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In silico Prediction of Angiogenesis-related Genes in Human Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and a typical hypervascular tumor. Therefore, it is important to find factors related to angiogenesis in the process of HCC malignancy. In order to find angiogenesis-related factors in HCC, we used combined methods of *in silico* prediction and an experimental assay. We analyzed 1457 genes extracted from cDNA microarray of HCC patients by text-mining, sequence similarity search and domain analysis. As a result, we predicted that 16 genes were likely to be involved in angiogenesis and then the effects of these genes were confirmed by hypoxia response element(HRE)-luciferase assay. For instant, we classified osteopontin into a potent angiogenic factor and coagulation factor XII into a significant anti-angiogenic factor. Collectively, we suggest that using a combination of *in silico* prediction and experimental approaches, we can identify HCC-specific angiogenesis-related factors effectively and rapidly.

Keywords: HCC, hepatocellular carcinoma, bioinformatics, angiogenesis, hmmer, blast

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and causes significant medical expense and mortality (Feitelson *et al.*, 2002). There are several reports on many risk factors involved in HCC. These risk factors include toxin exposure, chronic viral infection, and cirrhosis (Anders *et al.*, 2003). Although all the risk factors may cause direct damage to the genomic DNA and further induce hepatic carcinogenesis, the molecular mechanism remains to be studied (Tsai *et al.*, 2003).

Generally, HCC is a hypervascular tumor - especially large and advanced HCC are richly supplied with blood vessels - with an extremely poor prognosis (Yamaguchi *et al.*, 1998; Mise *et al.*, 1996; Nakashima *et al.*, 1999). HCC tissues express lots of angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor (IGF)-II (Kim *et al.*, 1998; Park *et al.*, 1995). Angiogenesis is a process of new capillaries sprouting from pre-existing vessels, which is different from vasculogenesis by which endothelial cells arise from progenitor cell types (Risau, 1997). It is known that tumor growth and metastasis are angiogenesis-dependent (Folkman, 1990). Therefore, therapies targeting pathological angiogenesis could be highly specific and be associated with little toxicity (Eatock *et al.*, 2000). It is therefore important to find angiogenesis-related factors for the treatment of HCC.

In order to find angiogenesis-related factors in the progress of HCC, we focused our interests on *in silico* screening and a cell based assay. Scheme of work flow is shown in Figure 1. The cDNA microarray data (data not shown) were from the Center for Functional Analysis of Human Genome (Daejon, Korea) and composed of 1457 gene lists that were up- or down-regulated in HCC patients. Based on these microarray data, we carried out *in silico* screening approaches, text-mining, sequence similarity search and domain structure analysis. Then, we performed hypoxia-responsive element (HRE)-luciferase assays for evaluating angiogenic effects of *in silico* data. HRE-luciferase assay is a sensitive and simple method for evaluating an angiogenic activity. As a result, we could identify several genes as angiogenesis-related factors in human hepatocellualr carcinoma.

Methods

Text mining

First, 105 genes that had full length sequences and concurrently whose expression-fold ratios were larger than 2 or smaller than -2 were selected from HCC chip data. These ratios were considered to be significantly

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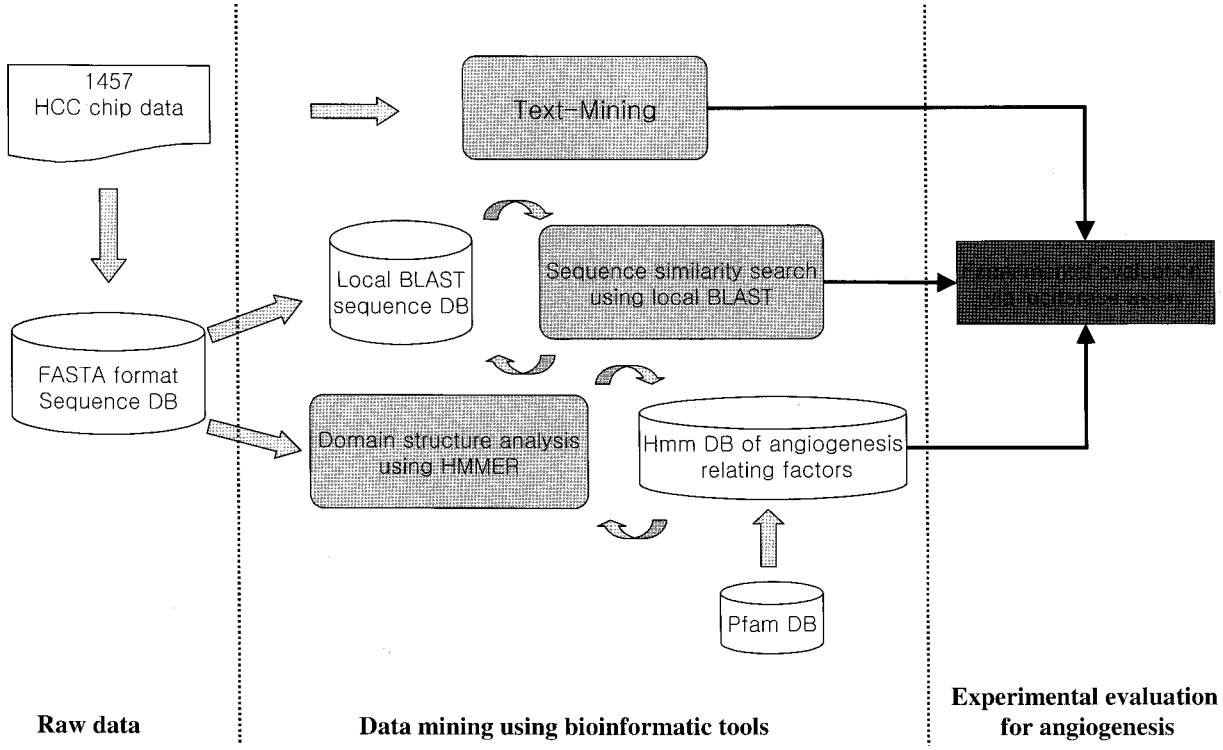


Fig. 1. Scheme of in silico data mining workflow. Workflow consists of two steps. First, angiogenesis-related genes were predicted by three in silico screenings based on microarray data, which are text-mining, sequence simirarity search, and domain analysis. Then, the angiogenesis-related activities of the predicted genes were assayed by luciferase assay.

different. Subsequently, we investigated angiogenesis-related effects of these genes which were reported in PubMed abstract information and could classify the genes into two groups – angiogenesis and anti-angiogenesis.

Sequence similarity search using local BLAST

We downloaded stand-alone BLAST 2.2.6 (binary file: blast-2.2.6-ia32-win32.exe) from NCBI's BLAST ftp server (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/release/2.2.6/). First, gene lists having Swiss-Prot (http://au.expasy.org/sprot/) accession numbers were parsed to Swiss-Prot database. Thereafter, FASTA-formatted sequences were generated, and sequence database were constructed using 'formatdb' in BLAST package. Then, the FASTA format sequences were aligned with angiogenesis-related factors using 'blastp' in BLAST package. In this process, we used the AngioDB (http://angiodb.snu.ac.kr) to draw the information regarding to angiogenesis. The AngioDB is a secondary database focused on angiogenesis-related factors (Sohn *et al.*, 2002).

Domain analysis using HMMER

HMMER program - HMMER 2.2g binaries for DOS/Cygwin environment version – was downloaded from HMMER official web site (http://hmmer.wsustl.edu/). For domain search, two angiogenesis-related domains (Kringle and ECM1) reported in Pfam database (http://pfam.wustl.edu/) were selected, and downloaded as a hmms seed format file. Then, domain database were constructed using 'hmmbuild' in HMMER package by adding three 'hmm' files to database. The domain DB was calibrated using 'hmmcalibrate'. Finally, sequence file of HCC chip data was searched by domain DB using 'hmmsearch'.

Cell culture

HepG2 cells were maintained in minimum essential medium (MEM) (Invitrogen, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS) (Invitrogen, Gaithersburg, MD, USA) and 1% antibiotics. For hypoxic conditions, cells were placed for 18 hours in a hypoxic chamber (Forma scientific, Inc., Marietta, OH) with 1% O_2, 5% CO_2, and 94% N_2 in a humidified atmosphere.

Transient transfection

The day before transfection, cells were seeded in 6-well plates so that they were about 50% confluent at the day of transfection. To carry out luciferase assay, plasmids were transfected to 2×10^5 HepG2 cells per well, with the

Table 1. Gene lists generated from HCC chip data via text-mining

Swiss-Prot ID	Unigene ID	Gene Name	Expressionstage	Fold ratio	Activity
O95997	Hs.252587	Securin (Pituitary tumor-transforming protein 1, PTTG 1)	Up in G23* HCC	2.24	
P10451	Hs.313	Osteopontin (Secreted phosphoprotein 1, SPP-1, OPN)	Up in G23* HCC	9.6	Angiogenic
P07355	Hs.217493	Annexin A2 (Annexin II, Lipocortin II,)	Up in HCC	2.28	
P01008	Hs.75599	Antithrombin-III (AT-III)	Down in G23* HCC	-3.89	
P55056	Hs.110675	Apolipoprotein C-IV (Apo-CIV)	Down in G23* HCC	-4.05	Anti-angiogenic
P02647	Hs.93194	Apolipoprotein A-I (Apo-AI)	Down in HCC	-8.23	

*G23: moderately and poorly differentiated stage of hepatocellular carcinoma

Table 2. Five sequences showing high sequence similarity with angiogenesis-related factors

Swiss-Prot ID	Unigene ID	Gene Name	Aligned protein*	Score	E – value**
Q02952	Hs.788	A-kinase anchor protein 12	AKAP12	2791	0
O60884	Hs.21189	DnaJ homolog subfamily A member 2 (DNAJA2)	Tid-1	111	5.00E-26
Q9UNF1	Hs.4943	Melanoma-associated antigen D2 (MAGED2)	Necdin	168	2.00E-43
Q12816	Hs.259802	Trophinin	Necdin	168	2.00E-43
Q9BQ84	Hs.7457	Melanoma-associated antigen D4 (Melanoma E1 antigen)	Necdin	149	2.00E-37

*Aligned proteins are included in AngioDB.
**E-value: parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size (the default value is 10, which mean that 10 matches are expected to be found merely by chance.)

proper combination of effector plasmids, pSV40promoter-EpoHRE-Luc reporter (1 μg), control plasmid pCMV-β-gal (0.5 μg) and pBOS-hHIF-1α (0.1 μg), pBOS-hARNT (0.1 μg), 1 μg of predicted genes, *i. e.*, pCNS-D2-osteopontin, pCNS-D2-apolipoprotein A-I, pT7T3Pac-apolipoprotein C-IV, pCNS-D2-antithrombin-III, pTZ18RP1-HAF, pCNS-D2-DNAJA2, and pCNS-PTTG1, and empty vectors of each predicted gene using 4 μg of Lipofectamine (Invitrogen, Gaithersburg, MD, USA).

Luciferase assay

Transiently transfected cells were harvested after treatment of hypoxia or normoxia for 18 hours and extracts were prepared using reporter lysis buffer (Promega, Madison, WI). Cell lysates were analyzed for luciferase activity using assay kit (Promega, Madison, WI) and luminometer (AutoLumat LB953, berthold) as manufacture's manual. β-galactosidase enzyme from extracts was also analyzed to correct relative luciferase units (RLU). Each condition was assayed three times, and the luciferase activity was calculated as RLU / β-galactosidase activity.

Results

Six genes selected by text-mining method

The microarray chip experiments of HCC patients were performed by the Center for Functional Analysis of Human Genome (Daejon, Korea). We selected 105 genes among the HCC microarray chip data. Those genes had expression-fold ratio and full-length clones. In this step, the data whose expression-fold ratio was larger than 2 or smaller than -2 were selected. Thereafter, we selected 6 genes reported on angiogenesis from the information of PubMed abstract. However, they have not been reported for angiogenic effects in HCC. So, we investigated angiogenic effects of those genes in a hepatoma cell line.

As shown in Table 1, the fold ratio of osteopontin (OPN) was 9.6, the highest of all data. Also, a securin (pituitary tumor-transforming protein 1) and annexin A2 were selected to be angiogenic and each fold-ratio of a securin and annexin A2 was 2.24 and 2.28, respectively. Securin has been known to play a role in pituitary tumors and stimulates fibroblast growth factor (FGF)-2-mediated angiogenesis (Ishikawa *et al.*, 2001). Annexin A2 has been known to be a predominant receptor for angiostatin and blocks angiostatin's activity (Tuszynski *et al.*, 2002).

As anti-angiogenic factors, two apolipoproteins and antithrombin-III were selected. Antithrombin-III is known to have a serpin (serine protease inhibitor) structure and inhibit angiogenesis (Corvol *et al.*, 2003). Apolipoproteins have been known to contain variable numbers of Kringle domains that share 61–75% homology with Kringle 4 of plasminogen (McLean *et al.*, 1987) and have effects of anti-angiogenesis and anti-tumor growth (Kim *et al.*, 2003). Isoform of apolipoprotein A1 has been reported to

Table 3. Angiogenesis-related domains from Pfam database

Aceesion Number	Domain Name	Description
PF00051	Kringle domain	Kringle domains have been found in plasminogen, hepatocyte growth factors, prothrombin, and apolipoprotein A. Structure is disulfide-rich, nearly all-beta.
PF05782	Extracellular matrix protein 1 (ECM1)	This family consists of several eukaryotic extracellular matrix protein 1 (ECM1) sequences. ECM1 has been shown to regulate endochondral bone formation, stimulate the proliferation of endothelial cells and induce angiogenesis. Mutations in the ECM1 gene can cause lipoid proteinosis, a disorder which causes generalized thickening of skin, mucosae and certain viscera. Classical features include beaded eyelid papules and laryngeal infiltration leading to hoarseness.

be a HCC-specific marker (Steel *et al.*, 2003).

Five genes showing high sequence similarity with three angiogenesis-related genes in the AngioDB

For the sequence alignment, we parsed 1457 HCC chip data to Swiss-Prot as FASTA format protein sequence file. Then, those sequences were aligned with angiogenesis-related factors in AngioDB. All options except for E-value (under 0.001) were of default values. Results from sequence alignment were shown in Table 2.

AKAP12 was found in HCC chip data. AKAP12 encodes a major cytoskeleton-associated protein kinase C substrate and kinase-scaffolding activities (Lin *et al.*, 1996). AKAP12 may be considered as anti-angiogenic factor *in vivo* and *in vitro* (Lee *et al.*, 2003). Tid-1 was aligned to DnaJ homolog subfamily A member 2 with score: 111, E-value: $5 \times e^{-26}$. Tid-1 is a RasGAP-binding protein known for a tumor suppressor gene (Trentin *et al.*, 2001). Moreover, Tid-1 was reported to inhibit angiogenesis by degradation of HIF-1α (Bae *et al.*, 2004). Necdin was aligned to three MAGE family genes. Necdin promotes differentiation and survival of neurons through its antagonistic interactions with E2F1 (Kobayashi *et al.*, 2002) and is expected to be a tumor suppressor (Taniura *et al.*, 1999).

Five proteins containing angiogenesis-related domains

We used the HMMER as a protein domain analysis tool. HMMER is a profile Hidden Markov Model- implemented program for protein sequence analysis. For the domain analysis, we searched angiogenesis-related domains in Pfam database. Then, we selected 2 protein domains of Kringle and ECM1 (Table 3).

Kringle domains have disulfide-rich, nearly all-beta structure (Pfam database, http://pfam.wustl.edu/), and are well known to have anti-angiogenic effects (Cao *et al.*, 2002). Kringle domains have been found in plasminogen, hepatocyte growth factor, prothrombin, and apolipoprotein A. Extracellular matrix protein 1 (ECM 1) domain family consists of several eukaryotic extracellular matrix protein 1 sequences. ECM1 domains play a role in the regulation of endochondral bone formation, proliferation of endothelial cells, and angiogenesis (Han *et al.*, 2001; Deckers *et al.*, 2001).

For the domain analysis, we downloaded HMMER program – HMMER 2.2g binaries for DOS/Cygwin environment version – from HMMER official web site and each of domain hmm files was added to angiodomain local DB. Finally, profile hmm DB were constructed. A hmm file is a score table composed of statistical values calculated by profiled model. Then, 1457 gene sequences from HCC chip data as FASTA format were parsed to profile hmm db (E-value < 0.001). As a result, we found that 5 proteins had angiogenesis-related domains (Table 4). Four proteins had Kringle domain under limited E-value in HCC data, which are plasminogen precursor, coagulation factor XII (HAF), urokinase-type plasminogen activator (uPA), and hepatocyte growth factor activator (HGFA). ECM 1 domain was found in ECM1 protein of 1457 gene sequences.

Angiogenesis-related effect of predicted genes by luciferase assay

In order to investigate whether the predicted genes are involved in angiogenesis, we determined transcription

Table 4. Five proteins containing angiogenesis-related domains

Swiss-Prot ID	Unigene ID	Gene Name	Compared domain	Fold ratio	Score	E - value
P00747	Hs.279766	Plasminogen	Kringle domain	-6.81	856.3	6.70E-258
P00748	Hs.75576	Coagulation factor XII (Hageman factor, HAF)	Kringle domain	-2.71	157.4	1.60E-47
P00749	Hs.1321	Urokinase-type plasminogen activator (uPA, U-plasminogen activator)	Kringle domain	-	135.9	4.80E-41
Q04756	Hs.77274	Hepatocyte growth factor activator (HGF activator, HGFA)	Kringle domain	-7.32	163.3	2.80E-49
Q16610	Hs.104	Extracellular matrix protein 1 (Secretory component p85)	ECM1	-3.03	1413.5	0

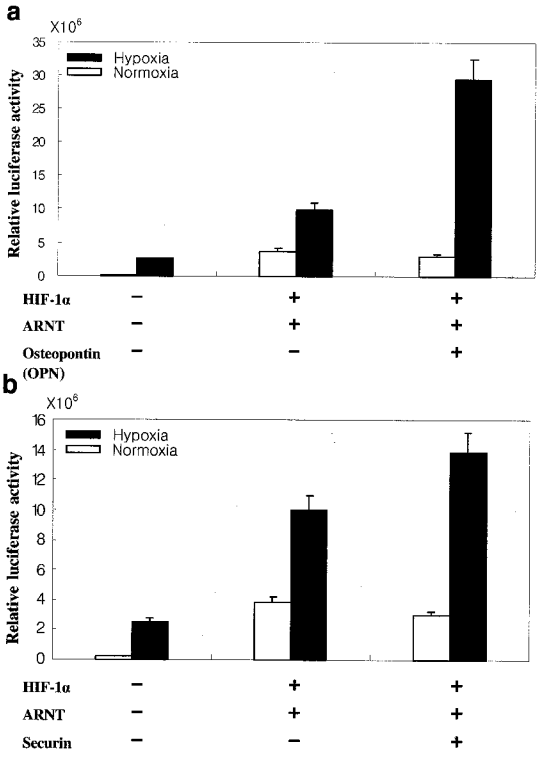
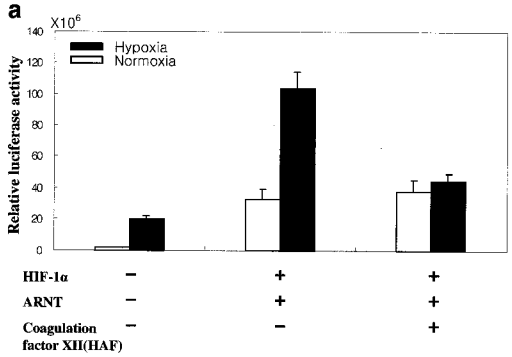


Fig. 2. Relative luciferase activity of predicted genes having angiogenic effects. HepG2 cells were transfected with 1 μg of (a) pCNS-D2- osteopontin (OPN) or (b) pCNS-securin in addition to pSV40pro-EpoHRE-Luc (1 μg), pBOS-hHIF-1α (0.1 μg), and pBOS-hARNT (0.1 μg). Transfected cells were incubated for 3 hours at 21% O_2, and then incubated at 21% O_2 or 1% O_2 for additional 18 hours.

activity of HIF-1α using the luciferase reporter system composed of pSV40 promoter-EpoHRE-Luc. To correct variable transfection efficiency, cells were co-transfected with β-gal plasmid constitutively expressing β-galactosidase under the control of the SV40 promoter and enhancer. Hypoxia response element (HRE) is a functional HIF-1α binding site of VEGF and essential for the activity of VEGF promoter under hypoxic condition (Levy *et al.*, 1995). To evaluate our *in silico* data, we



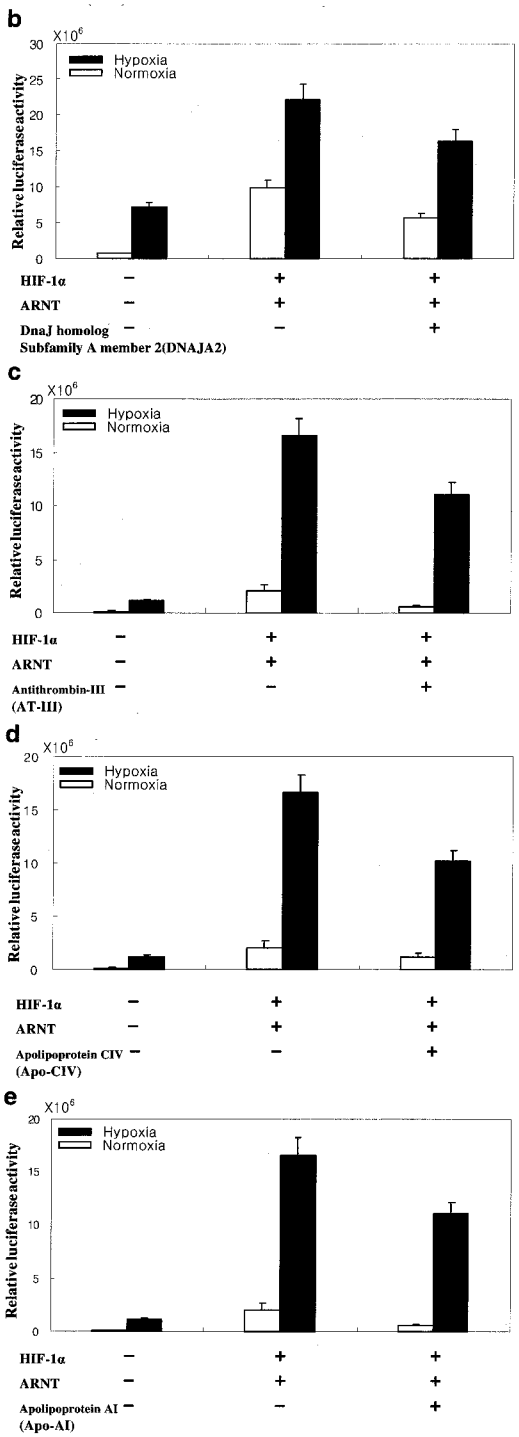


Fig. 3. Relative luciferase activity of predicted genes having anti-angiogenic effects. HepG2 cells were transfected with 1 μg of (a) pTZ18RP1-HAF or (b) pCNS-D2-DNAJA2 or (c) pCNS-D2-AT-III or (d) pT7T3Pac- ApoCIV or (e) pCNS-D2-ApoAI in addition to pSV40pro-EpoHRE-Luc (1 μg), pBOS-hHIF-1α (0.1 μg), and pBOS-hARNT (0.1 μg). Transfected cells were incubated for 3 hours at 21% O_2, and then incubated at 21% O_2 or 1% O_2 for additional 18 hours.

performed luciferase assays for representative genes from *in silico* data. Subsequently, we showed the results of the genes with significant differences (Figure 2 and 3). Osteopontin increased the relative luciferase activity (RLA) significantly (Figure 2a). Also, securin showed the increased tendency of RLA (Figure 2b). In Figure 3, genes which are expected to be anti-angiogenenic, coagulation factor XII (Figure 3a), DnaJ homolog subfamily A member 2 (Figure 3b), antithrombin-III (Figure 3c), apolipoprotein A-I (Figure 3e) and apolipoprotein C-IV (Figure 3d), decreased RLA compared with HIF-1α -transfected cells.

Discussion

HCC is a well-known hypervascular tumor and expresses many angiogenesis-related factors. Because prognosis of HCC is very poor and molecular mechanism of HCC is very complicated, it is difficult to find a HCC-specific marker or an effective therapeutic target. Because of these difficulties, we performed bioinformatic approaches to analyze HCC chip data effectively and efficiently.

First of all, in text-mining results, 6 genes were selected as candidates. Among them, fold-ratio of OPN was 9.6 ,the highest of HCC chip data. OPN has been known to be a multifunctional phosphoprotein with angiogenic activity (Hirama *et al.*, 2003), and to be secreted by several cell types including osteoclasts, lymphocytes, macrophages, and tumor cells (Denhardt *et al.*, 1993). OPN was over-expressed in HBx infected metastatic HCC (Ye *et al.*, 2003) and intrahepatic metastasis (Pan *et al.*, 2003). In the luciferase assay (Figure 2a), OPN also showed significantly increased relative lucifease activity (RLA). Moreover, OPN is a secreted protein which can be detected in the blood. Therefore, OPN could be a potential angiogenic marker of HCC.

Second, in the sequence analysis, 5 genes were selected with significant statistic values. Among them, A-kinase anchor protein 12 (AKAP12) was found in AngioDB. AKAP12 is known to suppress angiogenesis *in vivo* and *in vitro*. DnaJ homolog subfamily A member 2 (DNAJA2) was aligned to Tid-1 which may suppress angiogenesis by degradation of HIF-1α. In the result of the luciferase assay, DNAJA2 showed an anti-angiogenic effect (Figure 3b).

Third, in the domain analysis, 5 genes including Kringle and ECM1 domain were selected with significant values. The domain structures used in our domain structure analysis were reported in Pfam database. Plasminogen is an anti-angiogenic factor containing a well-known anti-angiogenic protein, angiostatin, and urokinase type plasminogen activator participates in the activation of plasminogen. Hepatocyte growth factor activator (HGFA) and coagulation factor XII (Hageman Factor, HAF) participate in the activation of hepatocyte growth factor (HGF) when liver tissue injury occurs (Shimomura *et al.*, 1995; Miyazawa *et al.*, 1996). In addition, plasminogen and HGFA were down-regulated in other microarray analysis (Delpuech *et al.*, 2002) and in our HCC chip data (each fold-ratio was -6.81 and -2.71). Furthermore, HAF significantly decreased relative luciferase activity in our experimental result (Figure 3a). Therefore, these genes having Kringle domain may play an important role in injured HCC.

Collectively, of 1457 genes generated from cDNA microarray data of HCC patients, we found that 16 genes were likely to have angiogenesis-related functions through text-mining, sequence similarity search and domain analysis. Thereafter, the angiogenic effects of these genes were evaluated by the luciferase assay. Although more investigation on predicted genes is needed, the results of this work could provide the possibility that *in silico* prediction is useful for identifying novel factors for angiogenesis in HCC.

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