Integrative genomic analyses of secreted protein acidic and rich in cysteine and its role in cancer prediction

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Abstract. Secreted protein acidic and rich in cysteine (SPARC), also termed osteonectin or basement-membrane-40 (BM-40), is a matrix-associated protein that elicits changes in cell shape, inhibits cell-cycle progression and affects the synthesis of extracellular matrix (ECM). The final mature SPARC protein has 286 amino acids with three distinct domains, including an NH2-terminal acidic domain (NT), follistatin-like domain (FS) and C terminus domain (EC). The present study identified SPARC genes from 14 vertebrate genomes and revealed that SPARC existed in all types of vertebrates, including fish, amphibians, birds and mammals. In total, 21 single nucleotide polymorphisms (SNPs) causing missense mutations were identified, which may affect the formation of the truncated form of the SPARC protein. The human SPARC gene was found to be expressed in numerous tissues or organs, including in the bone marrow, whole blood, lymph node, thymus, brain, cerebellum, retina, heart, smooth muscle, skeletal muscle, spinal cord, intestine, colon, adipocyte, kidney, liver, pancreas, thyroid, salivary gland, skin, ovary, uterus, placenta, cervix and prostate. When searched in the PrognoScan database, the human SPARC gene was also found to be expressed in bladder, blood, breast, glioma, esophagus, colorectal, head and neck, ovarian, lung and skin cancer tissues. It was revealed that the association between the expression of SPARC and prognosis varied in different types of cancer, and even in the same cancer from different databases. It implied that the function of SPARC in these tumors may be multidimensional, functioning not just as a tumor suppressor or oncogene.

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Introduction

Secreted protein acidic and rich in cysteine (SPARC), also termed as osteonectin or basement-membrane-40, is a matrix-associated protein that elicits changes in cell shape, inhibits cell-cycle progression and affects the synthesis of extracellular matrix (ECM) (1). The human SPARC gene was initially cloned from a human placenta cDNA library (2). The final mature SPARC protein has 286 amino acids with three distinct domains, including an NH2-terminal acidic domain (NT), follistatin-like domain (FS) and C terminus domain (EC). The NT domain, spanning the first 52 amino acids (Ala1-Glu52), binds hydroxyapatite and calcium ions. This is followed by FS, which comprises the next 85 amino acids (Asn53-Pro137). This region contains several internal disulfide bonds that stabilize two weakly interacting nodules. The third domain, the EC, is 149 amino acids in length (Cys138-Ile286). It contains two EF-hand motifs that bind calcium with high affinity and is comprised almost entirely of β-helices.

SPARC binds fibrillar collagen and basal lamina collagen IV (3) and is associated with ECM assembly and fibrosis (4). SPARC has also been demonstrated to regulate the activity of matrix metalloproteinases (MMPs), a family of enzymes considered to be the primary mediators of ECM proteolysis and turnover. SPARC has also been demonstrated to modulate growth factor signaling mediated by cell surface receptors, including vascular endothelial growth factor receptor, basic fibroblast growth factor and transforming growth factor β1 (5). SPARC is also involved in activating odontoblasts during tooth development (6). SPARC upregulation in endothelial cells and fibroblasts may contribute to compensatory signaling for controlling angiogenesis (7).

Numerous clinical studies have revealed a correlation between SPARC expression, malignant progression and patient survival (8,9). However, SPARC has demonstrated seemingly contradictory effects on tumor progression in clinical correlative studies and in animal models (10-13). The capacity of SPARC to dictate the tumorigenic phenotype has

been attributed to its effects on the bioavailability and signaling of integrins and growth factors/chemokines. These molecular pathways contribute to a number of physiological events affecting malignant progression, including ECM remodeling, angiogenesis, immune modulation and metastasis (14-17). Thus, comprehensive investigation regarding whether SPARC is involved in various types of tumor formations is required.

In the present study, SPARC genes from humans, chimpanzees, macaques, orangutans, dogs, cows, horses, mice, rats, opossums, chickens, western clawed frog, zebrafish and fugu were identified by comparative genomic analyses. Conserved transcription factor-binding sites within promoter regions of human SPARC genes were then searched. The expression data, functional relevant single nucleotide polymorphisms (SNPs) and comparative proteomic analyses were conducted. Furthermore, meta-analysis of the prognostic value of SPARC genes in various types of cancer was also performed.

Materials and methods

Identification of novel SPARC genes in vertebrate genomes and integrative genomic analyses. SPARC genes were searched in the genome sequences of humans (*Homo sapiens*), chimpanzees (*Pan troglodytes*), macaques (*Macaca mulatta*), orangutans (*Pongo pygmaeus*), dogs (*Canis familiaris*), cows (*Bos taurus*), horses (*Equus caballus*), mice (*Mus musculus*), rats (*Rattus norvegicus*), opossums (*Monodelphis domestica*), chickens (*Gallus gallus*), frog (*Xenopus tropicalis*), zebrafish (*Danio rerio*) and fugu (*Takifugu rubripes*) by the method described prior to using the human SPARC (NM_003118.3) as queries. The assemblies used were human NCBI 36, chimpanzee CHIMP2.1, macaque MMUL 1.0, orangutan PPYG2, dog Canfam 2.0, cow Btau_4.0, horse Equ Cab 2, mouse NCBI m37, rat RGSC 3.4, opossum monDom5, chicken WASHUC2, frog JGI 4.1, zebrafish Zv8 and fugu FUGU 4.0. The identified putative SPARC genes were BLASTed against the nr database of GenBank to confirm that the best hits were SPARC genes (18-21). Conserved transcription factor-binding sites within promoter regions of the human SPARC gene was obtained from SABiosciences' proprietary database which combines Text Mining Application and data from the UCSC Genome Browser.

Comparative proteomic analyses of SPARC proteins. The amino acid sequences of SPARC were deduced from the identified SPARC genes and aligned using Clustal X 1.8 software (22). The phylogenetic tree of SPARC was obtained using maximum likelihood (ML; PHYML v2.4.4) (23) and neighbor-joining (NJ; MEGA 3.0) (24) methods, and the reliability of the tree was evaluated by the bootstrap method with 1,000 replications. The program Codeml implemented in the PAML 3.14 b software package was used to investigate whether Ikaros proteins are under positive selection (25). Six models of codon substitution, one-ratio (M0), NearlyNeutral (M1a), PositiveSelection (M2a), discrete (M3), β (M7) and β and ω (M8) were used in the analysis (26).

Functional relevant SNP evaluation of the human SPARC gene. Functional relevant SNPs of the human SPARC gene were identified as previously described (18-21). The SNPs were extracted from Ensembl (http://www.ensembl.org) and NCBI's SNPdb (http://www.ncbi.nlm.nih.gov). The SNPs that were able to disrupt exonic splicing enhancer/exonic splicing silencer (ESE/ESS) motifs and cause missense mutations were also identified.

In silico expression analyses of the human SPARC gene. Expressed sequence tags (ESTs) derived from the human SPARC gene were searched for using the BLAST programs as previously described (27-30). The human SPARC gene (NM_003118) was used as a query sequence for the BLAST programs. The expression profiles for normal human tissues were obtained from GeneAnnot (31) and ArrayExpress (32). Northern analysis of NCBI's uniGene dataset was also extracted (18-21). Furthermore, the protein expression of SPARC was obtained from the Systematic Protein Investigative Research Environment (SPIRE) (33) and the Model Organism Protein Expression Database (MOPED) (34).

Meta-analysis of the prognostic value of the SPARC gene in cancer. A database named 'PrognoScan' has been developed (35). This database is a large collection of publicly available cancer microarray datasets with clinical annotation, as well as a tool for assessing the biological association between gene expression and prognosis. PrognoScan employs the minimum P-value approach for grouping patients for survival analysis. PrognoScan provides a powerful platform for evaluating potential tumor markers and therapeutic targets and is publicly accessible at http://gibk21.bio.kyutech.ac.jp/PrognoScan/index.html. The human SPARC gene was inputted as queries and the data were collected for analysis.

Results

Comparative proteomics of SPARC proteins identified in vertebrate genomes. The SPARC genes were identified in the genome sequences of humans, chimpanzees, macaques, orangutans, dogs, cows, horses, mice, rats, opossums, chickens, western clawed frog, zebrafish and fugu. The refined phylogenetic trees using the identified SPARC proteins amino acid sequences by ML and NJ methods were almost identical (Fig. 1). The present study was unable to identify any site under positive selection, with any of the six models, in the SPARC proteins. Instead, the SPARC proteins were observed to be under purifying selection (data not shown).

Expression profile of the human SPARC gene. By searching for EST sequences, the human SPARC gene was found to be expressed in the eye, placenta, fetal brain, neuroblastoma, fetal liver and Lupski dorsal root ganglion. The investigation of available microarray experiments and 'virtual Northern blot' demonstrated a predominant expression of SPARC in the bone marrow, whole blood, lymph node, thymus, brain, cerebellum, retina, heart, smooth muscle, skeletal muscle, spinal cord, intestine, colon, adipocyte, kidney, liver, pancreas, thyroid, salivary gland, skin, ovary, uterus, placenta, cervix and prostate. When searched in the PrognoScan database, the human SPARC gene was also revealed to be expressed in

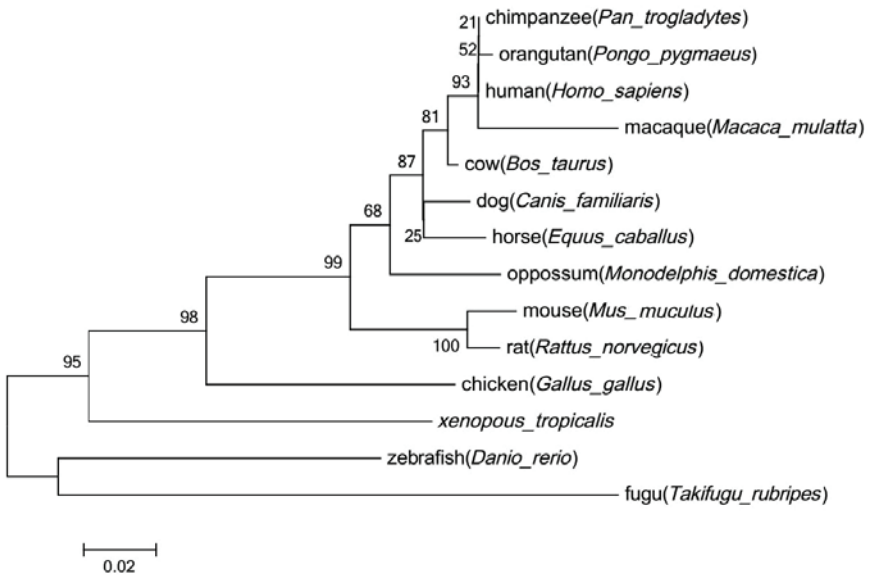

Figure 1. Phylogentic analysis of SPARC. SPARC genes were identified in the genome sequences of humans, chimpanzees, macaques, orangutans, dogs, cows, horses, mouses, rats, opossums, chickens, western clawed frog, zebrafish and fugu. The phylogenetic tree of the SPARC gene was obtained using maximum likelihood and neighbor-joining methods. It appeared that the primate SPARC gene clustered into one group, different from other SPARC genes. SPARC, secreted protein acidic and rich in cysteine.

bladder, blood, brain, breast, colorectal, eye, lung, ovarian, prostate, renal, skin and soft tissue cancer tissues. Among the protein expression databases SPIRE and MOPED, SPARC protein was highly expressed in blood plasma, blood monocyte, kidney HEK-293, liver and liver HuH-7 cancer cells; however, low levels of SPARC protein expression were observed in blood erythroleukemia, blood neutrophil, blood B-lymphocyte, blood T-lymph Jurkat, kidney urine, lung alveolar lavage, pancreatic cancer and prostate cancer tissues.

Comparative genomics of the human SPARC gene. Nkx2-5, Brachyury PPAR-α, AML1a and p53 regulatory transcription factor binding sites were identified in the SPARC gene upstream (promoter) region.

Functional relevant SNP evaluation of the human SPARC gene. In total, 471 available SNPs were identified in the human SPARC gene. Among these, 23 SNPs were functionally relevant, including two available alleles, which disrupted an existing ESE and 21 SNPs causing missense mutations (Table I).

Meta-analysis of the prognostic value of the human SPARC gene in cancer. When provided with the gene, PrognoScan exhibits a summary in table format of tests for the gene with columns for dataset, cancer type, subtype, end point, cohort, contributor, array type, probe ID, number of patient, optimal cut point, Pmin and Pcor. Among the databases that detected the expression of the SPARC gene, 27 out of 136 tests demonstrated an association between microarray expression in the SPARC gene and cancer prognosis (bladder cancer 0/4, blood cancer 5/18, brain cancer 0/8, breast cancer 4/38, colorectal cancer 8/18, eye cancer 2/2, lung cancer 3/31, ovarian cancer 4/13, prostate cancer 1/1, renal cancer 0/1, skin cancer 0/2 and soft tissue cancer 0/2) with a 5% significance level (Table II, 36-50). Among the five types of blood cancer, a higher expression of the SPARC gene was associated with a poor survival rate and was found in three acute myeloid leukemia (AML) cases (GSE12417-GPL96, GSE12417-GPL96 and GSE12417-GPL570). However, in B-cell lymphoma (GSE4475) and diffuse large B-cell lymphoma (DLBCL; TABM-346) cases, a lower expression of the SPARC gene was associated with a poor survival rate. Among the four types of breast cancer, a higher expression of the SPARC gene was associated with a poor survival rate and was identified in two cases (GSE11121 and E-TABM-158). However, a lower expression of the SPARC gene was associated with a poor survival rate in another two cases of breast cancer (GSE3494-GPL96). The present study revealed that a higher expression of the SPARC gene was associated with a poor survival rate in all eight colorectal cancer cases. In lung cancer cases, a higher expression of the SPARC gene was associated with a poor survival rate in all three types of lung cancer, including adenocarcinoma and non-small cell lung cancer (NSCLC). In addition, a lower expression of the SPARC gene was associated with a poor survival rate in four cases of ovarian cancer, two cases of eye cancer and one case of prostate cancer.

Discussion

SPARC, also known as osteonectin or BM-40, is a matrix-associated protein that elicits changes in cell shape, inhibits cell-cycle progression and affects the synthesis of the extracellular matrix (ECM) (1). In the present study, additional SPARC genes from 13 other vertebrate genomes

Table I. Functional relevant SNP evaluation of the human SPARC gene.

SNP ID	Chr 5 position	Sequence	Type	Amino acid change
rs707157	151047108(-)	TGCGGA/G/TACTGG	Missense	ND/Y
rs1053296	151047111(-)	GCATGC/GGGGAC	Missense	R/D
rs11542492	151049293(-)	TGCCAC/TAAAGT	Missense	T/I
rs11542497	151054219(-)	CCTGCA/CTGATG	Missense	H/P
rs11542498	151054198(-)	GGTGGA/GAGAAA	Missense	E/G
rs41290587	151051255(+)	AGGGAT/CCTGTA	Missense	/N/S
rs7433231	151045923(+)	TTACCC/TGTCAA	Missense	R/G
rs113617771	151052711(+)	CTCTTC/TGGTTT	Missense	K/E
rs141567625	151045999(+)	TCGAAG/TTCCCG	Missense	E/D
rs142207246	151051184(+)	GCACAC/TGCACA	Missense	M/V
rs142378176	151051171(+)	TGGTGA/GGGTCC	Missense	P/L
rs142717464	151043728(+)	AAAAGC/TGGGTG	Missense	H/R
rs146500464	151052741(+)	TTCTCC/TTACTT	Missense	R/G
rs147557671	151051145(+)	AAACTC/TGCCAA	Missense	K/E
rs185684862	151047110(+)	AGTCCC/TGCATG	Missense	Q/R
rs188911380	151043660(+)	GATGCC/TGAAGC	Missense	S/G
rs199591638	151051252(+)	GGCAGG/TGATCT	Missense	H/P
rs199655940	151043747(+)	CTCCAC/TGGGGA	Missense	M/V
rs200777949	151047030(+)	TACCCA/GCAGCT	Missense	R/W
rs201797309	151049318(+)	AGAGTC/TGAAGG	Missense	N/D
rs201856432	151045950(+)	CTGGCC/TGAACT	Missense	S/G
rs2304049	151047100(+)	TTCTTG/CAGCCA	ESE	
rs11542495	151049274(-)	GAGGGC/TACCAA	ESE	

In total, 471 available SNPs were identified in the human SPARC gene. Among these, 23 SNPs were functionally relevant, including two available alleles, which disrupted an existing ESE and 21 SNPs causing missense mutations. SNP, single nucleotide polymorphism; SPARC, secreted protein acidic and rich in cysteine; ESE, exonic splicing enhancer.

were identified and SPARC was found to exist in all types of vertebrates, including fish, amphibians, birds and mammals. Furthermore, all identified RON proteins contained NT, FS and EC domains. The phylogenetic tree demonstrated that SPARC is separated in the order fish, amphibians, birds and mammals. Primate SPARCs are almost the identical and clustered together. From the alignment and phylogenetic tree, mammalian SPARCs were observed to be conserved among vertebrate genomes, suggesting that the function of SPARC may be important physiologically for all the vertebrates in the long evolutionary process. Furthermore, this process was under purifying selection. It is in accordance with multiple biological functions that have been ascribed to this protein, including its involvement in tissue remodeling (51), morphogenesis (52,53) and bone mineralization (54).

Matrix metalloproteinases (MMP-2, -3, -7 and -13), plasmin and trypsin, have been demonstrated to cleave SPARC *in vitro*, producing a KGHK-containing fragment (15,55,56-58). The presence of the truncated form of the SPARC protein has been reported in hepatocellular carcinoma samples (59,60) and esophageal carcinoma (61). It appeared that truncated SPARC may have an important pro-angiogenic function in cancer. The present study identified 21 SNPs causing missense mutations, which may affect the formation of the truncated form of the SPARC protein. The effects of these SNPs on the physiological and pathological functions of SPARC requires further investigation.

SPARC was initially identified in bone and endothelial cells (62,63). It is also highly expressed in developing tissues, including the notochord (64), somites (65) and the embryonic skeleton (66), as well as in differentiating chondrocytes (67), megakaryocytes (68) and macrophages (69) at sites of tissue injury. The systematic analysis of SPARC expression in normal tissues and cancer samples has not been well studied. The present study revealed that the human SPARC gene was expressed in numerous tissues and organs, including the bone marrow, whole blood, lymph node, thymus, brain, cerebellum, retina, heart, smooth muscle, skeletal muscle, spinal cord, intestine, colon, adipocyte, kidney, liver, pancreas, thyroid, salivary gland, skin, ovary, uterus, placenta, cervix and prostate.

When searched in the PrognoScan database, the human SPARC gene was also revealed to be expressed in bladder, blood, breast, giloma, esophagus, colorectal, head and neck, ovarian, lung and skin cancer tissues. SPARC is differentially expressed in tumors and its surrounding stroma in various types of cancer in comparison with the normal tissue, yet, its pattern of expression is variable depending on the type of

Table II. Dataset content from PrognoScan demonstrated an association between microarray expression of SPARC and cancer prognosis.

Database	Case type	Subtype	Patient number	End point	Cut point	P-value	Prognosis	Reference
GSE12417-GPL96	Blood cancer	AML	163	Overall survival	0.86	0.04	2	66
GSE12417-GPL96	Blood cancer	AML	163	Overall survival	0.63	0.001	2	66
GSE12417-GPL570	Blood cancer	AML	79	Overall survival	0.78	0.014	2	66
GSE4475	Blood cancer	B-cell lymphoma	158	Overall survival	0.26	0.001	1	67
TABM-346	Blood cancer	DLBCL	53	Overall survival	0.38	0.033	1	68
GSE11121	Breast cancer		200	Distant metastasis free survival	0.6	0.045	2	69
E-TABM-158	Breast cancer		117	Disease specific survival	0.52	0.004	2	70
GSE3494-GPL96	Breast cancer		236	Disease specific survival	0.27	0.00017	1	71
GSE4922-GPL96	Breast cancer		249	Disease free survival	0.27	0.0054	1	72
GSE17536	Colorectal cancer		177	Disease specific survival	0.64	0.0001	2	73
GSE17536	Colorectal cancer		177	Overall survival	0.56	0.013	2	73
GSE17536	Colorectal cancer		177	Overall survival	0.64	0.007	2	73
GSE17536	Colorectal cancer		177	Disease free survival	0.58	0.01	2	73
GSE17536	Colorectal cancer		177	Disease free survival	0.66	0.0006	2	73
GSE17536	Colorectal cancer		177	Disease specific survival	0.56	0.0003	2	73
GSE14333	Colorectal cancer		226	Disease free survival	0.48	0.0063	2	74
GSE14333	Colorectal cancer		226	Disease free survival	0.56	0.0039	2	74
GSE22138	Eye cancer	Uveal melanoma	63	Distant metastasis free survival	0.56	0.0025	2	75
GSE22138	Eye cancer	Uveal melanoma	63	Distant metastasis free survival	0.59	0.0007	2	75
GSE31210	Lung cancer	Adenocarcinoma	204	Overall survival	0.83	0.048	2	76
GSE31210	Lung cancer	Adenocarcinoma	204	Relapse free survival	0.89	0.003	2	76
GSE8894	Lung cancer	NSCLC	138	Relapse free survival	0.36	0.044	2	77
GSE9891	Ovarian cancer		278	Overall survival	0.67	0.011	2	78
CSE9891	Ovarian cancer		278	Overall survival	0.59	0.006	2	78
GSE26712	Ovarian cancer		185	Disease free survival	0.88	0.009	2	79
GSE26712	Ovarian cancer		185	Overall survival	0.88	0.039	2	79
GSE16560	Prostate cancer		281	Overall survival	0.42	0.004	1	80

In total, 27 out of 136 tests demonstrated an association between microarray expression of the SPARC gene and cancer prognosis (bladder cancer 0/4, blood cancer 5/18, brain cancer 0/8, breast cancer 4/38, colorectal cancer 8/18, eye cancer 2/2, lung cancer 3/31, ovarian cancer 4/13, prostate cancer 1/1, renal cancer 0/1, skin cancer 0/2 and soft tissue cancer 0/2) with a 5% significance level (36-50). AML, acute myeloid leukemia; DLBCL, diffuse large B-cell lymphoma; NSCLC, non-small cell lung cancer; SPARC, secreted protein acidic and rich in cysteine.

cancer. In total, 27 out of 136 tests demonstrated an association between microarray expression of the SPARC gene and cancer prognosis (bladder cancer 0/4, blood cancer 5/18, brain cancer 0/8, breast cancer 4/38, colorectal cancer 8/18, eye cancer 2/2, lung cancer 3/31, ovarian cancer 4/13, prostate cancer 1/1, renal cancer 0/1, skin cancer 0/2 and soft tissue cancer 0/2) with a 5% significance level.

SPARC mRNA was significantly overexpressed in pancreatic cancer; however, not in cancer of the papilla of Vater (8). SPARC was demonstrated to be associated with drug resistance in ovarian cancer (70). In addition, SPARC induced the migration of glioblastoma cell lines (10) and the downregulation of SPARC expression inhibited cell migration and invasion in malignant gliomas (11). However, other studies suggested that SPARC induced endoplasmic reticulum stress leading to autophagy-mediated apoptosis in neuroblastoma (12), and RNA interference against SPARC promoted the growth of malignant glioma cells (13). The aberrant methylation of SPARC was identified in human laryngeal and hypopharyngeal carcinomas (71). In addition, SPARC was observed to be involved in the transformation of hamster oral mucosa from precancerous lesions to squamous cell carcinoma (72,73). SPARC protein expression was also observed to be markedly induced in the supernatants of co-cultured astrocytes (74). Despite transcriptional silencing by aberrant hypermethylation of the CpG-rich region in endometrial carcinoma, the SPARC protein remained overexpressed (9). Furthermore, microRNA-29a was able to suppress cell proliferation by targeting SPARC in hepatocellular carcinoma (75).

This suggested that the expression of SPARC was associated with the prognosis of numerous types of cancer, including hematological and solid cancers. The underlying mechanisms of SPARC involved in the process of these tumors requires further investigation. It is important to note that the association between the expression of SPARC and prognosis varied in different types of cancer, even in the same cancer from different databases. It implied that the function of SPARC in these tumors may be multidimensional, functioning not just as a tumor suppressor or oncogene.

Nkx2-5, Brachyury PPAR-α, AML1a and p53 regulatory transcription factor binding sites were identified in the SPARC gene upstream (promoter) region. Nkx2-5 encodes a homeobox-containing transcription factor. This transcription factor functions during heart formation and development. It was also revealed that Nkx2-5 is the key transcription factor regulating its genomic neighborhoods differently between the tumor types (76). The p53 gene is mutated in approximately half of all types of human tumor. p53 is a transcription factor and its activity gives rise to a variety of cellular outcomes, most notably cell cycle arrest and apoptosis, eliminating cancer-prone cells from the replicative pool (77-79). These two tumor-related transcriptional factors may be involved in the effect of SPARC on various types of tumor.

The present study demonstrated that the association between the expression of SPARC and prognosis varied in different types of cancer. However, the specific functions of SPARC in the majority of tumors remain to be elucidated. Further studies are required to focus on its different behaviors in different types of cancer and its potential relative pathways, including MMPs.

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