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14. ABSTRACT 12(S)-hydroxyeicosatetraenoic [12(S)-HETE], a lipoyxygenase metabolite of arachidonic acid, has been demonstrated to enhance metastatic capacity during tumor progression via its receptor. Using cloning and binding strategy, we have identified a G-protein coupled receptor, GPR31, as a 12(S)-HETE receptor. In this project, we aimed to characterize the biochemical properties of GPR31 and study the role of GPR31 in tumor survival and metastasis. We have constructed a secondary structure model for GPR31 and using the structural characteristics, we designed two peptides to produce antibodies that against both sides of this membrane protein. We have analyzed the expression of GPR31 in cell lines and human prostate specimens. The GPR31 protein was expressed in various cancer cells and stained in prostate cancer tissue. In order to further characterize this receptor, we designed a series experiments to determine its biochemical properties. Binding experiments with or without GPR31 expressed in Chinese hamster ovary cells demonstrated that this receptor has a Kd value of 4.8 for [3H]12(S)-HETE specific binding with Bmax value of 38.3 pmol/mg protein. We also purified the two antibodies from immunized rabbit antisera. This project will provide significant insights into the process of prostate cancer progression and metastasis.						
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Introduction

Prostate cancer is the most commonly diagnosed cancer in the Western World and is the second leading cause of male cancer death(1). Advances in surgical techniques and radiotherapy, coupled with the early detection from the biochemical determination of prostate specific antigen, has led to a significant decrease in mortality from this disease in recent years. However while these therapies are effective in organ-confined disease, metastasis, especially to bone, is the main reason for prostate cancer patient death. Therefore understanding the underlying mechanisms of prostate cancer cell metastasis is important for rational intervention and treatment. Researchers in Dr. Honn's lab found that the level of platelet-type 12-LOX expression was correlated with the tumor stage and grade(2-4). 12-LOX mRNA levels were elevated in prostate cancer cells and the expression associated with poor differentiation and invasiveness of prostate cancer. Overall, 46 of 122 evaluable patients (38%) showed increased levels of 12-LOX mRNA in prostate cancer tissues compared with the matching normal tissues(2). This clinical study was corroborated by animal model experimentation where overexpression of the platelet-type 12-LOX in human prostate cancer PC-3 cells resulted in significantly enhanced angiogenesis and tumor growth(4). As a result of the above-mentioned clinical study, research has focused on the platelet-type 12-LOX and its metabolite of arachidonic acid, i.e. 12(S)-hydroxyeicosatetraenoic [12(S)-HETE]. A number of studies suggest that 12(S)-HETE enhances the metastatic capacity during tumor progression. It evokes a wide variety of cellular responses and influences tumor progression (5). This eicosanoid stimulates several steps of tumor invasion and motility by inducing alterations in the cancer cell cytoskeleton (6, 7), thereby enhancing tumor cell motility (8). Exogenously added 12(S) HETE enhances cancer cells to secrete proteinases (9, 10) and vascular endothelial growth factor (11, 12). 12(S)-HETE treatment of cancer cells also enhanced the expressions of integrins (6, 13) and fibronectin (14), which prolong cell survival (15). In endothelial cells, 12(S)-HETE induces the non-destructive retraction of monolayers (16) and promotes tumor cell adhesion (17). The motility of isolated endothelial cells and tube formation is enhanced by 12(S)-HETE (4). Obviously, 12(S)-HETE plays an important role in the expansion and metastasis of prostate cancer.

There is strong evidence that 12(S)-HETE signaling involves G-proteins. Work in our lab provided evidence that regulation of PKC-alpha by 12(S)-HETE and 13(S)-HODE is through GPCR-mediated hydrolysis of inositol phospholipids (18). Studies in our lab suggest that 12(S)-HETE receptor may stimulate a protein tyrosine phosphatase as well as a protein tyrosine kinase, i.e. Src (19). Additionally, Hampson and Grimaldi found that 12(S)-HETE attenuates glutamate-induced calcium influx into neurons via a pertussis toxin-sensitive mechanism, suggesting that it acts *via* a G-protein-coupled receptor(20). These results suggest that 12(S)-HETE receptor is G-protein-coupled receptor.

G protein coupled receptors (GPCRs) are a large family of seven-transmembrane proteins. On the basis of sequence comparisons, human GPCRs can be divided into three groups (A, B and C). Besides their conserved secondary domain structure, these GPCR families do not share significant sequence homologies. We conducted a computer search of GPCRs for lipid derived messengers. A total of 35 GPCRs belong to the lipid-derived

messenger GPCRs, which fall into group A (rhodopsin-like) sub-family. Based on this information, we hypothesize that eicosanoids (such as leukotrienes, prostaglandins, and cannabinoids) receptors, including 12(S)-HETE receptor, should fall into group A sub-family. Therefore, we have cloned 20 orphan GPCRs (whose ligands are unknown) from human prostate cancer cells. The cDNAs were subcloned into pcDNA3.1 expression vector, which were transfected into Chinese Hamster Ovary cells. Then the transfectants were evaluated for [³H]12(S)-HETE binding. Through this approach we identified a receptor for 12(S)-HETE, G-protein coupled receptor 31 (GPR31). We have sequenced the full-length GPR31 cDNA cloned from PC3 cells and subcloned the cDNA into mammalian expression vector pcDNA3.1. The expression of this receptor was detected in various cancer cell lines and prostate cancer tissue. In this project, we aimed to characterize the biochemical properties of GPR31 and study the role of GPR31 in tumor survival and metastasis.

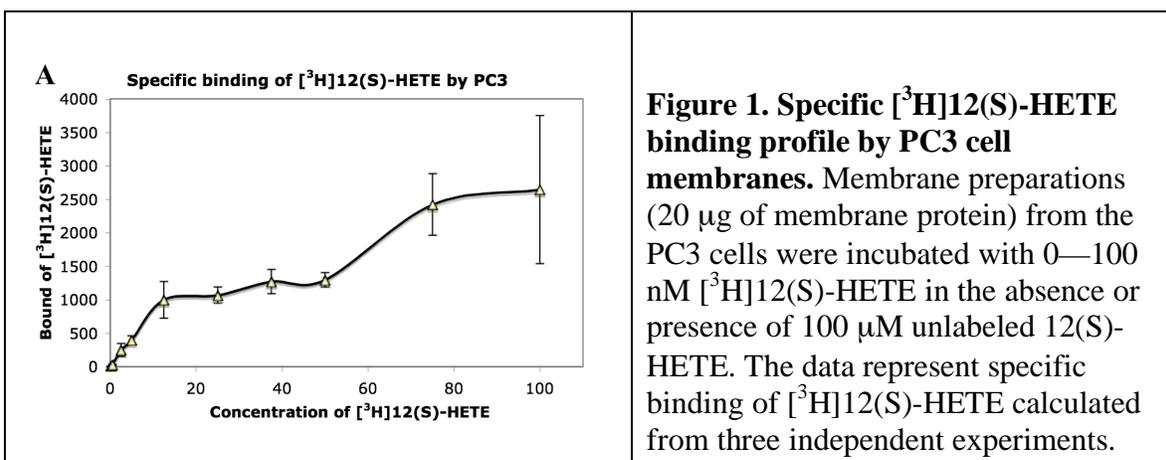
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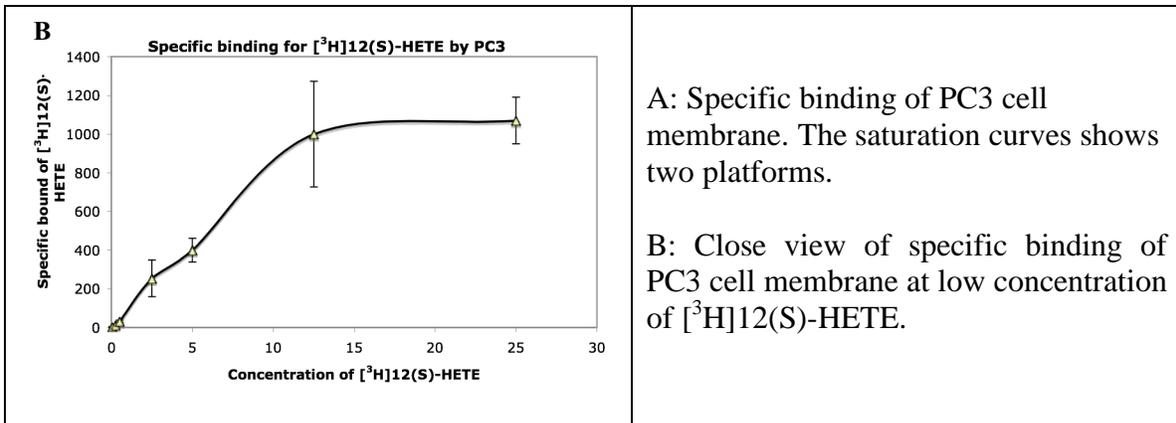
In order to characterize the binding ability of GPR31 to its ligand 12(S)-HETE, we designed a series binding experiments to determine its K_d and B_{max} values. First, we determined the [^3H]12(S)-HETE binding profile for PC3 cells in which we cloned the receptor GPR31. Second, we determined the [^3H]12(S)-HETE binding profile for Chinese hamster ovary (CHO) cells. The results indicate that this cell line has no endogenous [^3H]12(S)-HETE binding ability. Thereafter, we transfected CHO cells with mammalian expressed construct, pcDNA3.1/GPR31 and examined the binding ability using the membranes from the transfected CHO cells. We assumed that any specific [^3H]12(S)-HETE binding should be contributed by the expressed GPR31 receptor. The detail experiment and results are as followings:

Radioligand binding and determination of the K_d and B_{max} values for GPR31:

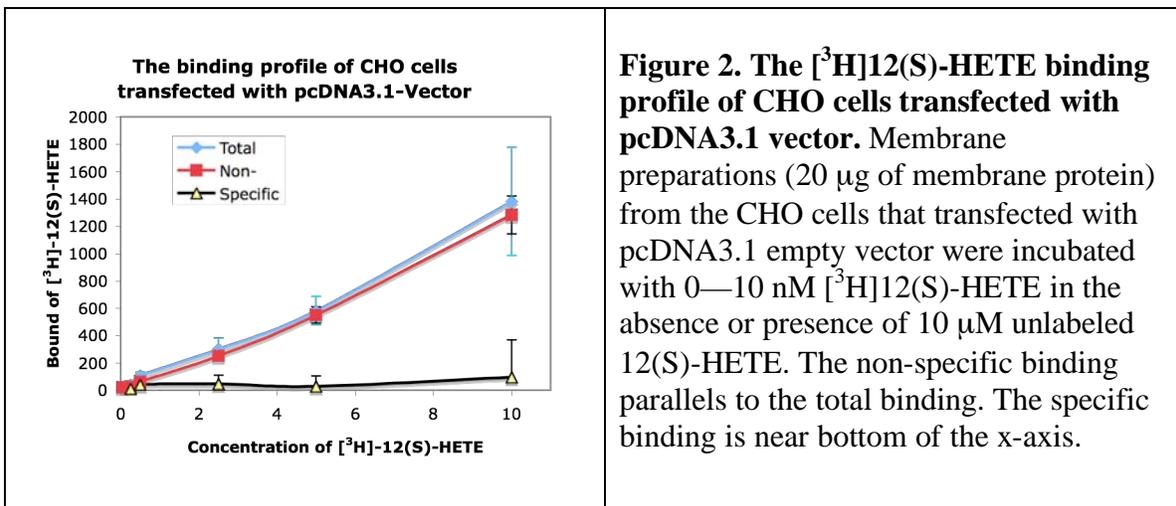
The binding assays were performed with cell membranes. The membranes from PC3 cells, CHO cells or transfected cells were prepared as described by Yokomizo et al. (21) Binding assays were initiated by the addition of various concentrations of [^3H]12(S)-HETE in the binding buffer. For total binding samples, the membrane was treated with various concentrations of [^3H]12(S)-HETE at room temperature. For the nonspecific binding samples, [^3H]12(S)-HETE plus 1000-fold excess non-labeled 12(S)-HETE were co-incubated. After 60 min incubation, the sample was transferred onto Whatman GF/C glass fiber filter and was quickly rinsed with wash buffer. Filters were then place in separate scintillation vials contain 2 ml of scintillation liquid, UltimaGold (PerkinElmer, MA) and the radioactivity was determined by a liquid scintillation counter.

First, we determined the specific [^3H]12(S)-HETE binding by PC3 cell membranes. As shown in Figure 1, the saturation curve appears to be biphasic. This result is consistent with our previous conclusion that two types of receptors, a high affinity and a low affinity 12-HETE receptors are expressed in cells (19).





In order to determine the binding values for cloned receptor, GPR31, we chose CHO cells as model system. Figure 2 shows the result of 12(S)-HETE binding profile of the membrane from the CHO cells transfected with pcDNA3.1 empty vector. The specific binding curve that is at bottom of the x-axis reveals that CHO cells have no endogenous 12(S)-HETE receptor.



Further experiments were carried out to determine the affinity of [³H]12(S)-HETE binding to cloned GPR31. Figure 3 shows the [³H]12(S)-HETE binding profile of the membrane from CHO cells transfected with pcDNA3.1/GPR31. Analysis of binding data revealed that cloned GPR31 has K_d values of 4.8 ± 0.12 nM with B_{max} value of 38.3 ± 0.23 pmol/mg protein.

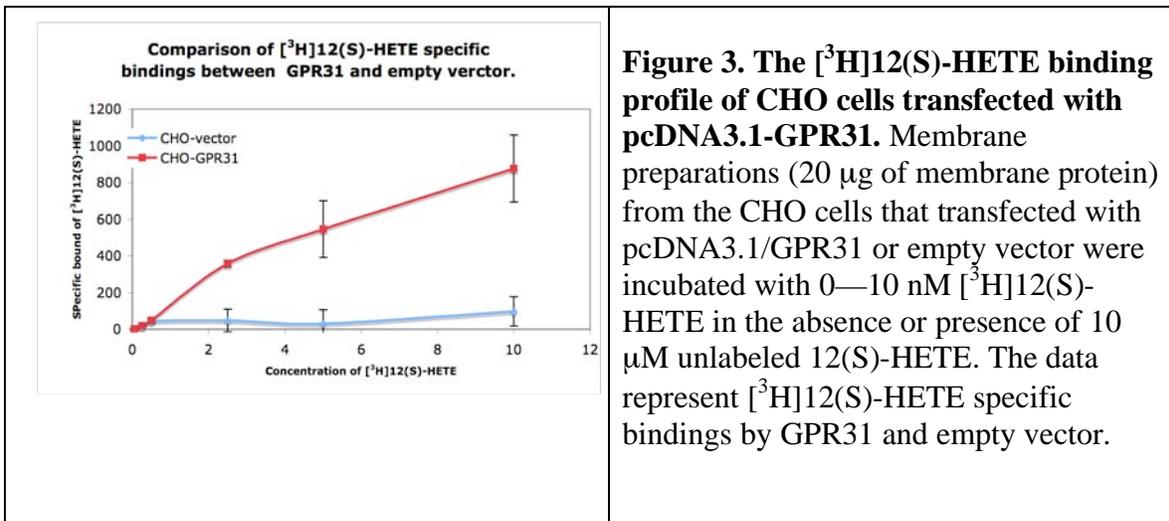


Figure 3. The $[^3\text{H}]12(\text{S})\text{-HETE}$ binding profile of CHO cells transfected with pcDNA3.1-GPR31. Membrane preparations (20 μg of membrane protein) from the CHO cells that transfected with pcDNA3.1/GPR31 or empty vector were incubated with 0—10 nM $[^3\text{H}]12(\text{S})\text{-HETE}$ in the absence or presence of 10 μM unlabeled 12(S)-HETE. The data represent $[^3\text{H}]12(\text{S})\text{-HETE}$ specific bindings by GPR31 and empty vector.

It is reported that BLT2 has 12(S)-HETE binding ability (21). Therefore, we use pcDNA3.1/BLT2 as a control construct to do a similar binding experiment. The result indicates that BLT2 binds 12(S)-HETE (Figure 4A). However, Its binding potential is much low than that of GPR31 (Figure 4B).

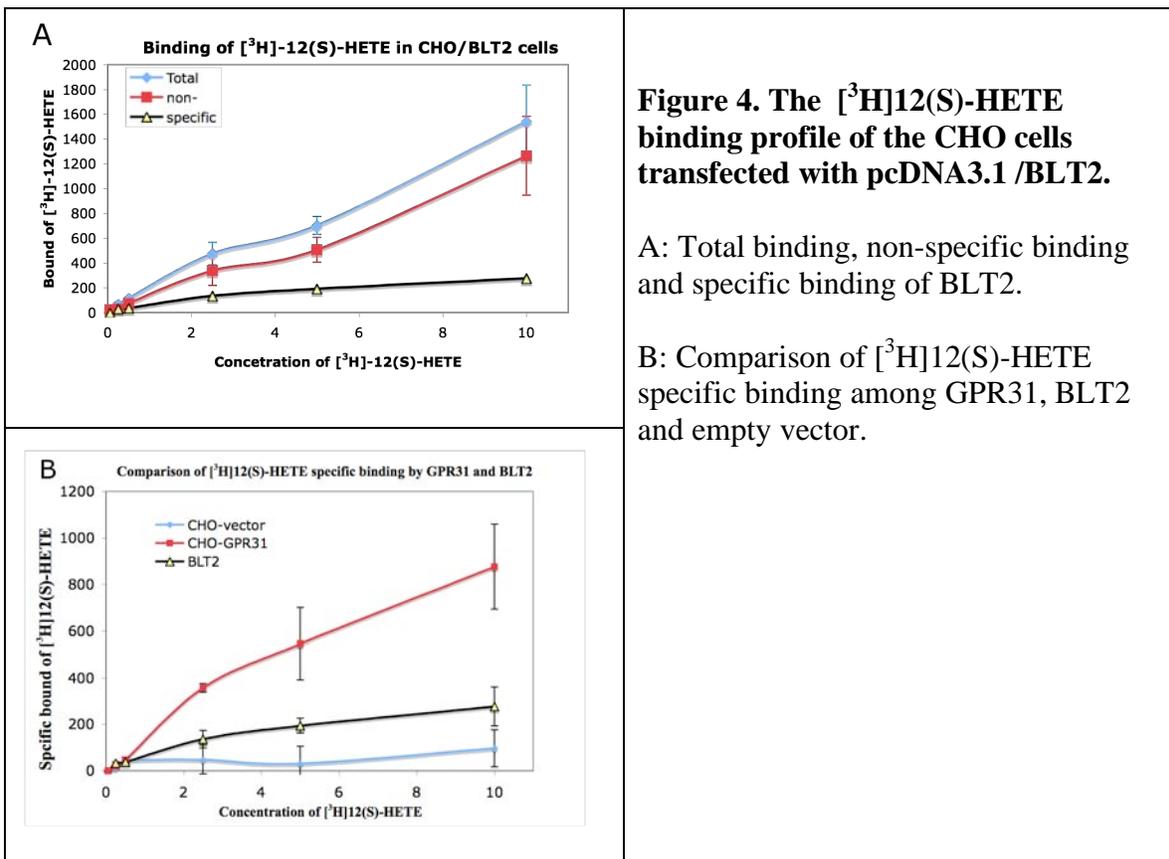


Figure 4. The $[^3\text{H}]12(\text{S})\text{-HETE}$ binding profile of the CHO cells transfected with pcDNA3.1 /BLT2.

A: Total binding, non-specific binding and specific binding of BLT2.

B: Comparison of $[^3\text{H}]12(\text{S})\text{-HETE}$ specific binding among GPR31, BLT2 and empty vector.

Further, we examined the inhibition abilities that various eicosanoids of [³H]-12(S)-HETE binding to the membrane fraction of CHO cells transfected with pcDNA3.1/GPR31. The results indicates that the non-radiolabeled 12(S)-HETE has the highest potential to replace bound [³H]-12(S)-HETE in the membrane from pcDNA3.1/GPR31 transfected CHO cells. 5-oxoETE and 5(S)-HETE also showed significant inhibition of [³H]-12(S)-HETE binding.

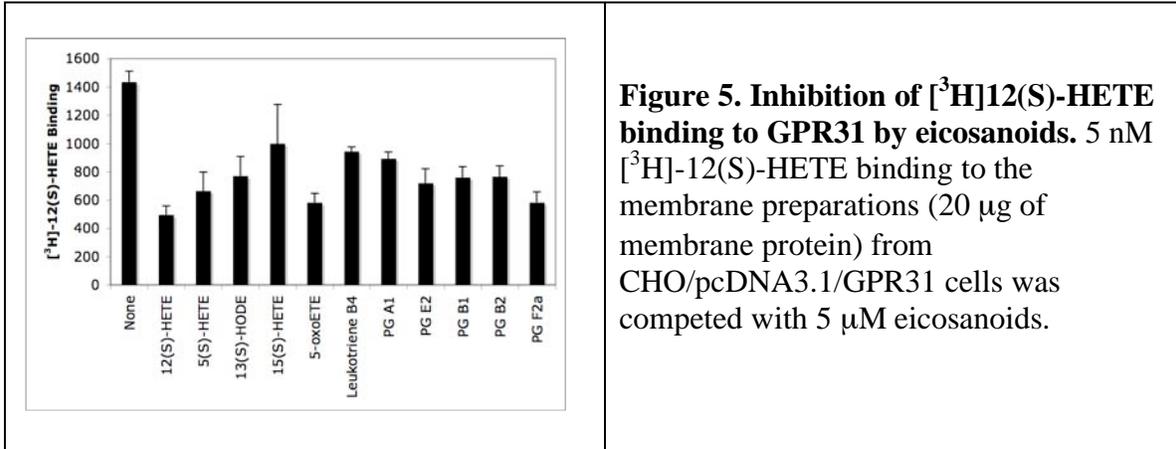


Figure 5. Inhibition of [³H]-12(S)-HETE binding to GPR31 by eicosanoids. 5 nM [³H]-12(S)-HETE binding to the membrane preparations (20 µg of membrane protein) from CHO/pcDNA3.1/GPR31 cells was competed with 5 µM eicosanoids.

Purification of antibodies for GPR31:

As mentioned in the reporter last year, we have synthesized two peptides that were used as antigens to immunized rabbits to produce anti-GPR31 serums. When we obtained the serums, we purified these serums used protein G. The results are shown in Figure 6 and 7. We will use these purified antibodies to determine the distributions of GPR31 both in cell level and tissue level.

Figure 6. Purification of antibody for anti GPR31 C-terminal antibody.

Lane M: Protein marker
 Lane 1: Rabbit anti-GPR31 250G31 bleed#3
 Lane 2: Serum flowthrough
 Lane 3: Purified antibody

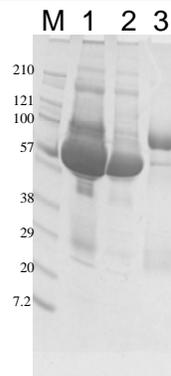
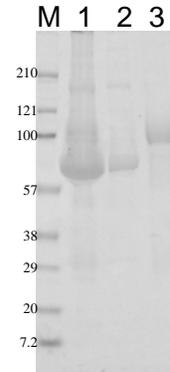


Figure 7. Purification of antibody for anti GPR31 secondary extracellular loop antibody.

Lane M: Protein marker
Lane 1: Rabbit anti-GPR31 B2849 bleed#2
Lane 2: Serum flowthrough
Lane 3: Purified antibody



Immunohistochemical analysis of GPR31:

After we obtained rabbit anti-serum that is against the secondary extracellular loop of GPR31, we have performed the immunohistochemical experiments using human prostate cancer specimens. The results indicate that GPR31 is weakly detected in benign gland, but strong staining were detected in neoplastic gland and differentiated tumors (Gleason grade3/4) (Figure 8).

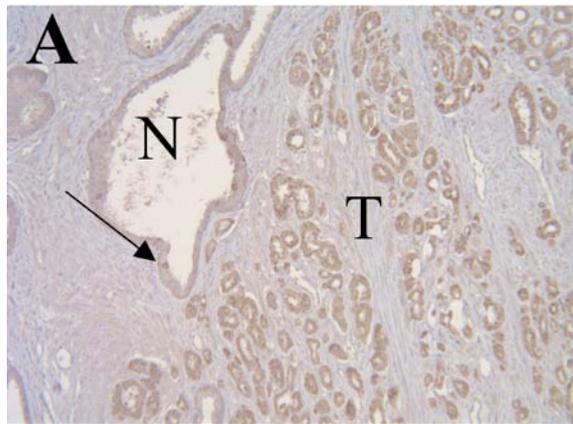
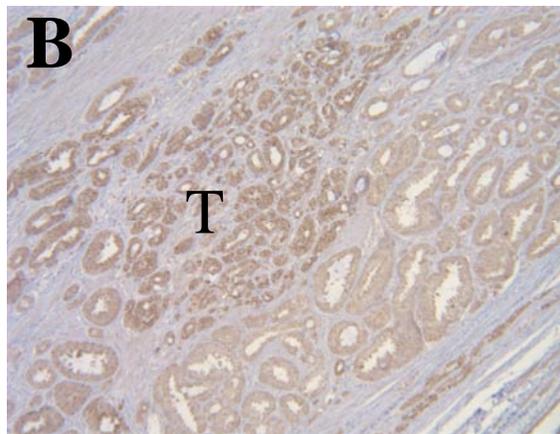


Figure 8. Immunohistochemical analysis of GPR31 in two cases of human prostate cancer tissue specimens with antibody against the secondary extracellular loop of GPR31.

A: Benign gland (N: left corner) with rare cells (arrow) demonstrating staining, and neoplastic glands (T: Gleason grade 3/4) showing intense brown staining, 10X.



B: Strong staining in an area (T: upper-left) with mixed Gleason 3/4 tumor area, weak staining in an area (lower-right) with Gleason grade 3 tumor, 10X.

Key research accomplishments:

- (1) We have performed a series of experiments to determine the biochemical characteristics for GPR31. The radio labeled ligand binding experiments revealed that GPR31 have a Kd value of 4.6 nM and Bmax value of 25 pmol/mg protein.
- (2) We have purified two antibodies for GPR31 from antiserums of immunized rabbits protein G.
- (3) Immunohistochemical analysis reveal that GPR31 was strong expressed in neoplastic gland and differentiated tumors.

Conclusions

It has been well known that 12(S)-HETE promotes cell adhesion, cell migration and inhibits apoptosis. There is biochemical evidence that the diverse effects of 12(S)-HETE are mediated via its receptors. We cloned and characterized a G-protein coupled receptor, GPR31, from human prostate cancer cells. Our findings suggest that GPR31 is expressed in various cancer cells and prostate cancer tissues. Binding experiments demonstrate that this receptor has a Kd value of 4.8 nM and Bmax value of 38.3 pmol/mg protein for [³H]12(S)-HETE binding.

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