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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 in benign and malignant tissues from the same organ/tissue were then compared using the Student's 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 benign and malignant tissues. Among the five S1PRs, S1PR5 has the highest expression level (in either the 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 scores for 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.

1. 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 and Milstien, 2003). S1P, an active lipid metabolite, was previously thought to influence intracellular functions directly (Olivera and Spiegel, 1993; Spiegel, 1999; Zhang et al., 1991). 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 and Maciag (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

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systems that include: the central nervous system, immune system (Garris et al., 2014; Soliven et al., 2011), and cardiovascular system (Schmouder et al., 2012), and in embryonic development (Hiraga et al., 2006; Kondo et al., 2014). Further, emerging evidence indicates that S1P/S1PRs play significant roles in the proliferation, progression, survival and therapeutic response to treatment in cancer (Kunkel et al., 2013; Pyne and Pyne, 2010, 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., 2013). 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 the 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 (Takuwa et al., 2012; Van Brocklyn et al., 2002). This fact implies that while the effects of one activated S1PR on cancer are 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 human cell lines (Chae et al., 2004; Gräler et al., 1998; Im et al., 2000; Liu and Hla, 1997; Zhang et al., 1999). The systemic distribution and subcellular localization of each S1PR protein molecule in benign and malignant human tissues have 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.

2. Material and methods

2.1. 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. 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.

2.2. Immunohistochemistry (IHC) and scoring system

The protocol for immunohistochemical staining and scoring system was 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 a microscope. The expression level of each S1PR was scaled with a 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%,

Table 1
S1PR 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