OY-TES-1 may regulate the malignant behavior of liver cancer via NANOG, CD9, CCND2 and CDCA3: A bioinformatic analysis combine with RNAi and oligonucleotide microarray

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Abstract. Given its tumor-specific expression, including liver cancer, OY-TES-1 is a potential molecular marker for the diagnosis and immunotherapy of liver cancers. However, investigations of the mechanisms and the role of OY-TES-1 in liver cancer are rare. In the present study, based on a comprehensive bioinformatic analysis combined with RNA interference (RNAi) and oligonucleotide microarray, we report for the first time that downregulation of OY-TES-1 resulted in significant changes in expression of NANOG, CD9, CCND2 and CDCA3 in the liver cancer cell line BEL-7404. NANOG, CD9, CCND2 and CDCA3 may be involved in cell proliferation, migration, invasion and apoptosis, yet also may be functionally related to each other and OY-TES-1. Among these molecules, we identified that NANOG, containing a Kazal-2 binding motif and homeobox, may be the most likely candidate protein interacting with OY-TES-1 in liver cancer. Thus, the present study may provide important information for further investigation of the roles of OY-TES-1 in liver cancer.

Introduction

Surgical resection is the primary mode of choice in the treatment of liver cancer, while the 5-year recurrence rate after resection is as high as 35.4-43.5% (1). The poor prognosis associated with liver cancer has prompted the identification and development of new diagnostic markers and therapeutic strategies. Immunotherapy is a potentially attractive option for patients with liver cancer. Cancer/testis (CT) antigens are potential immunotherapeutic targets in many types of cancers including liver cancer due to their expression pattern, which is restrictively expressed in the testes, yet aberrantly expressed by a variety of malignancies (2-8). OY-TES-1 has been defined as the 23rd member of the CT antigen family, called CT23 (9-12). OY-TES-1 was originally identified to be the human homologue of pro-acrosin binding protein (ACRBP), a tyrosine phosphorylated protein related to capacitation, the sp32 precursor in mouse (13). Spontaneous humoral response against OY-TES-1 has been detected in patients with different tumors including liver cancer (9). An HLA-A24-binding OY-TES-1 peptide recognized by CD8 T cells was identified, and T-cell cytotoxicity was observed against an OY-TES-1 mRNA-expressing lung tumor cell line *in vitro* (14). The above studies imply that OY-TES-1 is an attractive target for antigen-specific immunotherapy in cancers due to its immunogenic traits in humans (9,14). In another study in ovarian cancer cells, a mitotic spindle protein NuMA was identified as an ACRBP-interacting protein (12). ACRBP depletion resulted in mitotic errors and reduced proliferative fitness that could be rescued by NuMA co-depletion. This indicates that ACRBP could normalize the perturbed mitotic infrastructure responsible for disease-promoting genetic variation. In our previous report, we demonstrated that OY-TES-1 was expressed in human mesenchymal stem cells (MSCs) at both the mRNA and protein levels, and downregulation of OY-TES-1 expression in these MSCs caused cell growth inhibition, cell cycle arrest, apoptosis induction and migration ability attenuation (15). However, whether OY-TES-1 is involved in the biological function of liver cancer remains undetermined. In the present study, we applied bioinformatic analysis combined with a molecular biology assay to investigate the biological function and protein interaction of OY-TES-1 in liver cancer. Our data indicated that OY-TES-1 regulates biological processes of liver cancer cells via NANOG, CD9, CCND2 and CDCA3.

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Materials and methods

Motif and domain-domain interaction analysis. The motif analysis of OY-TES-1 protein was performed with SSDB Motif Search in Kyoto Encyclopedia of Genes and Genomes (KEGG) online database (http://www.kegg.jp/). The protein domain interactions were analyzed by DOMINE online database (16) (http://domine.utdallas.edu/cgi-bin/Domine) and the Pfam protein families database (17), respectively. KEGG is a database resource for understanding high-level functions and utilities of the biological system from molecular-level information, particularly large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies. With KEGG motif search, a domain of unknown function with peptide fragment usually can be found (18). DOMINE is a database of known and predicted protein domain (domain-domain) interactions, which are predicted by 13 different computational approaches using Pfam domain definitions. DOMINE contains a total of 26,219 domain-domain interactions (among 5,410 domains) out of which 6,634 are inferred from PDB entries, of which 2,989 interactions are high-confidence predictions (HCPs) (16,17).

Co-expressing gene analysis in liver cancer through ONCOMINE. To identify significant OY-TES-1-co-expressing genes in liver cancer, we searched for all relevant, publically available microarray datasets in online cancer microarray gene expression database, ONCOMINE (https://www.ONCOMINE.org/resource/main.html) (19). ONCOMINE database is a bioinformatics initiative aimed at collecting, standardizing, analyzing and delivering cancer transcriptome data to the biomedical research community. The analysis has identified the genes, pathways and networks deregulated across 18,000 cancer gene expression microarrays, spanning the majority of cancer types and subtypes (19). As there are often many hundreds of tumor samples/microarrays within a single multi-array result from co-expressing genes can be analyzed. ONCOMINE database provides a potentially significant list of co-expressing genes, which is important to define pathways in which the gene of interest is involved (20).

Co-expressing gene annotation through gene ontology (GO) annotator. GO annotator uses text-mining methods to extract GO terms from scientific studies and provides this information along with a GO term from an uncurated annotation; thus, it provides not only facts but also their evidence (21). Based on the GO annotation, we searched each proliferation, migration, invasion or apoptosis GO term for the genes with high correlation and frequency to OY-TES-1 co-expression in the GO database.

Co-expressing gene literature co-occurrence through COREMINE and PubMed. The OY-TES-1-co-expressing genes with GO terms of cell proliferation, adhesion, migration and apoptosis in liver cancer were fed to a literature co-occurrence tool-COREMINE online tool (http://www.coremine.com/medical/#search) (22). COREMINE medical is a gene/protein database and web-based tool for literature mining. It develops automated extraction of experimental and theoretical knowledge of biomedicine from publicly available gene and text databases to create a gene-to-gene co-citation network for human genes in MEDLINE records (22). The systematic search of the literature was performed with PubMed for studies addressing association among liver cancer, OY-TES-1 and OY-TES-1 interacting proteins.

Oligonucleotide microarray analysis combined with RNAi. OY-TES-1 was downregulated in the liver cancer cell line BEL-7404 using small interfering RNA (siRNA) with X-tremeGENE siRNA transfection reagent (Roche Diagnostics). OY-TES-1 siRNA and a scrambled siRNA were synthesized by Shanghai GenePharma Co., Ltd. The sequences of the siRNAs and experimental procedure were previously described by Cen *et al* (15). Total RNA extracted from non-siRNA-treated cells and siRNA-OY-TES-1-treated cells was used for genome-wide expression analysis with the Human Whole Genome 6x44K Microarray (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer's protocol (23). Data quality check and analysis were conducted using SBC analysis system (Agilent Technologies). p-value was calculated when duplicates were used in the experiment, and differentially expressed genes were selected by p-value (<0.05) (24).

Generation of biological interaction network through GeneMANIA. Candidate genes selected from the oligonucleotide microarray assay above were fed into a curated protein interaction network system-GeneMANIA (http://www.genemania.org/), which is a fast web-based tool and database for predicting gene function based on multiple networks derived from different genomic or proteomic data/sources with great accuracy (25). With the GeneMANIA a gene/protein-gene/protein interaction network of OY-TES-1 was generated.

Results

Four motifs were identified in OY-TES-1. Following a search for 'OY-TES-1' in the KEGG online database, four motifs, Kazal-1 and -2, PBP-sp32 and TFIIF-α, were found in human OY-TES-1 on the dataset of hsa:84519 (Table I; Fig. 1). The Kazal motif contains two patterns, Kazal-1 and -2. The amino terminal segment of both Kazal motifs can bind to the active site of target proteases resulting in functional inhibition (Table I). The family of Kazal-1 inhibitor proteins inhibits serine peptidases of the S1 family, such as trypsin and elastase (26,27), while the family of Kazal-2 inhibitor proteins inhibits serine peptidases of MEROPS, such as I1, I2, I17 and I31. However, Kazal-like domains are also seen in the extracellular part of agrins, which are unknown to be protease inhibitor (28). TFIIF-α, a subunit of transcription initiation factor IIF, or RNA polymerase II-associating protein 74 (RAP74) is the large subunit of transcription factor IIF. By interacting with the proteins containing interacted motifs as summarized in Table I, TFIIF-α plays an essential role in accurate initiation and stimulates elongation by RNA polymerase II (29). PBP-sp32 is a sperm-specific domain involved in packaging acrosin zymogen into acrosomal matrix (30). In general, OY-TES-1 interacts with the proteins containing TFIIF-α, Kazal-1 and -2 motifs or the proteins

Table I. The motifs of OY-TES-1 and NANOG, interacted motifs and motif-shared proteins according to database search[a].

Protein	Motif id	Location	Definition	E-value	Interacted motif	Motif-shared proteins
OY-TES-1	pf:Kazal_1	474-504	Kazal-type serine protease inhibitor domain	0.05	TGF-β, Kazal-1, Peptidase-S8, Trypsin, FOLN, SPARC-Ca_bdg, efhand, Laminin-EGF, Thyroglobulin_1, EGF, Kunitz-BPTI, ig, Laminin-G_1, Ldl_recept_a, Sushi, TSP-1, zf-C_2H_2, SRCR, PDZ, SEA, MACPF, OATP	AGRIN, CPAMD8, FST, FSTL3, FSTL4, FSTL5, IGFBPL1, SMOC1, SPARC, SPARCL1, SPINK1, SPINK2, SPINK4, SPINK5, SPINK5L2, SPINK5L3, SPINK6, SPINK7, SPINK9, TMEFF1, TMEFF2
	pf:Kazal_2	473-506	Kazal-type serine protease inhibitor domain	0.0075	TGF-β, Trypsin, Kazal-2, BTB, Homeobox, Arrestin_N, LIM, Arrestin-C	C6, CFI, FSTL1, FSTL3, HTRA1, HTRA3, HTRA4, IGFBP7, KAZALD1, LST3, RECK, SLC21A8, SLCO1A2, SLCO1B1, SLCO1B3, SLCO1C1, SLCO2A1, SLCO3A1, SLCO4A1, SLCO4C1, SLCO5A1, SLCO6A1, SMOC2, SPINK5, SPOCK1, SPOCK2, SPOCK3, WFIKKN1, WFIKKN2
	pf:TFIIF_α	197-263	Transcription initiation factor IIF, α subunit (TFIIF-α)	0.13	TFIIF_β, FCP1_C, Tax, FlhD, Ribosomal_L7Ae, HNF-1_N, TFIIF_α	TFIIF
	pf:PBP_sp32	1-240	Proacrosin binding protein sp32	9.30e-135	Unknown	OY-TES-1 (sp32/ACRBP)
NANOG	pf:Homeobox	97-152	Homeobox domain	7.80E-19	Homeobox, Pou, SRF-TF, SBP_bac_1, CUT, HNF-1B_C, HNF-1_N, PD-C2-AF1, HLH, Pkinase, RRM_1, zf-C2H2, PAX, WD40, MH2, EGF, Kazal_2	Pou family
	pf:Homez	110-147	Homeodomain leucine-zipper encoding, Homez	0.00024	Unknown	Unknown

[a]Search in the SSDB, DOMINE and Pfam database.

containing the interacted motifs of these 3 motifs. Thus, through these interactions, OY-TES-1 may perform its functions in regulating the biological behavior of tumor cells.

Sixty genes were found to co-express with OY-TES-1 in liver cancer. To investigate OY-TES-1-co-expressing genes in liver cancer, we queried the ONCOMINE database using

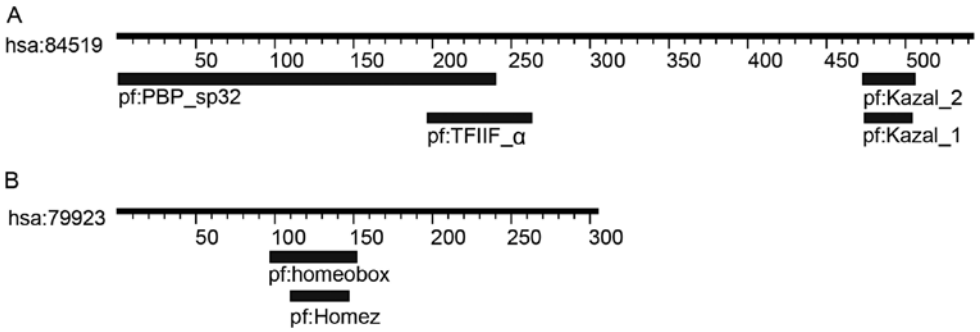

Figure 1. The motifs contained in (A) OY-TES-1 and (B) NANOG protein according to SSDB Motif Search. Black bars represent the location of each motif.

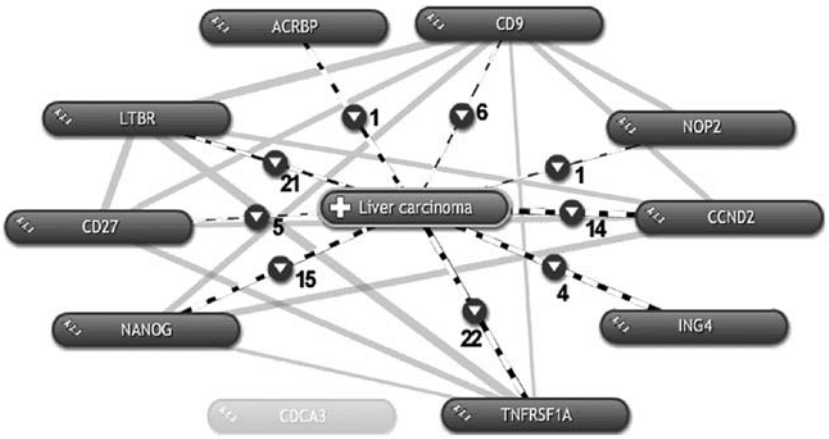

Figure 2. The co-occurrence analysis of OY-TES-1 (ACRBP) and its co-expressing genes to the exact key word expression 'liver carcinoma (aliases of 'liver cancer')' by COREMINE online tool search. Zebra line indicates the co-occurrence of the genes to the key word 'liver carcinoma', and the number near the line is the number of co-occurrences between the gene and 'liver carcinoma'; the light gray line indicates the co-occurrence of the genes between each other. ACRBP, acrosin binding protein.

a concept 'co-expression genes with OY-TES-1 expression in liver cancer'. There was a list of 5,051 genes in 9 datasets, namely Liver (Liao)-Cluster ID n9273 (17 genes); Multi-cancer (Beroukhim)-Cluster ID n9385 (85 genes), Cell Line (Rothenberg)-Cluster ID n9276 (207 genes), Cell Line (Wooster 2)-Cluster ID n9229 (209 genes), Cell Line (Barretina 2)-Cluster ID n9313 (229 genes), Liver cancer (Bittner Multi-cancer, 978 genes), Liver cancer (Wooster Cell Line 2, 1,875 genes), Liver cancer (Barretina Cell Line, 1,957 genes) and Liver cancer (Bittner Multi-cancer, 1,957 genes). As listed in Table II, 60 genes were co-expressed with OY-TES-1 at least in 5 of 9 datasets mentioned above, and the correlation between those genes and OY-TES-1 was >0.900.

Nine OY-TES-1 co-expressing genes may regulate biological processes. As the 60 genes identified above showed a correlation with OY-TES-1, we further predicted their function through GO annotator and COREMINE online tool search. As listed in Table III, we identified 9 genes: CD9 molecule (CD9), cyclin D2 (CCND2), CD27 molecule (CD27), cell division cycle-associated protein (CDCA3), inhibitor of growth family, member 4 (ING4), lymphotoxin-β receptor (LTBR), homeobox transcription factor Nanog (NANOG), nucleolar protein 2 homolog (NOP2) and tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A). These genes are involved in cell proliferation, adhesion, migration and/or apoptosis.

Eight OY-TES-1 co-expressing genes are co-occurring in liver cancer. According to the above search, 9 of the co-expressing OY-TES-1 genes are involved in the biological behavior of cells, but whether they are related to liver cancer remains unknown. Thus, these 9 genes and OY-TES-1 were further fed to COREMINE online tool search using 'liver carcinoma' as a key word. As shown in Fig. 2, co-occurrence with liver cancer was demonstrated for OY-TES-1 and 8 of the 9 genes except for CDCA3, and these 8 genes also co-occur with each other. Among those 8 genes, CD27, ING4, LTBR and TNFRSF1A are known to be involved in apoptosis; CD27 negatively regulates the apoptotic process (31-34), while ING4 (35-37), LTBR (38) and TNFRSF1A (39) positively regulate the apoptotic process. CD9 is also considered to regulate migration and adhesion of cells (40,41). In addition, CD9 and ING4 are thought to negatively regulate cell proliferation (35,44). The others, CCND2 (45-51), NANOG (52-54) and NOP2 (55,56), positively regulate cell proliferation. With regard to CDCA3, a G_1 phase controlling gene which prevents G_1 arrest, there is no current literature that shows that it is involved in liver cancer. However, considering that CDCA3 has a high expression frequency and a high co-expression correlation with OY-TES-1 in liver cancer datasets, further investigation of CDCA3 is needed. To date, there is no report of the involvement of OY-TES-1 in apoptosis, migration, adhesion and cell proliferation of liver cancer. We here demonstrated that

Table II. Genes co-expressing with OY-TES-1 at least in 5 out of 9 datasets.

Gene	Correlation[a]	Freq[b]	Gene	Correlation	Freq
EMG1	0.980±0.000	7/9	CLSTN3	0.964±0.028	5/9
CD9	0.980±0.005	7/9	C1RL	0.964±0.028	5/9
ZNF384	0.989±0.008	7/9	COPS7A	0.990±0.009	5/9
SCNN1A	0.971±0.014	7/9	LAG3	0.986±0.006	5/9
C12orf53	0.991±0.010	7/9	DPPA3	0.951±0.018	5/9
CLEC4A	0.951±0.018	6/9	ATN1	0.982±0.003	5/9
ENO2	0.983±0.003	6/9	RIMKLB	0.907±0.000	5/9
CD27	0.977±0.006	6/9	USP5	0.988±0.007	5/9
MFAP5	0.907±0.000	6/9	C12orf57	0.982±0.003	5/9
FOXJ2	0.951±0.018	6/9	TAPBPL	0.977±0.006	5/9
LPAR5	1.000±0.001	6/9	C1R	0.964±0.028	5/9
NCAPD2	0.977±0.006	6/9	LTBR	0.971±0.014	5/9
VAMP1	0.977±0.006	6/9	LEPREL2	0.988±0.007	5/9
C1S	0.976±0.007	5/9	TPI1	0.988±0.007	5/9
ITFG2	0.861±0.015	5/9	NOP2	0.996±0.004	5/9
ING4	0.997±0.004	5/9	GNB3	0.988±0.007	5/9
PHB2	0.982±0.003	5/9	MLF2	0.985±0.005	5/9
NANOG	0.951±0.018	5/9	RBP5	0.964±0.028	5/9
CDCA3	0.988±0.007	5/9	LRRC23	0.983±0.006	5/9
PTPN6	0.982±0.003	5/9	LPCAT3	0.976±0.007	5/9
CLEC4C	0.951±0.018	5/9	PLEKHG6	0.971±0.014	5/9
SLC2A14	0.922±0.009	5/9	GAPDH	0.984±0.011	5/9
TNFRSF1A	0.971±0.014	5/9	GDF3	0.951±0.018	5/9
AICDA	0.907±0.000	5/9	IFFO1	0.988±0.012	5/9
SLC2A3	0.922±0.009	5/9	CD4	0.976±0.017	5/9
FAM90A1	0.943±0.016	5/9	CHD4	0.999±0.001	5/9
NECAP1	0.951±0.018	5/9	PTMS	0.986±0.006	5/9
CCND2	0.956±0.022	5/9	A2ML1	0.907±0.000	5/9
GPR162	0.988±0.007	5/9	C3AR1	0.951±0.018	5/9
SPSB2	0.988±0.007	5/9	MRPL51	0.977±0.006	5/9

[a]Correlation (mean ± SD) between expression of the candidate genes and OY-TES-1 is >0.900. [b]Frequency of the candidate gene co-expressing with OY-TES-1 in the 9 datasets, searched in the ONCOMINE online database.

OY-TES-1 is co-expressed with 9 genes (CD9, CCND2, ING4, CDCA3, NANOG, NOP2, CD27, LTBR and TNFRSF1A) with a high correlation and frequency, inferring that OY-TES-1 may be involved in the cell adhesion/migration regulated by CD9, cell proliferation mediated by CD9, CCND2, ING4, CDCA3, NANOG and NOP2, and apoptosis modulated by CD27, ING4, LTBR and TNFRSF1A in liver cancer, respectively.

Four candidate genes are significantly altered by OY-TES-1 downregulation. As the above results identified 9 OY-TES-1-co-expressing genes with functions of cell proliferation, adhesion, migration and/or apoptosis, we further screened an oligonucleotide microarray following OY-TES-1 down-regulation in a liver cancer cell line. It was found that a total of 8,870 genes were significantly altered ($p<0.05$) in the siRNA-OY-TES-1-treated cell as compared with the control. Notably, these 9 OY-TES-1 co-expressing genes (CD9, CCND2, CDCA3, NANOG, ING4, NOP2, CD27, LTBR and TNFRSF1A) revealed a differential expression profile. CD9, CCND2 and CDCA3 were upregulated, whereas NANOG was downregulated. Another 5 genes had no expression change ($p>0.05$, Table IV). Furthermore, after searching the motif of CD9, CCND2, CDCA3 and NANOG in SSDB, DOMINE and Pfam database, an interacted motif of Kazal-2 contained in OY-TES-1, homeobox, was found in human NANOG on the dataset of hsa:79,923 (Table I; Fig. 1). Therefore, NANOG may be considered as the most likely candidate protein interacting with OY-TES-1 in liver cancers.

OY-TES-1 may be functionally related to NANOG, CD9, CCND2 and CDCA3 by various interactions. Due to the unclear functions of OY-TES-1 and its co-expressing proteins, OY-TES-1, NANOG, CDCA3, CD9 and CCND2 were fed into GeneMANIA to predict their functions and interactions. As shown in Fig. 3, OY-TES-1, NANOG, CD9, CCND2 and CDCA3 were co-expressed, co-localized, physically and genet-

Table III. The biological process annotation of OY-TES-1-co-expressing genes by GO annotator.

Gene	GO ID	Qualified GO term	Evidence	Ref.
CD9	GO:0007155	Cell adhesion	IDA	(38)
	GO:0008285	Negative regulation of cell proliferation	IEA	No
CCND2	GO:0007049	Cell cycle	IEA	No
	GO:0045737	Positive regulation of cyclin-dependent protein kinase activity	IDA	(43)
	GO:0051301	Cell division	IEA	No
CD27	GO:0006917	Induction of apoptosis	ISS	No
	GO:0008588	Release of cytoplasmic sequestered NF-κB	NAS	(29)
	GO:0043066	Negative regulation of apoptotic process	ISS	No
	GO:0043154	Negative regulation of cysteine-type endopeptidase activity involved in apoptotic process	IDA	(29)
CDCA3[a]	GO:0007067	Mitosis	IEA	No
	GO:0051301	Cell division	IEA	No
ING4	GO:0006915	Apoptotic process	IDA	(33)
	GO:0007050	Cell cycle arrest	IDA	(33)
	GO:0008285	Negative regulation of cell proliferation	IDA	(33)
	GO:0043065	Positive regulation of apoptotic process	IDA	(34)
LTBR	GO:0006915	Apoptotic process	IEA	No
	GO:2001238	Positive regulation of extrinsic apoptotic signaling pathway	IMP	No
NANOG	GO:0008283	Cell proliferation	IMP	(50)
NOP2	GO:0008284	Positive regulation of cell proliferation	TAS	(53)
TNFRSF1A	GO:0006915	Apoptotic process	TAS	No
	GO:0042981	Regulation of apoptotic process	IEA	No
	GO:0043123	Positive regulation of I-κB kinase/NF-κB cascade	IEP	(37)

GO, gene ontology; Ref., reference; IDA, inferred directly from assay; IEA, inferred from electronic assay; ISS, inferred from sequence or structural similarity; TAS, traceable author statement; IMP: inferred from mutate phenotype; IPI, inferred from physical interaction. [a]Did not co-occur in liver cancer.

ically interacted, and/or shared protein domains and pathways with each other and a number of other proteins, such as CCND3, CDK4, CDK6, CD44, ITGA2, ITGA3, ITGB1, ESRRB, EGR1, PITX2, REST, CDKN2C and WEE1 (Table V). Therefore, it can be suggested that OY-TES-1, NANOG, CDCA3, CD9 and CCND2 may be functionally related. Although OY-TES-1 was considerably less interactive with other proteins involving in cell proliferation, adhesion, migration and apoptosis in comparing the results, it contains a Kazal-2 domain that could bind with the homeobox domain shared by NANOG and PITX2. Thus, we added interactions between OY-TES-1, NANOG and PITX2, and predicted these interactions with cell proliferation, adhesion, motility and apoptosis in liver cancer. Based on the annotated functions in accordance with the GeneMANIA network, OY-TES-1, NANOG, CD9, CCND2 and CDCA3, along with other proteins listed in Table V, may play important roles in the regulation of cell adhesion, the cell cycle, kinase activity, apoptosis (or anoikis) and DNA binding.

Discussion

Functional prediction of genes/proteins based on bioinformatic analysis is a feasible and valuable technique for the mining of gene/protein functions, and many large-scale networks of protein interactions within the cell have made it possible to multi-dimensionally study the functions in the context of a network (57). Thus, mining and exploring potentially OY-TES-1-interacting genes via bioinformatic methods would be a first, necessary, feasible and reasonable way to reveal its function in liver cancer. Based on the motif, co-expression profile, GO and literature co-occurrence analysis, we found 60 genes to be co-expressing with OY-TES-1 in liver cancer, and 9 out of 60 of these genes are involved in cell proliferation, adhesion (migration) and/or apoptosis. OY-TES-1 and 8 out of 9 genes were found to co-occur in liver cancer, and these 8 genes co-occur with each other. Furthermore, with RNAi and oligonucleotide microarray analysis, we confirmed that, of these 9 genes, expression of CD9, CCND2 and CDCA3 was significantly increased, and NANOG was markedly decreased. The expression levels of the other 5 genes did not change when OY-TES-1 was suppressed in liver cancer cells ($p>0.05$, Table IV). GeneMANIA network analysis demonstrated that OY-TES-1, NANOG, CD9, CCND2 and CDCA3 were co-expressed, co-localized, physically and genetically interacted and/or shared protein domains and pathways with each other (Fig. 3). Annotated functions (Table V) suggested that OY-TES-1 may

Table IV. Expression profile of OY-TES-1 co-expressing candidate genes and their interacing genes by OY-TES-1 suppression in the cell line BEL-7404[a].

Gene symbol	Gene name	Biological function of encoded protein	Fold-change	P-value
OY-TES-1 co-expressing candidate genes				
CD9	CD9 molecule	Negative regulation of cell proliferation; suppressor of cancer cell motility and metastasis	2.2263	2.00E-04
CCND2	Cyclin D2	Positive regulation of cell proliferation. Regulatory subunit of CDK4 or CDK6, required for cell cycle G_1/S transition	2.0956	0.0017
CDCA3	Cell division cycle associated 3	F-box-like protein required for entry into mitosis. Acts by participating in E3 ligase complexes that mediate the ubiquitination and degradation of WEE1 kinase at G_2/M phase	2.0355	0.0208
NANOG	Nanog homeobox	Transcription regulator involvesin embryo stem (ES) cells proliferation and self-renewal. When overexpressed, promotes cells to enter into S phase and proliferation	0.4258	0.0064
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	Major receptor for the tumor necrosis factor-α, mediate apoptosis by activating NF-κB	1.2090	0.065
NOP2	NOP2 nucleolar protein homolog	Positive regulation of cell proliferation; increase nucleolar activity associated with cell proliferation	1.2410	0.1695
ING4	Inhibitor of growth family, member 4	Tumor suppressor protein, involves in the TP53-dependent regulatory pathway; negative regulation of cell proliferation, positive regulation of apoptotic process	1.2089	0.1978
LTBR	Lymphotoxin β receptor	Receptor for heterotrimeric lymphotoxin and TNFS14/LIGHT. Promotes apoptosis via TRAF3 and TRAF5	1.3154	0.2497
CD27	CD27 molecule	Receptor for CD70/CD27L. Negative regulation of cysteine-type endopeptidase activity involved in apoptotic process	0.8403	0.3803
Interacting genes of OY-TES-1-co-expressing candidate genes				
CCND3	Cyclin D3	Regulatory component of the cyclin D3-CDK4 (DC) complex that inhibits members of the retinoblastoma (RB) protein family, and regulates the cell-cycle during G_1/S transition	1.4241	0.0032
CDK6	Cyclin-dependent kinase 6	Serine/threonine-protein kinase involved in the control of the cell cycle and differentiation; promotes G_1/S transition; negatively regulates cell differentiation	1.2173	0.0205
WEE1	WEE1 homolog	Negative regulator of entry into mitosis (G_2 to M transition) by protecting the nucleus from cytoplasmically activated cyclin B1-complexed CDK1	1.5128	0.0031
CD44	CD44 molecule	Receptor for hyaluronic acid (HA) and possibly matrix metalloproteinases (MMPs). Adhesion with HA plays an important role in cell migration, tumor growth and progression	1.2606	0.0055
ITGA2	Integrin, α2	Receptor for laminin, collagen, collagen C-propeptides, fibronectin and E-cadherin. It is responsible for adhesion of platelets and other cells, modulation of collagen and collagenase gene expression	2.1047	0.0084

Table IV. Continued.

Gene symbol	Gene name	Biological function of encoded protein	Fold-change	P-value
ITGB1	Integrin, β1	Membrane receptors involved in cell adhesion and recognition in a variety of processes including embryogenesis, hemostasis, tissue repair, immune response and metastatic diffusion of tumor cells	1.2910	0.0297
ITGA5	Integrin, α5	Receptor for fibronectin and fibrinogen. Enhance angiogenesis in Kaposi's sarcoma lesions when HIV-I infected	2.0167	0.0029
EGR1	Early growth response 1	Transcriptional regulator recognizes and binds to EGR-site. Activates the transcription of target genes whose products are required for mitogenesis and differentiation	2.3115	1.00E-04

[a]Detected by oligonucleotide microarrays.

Table V. The biologic process annotated functions of OY-TES 1-co-expressing proteins and their interacting proteins in the GeneMANIA network.

GO annotation	FDR (n/a)	Genes/proteins in the network
Cyclin-dependent protein kinase holoenzyme complex	2.93E-05	CCND2, CCND3, CDK4, CDK6
Cell-matrix adhesion	1.47E-03	CD9, CD44, CDK6, ITGA2, ITGA3, ITGB1
Cell-substrate adhesion	5.14E-03	CD9, CD44, CDK6, ITGA2, ITGA3, ITGB1
Transcription regulatory region sequence-specific DNA binding	1.77E-02	NANOG, ESRRB, EGR1, PITX2, REST
G_1 phase/G_1 phase of mitotic cell cycle	6.28E-02	CDKN2C, CDK4, CDK6
Regulation of cyclin-dependent protein serine/threonine kinase activity	1.46E-01	CCND2, CCND3, CDKN2C
G_1/S transition of mitotic cell cycle	1.68E-01	CDCA3, CDK6, CDK4, WEE1, CDKN2C
Negative regulation of anoikis	2.06E-01	ITGB1, ITGA5

GO, gene ontology; FDR, false discovery rate.

participate in tumor cell proliferation, migration, invasion and apoptosis through regulation of CCND2, CDCA3, CD9 and NANOG.

Both CCND2 and CDCA3 are G_1 phase controlling genes. CCND2 overexpression is associated with the tumorigenesis and progression of various types of cancers including liver cancers by affecting the cell cycle, particularly in the G_1 phase (G_1 cell cycle transition) with G_1 CCND2/cyclin-dependent kinase (CDK)4 (or 6) complexes (58-61). Exhibiting a difference with CCND2, CDCA3 can increase the capacity of proliferation by preventing G_1 arrest via decreased expression of the CDK inhibitor (CDKI) (62,63). In the present study, downregulation of OY-TES-1 in BEL-7404 cells was accompanied by an increase in CCND2 and CDCA3 as well as their interacting genes CCND3 and CDK6 ($p<0.05$, Table IV, Fig. 3), which are able to accelerate cell proliferation by promoting G_1/S transition, CDK activity regulation or cyclin/CDK complex formation (60,61,64). However, as a negative regulator of entry into mitosis (G_2 to M transition) (65), WEE1 was significantly increased ($p<0.05$) (Table IV, Fig. 3); the other cell cycle involved genes CD4 and CDKN2C were not altered (data not shown). Therefore, it is reasonable to infer that downregulation of OY-TES-1 may accelerate the cell cycle and promote proliferation in liver cancer cells through increased expression of CCND2, CDCA3 and their interacing genes CCND3 and CDK6.

CD9 and NANOG are also thought to be associated with the malignant behavior of cells. The absence and low expression of CD9 in small cell lung cancer may contribute to the highly invasive and metastatic phenotype, while ectopic expression of CD9 reduced cell proliferation and motility, attenuated metastasis (66,67) and promoted apoptosis (68,69). Therefore, CD9 has been regarded as an important tumor progression suppressor (70). To date, there is paucity in the research of the correlation between CD9 and liver cancer. As regard to NANOG, it is one of the most important core markers of cancer stem cells (CSCs) due to its capacity to maintain pluripotency, regulate proliferation and prevent differentiation (71,72). NANOG-positive CSCs in liver cancer exhibit drug resistance and a high capacity for tumor invasion

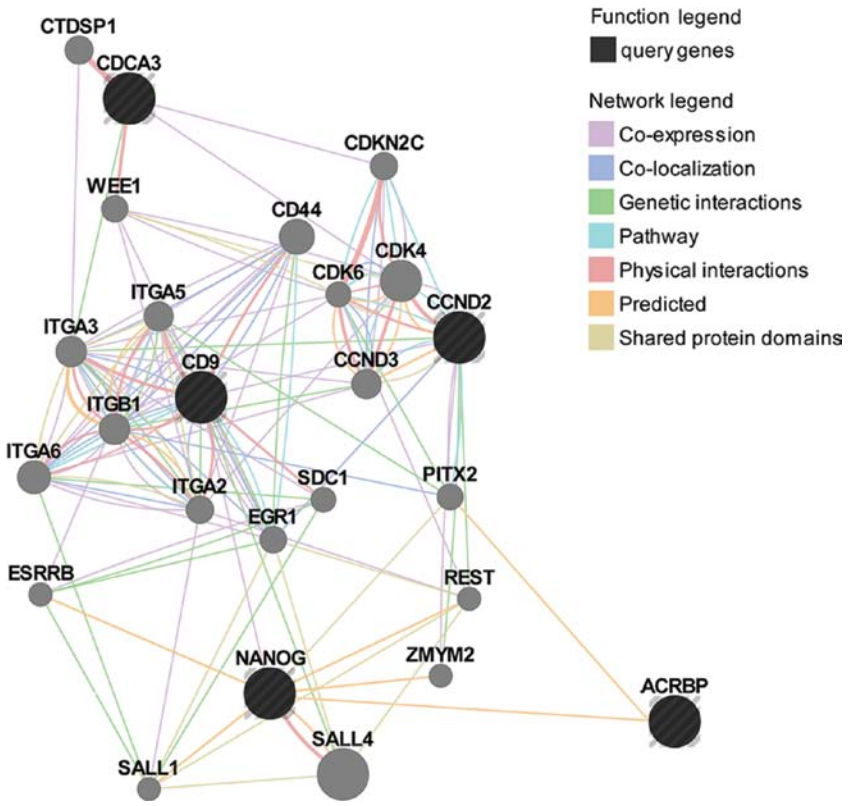

Figure 3. Function prediction of OY-TES-1 (ACRBP) and OY-TES-1-co-expressing proteins using GeneMANIA. The type of interaction between genes/proteins is illustrated as indicated in the network legend.

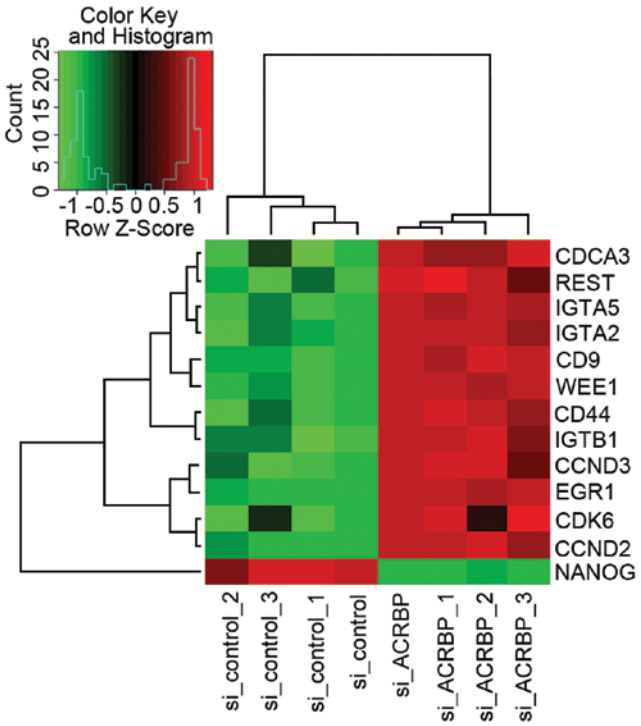

Figure 4. Transcriptional profiling of OY-TES-1 (ACRBP)-associated genes between 3 independent Si_control and 3 independent Si_OY-TES-1 groups of the BEL-7404 cell line, as detected using Agilent oligonucleotide microarray. Si_ control and Si_ OY-TES-1 are the average of 3 independent Si_control and 3 independent Si_OY-TES-1 groups, respectively. Highly expressed genes are shown in red, whereas genes that are expressed at lower levels compared with the Si_control group are displayed in green. A brighter color means a greater difference from the Si_control group compared with a darker color. ACRBP, acrosin binding protein.

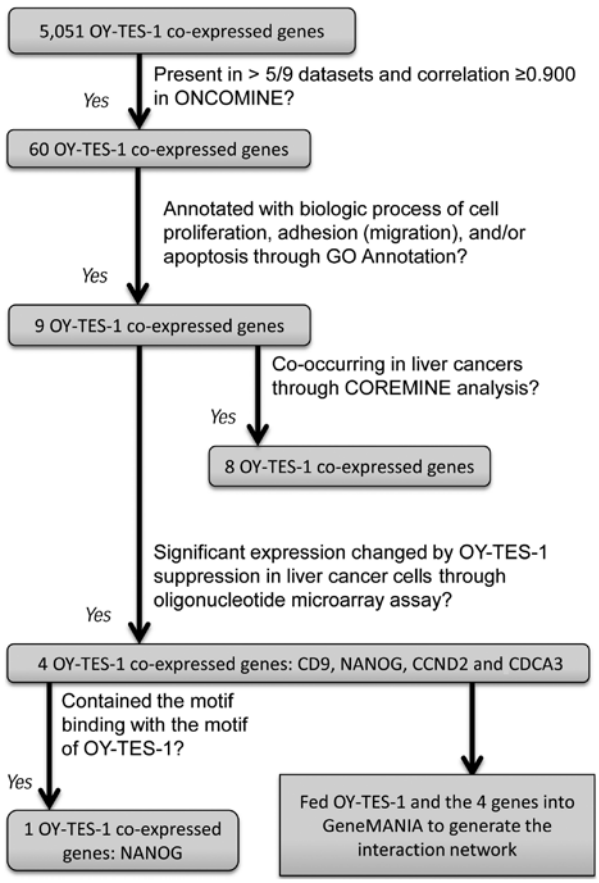

Figure 5. Workflow for selecting OY-TES-1-interacting protein candidates in liver cancers through bioinformatic methods and oligonucleotide microarray assay.

and metastasis (73,74). The same situation is present in other cancers. For example, upregulation of NANOG enhances malignant behaviors in esophageal cancer (52,75); adversely, its downregulation causes inhibitive effects on ovarian and gastric cancer (76). Here, we demonstrated that suppression of OY-TES-1 in a cancer cell line significantly increased expression of CD9 and its interacting genes (CD44, ITGA2, ITGB1 and ITGA5), which negatively regulate proliferation and migration in cancer cells (40-43). Meanwhile, we also found a decrease in NANOG and elevation in EGR1 which interacts with NANOG (Fig. 4; Table IV). EGR1 is thought to be a cancer suppressor (77). There was no change in the other genes listed in Table V, which are involved in cell differentiation and proliferation and are related with CD9 or NANOG. Notably, in the present study downregulation of OY-TES-1 in liver cancer cells caused two opposite effects, namely, promotion of cell proliferation with increase in CCND2 and CDCA3, and inhibition of cell proliferation with CD9 upregulation and NANOG downregulation. Therefore, it was speculated that OY-TES-1 may play multiple roles in liver cancer. Experiments should be conducted to elucidate the function of OY-TES-1 with CD9, NANOG, CCND2, CDCA3 and their interacted proteins in the future.

Collectively, as shown in Fig. 5, we first report that OY-TES-1 suppression results in significant expression changes of its co-expressing genes, CCND2, CDCA3, CD9 and NANOG. As it contains a Kazal-2-interacting motif, homeobox, NANOG may be considered to be the most likely candidate protein interacting with OY-TES-1 in liver cancer. Thus, the present study may set the stage for further investigation of the role of OY-TES-1 in liver cancer.

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