUUGCUCUCUGGCUCU ($S1P_2$ siRNA); UUCUCCGAACGUGUCACGUUU (NS siRNA). For adenoviral transduction, cells were infected with adenovirus containing GFP, $S1P_1$ or $S1P_2$ for 16–24 h (100 multiplicity of infection) before different assays were done.

Quantitative real-time PCR

Total RNA was isolated from WiT49 cells treated with S1P (Biomol) under different conditions or Wilms tumor tissues using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Then cDNA was generated from 1 μg RNA in the presence of random hexamer primers, deoxynucleoside triphosphates and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Primers were designed using Primer Express™ 2.0 (Applied Biosystems) according to the software guidelines. Sequences were as follows: 5′-GTG CAA CAC TTG AGT GGC TAT-3′ (forward) and 5′-AGC AAT TTG CCT GGT GAA TGA T-3′ (reverse) for the COX-2 gene, 5′-GGC CTA GCC AGT TCT GAA AGC -3′ (forward) and 5′-GCG TTT CCA GCG TCT CCT T-3′ (reverse) for the $S1P_2$ gene, 5′-TGCACCACCAACTGCTTAGC-3′ (forward) and 5′-GGCATGGACTGTGGTCATGAG-3′ (reverse) for the GAPDH gene and 5′-GACAGGATGCAGAAGGAGATTACT-3′ (forward) and 5′-TGATCCACATCTGCTGGAAGGT-3′ (reverse) for the β-Actin gene. Real-time PCR was performed using SYBR Green I DNA binding dye technology on an ABI Prism 7900 HT Sequence Detection System (PE Applied Biosystems). Results were expressed relative to the internal control gene β-Actin or GAPDH.

Western blot analysis

WiT49 cells were treated with S1P with or without JTE-013 (Tocris Bioscience) under different conditions. Then they were washed with ice-cold PBS and homogenized in RIPA buffer. Samples were centrifuged at 14,000 g for 20 min at 4 °C, and protein concentrations of supernatants were determined by BCA protein assay Kit (Pierce). Equal amounts of protein were separated on 8% SDS-PAGE and blotted to nitrocellulose membranes. The membranes were incubated with the indicated primary antibodies (anti-COX-2 and anti-β-Actin, both from Santa Cruz), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was visualized by exposure to X-ray film using Pierce ECL Western Blotting Substrate, according to the manufacturer's instructions.

Prostaglandin E_2 (PGE_2) analysis

The level of PGE_2 in cell pellets was quantified by a highly precise and accurate assay employing gas chromatography/mass spectrometry utilizing stable isotope dilution methodology as described.[16]

Statistical Analysis

All experiments on cell lines were performed at least twice on separate occasions. The data are presented as means ± SD from a representative experiment. The statistical significance of differences between two groups was determined by Student's *t* test using Microsoft Excel software.

Results

S1P induced COX-2 expression in WiT49 cells

Previous reports have indicated that S1P signaling induces COX-2 expression. However, little is known about this pathway in Wilms tumor. Therefore, WiT49, a well-characterized Wilms tumor cell line,[15] was utilized. After treatment of WiT49 cells with different concentrations of S1P for 2 h, quantitative real-time PCR analysis showed that S1P induced COX-2 mRNA expression in a concentration-dependent manner with the maximal effect observed at 100 nM (fig. 1, *A*). Western blot analysis further confirmed the increase of COX-2 expression by S1P on the protein level (fig. 1, *B*). However, using FTY720 phosphate (FTY720-P), an agonist for all S1P receptors except $S1P_2$, we did not see such an induction (data not shown), which suggested that this effect might be mediated by $S1P_2$.

Overexpression of $S1P_2$ increased S1P-induced COX-2 expression and PGE_2 synthesis in WiT49 cells

To prove this notion, we overexpressed $S1P_2$ in WiT49 cells by adenoviral transduction. Consistent with our hypothesis, overexpression of $S1P_2$ into WiT49 cells dramatically increased the expression level of COX-2 mRNA and a further increase was seen with S1P stimulation by quantitative real-time PCR analysis (fig. 2, *A*). Western blot analysis further confirmed this result on COX-2 protein level while overexpression of $S1P_1$ had no such effect compared to that of GFP control cells (fig. 2, *B*). In addition, pretreatment of WiT49 cells overexpressing $S1P_2$ with $S1P_2$ selective antagonist JTE-013 [17] completely blocked S1P-induced COX-2 expression (fig. 2, *C*).

Prostaglandin E_2 (PGE_2) is the major prostaglandin product of COX-2 enzyme in many human tumors.[3] Functional assay measuring PGE_2 synthesis showed that cells overexpressing $S1P_2$ contained relatively abundant PGE_2 compared to GFP control cells in which the basal level of PGE_2 is undetectable in our assay system. Consistent with the data on COX-2 mRNA and protein, stimulation with S1P further increased PGE_2 synthesis in $S1P_2$-overexpressing cells. Moreover, it resulted in the amount of PGE_2 in GFP control cells becoming detectable (fig. 2, *D*).

Downregulation of $S1P_2$ reduced S1P-induced COX-2 expression in WiT49 cells

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To further confirm the role of S1P/$S1P_2$ signaling on COX-2 expression, we used siRNA technology to downregulate $S1P_2$ expression in WiT49 cells. Quantitative real-time PCR analysis proved that the siRNA against $S1P_2$ was significantly effective in reducing the mRNA level of $S1P_2$ (fig. 3, *A*). Treatment of WiT49 cells with this $S1P_2$ siRNA potently inhibited S1P-induced COX-2 mRNA and protein expression (fig. 3, *B* and *C*). Taken together, all the above data clearly demonstrated that S1P-induced COX-2 expression was mediated by $S1P_2$.

$S1P_2$ mRNA was increased in Wilms tumor

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Having shown the role of $S1P_2$ in S1P-induced COX-2 expression *in vitro* and based on the finding that COX-2 was extensively expressed in Wilms tumor,[14, 18] we were interested in knowing whether this pathway also exists *in vivo*. Therefore, quantitative real-time PCR analysis was performed to detect the expression level of COX-2 and $S1P_2$ mRNA in 10 Wilms tumor specimens. Compared to their matched normal tissues, COX-2 mRNA was slightly increased in Wilms tumor specimens while $S1P_2$ mRNA was greatly increased ($P<0.01$; fig. 4), which indicated that S1P/$S1P_2$/COX-2 pathway may also exist *in vivo*.

Discussion

Wilms tumor is the most common malignant renal tumor in children. Although it has a relatively high cure rate, which is achieved by surgery, chemotherapy and radiotherapy, some children with tumors that harbor adverse biologic features still succumb to their disease.[19] Moreover, current therapies are usually associated with significant late sequelae. To date, our knowledge of the mechanisms leading to Wilms tumor progression and metastasis is limited. Therefore, a better understanding of the stimuli and signaling pathways involved in Wilms tumor progression is needed in order to develop future therapeutic strategy.

Recently, S1P signaling has been reported to induce COX-2 expression in different cell types.[8–13] However, it is unknown whether this effect also exists in human cancers, such as Wilms tumor. We detected the effect of S1P on COX-2 expression in WiT49 cells and found that S1P induced COX-2 mRNA and protein expression concentration-dependently (fig. 1). S1P displays diverse cellular functions by interaction with its five specific receptors $S1P_{1-5}$, in which $S1P_1$ mainly couples Gi protein while $S1P_2$ couples $G_{12/13}$ protein.[5] In rheumatoid arthritis synoviocytes, Kitano et al. found that S1P-induced COX-2 expression was sensitive to pertussis toxin, an inhibitor of the Gi protein,[11] in accordance with Kim et al.'s findings in human amnion-derived WISH cells.[10] However, in mouse embryonic fibroblast cells, S1P-induced COX-2 expression was specifically regulated by $G\alpha_{12}$.[9] These findings indicated that S1P-induced COX-2 expression might be cell type-specific.

To delineate which S1P receptor was responsible for S1P-induced COX-2 expression in Wilms tumor, we used different approaches. FTY720-P, an S1P analogue that binds all S1P receptors except $S1P_2$, could not induce COX-2 expression suggesting that this effect might be mediated by $S1P_2$ signaling. Further, overexpression or downregulation of $S1P_2$ expression either increased or decreased COX-2 mRNA and protein expression confirming that $S1P/S1P_2$ signaling was required for COX-2 induction (figs. 2 and 3). In addition, the specific $S1P_2$ antagonist JTE-013 completely blocked S1P-induced COX-2 expression in cells overexpressing $S1P_2$ (fig. 2, *C*), further confirming the requirement of $S1P_2$ in COX-2 induction by S1P. PGE_2 is the principle metabolite of COX-2 enzyme. As expected, the cells overexpressing $S1P_2$ produced much more PGE_2 than GFP control cells. And stimulation with S1P led to a further increase (fig. 2, *D*). Taken together, these data clearly demonstrate that $S1P_2$ was responsible for S1P-induced COX-2 expression and its downstream molecule PGE_2 synthesis *in vitro*.

Previous reports have shown that COX-2 was ubiquitously expressed in human Wilms tumor.[14, 18] Consistent with these findings, our study on 10 Wilms tumor specimens also showed that COX-2 mRNA was extensively expressed in Wilms tumor specimens. Interestingly, we also found that among the four S1P receptors ($S1P_{1-3,5}$) that Wilms tumor expressed (data not shown), $S1P_2$ was significantly upregulated in Wilms tumor, which indicate that $S1P/S1P_2$/COX-2 pathway may also have physiopathological relevance *in vivo*.

In summary, our study for the first time clearly demonstrated that S1P induced COX-2 expression and subsequent PGE_2 synthesis in WiT49 cells, and this effect was mediated by

$S1P_2$, which extends the biological function of $S1P_2$ in human cancers and provides the biochemical basis for the development of inhibitors targeting S1P/COX-2 signaling pathway. In previous studies $S1P_2$ was usually regarded as a negative modulator in tumor progression.[20] Here for the first time we suggest that $S1P_2$ might act as a positive tumor modulator and thus promote tumor progression. Further work in other human cancers will allow us to better understand the role of $S1P_2$ in tumor progression.

Conclusions

This study for the first time clearly demonstrated that S1P induced COX-2 expression and subsequent PGE_2 synthesis in WiT49 cells, and this effect was mediated by $S1P_2$, which extends the biological function of $S1P_2$ in human cancers and provides the biochemical basis for the development of inhibitors targeting S1P/COX-2 signaling pathway.

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Abbreviations

COX	cyclooxygenase
PGs	prostaglandins
S1P	sphingosine-1-phosphate
FTY720-P	FTY720-phosphate
siRNA	small interfering RNA
NS	non-specific
PGE_2	prostaglandin E_2

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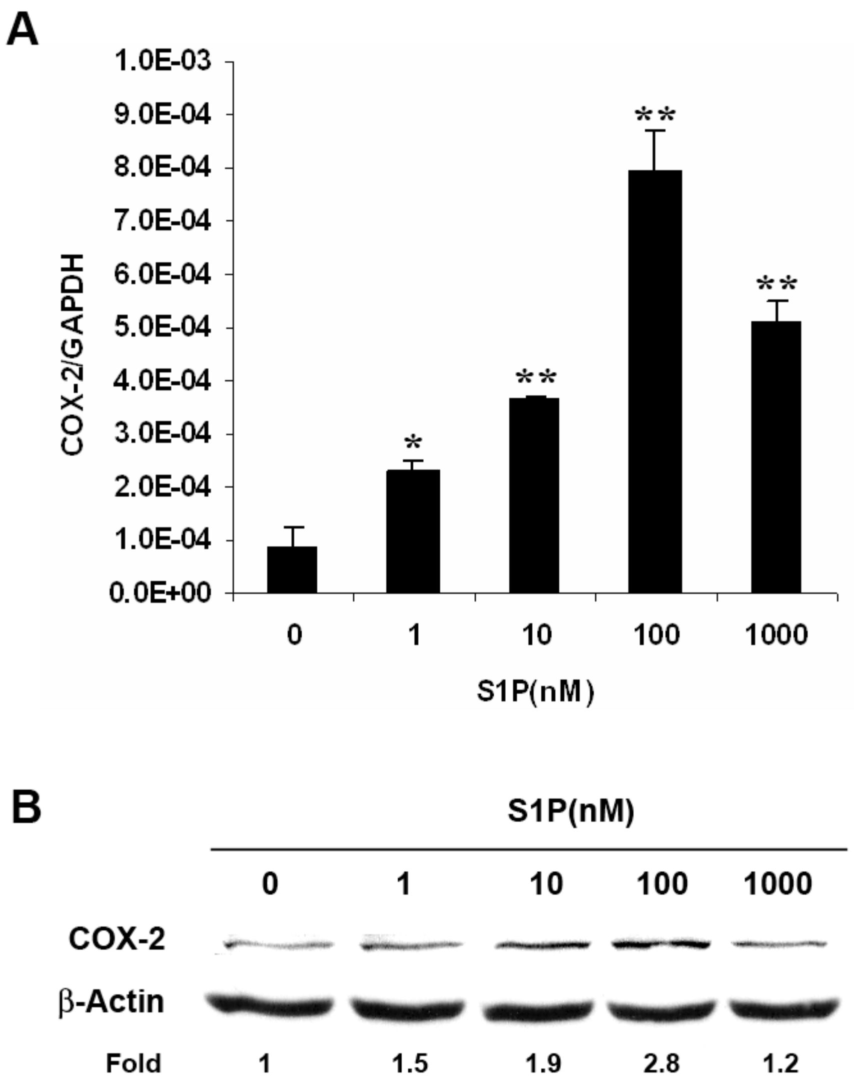

Fig. 1.

S1P induced COX-2 expression in WiT49 cells. WiT49 cells were serum starved for 24 h and then treated with different concentrations of S1P for 2 h before quantitative real-time PCR (*A*) and western blot analysis (*B*) were done. The mRNA expression of COX-2 was normalized to that of GAPDH and presented as means ± SD. *, $P<0.05$, **, $P<0.01$ *versus* without S1P.

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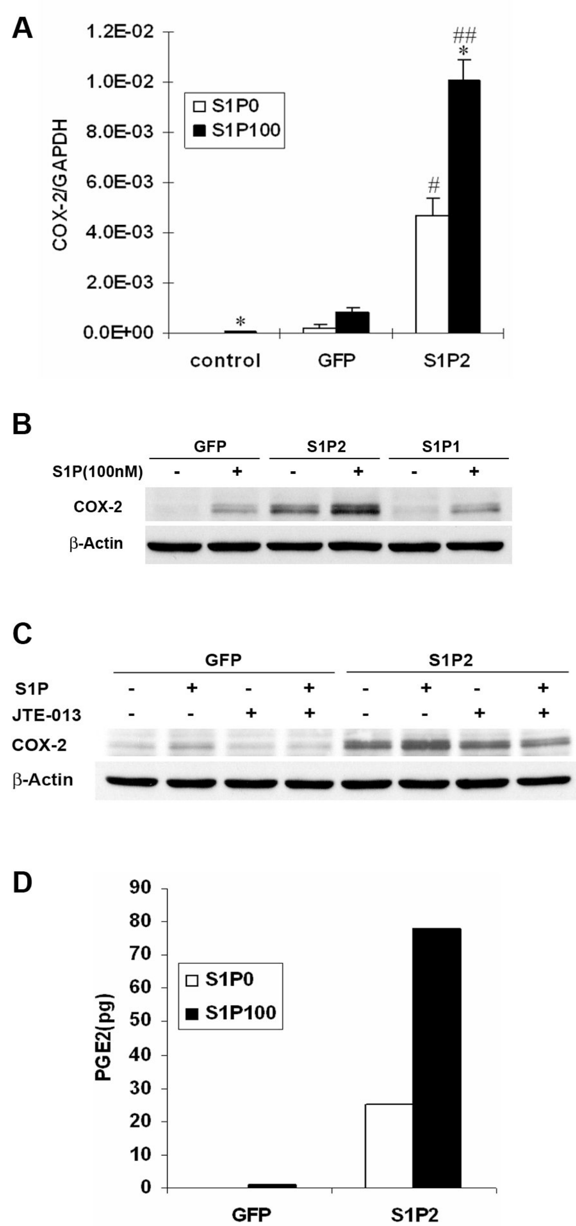

Fig. 2.

Overexpression of $S1P_2$ increased COX-2 and PGE_2 synthesis in WiT49 cells. WiT49 cells were infected with adenovirus containing GFP, $S1P_2$ or $S1P_1$ with MOI 100. After 16–24 h, cells were serum starved for 24 h and then stimulated with 100 nM S1P for 2 h before quantitative real-time PCR (*A*) and western blot analysis (*B*) were done. *, P<0.05 *versus* without S1P; $^{\#}$, P<0.05, $^{\#\#}$, P<0.01 *versus* corresponding GFP control. *C*, WiT49 cells overexpressing $S1P_2$ or GFP were serum starved and pretreated with 1 μM JTE-013 for 30 min before stimulation with 100 nM S1P for another 2 h. Then western blot analysis was done. *D*, WiT49 cells were seeded at the cell density of $1x10^6$ cells per dish (60 mm). After attachment, they were infected with adenovirus containing $S1P_2$ or GFP with MOI 100 for 16 h followed by serum starvation for another 24 h. Then the cells were treated with 100 nM

S1P for 2 h and collected for PGE_2 analysis. Data shown are representative of two independent experiments.

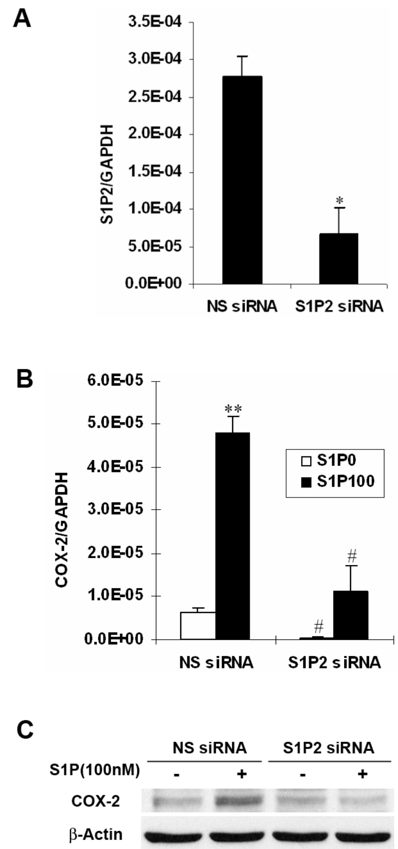

Fig. 3.

Downregulation of $S1P_2$ reduced S1P-induced COX-2 expression in WiT49 cells. *A*, WiT49 cells were transfected with 100 nM $S1P_2$ siRNA or NS siRNA, harvested 48 h later and assayed for the level of $S1P_2$ mRNA by quantitative real-time PCR. *, $P<0.05$ *versus* NS siRNA. *B* and *C*, After transfection with $S1P_2$ siRNA for 24 h, WiT49 cells were serum starved for 24 h and then stimulated with 100 nM S1P for another 2 h before quantitative real-time PCR (*B*) and western blot analysis (*C*) were done. **, $P<0.01$ *versus* without S1P; $^{\#}$, $P<0.05$, $^{\#\#}$, $P<0.01$ *versus* corresponding NS siRNA control.

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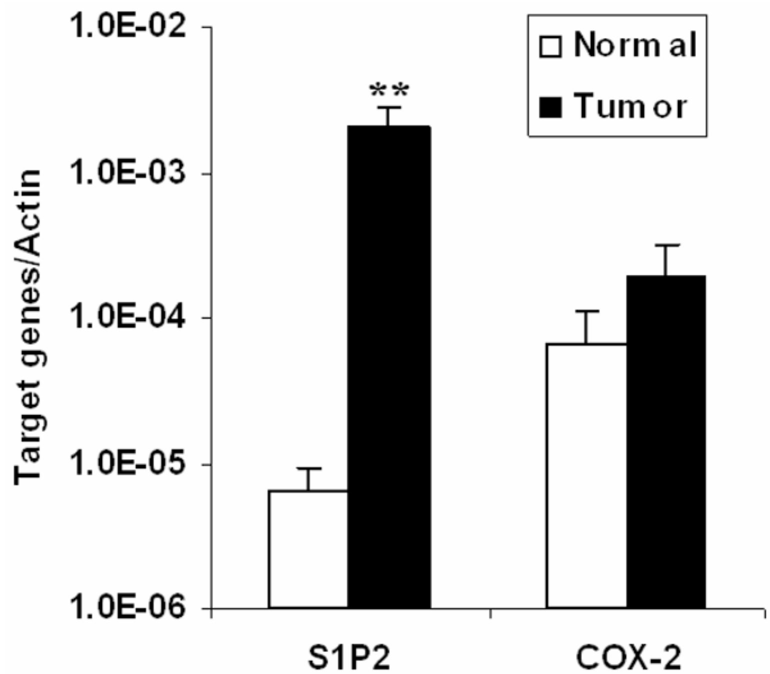

Fig. 4.

Relative mRNA expression of $S1P_2$ and COX-2 in Wilms tumor and their matched normal tissues. Quantitative real-time PCR was performed in 10 Wilms tumor tissues and their matched normal tissues. The mRNA expression of $S1P_2$ and COX-2 was normalized to that of the housekeeping gene β-Actin and presented as means ± SE from 10 samples..**, $P<0.01$ *versus* that of their matched normal tissues.