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Systemic distribution, subcellular localization and differential expression of sphingosine-1-phosphate receptors in benign and malignant human tissues

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Abstract

Aims—Five sphingosine-1-phosphate receptors (S1PR): S1PR1, S1PR2, S1PR3, S1PR4 and S1PR5 (S1PR1-5) have been shown to be involved in the proliferation and progression of various cancers. However, none of the S1PRs have been systemically investigated. In this study, we performed immunohistochemistry (IHC) for S1PR1-S1PR5 on different tissues, in order to simultaneously determine the systemic distribution, subcellular localization and expression level of all five S1PRs.

Methods—We constructed tissue microarrays (TMAs) from 384 formalin-fixed paraffin-embedded (FFPE) blocks containing 183 benign and 201 malignant tissues from 34 human organs/systems. Then we performed IHC for all five S1PRs simultaneously on these TMA slides. The distribution, subcellular localization and expression of each S1PR were determined for each tissue. The data were then compared in benign and malignant tissues from the same organ/tissue using the student t-test. In order to reconfirm the subcellular localization of each S1PR as determined by IHC, immunocytochemistry (ICC) was performed on several malignant cell lines.

Results—We found that all five S1PRs are widely distributed in multiple human organs/systems. All S1PRs are expressed in both the cytoplasm and nucleus, except S1PR3, whose IHC signals are only seen in the nucleus. Interestingly, the S1PRs are rarely expressed on cellular membranes. Each S1PR is unique in its organ distribution, subcellular localization and expression level in

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benign and malignant tissues. Among the five S1PRs, S1PR5 has the highest expression level (either in nucleus or cytoplasm), with S1PR1, 3, 2 and 4 following in descending order. Strong nuclear expression was seen for S1PR1, S1PR3 and S1PR5, whereas S1PR2 and S1PR4 show only weak staining. Four organs/tissues (adrenal gland, liver, brain and colon) show significant differences in IHC scores for the multiple S1PRs (nuclear and/or cytoplasmic), nine (stomach, lymphoid tissues, lung, ovary, cervix, pancreas, skin, soft tissues and uterus) show differences for only one S1PR (cytoplasmic or nuclear), and twenty three organs/tissues show no significant difference in IHC score of any S1PR (cytoplasmic or nuclear) between benign and malignant changes.

Conclusion—This is the first study to evaluate the expression level of all S1PRs in benign and malignant tissues from multiple human organs. This study provides data regarding the systemic distribution, subcellular localization and differences in expression of all five S1PRs in benign and malignant changes for each organ/tissue.

Keywords

Sphigosine-1-phosphate (S1P) Receptors (S1PRs); Cancer; Tissue Microarrays (TMAs); Immunohistochemistry (IHC); Immunocytochemistry (ICC); Adrenal gland; Liver; Neural System; Colon; Stomach; Lymphoid tissues; Lung; Ovary; Cervix; Pancreas; Skin; Soft tissues; Uterus

Introduction

Sphingosine-1-phosphate (S1P) is formed by the phosphorylation of sphingosine by two sphingosine kinases (SphK1 and SphK2) and degraded by S1P lyase and several phosphatases (Spiegel et al, 2003). S1P, an active lipid metabolite, was previously thought to influence intracellular functions directly (Zhang et al, 1991; Spiegel, 1999; Olivera et al, 1993). It is now recognized that S1P is a common ligand for several G protein-coupled receptors (GPCRs). These S1P-specific receptors are now named S1P receptors (S1PRs). A total of five subtypes of S1PRs (S1PR1-5) have been identified since the first S1PR was discovered by Hla T et al (1990). During the past two decades, S1P and its specific receptors were found to be involved in a wide range of pathophysiological processes (Maceyka et al, 2012) in multiple organ systems that include: the central nervous system, immune system (Soliven et al, 2011; Garris et al, 2014), cardiovascular system (Schmouder et al, 2012), and in embryonic development (Kondo et al, 2014; Hiraga et al, 2006). Further, emerging evidence indicates that S1P/S1PRs play significant roles in the proliferation, progression, survival and therapeutic response to treatment in cancer (Pyne et al, 2010; Kunkel et al, 2013; Pyne et al, 2013).

All S1PRs have been correlated with pathogenesis and progression in cancers. In one study of 304 patients with estrogen receptor-positive (ER^+) breast cancer, concurrently high expression of nuclear SphK1 with membrane S1PR1 and cytoplasmic S1PR3 expression were associated with shorter disease-specific survival. In the same study population, elevated expression of nuclear S1PR2 was associated with improved patient prognoses, which was linked to a reduction in the nuclear localization of SphK1 (Okhotsk et al, 2012). In another study of 140 patients with ER^- breast cancer, high expression of S1PR4 and

SphK1 was associated with shorter disease-free and disease-specific survival (Okhotsk et al, 2012). S1PR3 was markedly up-regulated in a subset of lung adenocarcinoma cells. Binding of S1P with S1PR3 on the surface of these cancer cells increases the expression of epidermal growth factor receptor (EGFR) via Rho kinase (ROCK) pathway, which enhances the proliferation and anchorage-independent growth of these cancer cells (Zhang et al, 2013). In colon cancer, S1PR1 is up-regulated by persistent activation of NF-KB and STAT3 transcription factors, resulting in colitis-associated colon cancer (Liang et al, 2013). In addition, S1PR2-induced ERK phosphorylation up-regulates CD44, a cancer stem cell marker in HCT116 human colon carcinoma cells (Kawahara et al, 2013). In prostate cancer, activity of S1PR5 induces autophagy through generating endoplasmic reticulum stress in human prostate cancer PC-3 cells (Huang et al, 2014). Additionally, binding of S1P to S1PR2 may sequentially activate phosphatidylinositol 3-kinase (PI3K) and oncogenic kinase Akt pathways in Du-145 prostate cancer cells (Beckham et al, 2013).

S1P is a common ligand for all five S1PRs, but each S1PR subtype is coupled with a unique set of G proteins, $G_{i/o}$, G_q, or $G_{12/13}$ (Windh et al, 1999). Thus, binding of S1P with different S1PR subtypes may result in activation of various classical pathways downstream of different G proteins. In a given tissue/cell type, the impact on the fate of cancer cells of S1P via activation of S1PRs may reflect a summation of the various counteracting signal inputs by multiple activated S1PR subtypes. For example, S1PR1 mediates stimulation of cell proliferation through the $G_{i/o}$-mediated signaling pathways including PI3K/Akt and ERK, whereas S1PR2 mediates inhibition of cell proliferation through mechanisms involving $G_{12/13}$ /Rho/Rho kinase/PTEN-dependent Akt inhibition. Whether or not cancer cells migrate and proliferate depends on the net effect of downstream signaling pathways of S1PR1 and S1PR2 (Van et al, 2002; Takuwa et al, 2012). This fact implies that while the effects of one activated S1PR on cancer is being investigated, the synergistic/ antagonistic effects from the other S1PRs that are simultaneously activated by binding S1P should also be considered.

Distinctive patterns of distribution in various tissues for each S1PR have been previously mapped principally by semi-quantitative PCR techniques, Northern blots, in situ hybridization, and Western blots (Goetzl et al, 2002). S1PR1, 2 and 3 are found in multiple organs/tissues, whereas the expression of S1PR4 and S1PR5 may be limited to hematopoietic and lymphatic tissues, and central nervous system tissues, respectively. In addition, the data on S1PR tissue distribution patterns were mostly obtained from rodents (mouse and rat) or in human cell lines (Liu et al, 1997; Zhang et al, 1999; Chae et al, 2004; Gräler et al, 1998; Im et al, 2000). The systemic distribution and subcellular localization of each S1PR protein molecule in benign and malignant human tissues has been understudied. The expression level of the five different S1PRs has not been compared simultaneously for a given tissue pair of benign and malignant changes.

This study performed immunohistochemistry (IHC) on approximately 400 human benign and malignant tissues and immunocytochemistry (ICC) on different cell lines, to simultaneously determine the systemic distribution and subcellular localization of each S1PR in major human organs/tissues, to compare the expression levels of each S1PR between benign and malignant changes in each individual organ/tissue, and to provide

information for future studies on the interacting effects of different S1PRs on cancer biology.

Material and methods

Tissue Collection and Tissue Microarray (TMA) Construction

This study was approved by the University of Mississippi Medical Center (UMMC) Institutional Review Board. All participants are patients enrolled into UMMC. Three hundred and eighty-four formalin-fixed and paraffin-embedded (FFPE) benign and malignant specimens from 36 different human organs/tissues were obtained from archives in the Department of Pathology at UMMC. Histological features and pathological diagnosis were confirmed by pathologists on the original hematoxylin and eosin (H&E) stained slides. Areas of interest were selected on the original H&E stained slides and topographically correlated with the corresponding FFPE blocks. One, 1-mm cylindrical core from each area of interest on the primary FFPE block was punched and transferred to composite paraffin blocks to construct TMAs using a Beecher MTA1 manual tissue arrayer. The resulting TMA blocks were heated at 40°C for 4 h in order to fuse transferred cores with composite paraffin block. Each composite TMA block was sectioned at 5 μm in thickness. One slide from each TMA block was stained with H&E, in order to re-confirm the histological findings and pathological diagnosis. The remaining slides were used for immunohistochemistry (IHC) study.

Immunohistochemistry (IHC) and scoring system

The protocol for immunohistochemical staining and scoring system were used as described previously (Zhou et al, 2012). Briefly, the TMA slides were deparaffinized in a 56°C oven overnight the day before performing immunohistochemistry staining. The TMA slides were further deparaffinized in xylene and rehydrated through graded ethanol. Antigens were retrieved with antigen retrieval solution (Citric-plus, BioGenex, Fremont CA), and endogenous peroxidase was quenched with 3% hydrogen peroxide for 30 min. Blocking serum corresponding to each primary antibody in ABC kits (Vector Laboratories, Burlingame, CA) was incubated at room temperature for 1 h to block nonspecific binding sites. Then, the slides were incubated with primary antibodies (anti- S1PR1-5, respectively, Table 1) for 2 h at room temperature for polyclonal antibodies, or, overnight at 4°C for monoclonal antibodies. The slides for negative controls were incubated with the blocking serum, rather than primary antibodies. Following extensive washing in phosphate-buffered saline (PBS), antigen-antibody complexes were detected using the ABC Elite kits and NovaRed peroxidase substrate kits (Vector Laboratories, Burlingame, CA). Then, the slides were counterstained and mounted.

Subcellular localizations for each S1PR were observed under microscope. The expression level of each S1PR was scaled with final IHC score for each of subcellular localization as described previously (Zhou et al, 2012). Briefly, the score for the extent of the IHC-stained areas was set as 0 for no IHC signal at all, 1 for <10%, 2 for 10% to 50%, and 3 for >50% of interested cells stained. The score for IHC intensity was also scaled as 0 for no IHC signal, 1 for weak, 2 for moderate, and 3 for strong IHC signals. The final IHC score used in the

analysis was calculated by multiplying the extent score and intensity score, with a maximum score of 9.

Cell culture and Immunocytochemistry (ICC)

The cell lines used in this study include HCC cell line Hep G2, prostate cancer cell lines 22RV1, LNCaP and LNcap-95, and colon cancer cell line DLD-1. All cell lines were obtained from ATCC and maintained according to the corresponding manufactory instructions. The cell suspension of each cell line was gently placed on Poly-L-Lysing coated (PLLC) coverslips (Neuvitro Corporation) in the bottom of each well of 6-well culture plates. The cells were then incubated at 37°C in 5% CO_2. When cells on the coverslips reached 50–80% confluence, the media in the wells were removed, and cells on the coverslips were washed with PBS 3 times, fixed with 4% formalin/PBS for 20min and washed with PBS 3 times again (if ICC was to be performed later, the cells on coverslips were kept in the 6-wells plates, soaked in 75% ethanol and sealed with paraffin film). To perform ICC, the cells on the coverslips were incubated with permealizing solution (2%BSA and 0.25% Tritonx-100 in PBS) for 30 min, blocking serum for 1 h, and primary antibodies (anti S1PR1-S1PR5) for 2 h at room temperature (or overnight at 4°C for monoclonal antibodies). Then, the antigen-antibody complexes were detected using the ABC Elite kits and NovaRed peroxidase substrate kits (Vector Laboratories, Burlingame, CA). Finally, the cells on each PLLC coverslip were counterstained with hematoxylin and face-down mounted on a glass slide. Under light microscope, the extent and subcellular localization of each S1PR was observed.

Statistical analysis

A 2-sample *t* test in SPSS22 software was performed to compare the mean IHC scores for each S1PR between benign versus malignant tissues overall and in a given organ/system. The Fisher Exact Test was used to compare mean IHC scores in vascular smooth muscle of fetal umbilical vein and adult artery. The difference in expression and subcellular localization of each S1PR for the paired groups was considered significant if the 2-sided *P* value was less than 0.05. The extent and subcellular localization of each S1PR determined by ICC in each cell line were recorded. These data were not statistically analyzed, but used to confirm the extent and subcellular localization of each S1PR determined by IHC in corresponding tissues.

Results

Subcellular localizations and differences in the expression levels of S1PRs between overall benign and malignant changes

IHC for S1PR1-5 was performed on 384 specimens: 183 benign and 201 malignant. The extent and subcellular localizations of the IHC signals were determined for each S1PR. The IHC signals for all S1PRs were found in both nuclei and cytoplasm, except of S1PR3, whose IHC signal is not present in cytoplasm. Among the five S1PRs, S1PR5 has the highest expression level (either in nuclei, or in cytoplasm), with S1PR1, 3, 2 and 4 following in descending order. S1PR1, 3 and 5 are predominantly expressed in nuclei, conversely, S1PR2 and 4 are mainly presented in cytoplasm. IHC signals are rarely seen on cell membranes for

all S1PRs. All S1PRs have higher nuclear IHC scores in the malignant specimens (201) as compared with those in benign specimens (183). The IHC score for nuclear S1PR3 was significantly higher in malignant tissues than in benign tissues (p=0.000019, Figure 1).

Systemic distribution of S1PRs and their differential expression between benign and malignant changes in different organs/tissues

Each S1PR is distributed in a different spectrum of organs/tissues, and differentially expressed in the cytoplasm and nucleus when comparing benign to malignant changes in the same organ/tissue. For S1PR1, both nuclear S1PR1 (S1PR1N) and cytoplasmic S1PR1 (S1PR1C) are seen in all 36 detected organs/tissues. S1PR1N has a very high expression pattern (IHC score ≥ 3) in 33 of 36 (33/36) organs/tissues, and has a low expression pattern (IHC score <3) in 3/36 organs/tissues: spleen, bone and myocardium. S1PR1C has a high expression pattern in 9/36, and a low expression pattern in 27/36 organs/tissues. For S1PR2, both nuclear S1PR2 (S1PR2N) and cytoplasmic S1PR2 (S1PR2C) have low expression patterns. S1PR2N was seen in 26/36 and S1PR2C found in 32/36 organs/tissues. For S1PR3, nuclear S1PR3 (S1PR3N) had a high expression pattern in 32/36 organs/tissues, and no expression (no IHC signal) is seen in bone, adult arterial smooth muscle, skeletal muscle and myocardium. S1PR3C is not expressed in any organ/tissue. Nuclear S1PR4 (S1PR4N) has a low expression pattern, and it is present in only 10/36 organs/systems. Cytoplasmic S1PR4 (S1PR4C) varies in its expression levels, from no expression in 7/36 organs/tissues, low expression pattern in 26/36 organs/tissues, and high expression pattern in 3/36 organs/tissues. The expression pattern of S1PR5 is similar to that of S1PR1: nuclear S1PR5 (S1PR5N) is highly expressed in 34/36 organs/tissues, low expression pattern in bone, and no expression in myocardium. Cytoplasmic S1PR5 (S1PR5C) has low expression pattern in 32/36 organs/tissues, and high expression patterns in 4/36 organs/tissues (Figure 2).

Shown in Figure 3 depicts representative IHC staining in paired benign and malignant changes in same organ/system, except for Figure 3A-1 and 3A-2, which shows the difference between IHC staining on vascular smooth muscle of fetal umbilical vein (3A-1, n=2) and adult artery (3A-2, n=4). It is interesting that S1PR3N is highly expressed in vascular smooth muscle of fetal umbilical vein with a mean IHC score of 6, however, it is not expressed in vascular smooth muscle of adult artery (arterial media) at all (mean IHC score 0, p=0.067). This development-related expression pattern of S1PR3N in vascular smooth muscle cells is not observed in other S1PRs.

Each organ/tissue differentially expresses S1PRs: in subcellular localization for each S1PR, and in the expression level of each S1PR between benign and malignant changes. Liver and colon are 2 out of 4 organs/tissues that show significant differences in the expression levels of multiple nuclear and cytoplasmic S1PRs between benign and malignant changes.

In liver, the IHC scores for all nuclear S1PRs (except S1PR4N, which is not expressed in either benign or malignant liver), are exclusively higher in hepatocellular carcinoma (HCC, n=13) than in benign liver (n=7). In benign vs malignant liver, the IHC scores are 2.5±1.1 vs. 6.38±1.6, p=0.000051 for S1PR1N; 0 vs. 0.23±0.83, p=0.48 for S1PR2N; 0.71±1.68 (Figure 3B-1) vs. 4.65±2.76 (Figure 3B-2), p=0.0031 for S1PR3N; and 2.0±1.82 vs. 6.81±1.32, p=0.0000047 for S1PR5N. In contrast, the IHC scores for all cytoplasmic S1PRs

(except S1PR3C, which is not detected in any organ/tissue, including benign and malignant liver) are lower in HCC (n=13) than in benign liver (n=7). In benign vs malignant liver, the IHC scores are 3.25±1.13 vs. 2.08±0.76, p=0.016 for S1PR1C; 2.79±057 (Figure 3B-3) vs. 1.38±1.14 (Figure 3B-4), p=0.0072 for S1PR2C; 2.71±0.98 vs. 0.38±0.68, p=0.00025 for S1PR4C; and for 4.75±1.47 vs.2.08±0.98, p=0.00019 S1PR5C. Thus, cytoplasmic and nuclear expressions of all S1PRs are decreased in HCC as compared to benign liver. This concordance may imply an importance of nuclear translocation of S1PRs in the pathogenesis of HCC.

In colon, significantly higher IHC scores for multiple S1PRs are exclusively seen in colon cancer (n=16) as compared with benign colon ((n=8), regardless of expression site. For example, the IHC score of nuclear expressed S1PR3 (S1PR3N) is 5.19±1.83 in benign colon (Figure 3C-1), and 7.03±0.9 in colon cancer (Figure 3C-2), p=0.003; further, the IHC score of cytoplasmic expressed S1PR2 (S1PR2C) is 0 in benign colon (Figure 3C-3), and 2.09±1.02 in colon cancer (Figure 3C-4), p=0.000003. The difference in subcellular expression of S1PRs between colon and liver may suggest different mechanisms in carcinogenesis.

Nine organs/tissues show significant difference in IHC score of only one S1PR (cytoplasmic or nuclear) between benign and malignant changes. Taking stomach as an example: cytoplasmic expressed S1PR4 (S1PR4C) is the only S1PR that shows significant difference in IHC score between benign gastric changes (3.5±2.26, Figure 3D-1) and gastric adenocarcinoma (0, Figure 3D-2), p=0.00002. Significant change in single S1PR (nuclear or cytoplasmic) could be essential for the pathogenesis of a given tumor. No statistically significant difference of IHC scores of any S1PR (cytoplasmic or nuclear) is observed between benign and malignant changes in 23/36 organs/tissues, including breast (Figure 3E1–2) and prostate (Figure 3F1–2). However, these results need to be reconfirmed with an increasing in sample size in these organs/tissues. Listed in Table 2 are all nuclear or cytoplasmic S1PRs that have significant differences in their expression levels between benign and malignant changes in different organs/tissues.

Subcellular localizations of S1PRs in cell lines

Immunocytochemistry (ICC) for S1PR1-5 was performed on several malignant cell lines to confirm subcellular localizations of each S1PR determined by IHC. Shown in Figure 4 is representative ICC for a few S1PRs in several cell lines. The subcellular localizations and expression level of all S1PRs determined by ICC in these malignant cell lines are same as those determined by IHC in corresponding malignant tissues.

Similar to IHC, apparent ICC signal for all S1PRs has not been observed on cellular membrane of all detected cell lines.

Discussion

One of the distinctive features for S1PRs is that they share a common ligand, S1P. While endogenous S1P molecules are transported to the surface of cellular membrane, multiple S1PRs could be activated simultaneously. Thus, the impact of the S1P/S1PR axis on the

behaviors of cancer cells could be a net outcome of interaction among all activated S1PRs. To investigate the roles of the S1P/S1PRs axis in the oncogenesis and progression of, and explore preventive and therapeutic interventions for cancers, it is critical to understand not only systemic distribution, subcellular localization and difference in the expression level between benign and malignant changes for a given S1PR, but also relative expression level of all S1PRs that are possibly activated by S1P simultaneously in a given tissue type with benign or malignant change. In this study, we performed IHC for S1PR1-5 on TMAs containing 384 human benign and malignant tissues from various organs/tissues. The results indicated that all 5 S1PRs (cytoplasmic, and/or nuclear) are widely distributed in benign and/or malignant tissues of multiple systems/organs. However, each S1PR is different from others in its systemic distribution, subcellular localization, and difference in the expression level between benign and malignant tissues. In both overall benign (183) and malignant (201) specimens, S1PR1N, S1PR3N, and S1PR5N belong to high expression pattern (IHC score ≥3); the rest have a low expression pattern (IHC score<3) in most organs/tissues. All 5 S1PRs, especially for S1PR1, 3 and 5 have a higher nuclear expression in overall malignant tissues than in overall benign tissues. These results imply that nuclear translocation of S1PRs could be a common step critical in oncogenesis and progression of cancers.

S1PR5 is the latest identified S1PR (Im et al, 2000; Im et al, 2001). S1PR5 was initially thought to be mainly distributed in the nervous system. Later studies have suggested that S1PR5 is associated with proliferation and migration of cancer cells in gastric and esophageal malignant cell lines (Yamashita et al, 2006; Hu et al, 2010). As compared with other S1PRs, S1PR5 has been even less studied in its systemic distribution, subcellular localization and correlation with cancers. This study revealed that S1PR5 is expressed in all detected organs/tissues. Especially, nuclear S1PR5 (S1PR5N) which has the highest expression of all the S1PRs in both benign and malignant tissues, as compared with other S1PRs (either cytoplasmic, or nuclear). The expression level S1PR5N is significantly higher in malignancies in lymphoid, hepatic, cervical and cutaneous systems/organs, whereas S1PR5C expression is statistically higher in benign liver and lung tissues, as compared with their malignant counterparts. The increased expression of S1PR5N over S1PR5C in malignancies of multiple organs/tissues further supports the notion that nuclear translocation of S1PRs is critical in the pathogenesis or progression of cancers. Further in-depth investigation of S1PR5 in malignancy may be warranted with these new discoveries.

One S1PR may have different biological effects on different cancers; as well, biological behaviors of one cancer may be influenced by multiple activated S1PRs. For example, activated S1PR2 inhibits motility of GNS-334 glioblastoma cells (Malchinkhuu et al, 2008); in contrast, activated S1PR1 stimulates migration of fibrosarcoma cells (Fisher et al, 2006). Functional studies demonstrating the interaction among S1PRs is outside of the scope of this study, however, we found wide variation in the combinations of different expression levels of nuclear and cytoplasmic S1PRs in different malignancies. For example, the expression levels of cytoplasmic S1PR2 and S1PR5 are lower in in hepatocellular carcinoma, but they are higher in colon cancer, as compared with their benign counterparts, respectively. The average IHC score for S1PR4C was very low in overall benign (0.87) and malignant (0.66) tissues. However, it is significantly higher in benign adrenal gland (3.90), stomach (3.5) and liver (2.17), as compared with pheochromocytoma (0.83), gastric adenocarcinoma (0) and

HCC (0.38), respectively. Whether loss of cytoplasmic expression of S1PR4 relates to pathogenesis in these tumors warrants further investigation.

Qu et al (2012) reported that strong S1PR3 expression (western blot and IHC) was detected in smooth muscle of abdominal aortic aneurysms, but not in that of normal aortas. In agreement with this report, our study showed that S1PR3 (nuclear and cytoplasmic) is not detected in smooth muscle of adult human aorta. However, nuclear S1PR3 is highly expressed in smooth muscle of fetal umbilical veins. These results together suggest that S1PR3 plays important roles in the development of vascular smooth muscle cells, in terms of proliferation and migration. It remains to be further investigated whether S1PR3 plays similar roles in pathological angiogenesis in cancer.

Previous studies suggest that S1PRs play a role in breast cancer (Sukocheva et al, 2013) and prostate cancer (Sekine et al, 2011). Our results revealed that the expression levels of all S1PRs (both nuclear and cytoplasmic) are very similar in both benign and malignant breast and prostate cancers. These results need to be further confirmed in a larger series.

S1PRs belong to G-protein-coupled receptors, which are integral membrane proteins that possess seven membrane-spanning domains or transmembrane helices. However, membranous localization of all S1PRs is barely seen either by IHC in FFPE human tissues or by ICC in malignant cell lines. This could be because S1PRs are rapidly internalized into cytoplasmic vesicles upon activation by S1P (Licht et al, 2003).

Conclusion

Sole immunostaining in methods, small sample size in each organ/tissue, and lack of functional study for each S1PR are the limitations of this study. Nevertheless, it is first to perform IHC for all S1PRs simultaneously on benign and malignant tissues from the entire spectrum of human organs/tissues. This study has determined the systemic distribution, subcellular localization and expression differences among various tissues, and, further, between benign and malignant changes of the same human tissue/organ for each S1PR. This data can serve as a basis for future studies examining the role of S1P/S1PR axis both in development and in neoplasia.

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References

Beckham TH, Cheng JC, Lu P, Shao Y, Troyer D, Lance R, Marrison ST, Norris JS, Liu X. Acid ceramidase induces sphingosine kinase 1/S1P receptor 2-mediated activation of oncogenic Akt signaling. Oncogenesis. 2013; 2:e49. [PubMed: 23732709]

Chae S, Proia R, Hla T. Constitutive expression of the S1P1 receptor in adult tissues. Prostaglandins Other Lipid Mediat. 2004; 73(1–2):141–150. [PubMed: 15165038]

Fisher K, Pop A, Koh W, Anthis N, Saunders W, Davis G. Tumor cell invasion of collagen matrices requires coordinate lipid agonist-induced G-protein and membrane-type matrix metalloproteinase-1-dependent signaling. Mol Cancer. 2006; 5:69. [PubMed: 17156449]

Garris CS, Blaho VA, Hla T, Han MH. Sphingosine-1-phosphate Receptor 1 (S1P1) Signaling in T cells: Trafficking and Beyond. Immunology. 2014; 43(1):15–24. Genomics. 10.1111/imm.12272

Goetzl E, Graeler M, Huang M, Shankar G. Lysophospholipid growth factors and their G protein-coupled receptors in immunity, coronary artery disease, and cancer. Scientific World Journal. 2002; 2:324–338. [PubMed: 12806020]

Gräler M, Bernhardt G, Lipp M. EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. Genomics. 1998; 53(2): 164–169. [PubMed: 9790765]

Hiraga Y, Kihara A, Sano T, Igarashi Y. Changes in S1P1 and S1P2 expression during embryonal development and primitive endoderm differentiation of F9 cells. Biochem Biophys Res Commun. 2006; 344(3):852–858. [PubMed: 16631609]

Hla T, Maciag T. An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. J Biol Chem. 1990; 265(16): 9308–9313. [PubMed: 2160972]

Hu W, Li L, Jing B, Zhao Y, Wang C, Feng L, Xie Y. Effect of S1P5 on proliferation and migration of human esophageal cancer cells. World J Gastroenterol. 2010; 16(15):1859–1866. [PubMed: 20397263]

Huang Y, Chang C, Tang C, Lin Y, Ju T, Huang W, Lee H. Extrinsic sphingosine 1-phosphate activates S1P5 and induces autophagy through generating endoplasmic reticulum stress in human prostate cancer PC-3 cells. Cell Signal. 2012; 26(3):611–618. [PubMed: 24333325]

Im D, Clemens J, Macdonald T, Lynch K. Characterization of the human and mouse sphingosine 1-phosphate receptor, S1P5 (Edg-8): structure-activity relationship of sphingosine1-phosphate receptors. Biochemistry. 2001; 40(46):14053–14060. [PubMed: 11705398]

Im D, Heise C, Ancellin N, O'Dowd B, Shei G, Heavens R, Rigby M, Hla T, Mandala S, McAllister G, George S, Lynch K. Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. J Biol Chem. 2000; 275(19):14281–14286. [PubMed: 10799507]

Kawahara S, Otsuji Y, Nakamura M, Murakami M, Murate T, Matsunaga T, Kanoh H, Seishima M, Banno Y, Hara A. Sphingosine kinase 1 plays a role in the upregulation of CD44 expression through extracellular signal-regulated kinase signaling in human colon cancer cells. Anticancer Drugs. 2013; 24(5):473–483. [PubMed: 23426175]

Kondo S, Bottos A, Allegood JC, Masson R, Maurer FG, Genoud C, Kaeser P, Huwiler A, Murakami M, Spiegel S, Hynes N. Memo has a novel role in S1P signaling and crucial for vascular development. PLoS One. 2014; 9(4):e94114. [PubMed: 24714781]

Kunkel GT, Maceyka M, Milstien S, Spiegel S. Targeting the sphingosine-1-phosphate axis in cancer, inflammation and beyond. Nat Rev Drug Discov. 2013; 12(9):688–702. [PubMed: 23954895]

Liang J, Nagahashi M, Kim EY, Harikumar KB, Yamada A, Huang WC, Hait NC, Allegood JC, Price MM, Avni D, Takabe K, Kordula T, Milstien S, Spiegel S. Sphingosine-1-phosphate links persistent STAT3 activation, chronic intestinal inflammation, and development of colitis-associated cancer. Cancer Cell. 2013; 23(1):107–120. [PubMed: 23273921]

Licht T, Tsirulnikov L, Reuveni H, Yarnitzky T, Ben-Sasson SA. Induction of pro-angiogenic signaling by a synthetic peptide derived from the second intracellular loop of S1P3 (EDG3). Blood. 2003; 102:2099–2107. [PubMed: 12763936]

Liu, C.; Hla, T. The mouse gene for the inducible G-protein-coupled receptor edg-1. 1997.

Maceyka M, Harikumar KB, Milstien S, Spiegel S. Sphingosine-1-phosphate signaling and its role in disease. Trends Cell Biol. 2012; 22(1):50–60. [PubMed: 22001186]

Malchinkhuu E, Sato K, Maehama T, Mogi C, Tomura H, Ishiuchi S, Yoshimoto Y, Kurose H, Okajima F. S1P(2) receptors mediate inhibition of glioma cell migration through Rho signaling pathways independent of PTEN. Biochem Biophys Res Commun. 2008; 366(4):963–968. [PubMed: 18088600]

Okhotsk J, Edwards J, Elsberry B, Watson C, Orange C, Mallon E, Pyne S, Pyne NJ. Identification of novel functional and spatial associations between sphingosine kinase 1, sphingosine 1-phosphate

receptors and other signaling proteins that affect prognostic outcome in estrogen receptor-positive breast cancer. Into J Cancer. 2013; 132(3):605–616.

Okhotsk J, Long JS, Orange C, Elsberry B, Mallon E, Doughty J, Pyne S, Pyne NJ, Edwards J. Expression of sphingosine 1-phosphate receptor 4 and sphingosine kinase 1 is associated with outcome in estrogen receptor-negative breast cancer. Br J Cancer. 2012; 106(8):1453–1459. [PubMed: 22460268]

Olivera A, Spiegel S. Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. Nature. 1993; 365(6446):557–560. [PubMed: 8413613]

Pyne NJ, Pyne S. Sphingosine-1-phosphate and cancer. Nature Rev Cancer. 2010; 10:489–503. [PubMed: 20555359]

Pyne S, Pyne NJ. New perspectives on the role of sphingosine 1-phosphate in cancer. Handb Exp Pharmacol. 2013; (216):55–71. [PubMed: 23563651]

Qu Z, Cheuk B, Cheng S. Differential expression of sphingosine-1-phosphate receptors in abdominal aortic aneurysms. Mediators Inflamm. 2012; 2012:643609. [PubMed: 22547907]

Schmouder R, Hariry S, David OJ. Placebo-controlled study of the effects of fingolimod on cardiac rate and rhythm and pulmonary function in healthy volunteers. Eur J Clin Pharmacol. 2012; 68(4): 355–362. [PubMed: 22071882]

Sekine Y, Suzuki K, Remaley A. HDL and sphingosine-1-phosphate activate stat3 in prostate cancer DU145 cells via ERK1/2 and S1P receptors, and promote cell migration and invasion. Prostate. 2011; 71(7):690–699. [PubMed: 20979115]

Soliven B, Miron V, Chun J. The neurobiology of sphingosine 1-phosphate signaling and sphingosine 1-phosphate receptor modulators. Neurology. 2011; 76(8 Suppl 3):S9–S14. [PubMed: 21339490]

Spiegel S. Sphingosine 1-phosphate: a prototype of a new class of second messengers. J Leukocyte Biol. 1999; 65:341–344. [PubMed: 10080537]

Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat Rev Mol Cell Biol. 2003; 4:397–407. [PubMed: 12728273]

Sukocheva O, Wadham C, Xia P. Estrogen defines the dynamics and destination of transactivated EGF receptor in breast cancer cells: role of S1P receptor and Cdc42. Exp Cell Res. 2013; 319(4):455–465. [PubMed: 23142484]

Takuwa Y, Okamoto Y, Yoshioka K, Takuwa N. Sphingosine-1-phosphate signaling in physiology and diseases. Biofactors. 2012; 38(5):329–337. [PubMed: 22674845]

Van Brocklyn J, Letterle C, Snyder P, Prior T. Sphingosine-1-phosphate stimulates human glioma cell proliferation through Gi-coupled receptors: role of ERK MAP kinase and phosphatidylinositol 3-kinase beta. Cancer Lett. 2002; 181(2):195–204. [PubMed: 12175535]

Windh R, Lee M, Hla T, An S, Barr A, Manning D. Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and H218/Edg-5 to the G(i), G(q), and G(12) families of heterotrimeric G proteins. J Biol Chem. 1999; 274(39):27351–27358. [PubMed: 10488065]

Yamashita H, Kitayama J, Shida D, Yamaguchi H, Mori K, Osada M, Aoki S, Yatomi Y, Takuwa Y, Nagawa H. Sphingosine 1-phosphate receptor expression profile in human gastric cancer cells: differential regulation on the migration and proliferation. J Surg Res. 2006; 130(1):80–87. [PubMed: 16183075]

Zhang G, Contos J, Weiner J, Fukushima N, Chun J. Comparative analysis of three murine G-protein coupled receptors activated by sphingosine-1-phosphate. Gene. 1999; 227(1):89–99. [PubMed: 9931453]

Zhang H, Desai NN, Olivera A, Seki T, Brooker G, Spiegel S. Sphingosine-1- phosphate, a novel lipid, involved in cellular proliferation. J Cell Biol. 1991; 114(1):155–167. [PubMed: 2050740]

Zhang W, Zhao J, Lee JF, Grating A, Jawed H, Lambic WL, Honan KV, Lee MJ. TS-1-mediated transcriptional up-regulation of CD44 is required for sphingosine-1-phosphate receptor subtype 3-stimulated chemo taxis. J Boil Chem. 2013; 288(45):32126–32137.

Zhou X, Lawrence TJ, He Z, Pound C, Mao J, Bigler S. The expression level of lysophosphatidylcholine acyltransferase 1 (LPCAT1) correlates to the progression of prostate cancer. Exp Mol Pathol. 2012; 92(1):105–110. [PubMed: 22101258]

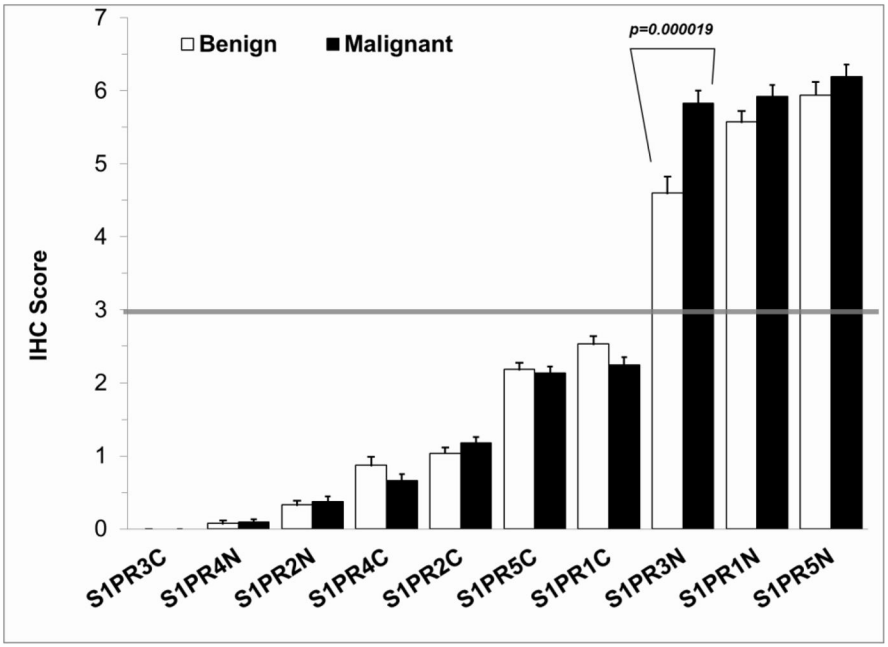

Figure 1.

Comparison of expression levels of sphingosine-1-phosphate (SIP) receptors (S1PRs) between benign and malignant tissues overall. The letter “C” represent cytoplasmic subcellular localization of S1PRs, and the letter of “N” represent nuclear subcellular localization of S1PRs.

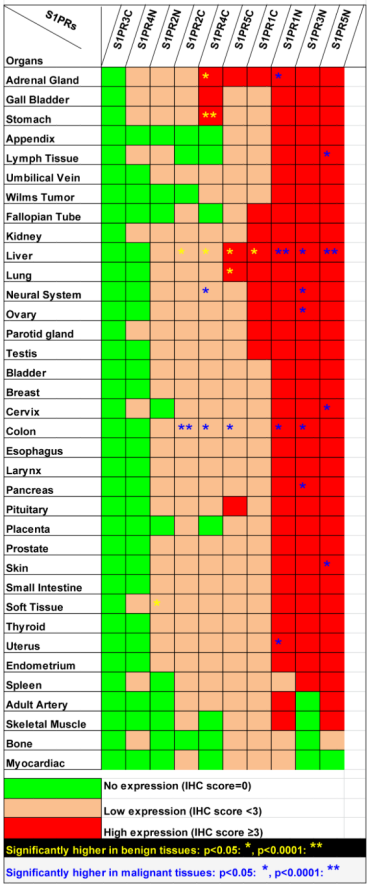

Figure 2.

Systemic distribution of sphingosine-1-phosphate (SIP) receptors (S1PRs) in human organs/tissues. The letter “C” represent cytoplasmic subcellular localization of S1PRs, and the letter of “N” represent nuclear subcellular localization of S1PRs.

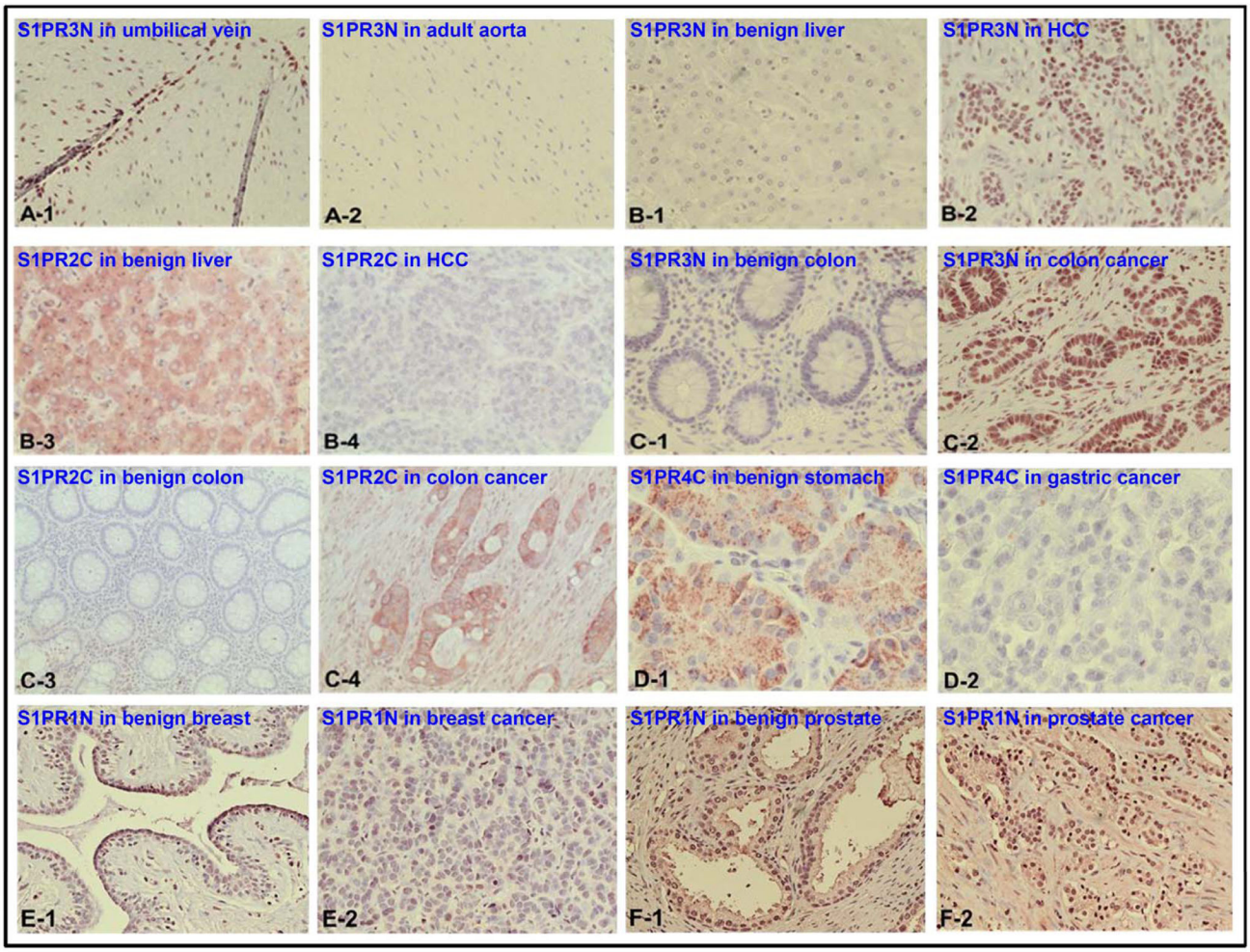

Figure 3.

Immunohistochemical staining for various sphingosine-1-phosphate (SIP) receptors (SIPRs) in different organs/tissues. **A:** Comparison of nuclear expressed S1PR3 (S1PR3N) between human vascular smooth muscle cells in fetal umbilical vein (A-1, IHC score: 6.0) and in adult aorta (A-2, IHC score: 0), p=0.067; **B:** Comparison of nuclear expressed S1PR3 (S1PR3N) in hepatocytes of human benign liver (B-1, IHC score: 0.71) and hepatocellular carcinoma (HCC, B-2, IHC score: 4.65), p=0.03, and comparison of cytoplasmic expressed S1PR2 (S1PR2C) between benign liver (B-3, IHC score: 2.79), and HCC (B-4, IHC score 1.38), p=0.0072; **C:** S1PR3N in human benign colon (C-1, IHC score: 5.19) and colorectal adenocarcinoma (C-2, IHC score:7.03), p=0.03, and S1PR2C in human benign colon (C-3, IHC score: 0) and colorectal adenocarcinoma (C-4, IHC score: 2.09), p=0.0000032; **D:** Comparison of cytoplasmic expressed S1PR4 (S1PR4C) in human benign stomach tissues (D-1, IHC score: 3.5) and gastric adenocarcinoma (D-2, IHC score: 0), p=0.00002; **E:** Comparison of nuclear expressed S1PR1 (S1PR1N) in human benign breast (E-1, IHC score: 5.83) and breast ductal adenocarcinoma (E-2, IHC score: 5.77), p=0.93; and **F:** comparison of S1PR1N in human benign prostate (F-1, IHC score: 7.88) and prostatic adenocarcinoma (F-2, IHC score: 8.0), p=0.85.

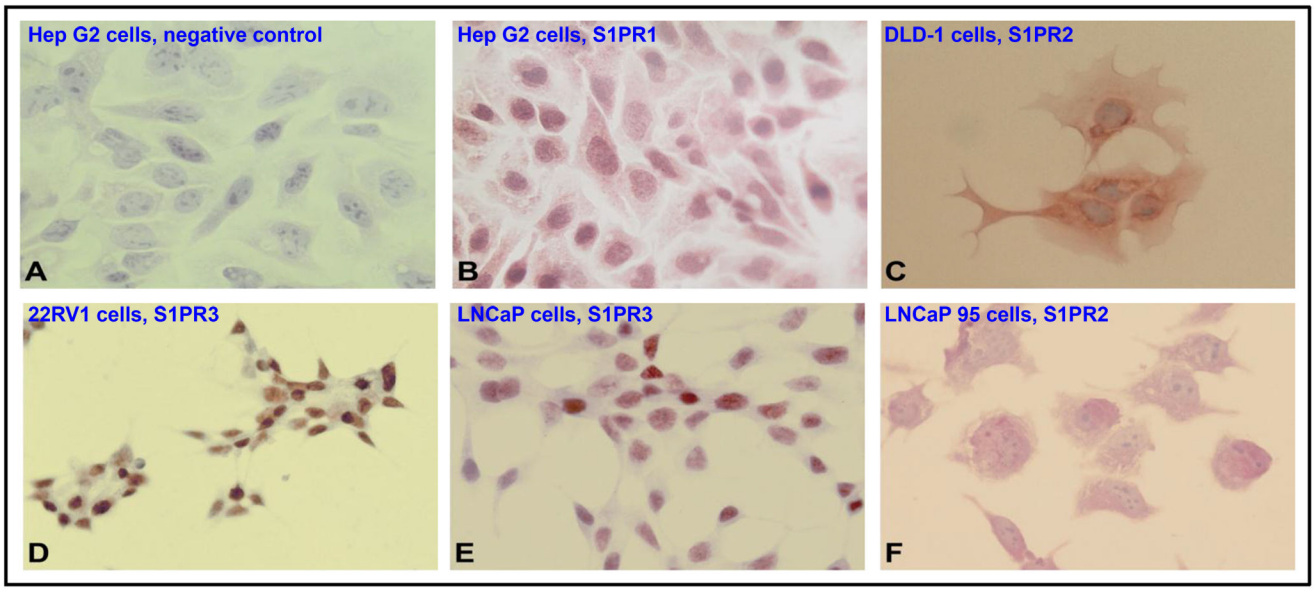

Figure 4.

Representative immunocytochemistry (ICC) for different sphingosine-1-phosphate (SIP) receptors (SIPRs) in different organs/tissues. **A:** Hep G2 cells (human hepatocellular carcinoma), negative control for ICC, 40×, no membranous, cytoplasmic and nuclear ICC signal is seen; **B:** Hep G2 cells, 40×, ICC signals for S1PR1 are seen in nuclei (strong) and cytoplasm (moderate); **C:** DLD-1 cells (Human colorectal adenocarcinoma), 40×, ICC signals for S1PR2 are seen in cytoplasm (weak); **D:** 22RV1 cells (human prostatic adenocarcinoma), 40×, ICC signals for S1PR3 are seen in nuclei (strong); **E:** LNCaP cells (androgen-sensitive human prostatic adenocarcinoma), 40×, ICC signals for S1PR3 are seen in nuclei (moderate to strong) and in cytoplasm (weak); **F:** LNCaP 95 (androgen-independent human prostatic adenocarcinoma), 40×, ICC signals for S1PR2 are seen in cytoplasm (weak).

Table 1

S1PRs antibodies used for IHC and ICC stains

Antibody	Catalog#	Location of Immunogen	Manufacturer	Dilution
EDG1(S1P1)	AB11424	C-terminal	Abcam, Inc, MA	1:200
EDG5(S1P2)	MABC95	C-terminal	EMD Millipore, MA	1:100
EDG3(S1P3)	AB150573	N-terminal	Abcam, Inc, MA	1:1600
EDG6(S1P4)	MABC97	C-terminal	EMD Millipore, MA	1:300
EDG8(S1P5)	G089	C-terminal	Assay Biotech, CA	1:800

Table 2

S1P receptor (S1PR) expression levels (IHC Score) that are statistically between benign and malignant changes in different organs/tissues

Organ/Tissue	S1P Receptor	Benign Changes			Malignant Changes			p Value
		n	Mean	SD	n	Mean	SD	
Adrenal Gland	S1PR1N*	5	4.20	1.96	3	7.50	1.50	.047
	S1PR4C**	5	3.90	1.34	3	.83	.76	.012
Cervix	S1PR5N	5	5.60	1.39	4	7.88	.75	.022
Colon	S1PR1N	9	5.11	1.73	16	6.75	1.73	.033
	S1PR2C	9	.00	.00	16	2.09	1.02	.0000032
	S1PR3N	8	5.19	1.83	16	7.03	.90	.003
	S1PR4C	8	.00	.00	16	.38	.43	.023
	S1PR5C	9	1.50	.00	16	2.25	.95	.028
Liver	S1PR1C	6	3.25	1.13	13	2.08	.76	.016
	S1PR1N	6	2.50	1.10	13	6.38	1.60	.000051
	S1PR2C	7	2.79	.57	13	1.38	1.14	.0072
	S1PR3N	7	.71	1.68	13	4.65	2.76	.0031
	S1PR4C	6	2.17	.98	13	.38	.68	.00025
	S1PR5C	6	4.75	1.47	13	2.08	.98	.00019
	S1PR5N	6	2.00	1.82	13	6.81	1.32	.0000047
Lymph Tissue	S1PR5N	2	5.25	1.06	2	9.00	.00	.04
Lung	S1PR5C	4	3.75	1.50	8	1.69	.53	.005
Neural System	S1PR3N	9	2.06	2.30	3	7.00	.87	.005
	S1PR4C	9	.11	.33	3	1.83	2.36	.04
Ovary	S1PR3N	4	3.38	2.56	4	7.50	1.22	.03
Pancreas	S1PR3N	9	5.17	1.77	9	6.83	1.52	.048
Skin	S1PR5N	4	5.63	1.89	7	8.57	1.13	.009
Soft Tissue	S1PR2N	2	2.50	.71	8	.19	.53	.001
Stomach	S1PR4C	6	3.50	2.26	13	.00	.00	.00002
Uterus	S1PR1N	4	5.25	.87	3	7.50	.00	.007

*Letter N represents nuclear;

** letter C represents cytoplasmic.